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Influence of N-Methylation and Conformation on Almiramide Anti-Leishmanial Activity

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Abstract: The almiramide N-methylated lipopeptides exhibit promising activity against trypanosomatid parasites. A structure–activity relationship study has been performed to examine the influences of N-methylation and conformation on activity against various strains of leishmaniasis protozoan and on cytotoxicity. The synthesis and biological analysis of twenty-five analogs demonstrated that derivatives with a single methyl group on either the first or fifth residue amide nitrogen exhibited greater activity than the permethylated peptides and relatively high potency against resistant strains. Replacement of amino amide residues in the peptide, by turn inducing α-amino γ-lactam (Agl) and N-aminoimidazalone (Nai) counterparts, reduced typically anti-parasitic activity; however, peptide amides possessing Agl residues at the second residue retained significant potency in the unmethylated and permethylated series. Systematic study of the effects of methylation and turn geometry on anti-parasitic activity indicated the relevance of an extended conformer about the central residues, and conformational mobility by tertiary amide isomerization and turn geometry at the extremities of the active peptides.

Keywords: almiramide; leishmaniasis, N-methylated peptide (List three to ten pertinent keywords specific to the article; yet reasonably common within the subject discipline.)

1. Introduction

Neglected tropical diseases caused by trypanosomatid protozoan infections, such as human African trypanosomiasis, Chagas disease and leishmaniasis, impact significantly on public health, especially in tropical countries, where their influences are compounded due to poverty, environment, and drug resistance [1–3]. Leishmaniasis is caused by an intracellular protozoan belonging to the genus Leishmania (L.) and transmitted by the phlebotomine sandflies [4]. Upon mammal host infection by way of a sand fly bite, the parasites enter the blood cells in the so-called promastigote stage, then begin to multiply in the amastigote stage spreading to other cells and tissues. Depending on the species, the parasites may disperse via blood and lymph fluid to other body sites, such as the skin and major organs.

With over 1 million new cases occurring annually, around 12 to 15 million people worldwide are infected with leishmaniasis [5,6], which presents as four main forms: cutaneous, mucocutaneous, visceral and post Kala-azar dermal leishmaniasis (CL, MCL, VL and PKDL) [7]. Leishmaniasis causes enlargement of the spleen and liver, anemia [8], skin
lesions [9], and destruction of mucous membranes [10]. In addition, controlling co-infection with HIV and visceral leishmaniasis has become a serious challenge [11].

In the absence of a vaccine, chemotherapy is the main tool to treat leishmaniasis, but only a few drugs are used clinically [12]. Pentavalent antimonial drugs, such as sodium stibogluconate and meglumine antimoniate, were first-line defenses against leishmania since the 1940s [13], but are now failing as treatments due to resistance [14]. Amphotericin B, which was discovered in the late 1950s, became an alternative drug for treating leishmaniasis patients [15]. Miltefosine was the first oral anti-leishmanial agent and has been used to treat VL patients since 2002 [16]. High cost and side effects have; however, limited the use of amphotericin B and miltefosine [17]. The aminoglycoside antibiotic paromomycin has been used to treat leishmaniasis [18], with variations in efficacy contingent upon geographic region [19].

The need for new anti-leishmanial agents is critical due to limitations in availability of effective drugs and the rise of drug resistant strains of leishmaniasis [20]. In the search for antiparasitic agents, selectivity and potency (μM) against certain Leishmania strains have been observed using chromone natural products [21] and artemisinin-inspired analogs [22]. Moreover, notable differences in ribosomal structure were exploited to conceive paromomycin derivatives with potent anti-leishmanial activity and low otoxicity [23]. Other anti-leishmanial agents under investigation include mitochondrial cytochrome bc1 and pteridine reductase 1 inhibitors, as well as aryl nicotinic acid derivatives [20]. In addition, the kinetoplastid-selective proteasome inhibitor LXE408 which exhibited efficacy in murine CL and VL models has recently entered Phase 1 human clinical trials [24].

Almiramides A–C (1–3, Figure 1) are N-methylated linear lipopetides isolated from the marine cyanobacterium *Lyngbya majuscula* [25]. Certain almiramide analogs have exhibited low μM anti-parasitic activity against the causative pathogen of fatal leishmaniasis, as well as a high therapeutic index [25]. Almiramide derivatives are attractive leads for developing agents against leishmaniasis, in part due to their likely mechanism of action involving disruption of the vital energy machinery proteins of the glycosome [26]. A peroxisome-related organelle without mammalian counterpart, the glycosome performs vital metabolic processes in trypanosomatids [27,28]. In search of glycosome targeting agents, the interface of two key transport proteins [peroxin (PEX)14–PEX5] has been blocked by pyrazolo[4,3-c]pyridines which killed effectively the causative trypanosomatids of Human African trypanosomiasis and Chagas disease, *Trypanosoma brucei* and *Trypanosoma cruzi*, and had low mammalian cell cytotoxicity [29,30]. Moreover, an examination of almiramide analogs in affinity capture, and fluorescent microscopy experiments on *T. brucei*, identified the glycosome proteins PEX11 and glycosomal integral membrane protein-5a (GIM5A) as likely targets [26].

Preliminary structure–activity relationship studies on the almiramides have indicated the importance of the unsaturated lipophilic terminus. Almiramides B and C (2 and 3, Figure 1) with 2-methyl-7-octenoyl and -7-octenoyl tails were reported to exhibit low μM activity against *Leishmania donovani*, but their 2-methyl-7-oxooctenoyl counterpart almiramide A (1) was inactive [25]. Employment of a 6-heptyl linolephil terminus and N-methylvaline in acid 12 and dimethyl amide 13 gave peptides with μM IC50 values against *L. donovani*, and improved therapeutic indices with relatively lower cytotoxicity (CC50) against mammalian Vero kidney cells: selectivity index (CC50/EC50) 12 = 13 (50.2) > 2 (21.8) > 3 (17.4) [25]. In this series, the significance of the peptide C-terminal was illustrated by the methyl ester counterpart (e.g., 14) of peptide acid 12, which exhibited a significant loss of anti-parasitic activity and a relative gain in Vero cell cytotoxicity [25]. In addition, peptide 2 and novel almiramides D–H (4–8) possessing the common 2-methyl-7-octenoyl N-terminus and a distinct N-(Me)Ile residue were isolated together from marine benthic cyanobacteria in a study that found 2 and 9 exhibited toxicity against human gingival fibroblast cells [31].
The active almiramide conformation has been examined by replacement of the Val-α Ala dipeptide moiety with a mannose-derived furanose amino acid in analogs 9–11. Sugar-peptide hybrids 9–11 exhibited a similar activity and selectivity index as miltefosine against intra-macrophage amastigotes of \textit{L. donovani} and Vero cells [32]. Conformational analysis of sugar-peptide hybrids 9 and 11, by a combination of computational and NMR spectroscopic methods, concluded a potential bent structure from a characteristic nuclear Overhauser effect correlation between the phenylalanine N-methyl group and the d-proton of the furanose amino acid residue [32].

The intriguing potential of the almiramides for the development of anti-parasitic agents has prompted a more detailed study to ascertain the features responsible for their potency and selectivity. In spite earlier efforts, limited knowledge exists of the relevance of N-methylation and conformation for almiramide activity against parasite and host cells. Among the reported almiramide analogs, peptides having only two N-methyl residues [e.g., 5, Val(Me)\textsuperscript{1} and Ile(Me)\textsuperscript{4}] to completely permethylated amides (e.g., acid 12) have...
been respectively isolated and synthesized. To the best of our knowledge, the activity of unmethylated and singly methylated almiramide peptides has yet to be reported nor has the effectiveness of almiramide analogs against resistant strains of Leishmania been examined. A systematic study of almiramide peptides has now been performed to shine light on the importance of tertiary amides and turn conformers for activity against Leishmania and resistant strains.

2. Results and Discussion

2.1. Chemistry

Commencing with the potent and selective lead peptide 12, a systematic study of the influence of N-methyl groups on each of the five amides was begun by the preparation of the corresponding unmethylated peptide acid 15 (Figure 2). Considering that long chain secondary amide derivatives of 12 retained significant anti-parasitic activity and offered potential for the synthesis of conjugates to study mechanism of action [26], the corresponding 1,6-hexanediamine amides 16 and 17 were examined for comparison with permethylated and unmethylated acids 12 and 15. Subsequently, an N-methyl scan was performed on amide 1Agl to provide singly methylated analogs 18–22. For systematic solid-phase syntheses of N-methyl peptides 18–22, the required N-methyl amino acids (Fmoc-Ala(Me)-OH, Fmoc-Phe(Me)-OH and Fmoc-Val(Me)-OH) were respectively prepared in solution, according to literature protocols featuring acid-catalyzed condensation of an Fmoc-protected amino acid with paraformaldehyde, to form an oxazolidinone followed by reduction with triethyl silane and trifluoroacetic acid [33].

In the study of biologically active peptides, α-amino γ-lactam residues (Agl residues, Figure 2) so-called Freidinger-Veber lactams have been commonly used to restrict backbone ω and ψ dihedral angles to favor β-turn conformers, in which the Agl residue situates at the i + 1 position [34,35]. The related N-amino-imidazol-2-one (Nai) residues offer similar backbone constraint with potential to add substituents at the heterocycle 4- and 5-positions to mimic side chain function with constrained χ dihedral angle geometry [35,36]. A biologically active almiramide bent conformer has been proposed based on the activity of sugar-peptide hybrids 9–11 [32]. To probe this hypothesis further, Agl and (5-Me)Nai residues were used to replace systematically the first four residues in the sequences of peptides 12 and 15–17 (Figure 3). For example, six Agl almiramide derivatives (e.g., 23–28) were respectively prepared by replacing valine (e.g., Val1, Val2, Val3) with the heterocycle in acids 12 and 15. The first four residues of permethylated and unmethylated peptide amides 16 and 17 were also systematically replaced by Agl residues to provide peptides 29–35. Moreover, (5-Me)Nai residues were respectively used to replace Val1 and Val2 of peptide acid 15 in peptides 36 and 37.

For the Agl scan, Fmoc-Agl dipeptides [Fmoc-Agl-Val-OH, Fmoc-Agl-Ala-OH and Fmoc-Agl-Phe-OH (38a–c), supporting information] were respectively synthesized by a modification of the original Freidinger-Veber protocol, as recently reported for the preparation of the valine dipeptide [34,37]. In brief, N-(Boc)methionyl dipeptide tert-butyl esters were treated with iodomethane to prepare the corresponding sulfonium ion intermediate, which on treatment with NaH underwent intramolecular N-alkylation to furnish N-(Boc)Agl dipeptide tert-butyl esters (e.g., 39a–c, supporting information). The carbamate and ester groups were removed using trifluoroacetic acid (TFA) in dichloromethane and the Fmoc group was installed using N-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) and sodium carbonate in aqueous acetone to provide Agl dipeptides 38. During the cyclization to form Boc-Agl-Phe-Oh-Bu (39c, supporting information), ester epimerization occurred providing an inseparable 2:1 mixture of diastereomers, which was used to prepare the separable mixture of Agl-Phe peptide 32 and Agl-D-Phe isomer R-32. The corresponding Nai-dipeptide ester [Fmoc-(5-Me)Nai-Val-Oh-Bu (40, supporting information)] was synthesized from Fmoc-azaGly-Val-Oh-Bu (41, supporting information), by a route featuring oxidation to the corresponding azopeptide using N-bromosuccinimide
(NBS) and lutidine in dichloromethane, proline-catalyzed alkylation with propionaldehyde, and dehydration using p-toluenesulfonic acid in chloroform [38]. Ester solvolysis using TFA in dichloromethane gave the Fmoc-(5-Me)Nai-Val-OH which without purification was coupled onto resin.

![Chemical structure](image)

| Peptide | R¹ | R² | R³ | R⁴ | R⁵ | X             |
|---------|----|----|----|----|----|---------------|
| 15      | H  | H  | H  | H  | H  | OH            |
| 16      | H  | H  | H  | H  | H  | NH(CH₂)₆NH₂  |
| 17      | Me | Me | Me | Me | Me | NH(CH₂)₆NH₂ |
| 18      | Me | H  | H  | H  | H  | NH(CH₂)₆NH₂ |
| 19      | H  | Me | H  | H  | H  | NH(CH₂)₆NH₂ |
| 20      | H  | H  | Me | H  | H  | NH(CH₂)₆NH₂ |
| 21      | H  | H  | H  | Me | H  | NH(CH₂)₆NH₂ |
| 22      | H  | H  | H  | H  | Me | NH(CH₂)₆NH₂ |

Figure 2. N-Methyl scan of almiraamide analogs.

![Chemical structures](image)

23: HCC(CH₂)₄CO-Agl-Val-Val-Ala-Phe-OH
24: HCC(CH₂)₄CO-Val-Agl-Val-Ala-Phe-OH
25: HCC(CH₂)₄CO-Val-Val-Agl-Ala-Phe-OH
26: HCC(CH₂)₄CO-Agl(5-Me)Nai-Val-Val-Ala-Phe(5-Me)OH
27: HCC(CH₂)₄CO-Val-Agl(5-Me)Nai-Val-Ala(5-Me)Phe(5-Me)OH
28: HCC(CH₂)₄CO-Val-Val-Agl(5-Me)Nai-Ala-Phe(5-Me)OH
29: HCC(CH₂)₄CO-Val-Val-Ala-Phe-NH(CH₂)₆NH₂
30: HCC(CH₂)₄CO-Val-Agl-Val-Ala-Phe-NH(CH₂)₆NH₂
31: HCC(CH₂)₄CO-Val-Val-Agl-Ala-Phe-NH(CH₂)₆NH₂
32: HCC(CH₂)₄CO-Val-Val-Agl-Phe-NH(CH₂)₆NH₂
33: HCC(CH₂)₄CO-Agl(5-Me)Nai-Val-Val-Ala-Phe(5-Me)NH(CH₂)₆NH₂
34: HCC(CH₂)₄CO-Val-Agl(5-Me)Nai-Val-Ala(5-Me)Phe(5-Me)NH(CH₂)₆NH₂
35: HCC(CH₂)₄CO-Val-Val-Agl(5-Me)Nai-Ala-Phe(5-Me)NH(CH₂)₆NH₂
36: HCC(CH₂)₄CO-(5-Me)Nai-Val-Val-Ala-Phe-OH
37: HCC(CH₂)₄CO-Val-(5-Me)Nai-Val-Ala-Phe-OH

Figure 3. Examination of turn conformation using Agl and Nai residues.
As illustrated by the synthesis of Agl analog 24, peptides 15–37, all were synthesized using conventional Fmoc/t-Bu solid phase peptide synthesis (SPPS) methods starting from chlorotriyl resin (CTC resin, Scheme 1) [25,39]. N-Methyl peptide amides 18–22 were synthesized on CTC resin modified with a 1,6-diaminohexane linker. 1,6-Diaminohexane was loaded onto the CTC resin using N,N'-diisopropylethylamine.

\[
\text{H-Ala-Phe}\quad \text{DIC, HOBt, NMP}
\]

\[
\text{Fmoc-Agl-Val-OH}\quad 38a
\]

1) 20% piperidine, DMF
2) Fmoc-Val-OH, DIC, HOBt, NMP
3) 20% piperidine, DMF

\[
\text{HCC(CH}_2)_4\text{CO}_2\text{H}, \text{DIC, HOBt, NMP}
\]

\[
\text{F}_3\text{CO}_2\text{H}:\text{Et}_3\text{SiH}:\text{H}_2\text{O} (95:2.5:2.5)
\]

\[
\text{HCC(CH}_2)_4\text{CO-Val-Agl-Val-Ala-Phe-}OH
\]

\[
\text{HCC(CH}_2)_4\text{CO-Val-Agl-Val-Val-Phe-NH(CH}_2)_6\text{NH}_2
\]

\[
\text{= a, O-CTC resin; b, HN(CH}_2)_6\text{NH-CTC resin}
\]

\[
\text{Fmoc-Agl-Val-Ala-Phe-}OH
\]

\[
\text{H-Val-Agl-Val-Ala-Phe-}OH
\]

\[
\text{HCC(CH}_2)_4\text{CO-Val-Agl-Val-Ala-Phe-}OH
\]

\[
\text{HCC(CH}_2)_4\text{CO-Val-Agl-Val-Phe-NH(CH}_2)_6\text{NH}_2
\]

\[
\text{24}
\]

\[
\text{30}
\]

Scheme 1. Representative SPPS (solid phase peptide synthesis) synthesis of almiramides derivatives 24 and 30.

N-(9-Fluorenylmethoxycarbonyl)phenylalanine was coupled to CTC resin using N,N'-diisopropylethylamine. Alternatively, Fmoc-Phe-OH and Fmoc-Phe(Me)-OH were coupled to 1,6-diaminohexane CTC resin using N,N'-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) in NMP. After Fmoc group removals with a 20% solution of piperidine in DMF, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Agl dipeptide acids 38a-c, Fmoc-(5-Me)Nai-Val-OH and 6-heptynoic acid, all were respectively coupled using a similar DIC/HOBt protocol. The N-methyl residues (e.g., Fmoc-Ala(Me)-OH and Fmoc-Val(Me)-OH) were coupled using 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) and N-methylmorpholine in DMF [40]. Peptide cleavage was performed using a TFA/TEA/H$_2$O (95:2.5:2.5) cocktail. Final peptides were precipitated with diethyl ether (EtO) and purified by HPLC on a C$_{18}$ column (Table 1). Peptide acids (e.g., 24) were permethylated using sodium hydride and iodomethane in THF (Scheme 2) [25]. As illustrated for the synthesis ofamide 34, unmethylated and permethylated peptide amides 16 and 17, as well as Agl peptide amides 29-35, all were prepared by coupling the corresponding peptide acids (e.g., 27) to 1,6-diaminohexane using N,N'-dicyclohexylcarbodiimide and 4-dimethylaminopyridine in dichloromethane.
Scheme 2. Representative permethylation of peptide acid 24 and coupling to 1,6-diaminohexane to prepare peptide amide 34.

Table 1. Purity, retention times, and mass of almiramide derivatives.

| #  | Sequences                                      | RT CH₃OH | CH₃CN | Purity at 214 nm | MS [M + 1]          |
|----|-----------------------------------------------|---------|-------|------------------|---------------------|
| 12 | R-Val(Me)-Val(Me)-Val(Me)-Ala(Me)-Phe(Me)-OH  | 6.83    | 7.19  | >99              | 734.4463            |
| 15 | R-Val-Val-Ala-Phe-OH                          | 8.83    | 7.44  | >99              | 642.3861            |
| 16 | R-Val-Val-Ala-Phe-NH(CH₂)₆NH₂                 | 7.33    | 6.52  | >99              | 740.5067            |
| 17 | R-Val(Me)-Val(Me)-Val(Me)-Ala(Me)-Phe(Me)-NH(CH₂)₆NH₂ | 8.95    | 7.81  | >99              | 810.5852            |
| 18 | R-Val(Me)-Val-Ala-Phe-NH(CH₂)₆NH₂             | 8.42    | 6.90  | >99              | 754.5225            |
| 19 | R-Val-Val(Me)-Val-Ala-Phe-NH(CH₂)₆NH₂        | 7.86    | 6.15  | >99              | 754.5225            |
| 20 | R-Val-Val-Val(Me)-Ala-Phe-NH(CH₂)₆NH₂        | 7.91    | 6.57  | >99              | 754.5225            |
| 21 | R-Val-Val-Ala(Me)-Phe(NH(CH₂)₆)NH₂           | 7.61    | 6.44  | >99              | 754.5225            |
| 22 | R-Val-Val-Ala-Phe(Me)-NH(CH₂)₆NH₂            | 7.62    | 6.62  | >99              | 754.5225            |
| 23 | R-Agl-Val-Val-Ala-Phe-OH                     | 7.11    | 6.71  | >99              | 626.3548            |
| 24 | R-Agl-Val-Ala-Phe-OH                         | 7.55    | 6.87  | >99              | 626.3548            |
| 25 | R-Val-Agl-Ala-Phe-OH                         | 7.94    | 7.17  | >99              | 626.3548            |
| 26 | R-Agl(Me)-Val-Val-Val(Me)-Ala(Me)-Phe(Me)-OH | 8.69    | 7.95  | >99              | 682.4174            |
| 27 | R-Agl(Me)-Val-Val-Val(Me)-Ala(Me)-Phe(Me)-OH | 9.12    | 8.26  | >99              | 704.3993            |
| 28 | R-Val(Me)-Val(Me)-Agl(Me)-Ala-Phe(OH)        | 9.01    | 8.43  | >99              | 704.3993            |
| 29 | R-Agl-Val-Val-Ala-Phe-NH(CH₂)₆NH₂            | 6.72    | 6.00  | >94              | 724.4756            |
| 30 | R-Val-Agl-Val-Ala-Phe-NH(CH₂)₆NH₂            | 6.83    | 5.98  | >96              | 724.4756            |
| 31 | R-Val-Agl-Ala-Phe-NH(CH₂)₆NH₂                | 7.50    | 6.44  | >99              | 724.4756            |
| 32 | R-Val-Val-Agl-Phe-NH(CH₂)₆NH₂                | 5.31    | 6.42  | >92              | 752.5069            |
| R-32| R-Val-Val-Agl-D-Phe-NH(CH₂)₆NH₂              | 5.07    | 6.42  | >94              | 752.5069            |
2.2. Bioactivity

The biological activity of peptides 15–37 was assessed against the *L. infantum* wild-type strain (WT), as well as mutants resistant to the common anti-leishmanial agents, such as antimony (Sb\textsuperscript{2+}), amphotericin B (AmB) and miltefosine (MF): Sb\textsuperscript{2000.1}, AmB\textsuperscript{1000.1} and MF\textsuperscript{200.5}. Activity against *Leishmania* promastigotes was determined by monitoring the replication of parasites after 72 h of incubation at 25 °C in the presence of increasing concentrations of the different peptides 12–37 and reported as EC\textsubscript{50} (Table 2). In general, almiramide peptides 12–37 exhibited activity against wild type and resistant strains in the range of 5–300 μM. The greatest potencies were observed using analogs against the amphotericin B resistant strain (e.g., AmB\textsuperscript{1000.1}).

Table 2. Structure, bioactivity of almiramide derivatives on different strains of *Leishmania* (EC\textsubscript{50}) and cytotoxicity of peptides 12–39.

| #  | Sequence (R = HCC(CH\textsubscript{3})) | Ldi WT \( \mu \text{M} \) | Ldi Sb-Res \( \mu \text{M} \) | Ldi MF-Res \( \mu \text{M} \) | Ldi AmB-Res \( \mu \text{M} \) | CC\textsubscript{50} \( \mu \text{M} \) | Selectivity Index |
|----|----------------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| 12 | R-Val(Val)-Val(Val)-Val(Val)-Val(Val)-Ala(Me)-Phe(Me)-OH | 68.33 | 70.69 | 75.60 | 79.59 | 281 | 4.1 |
| 15 | R-Val(Val)-Val(Val)-Phe(Me) | 44.69 | 60.26 | 50.77 | 46.38 | 316 | 7.0 |
| 16 | R-Val(Val)-Val(Val)-Val(Val)-Ala(Me)-Phe(Me)-OH | 23.38 | 30.34 | 18.09 | 20.76 | 446 | 19.0 |
| 17 | R-Val(Val)-Val(Val)-Val(Val)-Ala(Me)-Phe(Me)-OH | 48.42 | 60.18 | 25.46 | 5.60 | 338 | 6.9 |
| 18 | R-Val(Val)-Val(Val)-Val(Val)-Ala(Me)-Phe(Me)-OH | 36.70 | 39.65 | 12.12 | 5.03 | 177 | 4.8 |
| 19 | R-Val(Val)-Val(Val)-Ala(Me)-Phe(Me)-OH | 81.17 | 78.91 | 89.23 | 70.26 | 316 | 3.9 |
| 20 | R-Val(Val)-Val(Val)-Ala(Me)-Phe(Me)-OH | 53.86 | 40.25 | 60.64 | 57.18 | 281 | 5.2 |
| 21 | R-Val(Val)-Val(Val)-Ala(Me)-Phe(Me)-OH | 75.83 | 79.78 | 63.53 | 65.66 | 1440 | 18.9 |
| 22 | R-Val(Val)-Val(Val)-Ala(Me)-Phe(Me)-OH | 29.04 | 25.02 | 27.76 | 25.91 | 446 | 15.3 |
| 23 | R-Val(Val)-Val(Val)-Ala(Me)-Phe(Me)-OH | 225.1 | 240.6 | 231.5 | 211.7 | 363 | 1.61 |
| 24 | R-Val-Val(Val)-Val(Val)-Ala(Me)-Phe(Me)-OH | 248.1 | 251.1 | 237.5 | 261.1 | 398 | 1.6 |
| 25 | R-Val(Val)-Val(Val)-Ala(Me)-Phe(Me)-OH | N.D. | N.D. | N.D. | N.D. | 416 | - |
Examining peptide potency (EC₅₀) against wild type *L. infantum*, the 1,6-diaminohexane amides (e.g., 16 and 17) were respectively more potent than the acid counterparts (e.g., 15 and 12). In the Agl series, amides 29–31 and 33–35 were similarly more active than their acid counterparts 23–25 and 26–28. Moreover, the unmethylated analogs (e.g., 15, 16, 29 and 31) were typically more potent than the respective permethylated counterparts (e.g., 12, 17, 33 and 35), except in the case of Agl² analogs 30 and 34. Comparison of the activity of unmethylated 1,6-diaminohexane amide 16 with singly methylated counterparts 18–22 illustrated decreasing potency in the order of 16 > MePhe² 22 > MeVal¹ 18 > MeVal² 20 > MeAla¹ 21 > MeVal¹ 19. Considering the Agl analogs as constrained versions of the corresponding N-methyl dipeptide amides, except in the case of MePhe 22 which was significantly more potent than Agl-Phe 32, the more restricted Agl analogs 29–31 were slightly more active than their N-methyl counterparts 19–21. Activity against *Leishmania* dropped significantly in the case of the Agl peptide acids (23–28) and their (5-Me)Nai counterparts (36 and 37).

Many of the structure–activity relationships, that were observed in the wild-type strain, were also found in the resistant strains. For example, the amides were more potent than their acid counterparts. The nonmethylated analogs were also typically more potent than their permethylated analogs; however, in the case of the amphotericin B resistant
strain (e.g., AmB1000.1), permethylated analogs 17 and 35 were respectively 4- and 1.4-fold more active than nonmethylated counterparts 16 and 31, and in the case of the miltefosine resistant strain (e.g., MF200.5), 35 was 1.5-fold more active than 31. Although the order of potency for nonmethylated 16, MeVal 18 and MePhe 22 was contingent on the tested resistant strain, analogs with no tertiary amide and methylation at the C- and N-terminal residues were consistently more active than peptides 19–21 with N-methylation in the central residues. The potency of MePhe 22 was greater than Agl-Phe 32 for all strains tested. The Agl constraint at other positions tended to slightly favor activity compared to the tertiary amide counterpart in the antimony resistant strain (e.g., Sb2000.1) as was observed in wild type; however, the lactam analogs were significantly less active than the respective N-methyl counterparts against the miltefosine and amphotericin B strains.

In addition, preliminary investigations of peptides (e.g., 12–22), which had notable potency against Leishmania (EC50 = 5–90 µM), detected no activity against T. brucei and T. cruzi (data not shown).

The host cytotoxicity was evaluated by monitoring the survival rates of murine LM-1 macrophage at different concentrations of peptides 15–37 (0.0001 to 100 mM) over 24 h. Most analogs exhibited CC50 values between 170–450 µM. Exceptionally, MeAla peptide amide 21 was 3.2- to 8-fold less toxic than the other peptide analogs. Comparing macrophage toxicity with potency against L. infantum, the selectivity index (SI) for MeAla peptide amide 21 was relatively high (SI = 19.1) and comparable with the most potent peptide (e.g., 16, SI = 19.2). In comparisons of macrophage toxicity with potency against the AmB resistant Leishmania strain, permethylated and MeVal peptide amides 17 and 18 exhibited, respectively, therapeutic indices of 64 and 36.

3. Discussion

The structure–activity relationship studies obtained in wild type and resistant strains of L. infantum reflect likely a combination of ability to engage the target and pharmacokinetic properties that may influence peptide availability. The molecular targets of almira-mide C have been studied using a combination of photo-affinity and fluorescent probes in T. brucei, and suggested to include integral membrane proteins found in glycosomes (e.g., GIM5 and PEX11), which are specific to kinetoplastid parasites [26]. Responsible for the first seven steps of glycolysis, the glycosome is a peroxisome-related organelle essential for parasite survival in the bloodstream stage [41]. Notably, translocation across the glycosomal membrane implicates transporter and pore-forming proteins [42], which may differ contingent on species and be modified in resistant strains.

Resistant strains of Leishmania emerge by different mechanisms which contingent on the drug act commonly to reduce the active concentration inside the parasite by either decreasing uptake, increasing efflux or inhibiting activity [43]. Leishmania antimonial resistance is associated with thiol metabolism to prevent reduction of the SbIV to more active SbIII, and to sequester antimony in thiolate complexes amenable for efflux [44]. Miltefosine resistance is commonly associated with mutations in the Leishmania miltefosine transporter (LMT), a P-type ATPase responsible for the translocation of phospholipids, as well as overexpression of ABC transporters [44]. Amphotericin B resistant L. donovani promastigotes have been shown to feature substitution of ergosterol for another sterol, which alters the fluidity and AmB binding affinity in the cell membrane [45].

Although the conformational preferences of the almira-mides alone and target bound have yet to be described, information gleaned from related peptides and their N-methyl and lactam counterparts offers a lens through which to interpret the structure–activity relationships. For example, the circular dichroism spectrum of the (Val-Val-Val-Ala)15 oligomer in a mixture of hexafluoro-2-propanol and trifluoro ethanol indicated a curve shape typical of an extended β-sheet structure [46]. The corresponding Val-Val-Val-Ala region in nonmethylated almira-mide analogs 15 and 16, as well as MePhe analog 22 may likely adopt an extended β-strand conformer. Introduction of N-methyl groups causes significant consequences on peptide conformation, due in part to creation of a tertiary
amide which loses a potential NH hydrogen-bond donor and may adopt energetically similar cis and trans isomers [47]. Computational analysis of the N'-methyl amides of N-acetyl-N-methyl- and N-acetyl-alanine indicated that repulsive interactions between the N-methyl and carbonyl oxygen moieties of the former abolished the low-energy minimum β-conformer adopted by the latter [48]. In cyclic peptides, N-methyl residues have also induced backbone $f$ and $\psi$ dihedral angles consistent with β- and γ-turn conformers [49], as well as altered side chain $\chi$ geometry [50]. N-Alkylation of the central amide of the hairpin inducing D-Pro-Aib dipeptide has also been shown by variable temperature CD spectroscopy to reinforce the central turn conformer and enhance the stability of the folded β-sheet peptide [51]. Introduction of N-methyl residues within the peptide chain causes likely a shift from an extended sequence to a dynamic series of cis- and trans-amide conformers exhibiting a preference for turn geometry, which in peptides 19–21 reduces activity. In MeVal$^1$ analog 18 and permethylated analogs 12 and 17, methylation at the N-terminal enables a cis-amide conformer in which the lipid tail may fold in the direction of the peptide. Such a geometry may improve membrane transport by hiding hydrophilic NH moieties and may account for the significantly improved activity of 17 and 18 against the amphotericin B resistant strains. To further examine the influence of N-methylation on conformation, the 1H NMR spectra of nonmethylated peptide 16 and N-methyl counterpart 18 were examined in DMSO-d$_6$ (supporting information). In contrast to peptide 16, which exhibited a narrow distribution of amide protons signals between 7.55 and 7.95 ppm characteristic of a linear conformer, the corresponding peaks were downfield shifted, dispersed between 7.65 and 8.35 ppm, and existed in isomeric pairs for MeVal$^1$ peptide 18, likely due the tertiary amides favoring cis- and trans-amide isomers of similar energy and local turn conformers with intramolecular hydrogen bonds.

The propensity for Agl bearing peptides to adopt type II and II' β-turns contingent on α-carbon stereochemistry has been demonstrated using spectroscopic and computational methods, as well as X-ray diffraction, which has also characterized crystals of lactam analog in extended conformers [52]. Relatively diminished activities of Agl analogs 29–35 compared to nonmethylated peptide 16 may again be due to the favored turn geometry. The slightly better activity exhibited by Agl analogs 29–31 in comparison to N-methyl counterparts 19–21 in wild type L. infantum may be attributed to the capacity of the amino lactam to stabilize trans-amide isomers. On the other hand, the notably better activity of MePhe analog 22 relative to Agl counterpart 32 indicates the attributes of greater conformational flexibility at the C-terminal. Similarly, greater conformational dynamics of N-methyl analogs compared to the Agl counterparts appear to be important for the relatively better activity of the former in resistant Leishmania strains.

4. Materials and Methods

4.1. Experimental Section

4.1.1. Leishmania Cultures and Antileishmanial Activity Determination

The L. infantum (MHOM/MA/67/ITMAP-263) wild-type stain (WT), as well as the resistant mutants Sb2000.1, AmB1000.1 and MF200.5 [53–58], resistant to antimony (Sb$^{III}$), amphotericin B (AmB) and miltefosine (MF), respectively, were grown in M199 medium at 25 °C supplemented with 10% fetal bovine serum, 5 μg/mL of haemin at pH 7.0 and 2000 μM Sb (Potassium antimonyl tartrate, Sigma-Aldrich), 200 μM of MF (Miltefosine, Cayman Chem., Michigan, USA) or 1 μM AmB (Amphotericin B solution, Sigma, Oakville, Canada). Antileishmanial values were determined in Leishmania promastigotes by monitoring the replication of parasites after 72 h of incubation at 25 °C in the presence of increasing concentrations of the different peptides by measuring $A_{600}$ using a Cytation 5 machine (BioTek, Vermont, USA). EC $_{50}$ values were calculated based on dose–response curves analyzed by non-linear regression with GraphPad Prism 8.4.3 software (GraphPad Software, La Jolla, CA, USA). An average of at least three independent biological replicates from independent cultures was performed for each determination.
4.1.2. LM-1 Macrophages and Cytotoxicity Determination

The LM-1 macrophages were grown in DMEM supplemented with 10% heat inactivated FBS. Cells at the concentration of 100,000 cells/mL were cultivated for 24 h in a 96-well plate (37 °C, 5% CO2). The culture medium was removed and fresh medium containing the appropriate drug and concentration was added to the cells, which were incubated for 24 h (37 °C, 5% CO2). Seven different concentrations were tested (100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 mM) as well as controls (without drugs). After 24 h, the culture medium was removed and replaced for fresh medium containing 10% Alamar Blue (Thermo Fisher Scientific, Massachusetts, USA) and incubated for 2 h (37 °C, 5% CO2). Readings at 570 and 600 nm (Asys UVM340 Plate reader, Biochrom, Cambridge, UK) were taken and analyzed according to the manufacturer protocol. Survival rates at different drug concentrations and CC50 values were calculated using the Excel software.

4.2. Materials

Anhydrous solvents (THF, DMF, CH3Cl, and NMP) were obtained by passage through solvent filtration systems (GlassContour, Irvine, CA, USA). Unless specified otherwise, all reagents from commercial sources were used as received. 2-Chlorotrityl chloride (CTC) resin (1.46 mmol/g, 100–200 mesh), N-(9-fluorenlymethoxy carbonyloxy)succinimide (Fmoc-Osu), N,N′-diisopropylcarbodiimide (DIC), all were purchased from ChemImpex; 4-dimethylaminopyridine (DMAP), N,N′-dicyclohexylcarbodiimide (DCC), iodomethane, N,N-diisopropylethylamine (DIEA), formic acid (FA), all were purchased from Aldrich; amino acids, such as Fmoc-Phe-OH, Fmoc-Ala-OH, Fmoc-Val-OH, and 6-heptynoic acid were purchased from GL Biochem, ChemImpex and Combi-blocks; solvents were obtained from Fisher. Sodium hydride (60% dispersion in mineral oil) was washed with hexane three times to remove oil prior to use. The N-methyl amino acids, Fmoc-Phe(Me)-OH, Fmoc-Ala(Me)-OH and FmocVal(Me)-OH, were prepared by according to the literature procedure and exhibited 1H NMR spectra data identical to that previously reported [33]. The Agl dipeptide, Fmoc-Agl-Val-OH was prepared according to the literature procedure and exhibited a 1H NMR spectrum identical to that previously reported [37].

Chromatography was on 230–400 mesh silica gel. Analytical thin-layer chromatography (TLC) was performed on glass-backed silica gel plates (Merck 60 F254). Visualization of the developed chromatogram was performed by UV absorbance or staining with ninhydrin. 1H and 13C NMR spectra were measured respectively in CDCl3 at 500 MHz and 126 MHz, and referenced to CDCl3 (7.26 and 77.0 ppm). Coupling constants, J values were measured in Hertz (Hz) and chemical shift values in parts per million (ppm). Specific rotations, [α]D were measured at 25 °C at the specified concentrations (c in g/100 mL) using a 1 dm cell on a PerkinElmer Polarimeter 589 and expressed using the general formula: [αD] = (100 × α)/(d × c). High resolution mass spectral analyses were obtained by the Centre Régional de Spectrométrie de Masse de l’Université de Montréal. Protonated molecular ions [M + H]+ and sodium adducts [M + Na]+ were used for empirical formula confirmation.

Almiramide peptide analog synthesis was performed using Fmoc-based solid-phase synthesis in an automated shaker commencing with 2-chlorotrityl chloride resin. All final peptides were purified on a preparative column (C18 Gemini column) using a gradient from pure water (0.1% FA) to mixtures with MeOH (0.1% FA) at a flow rate of 10 mL/min. Purity of peptides (>95%) was evaluated using analytical LC–MS on a 5 μM 50 mm × 4.6 mm C18 Phenomenex Gemini column in two different solvent systems: water (0.1% FA) with CH3CN (0.1% FA) and water (0.1% FA) with MeOH (0.1% FA) at a flow rate of 0.5 mL/min using the appropriate linear gradient.
**HCC(CH₂)₄CO-Val(Me)-Val(Me)-Val(Me)-Ala(Me)-Phe(Me)-OH (12)** Under argon, peptide 15 (200 mg) was dissolved in THF (40 mL), cooled to 0 °C, treated with NaH (600 mg, 80 eq.), stirred for 5 min, and treated dropwise with iodomethane (0.6 mL, 9 mmol, 30 eq.). The cooling bath was removed. The reaction mixture warmed to room temperature. After 2 h, more iodomethane (0.6 mL, 30 eq.) was added to the reaction mixture, which was stirred for 20 h. The suspension was quenched with water, concentrated to a reduced volume, and acidified to pH = 1 using 10% aqueous HCl. The acidified mixture was extracted 3 times with EtOAc. The organic layers were combined, dried over Na₂SO₄, filtered and evaporated to 174 mg of residue, from which part (87 mg) was used to make peptide 17 as described below, and the remainder was purified by HPLC on a C18 column using a gradient of 30% to 90% MeOH in H₂O to obtain peptide 12 (16 mg, 13%), which was shown to be >99% pure by LC-MS analysis [30−95% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 6.83 min].

**HCC(CH₂)₄CO-Val-Val-Val-Ala-Phe-OH (15)** Fmoc-Phe-OH (848 mg, 1.5 eq.) and DIEA (0.8 mL, 3 eq.) were added to a suspension of 2-chlorotrityl chloride resin (1 g, 200–400 mesh, 1% DBV) swollen in CH₂Cl₂ (25 mL). The mixture was shaken for 18 h, filtered and washed sequentially with CH₂Cl₂ (3 times for 1 min/wash) and DMF (3 times for 1 min/wash). The Fmoc group was removed upon treatment twice for 20 min with a 20% solution of piperidine in DMF (20 mL/g resin). Subsequently, Fmoc-Ala-OH (3 eq.) was coupled to the resin swollen in NMP (25 mL/g resin) using DIC (3 eq.) and HOBt (3 eq.). After shaking for 16 h, the coupling mixture was filtered, and the resin was washed as described above. Subsequent Fmoc group removal and coupling of Fmoc protected amino acid residues were performed using the above protocols. Complete coupling reactions were confirmed by LC–MS monitoring. After coupling of the last residue, the 6-heptynoic acid (3 eq.), DIC (3 eq.) and HOBt (3 eq.) were added to the resin. The mixture was shaken for 18 h, filtered and washed as previously described. Then, the linear peptide was cleaved from the solid support using a solution of TFA/TEA/H₂O (95/2.5/2.5). The volatiles were evaporated. The reduced volume was treated with diethyl ether. The precipitate was collected by centrifugation (1200 rpm), washed with ether and recollected by centrifugation (3 × 10 min). Removal of diethyl ether afforded a colorless solid [2], which gave 387 mg of residue, from which 200 mg was used to make peptide 12 as described below, and 60 mg purified by HPLC on C₁₈ column using a gradient of 30% to 90% MeOH in H₂O to obtain peptide 15 (24 mg, 12%), which was shown to be >99% pure by LC-MS analysis [30−95% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 8.33 min].
A suspension of 2-chlorotrityl chloride resin (250 mg, 20–400 mesh, 1% DBV) swollen in CH₂Cl₂ (25 mL) was treated with 1,6-diaminohexane (51 mg, 1.2 eq.) and DIEA (0.2 mL, 3 eq.), shaken for 24 h, filtered and washed sequentially with CH₂Cl₂ (3 times for 1 min/wash) and DMF (3 times for 1 min/wash). Subsequently, Fmoc-Phe-OH (3 eq.) was coupled to the resin swollen in NMP (25 mL/g resin) using DIC (3 eq.) and HOBt (3 eq.). After shaking for 16 h, the coupling mixture was filtered, and the resin was washed as described above. Subsequent Fmoc group removals, couplings of Fmoc protected amino acid residues, and acylation with 6-heptynoic acid, all were performed using the protocols described for the synthesis of peptide 15. The linear peptide was cleaved from the solid support using a solution of TFA/TEA/H₂O (95/2.5/2.5). Removal of the volatiles gave a colorless oil, which was purified by HPLC on a C₁₈ column using a gradient of 30% to 90% MeOH in H₂O to obtain peptide 16 as a white solid (8.8 mg, 3%), which was shown to be >99% pure by LC-MS analysis [30–95% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 7.33 min].

A solution of permethylated peptide 12 (87 mg) in dry CH₂Cl₂ (15 mL) was treated with 1,6-hexamethylenediamine (17 mg, 1.2 eq.), DMAP (4 mg, 0.3 eq.), and DCC (30 mg, 1.2 eq.), and stirred for 48 h at room temperature. The reaction mixture was filtered through Celite™ and washed with CH₂Cl₂. The filtrate and washings were evaporated under reduced pressure. The residue was purified by HPLC on a C₁₈ column using a gradient of 30% to 90% MeOH in H₂O. Evaporation of the collected fractions afforded amino hexanamide 17 as white solid (17 mg, 14%), which was shown to be >99% pure by LC-MS analysis [30–95% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 9.01 min].

As described for the synthesis of peptide 16, 1,6-diaminohexane (51 mg, 1.2 eq.) and DIEA (0.2 mL, 3 eq.) were reacted with 2-chlorotrityl chloride resin (250 mg, 200–400 mesh, 1% DBV) in CH₂Cl₂ (25
mL). The resulting amine resin was swollen in NMP (25 mL/g resin), treated with Fmoc-Phe-OH (3 eq.), DIC (3 eq.) and HOBt (3 eq.), shaken for 16 h, filtered, and washed as described above. The Fmoc group was removed upon treatment twice for 20 min with a 20% solution of piperidine in DMF (20 mL/g resin). After Fmoc group removal, the peptide was elongated, coupled to 6-heptynoic acid using HATU (3 eq.) in 0.4 NMM in DMF (25 mL/g resin), cleaved and purified as described for the synthesis of peptide 16. Evaporation of the collected fractions gave peptide 18 (8.2 mg, 3%), which was prepared and shown to be >99% pure by LC-MS analysis [30−95% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 8.42 min].

HCC(CH₃)₂CO-Val-Val(Me)-Val-Ala-Phe-NH(CH₃)₂NH₂ (19) Employing the protocol described above for the synthesis of peptide 18 using Fmoc-Val(Me)-OH (386 mg, 3 eq.) in the fourth coupling, peptide 21 (6.4 mg, 2%) was prepared and shown to be >99% pure by LC-MS analysis [30−95% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 7.86 min].

HCC(CH₃)₂CO-Val-Val-Val(Me)-Ala-Phe-NH(CH₃)₂NH₂ (20) Employing the protocol described above for the synthesis of peptide 18 using Fmoc-Val(Me)-OH (386 mg, 3 eq.) in the third coupling, peptide 20 (5.8 mg, 2%) was prepared and shown to be >99% pure by LC-MS analysis [30−95% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 7.91 min].

HCC(CH₃)₂CO-Val-Val-Val-Ala(Me)-Phe-NH(CH₃)₂NH₂ (21) Employing the protocol described above for the synthesis of peptide 18 using Fmoc-Ala(Me)-OH (355 mg, 3 eq.), peptide 21 (7.8 mg, 3%) was prepared and shown to be >99% pure by LC-MS analysis [30−95% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 7.61 min]
HCC(CH$_2$)$_4$CO-Val-Val-Val-Ala-Phe(Me)-NH(CH$_2$)$_2$NH$_2$ (22) Employing the protocol described above for the synthesis of peptide 22 using Fmoc-Phe(Me)-OH (528 mg, 3 eq.), peptide 22 (4.5 mg, 2%) was prepared and shown to be >99% pure by LC-MS analysis [30–95% MeOH (0.1% FA) in H$_2$O (0.1% FA), 14 min, RT 7.62 min].

HCC(CH$_2$)$_4$CO-Agl-Val-Val-Ala-Phe-OH (23) Employing the protocol described above for the synthesis of peptide 15 using Fmoc-Agl-Val-OH (462 mg, 3 eq.) in the third coupling, peptide 23 (7.5 mg, 3%) was prepared and shown to be >99% pure by LC-MS analysis [50–95% MeOH (0.1% FA) in H$_2$O (0.1% FA), 14 min, RT 7.11 min].

HCC(CH$_2$)$_4$CO-Agl-Val-Val-Ala-Phe-OH (24) Employing the protocol described above for the synthesis of peptide 15 using Fmoc-Agl-Ala-OH (359 mg, 3 eq.) in the second coupling, peptide 24 (8.4 mg, 3%) was prepared and shown to be >99% pure LC-MS analysis [50–95% MeOH (0.1% FA) in H$_2$O (0.1% FA), 14 min, RT 7.55 min].

HCC(CH$_2$)$_4$CO-Val-Val-Agl-Ala-Phe-OH (25) Employing the protocol described above for the synthesis of peptide 15 using Fmoc-Agl-Ala-OH (359 mg, 3 eq.) in the first
coupling, peptide 25 (9.1 mg, 3%) was prepared and shown to be >99% pure by LC-MS analysis [30–95% MeOH (0.1% FA) in H$_2$O (0.1% FA), 14 min, RT 7.94 min].

HCC(CH$_2$)$_2$CO-Agl(Me)-Val-Val(Me)-Agl(Me)-Val-Val(Me)-Agl(Me)-Ala-Phe(Me)-OH (26) Employing the protocol described above for the synthesis of peptide 12 using Fmoc-Agl-Val-OH (462 mg, 3 eq.) in the third coupling, followed by permethylation, peptide 26 (9.5 mg, 3%) was prepared and shown to be >95% pure by LC-MS analysis [50–90% MeOH (0.1% FA) in H$_2$O (0.1% FA), 14 min, RT 8.69 min].

HCC(CH$_2$)$_2$CO-Val(Me)-Agl(Me)-Val-Val(Me)-Agl(Me)-Ala-Phe(Me)-OH (27) Employing the protocol described above for the synthesis of peptide 12 using Fmoc-Agl-Val-OH (462 mg, 3 eq.) in the second coupling, followed by permethylation, peptide 27 (8.3 mg, 3%) was prepared and shown to be >95% pure by LC-MS analysis [50–90% MeOH (0.1% FA) in H$_2$O (0.1% FA), 14 min, RT 9.12 min].

HCC(CH$_2$)$_2$CO-Val(Me)-Val-Val(Me)-Agl(Me)-Agl(Me)-Phe(Me)-OH (28) Employing the protocol described above for the synthesis of peptide 12 using Fmoc-Agl-Ala-OH (359 mg, 3 eq.) in the second coupling, followed by permethylation, peptide 28 (6.7 mg, 2%) was prepared and shown to be >95% pure by LC-MS analysis [50–90% MeOH (0.1% FA) in H$_2$O (0.1% FA), 14 min, RT 9.01 min].
Employing the protocol described above for the synthesis of peptide 18 using Fmoc-Agl-Val-OH (462 mg, 3 eq.) in the fourth coupling, peptide 29 (7.5 mg, 3%) was prepared and shown to be >95% pure by LC-MS analysis [30−95% MeOH (0.1% FA) in H2O (0.1% FA), 14 min, RT 6.72 min].

Employing the protocol described above for the synthesis of peptide 18 using Fmoc-Agl-Val (462 mg, 3 eq.) in the third coupling, peptide 30 (8.4 mg, 3%) was prepared and shown to be >95% pure by LC-MS analysis [30−95% MeOH (0.1% FA) in H2O (0.1% FA), 14 min, RT 6.83 min].

Employing the protocol described above for the synthesis of peptide 18 using Fmoc-Agl-Ala-OH (359 mg, 3 eq.) in the second coupling, peptide 31 (9.1 mg, 3%) was prepared and shown to be >99% pure by LC-MS analysis [30−95% MeOH (0.1% FA) in H2O (0.1% FA), 14 min, RT 7.42 min].
Employing the protocol described above for the synthesis of peptide 18 using Fmoc-Agl-(D,L)-Phe-OH (1.03 g, 3 eq.), the mixture of two diastereoisomers was prepared and separated by HPLC on C18 column using a gradient of 50% to 90% MeOH in H₂O to obtain peptide 32 (1 mg, 1%), which was shown to be >95% pure by LC-MS analysis [50–90% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 5.31 min] and peptide 39 (0.9 mg, 1%), which was shown to be >95% pure by LC-MS analysis [50–90% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 5.07 min].

Employing the protocol described above for the synthesis of peptide 17 using Fmoc-Agl-Val-OH (462 mg, 3 eq.) in the fourth coupling, followed by permethylation and coupling to 1,6-diaminohexane, peptide 33 (9.5 mg, 3%) was prepared and shown to be >99% pure by LC-MS analysis [50–90% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 6.72 min].

Employing the protocol described above for the synthesis of peptide 17 using Fmoc-Agl-Val-OH (462 mg, 3 eq.) in the third coupling, followed by permethylation and coupling to 1,6-diaminohexane, peptide 34 (8.3 mg, 3%) was prepared and shown to be >95% pure by LC-MS analysis [50–90% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 6.86 min].
HCC(CH₂)₄CO-Val(Me)-Val(Me)-Agl(Me)-Ala-Phe(Me)-NH(CH₂)₂NH₂ \( (35) \)

Employing the protocol described above for the synthesis of peptide 17 using Fmoc-Agl-Ala-OH (359 mg, 3 eq.) in the second coupling, followed by permethylation and coupling to 1,6-diaminohexane, peptide 35 (6.7 mg, 2%) was prepared and shown to be >95% pure by LC-MS analysis [50–90% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 6.80 min].

HCC(CH₂)₄CO-(5-Me)Nai-Val-Val-Ala-Phe-OH \( (36) \)

Employing the protocol described above for the synthesis of peptide 15 using Fmoc-(5-Me)Nai-Val-OH (318 mg, 2 eq.) in the third coupling, peptide 36 (8.8 mg, 9%) was prepared and shown to be >99% pure by LC-MS analysis [50–90% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 5.34 min].

HCC(CH₂)₄CO-(5-Me)Nai-Val-Ala-Phe-OH \( (37) \)

Employing the protocol described above for the synthesis of peptide 15 using Fmoc-(5-Me)Nai-Val-OH (318 mg, 2 eq.) in the second coupling, peptide 37 (4.7 mg, 4%) was prepared and shown to be >99% pure by LC-MS analysis [50–90% MeOH (0.1% FA) in H₂O (0.1% FA), 14 min, RT 4.41 min].

5. Conclusions

The unsaturated lipid tail and the C-terminal acid and carboxamide functions of almiramide peptides have previously been shown to have relevance for activity against trypanosomatid parasites [24,25]. Moreover, active almiramide analogs possessing sugar amino acids were suggested to adopt bent structures [31]. The influences on anti-parasite activity of amide N-methylation and turn-inducing Agl and Nai residues within almiramide peptides have now been investigated in wild type and resistant strains of *L. infatum* and compared with macrophage cytotoxicity. Peptide amides exhibited consistently better activity than their C-terminal acid counterparts. Within a set of almiramide 1,6-diaminohexane amides, more potent peptides with relatively high selectivity were typically
obtained without methylation (e.g., 16) and with a single methyl group in MePhe\(^3\) almiramide 22 than with analogs possessing a methyl amide at other positions and with permethylated analog 17. On the other hand, permethylated and MeVal\(^1\) peptide amides 17 and 18 exhibited \(\mu\)M inhibitory activity against the amphotericin B resistant strain with high therapeutic indices. Replacement of amino acid residues by turn-inducing counterparts caused typically losses of activity against the L. infatum strains; however, permethylated Agl\(^1\) amide had similar activity and better selectivity against wild type L. infatum compared to permethylated analog 17. Although studies of the consequences of such structural modifications on metabolism and mechanism of action are in progress, conformers extended about the central residues and mobile at the extremities of the peptide may favor almiramide activity.

**Supplementary Materials:** The following are available online at www.mdpi.com/article/10.3390/molecules26123606/s1, synthesis procedure of Agl and Nai dipeptides; NMR Spectra; Ascertainment of purity by HPLC; Dose-response curves of Leishmania strains and LM-1 macrophage survival rate in the presence of peptides 12–37.

**Author Contributions:** Conceptualization, methodology, validation, resources, writing—review and editing, visualization, supervision, project administration, funding acquisition: M.O., C.F.-P. and W.D.I.; formal analysis, data curation: A.M.T.N.; investigation: A.M.T.N., S.B., N.D., C.D., A.C., E.F.F.; writing—original draft preparation: A.M.T.N.—All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by a New Frontiers in Research Fund Exploration Grant NRF-2018-00766 awarded to CFP, MO and WL; the Natural Sciences and Engineering Research Council (NSERC) of Canada Discovery Grant Program Projects #06647 (WL) and RGPIN-2017-04480 (CFP); the FRQNT Centre in Green Chemistry and Catalysis, Project #171310 (WL). The Canada Foundation for Innovation, grant number 37324 (CFP). AC and ND were supported by the Fonds de recherche du Québec—Nature et technologies (FRQNT).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** Authors want to thank M. Ouellette for the kind gift of the Sb2000.1, MF200.5 and AmB1000.1 drug-resistant strains to the CFP lab. We acknowledge the assistance of members of the Université de Montréal facilities: A. Fürtös, M. Ouellette, A. Fürtös, M. Ouellette (NMR spectroscopy). We acknowledge the assistance of members of the Université de Montréal facilities: A. Fürtös, M.-C. Tang, and L. Mahrouche (mass spectrometry, HPLC analyses), C. Malveau, P. Aguiar, and S. Bilodeau (NMR spectroscopy).

**Conflicts of Interest:** The authors declare no conflict of interest.

**Sample Availability:** Samples of the compounds are not available from the authors.

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