Antileishmanial Activity and Structure-Activity Relationship of Triazolic Compounds Derived from the Neolignans Grandisin, Veraguensin, and Machilin G

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Academic Editor: Marcello Iriti
Received: 26 May 2016; Accepted: 16 June 2016; Published: 20 June 2016

Abstract: Sixteen 1,4-diaryl-1,2,3-triazole compounds 4–19 derived from the tetrahydrofuran neolignans veraguensin 1, grandisin 2, and machilin G 3 were tested against Leishmania (Leishmania) amazonensis intracellular amastigotes. Triazole compounds 4–19 were synthetized via Click Chemistry strategy by 1,3-dipolar cycloaddition between terminal acetylenes and aryl azides containing methoxy and methylenedioxy groups as substituents. Our results suggest that most derivatives were active against intracellular amastigotes, with IC₅₀ values ranging from 4.4 to 32.7 µM. The index of molecular hydrophobicity (ClogP) ranged from 2.8 to 3.4, reflecting a lipophilicity/hydrosolubility rate suitable for transport across membranes, which may have resulted in the potent antileishmanial activity observed. Regarding structure-activity relationship (SAR), compounds 14 and 19, containing a trimethoxy group, were the most active (IC₅₀ values of 5.6 and 4.4 µM, respectively), with low cytotoxicity on mammalian cells (SI = 14.1 and 10.6). These compounds induced nitric oxide production by the host macrophage cells, which could be suggested as the mechanism involved in the intracellular killing of parasites. These results would be useful for the planning of new derivatives with higher antileishmanial activities.

Keywords: neglected diseases; biological activity; cytotoxicity; synthetic compounds; neolignan derivatives

1. Introduction

Cutaneous leishmaniasis (CL) is a parasitic infectious disease that affects the skin, cartilage and mucosa of the upper respiratory tract. The resulting ulcers can develop into destructive and disabling
injuries, making the illness a serious public health problem [1]. There have been between 0.7 and 1.3 million new annual cases throughout the world, and 95% occur in the Americas, Mediterranean basin, Middle East and Central Asia [2]. *Leishmania (Leishmania) amazonensis* Lainson and Shaw 1972 is one of the etiological agents of CL. It produces the typical localized lesions or the diffuse form of the disease, in which the parasite spreads due to impaired cell-mediated immune response. Thus, the lesions tend not to heal spontaneously and to be more resistant to treatment [3,4].

The first choice drugs for CL are the pentavalent antimonials, which are associated to hepato, cardio and nephrotoxicity [5]. When these drugs are ineffective or cannot be prescribed, drugs such as amphotericin B, pentamidine or paramomycin are indicated, despite their degree of toxicity [6]. Resistance and high cost are other aspects that lead to the urgent need for new therapeutic options arising from natural products [7–10]. However, these products may have undesirable properties such as high toxicity, low solubility and bioavailability, which can be minimized by the development of synthetic derivatives [11].

Cassamale et al. [12] have synthetized a series of triazole derivatives from the tetrahydrofuran neolignans veraguensin 1, grandisin 2, and machilin G 3. These compounds have been considered important scaffolds in molecular modification studies due to their antileishmanial and antichagasic activities [13–16]. Cassamale et al. [12] demonstrated the antileishmanial activity of these triazole derivatives on promastigote forms; now the present work shows their activity on *L. (L.) amazonensis* intracellular amastigotes, searching for structure-activity relationship information to support the development of new drug candidates for CL.

2. Results and Discussion

Synthetic derivatives of tetrahydrofuran neolignans 1–3 were classified as active (<20 µM), moderately active (20–50 µM), and potentially inactive (>50 µM) according to Upegui et al. [17]. Most compounds were considered active against *L. (L.) amazonensis* intracellular amastigotes (4, 6, 8, 9, 10, 11, 12, 14, and 19). Compounds 5, 7, 15, 16, 17, and 18 have been moderately active and 13 did not show potential activity on the parasites (Table 1).

The ability to inhibit the growth of parasites apparently depends on the presence and ratio of lipophilic/hydrophilic substituents on aromatic rings [18]. The transport of a compound across membranes may be influenced by the molecular hydrophobicity described by the octanol/water partition coefficient (ClogP) [19]. According to Lipinski’s Rule of Five [20–22], compounds with logP < 5 have better absorption and permeation in vivo. Daunes and D’Silva [23] verified that among a series of molecules, the ones with higher logP (>2.7) were the most active against trypanosomatids once entering the host cell more easily. In other words, the molecular hydrophobic character improves the antileishmanial activity, which may indicate that the active compound must interact with a target system such as an enzyme or receptor, where the binding site is generally hydrophobic.

In our study, synthetic derivatives showed lipophilicity 2.8 < ClogP < 3.4 (Table 1), reflecting an adequate lipophilicity for the transport across membranes and resulting in potent antileishmanial activity.

We observed that ring B containing the trimethoxy substituent has influenced the antileishmanial activity independently of the substituent on the ring A (compounds 6, 10, 14 and 19) (Table 1).

Grandisin derivative 6 was active on intracellular amastigotes (IC_{50} value of 9.4 µM), with the highest selectivity index. This compound was 66 times more toxic to amastigotes than to mammalian cells (Table 1). The substitution of tetrahydrofuran ring by triazolic ring may have resulted in increased activity, once it was described an IC_{50} value of 98.05 µM (42.4 µg mL^{-1}) for grandisin on *L. (L.) amazonensis* promastigotes [24]. Furthermore, its coefficient of hydrophobicity is lower than of its precursor 2 (2.8 and 3.7, respectively), which may have resulted in a better solubility and consequent *in vitro* activity. Indeed, more soluble grandisin derivatives have been synthetized in order to reduce its lipophilicity, which may limit *in vivo* studies [25]. It is important to note that the intracellular amastigote form is the target for drug candidates in the mammalian host.
Table 1. Molecular hydrophobicity, in vitro antileishmanial activity and cytotoxicity of 1,4-diaryl-1,2,3-triazole derivatives 4–19 of the neolignans 1–3.

| Compounds | R₁ | R₂ | R₃ | R₄ | R₅ | R₆ | ClogP[^1] | Intracellular Amastigotes IC₅₀ (µM)[^2] | J774.A1 Cells IC₅₀ (µM)[^3] | SI[^4] |
|-----------|----|----|----|----|----|----|----------|--------------------------------|-----------------|--------|
| 4         | -H | -OCH₃ | -H | -H | -OCH₃ | -H | 3.4      | 13.1                          | >622.8          | >66.2  |
| 5         | -OCH₃ | -OCH₃ | -H | -OCH₃ | -OCH₃ | -H | 3.1      | 21.3                          | 76.4            | 4.2    |
| 6         | -OCH₃ | -OCH₃ | -OCH₃ | -OCH₃ | -OCH₃ | -OCH₃ | 2.8      | 9.4                          | >662.8          | >66.2  |
| 7         | -OCH₂O- | -H | -OCH₂O- | -H | -OCH₂O- | -H | 2.9      | 20.4                          | 86.2            | 4.2    |
| 8         | -H | -OCH₃ | -H | -OCH₃ | -OCH₃ | -H | 3.3      | 16.0                          | 79.2            | 4.9    |
| 9         | -OCH₃ | -OCH₃ | -H | -H | -OCH₃ | -H | 3.3      | 18.3                          | 22.6            | 1.2    |
| 10        | -H | -OCH₃ | -H | -OCH₃ | -OCH₃ | -OCH₃ | 3.1      | 16.8                          | 65.4            | 3.9    |
| 11        | -OCH₃ | -OCH₃ | -OCH₃ | -H | -OCH₃ | -H | 3.1      | 16.5                          | 67.5            | 4.1    |
| 12        | -H | -OCH₃ | -H | -OCH₂O- | -H | -OCH₃ | 3.2      | 14.8                          | 96.5            | 6.5    |
| 13        | -OCH₂O- | -H | -H | -OCH₃ | -OCH₃ | -H | 3.2      | 50.7                          | 134.6           | 2.6    |
| 14        | -OCH₃ | -OCH₃ | -H | -OCH₃ | -OCH₃ | -OCH₃ | 2.9      | 5.6                           | 79.1            | 14.1   |
| 15        | -OCH₃ | -OCH₃ | -OCH₃ | -OCH₃ | -OCH₃ | -H | 2.9      | 29.2                          | 76.9            | 2.6    |
| 16        | -OCH₃ | -OCH₃ | -H | -OCH₂O- | -H | -OCH₂O- | 3.0      | 32.7                          | >768.5          | >23.5  |
| 17        | -OCH₂O- | -H | -OCH₃ | -OCH₃ | -OCH₃ | H | 3.0      | 29.9                          | 9.2             | 0.3    |
| 18        | -OCH₂O- | -H | -OCH₃ | -OCH₃ | -OCH₂O- | -H | 2.9      | 29.8                          | 66.9            | 2.2    |
| 19        | -OCH₂O- | -H | -OCH₃ | -OCH₃ | -OCH₂O- | -H | 2.9      | 4.4                           | 46.9            | 10.6   |
| Doxorubicin[^5] |                     |                     |                     |                     |             |                       | 0.5                          | 0.5             |        |
| Amphotericin B[^5] |                     |                     |                     |                     |             |                       | 0.7                          | 2.2             | 3.1    |

[^1]: ClogP, octanol/water partition coefficient;[^2]: IC₅₀, half maximum inhibitory concentration on intracellular amastigotes;[^3]: IC₅₀, half maximum inhibitory concentration on J774.A1 cells;[^4]: SI (selectivity index), IC₅₀ on mammalian cells/IC₅₀ on intracellular amastigotes;[^5]: Positive controls, amphotericin B for *L. (L.) amazonensis* and doxorubicin for J774.A1 cells. The data are representative of three independent experiments.
Treatment with compound 6 had significantly decreased the infection index at the concentration of 6.25 µg·mL⁻¹, reaching 98.6% reduction (p < 0.0001) at the concentration of 50 µg·mL⁻¹ (Figure 1A). 6 has induced an increased production of nitric oxide (NO) compared to untreated infected cells (control) (Figure 2A). Thus, NO production may be suggested as the mechanism of leishmanicidal action, especially because 6 does not seem to have a direct action on L. (L.) amazonensis once it was proven inactive on promastigote forms [12].

Figure 1. Effect of 1,4-diaryl-1,2,3-triazole derivatives (4–19) of the neolignans 1–3 on intracellular amastigotes. Peritoneal macrophages were infected with L. amazonensis and treated with different concentrations of the compounds. Infection index was calculated 24 h after treatment. Bars represent the mean ± SD of six replicates. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 for the different concentrations versus untreated cells (control) (Student’s t-test). (A) trimethoxy series; (B) dimethoxy series; (C) methoxy series; (D) methylenedioxy series (substitution pattern of ring A).

Figure 2. Nitric oxide release by L. amazonensis infected macrophages 24 h after treatment with 1,4-diaryl-1,2,3-triazole derivatives (4–19) of the neolignans 1–3. Bars represent the mean ± SD of six replicates. * p ≤ 0.05, ** p ≤ 0.01, for the different concentrations compared to untreated cells (control) (Student’s t-test). (A) trimethoxy series; (B) dimethoxy series; (C) methoxy series; (D) methylenedioxy series (substitution pattern of ring A).
The other compounds from trimethoxy series (with substituents on ring A) were active (11) and moderately active (15 and 18), but less selective, with important cytotoxicity on mammalian cells (Table 1). All of them were able to significantly decrease infection index, mainly 18, reaching 92.1% reduction (p < 0.0001) (Figure 1A). This compound, however, did not induce a significant production of NO. 11 acted variably on NO release, with a production lower than untreated cells at the lowest concentrations, but higher than control at the highest concentrations tested (Figure 2A).

Compound 5 with a dimethoxy group as substitution pattern on rings A and B (veraguensin 1 derivative) was moderately active on intracellular amastigotes (IC$_{50}$ value of 21.3 µM) with relative selectivity (SI = 3.6). Silva Filho et al. [16] have demonstrated the activity of veraguensin on L. donovani promastigote forms (48.3 µM; 18 µg·mL$^{-1}$). Once again we may evade that the insertion of triazole ring may have improved the antileishmanial activity due to reduction of octanol/water partition coefficient (3.1 (5) versus 4.2 (1)). Compound 5 has significantly reduced the infection index from the concentration of 12.5 µg·mL$^{-1}$ (Figure 1B). Furthermore, the mechanism of leishmanicidal action seems also to be independent of NO, once NO production was lower than control at the concentration of 25 µg·mL$^{-1}$ (Figure 2B). These data corroborate those obtained by Konishi et al. [26], who verified the inhibition of NO production from LPS-activated murine macrophages by three veraguensin’s position isomers.

The other compounds from dimethoxy series were active (9 and 14), excepting 16, which was moderately active but selective (IC$_{50}$ = 32.7 µM; SI > 23.5). Despite its moderate activity, 16 was able to significantly induce NO production (Figure 2B).

Compound 14 was active and selective (IC$_{50}$ = 5.6 µM; SI = 14.1) (Table 1), and reduced the infection index at all concentrations tested (86.4% at the highest concentration, p = 0.0003) (Figure 1B). It’s important to note that 14 was a hybrid from veraguensin 1 and grandisin 2, and it had the best activity of the whole dimethoxy series. Furthermore, it significantly induced NO production at the lowest concentrations tested, and this may be suggested as a possible mechanism of leishmanicidal action (Figure 2B).

Compounds from methoxy series showed IC$_{50}$ values from 13.1 to 16.8 µM (4, 8, 10, and 12, Table 1). Compound 4 was the most active of this series, and highly selective (IC$_{50}$ = 13.1 µM; SI = 66.9). As well as 12, this compound was able to induce an increase of NO production at the highest concentration tested (Figure 2C).

The most active compound on intracellular amastigotes (19) came from methylenedioxy series (IC$_{50}$ = 4.4 µM). Compound 19 is a hybrid from machilin G 3 and grandisin 2 and was also quite selective (SI = 10.6) (Table 1). 19 has significantly reduced the infection index and in addition it induced NO production twice as high than control at the highest concentrations tested (Figure 2D). Cassamale et al. [12] have demonstrated this compound as highly active against L. (L.) amazonensis promastigote forms (IC$_{50}$ = 7.2 µM), and this suggests its direct action on the parasite. On the other hand, position isomer 18 (IC$_{50}$ = 29.8 µM, Table 1) was less active than 19, indicating the role of minor structural differences on the antileishmanial activity of these compounds.

Compound 13 did not show potential activity and selectivity (Table 1). Despite this, 13 was able to induce an increase in NO production (Figure 2D), as well as its position isomer 12 (Figure 2C). We should point out that all compounds from methylenedioxy series have induced NO production in greater or lesser degree (Figure 2D). This implies that this group may be associated to cellular activation and/or cytotoxicity. For example, compound 17 (machilin G 3 analog) was toxic for the macrophages (SI = 0.3) with moderate antileishmanial activity (Table 1).

3. Materials and Methods

3.1. Triazole Derivatives of Neolignans

Sixteen 1,4-diaryl-1,2,3-triazole derivatives with substitution patterns found in the neolignans veraguensin 1, grandisin 2, and machilin G 3 (Figure 3) were tested. They were designed based on the
concept of the bioisosterism of rings, where the tetrahydrofuran core was substituted by a 1,2,3-triazole ring (Figure 4) [12].

![Figure 3. Structure of the neolignans veraguensin 1, grandisin 2, and machilin G 3.](image)

Triazole derivatives 4–19 were obtained via Click Chemistry strategy from 1,3-dipolar cycloaddition reactions between terminal acetylenes 25a–d and aromatic azides 27a–d with methoxy and methylenedioxy substitution patterns [12]. The synthesis of starting materials began by preparing aryl bromides 21a–c via a bromination reaction of 20a–c in the presence of NBS, p-TsOH, CH₂Cl₂ and SiO₂ [12,27]. Subsequently, a cross-coupling Sonogashira reaction between bromobenzenes 21a–c and 2-methyl-3-butyn-2-ol in the presence of PdCl₂(PPh₃)₂/CuI, Et₃N provided the acetylene alcohols 22a–c with 81%–86% yields, after 24 h reaction time [12,28].

Retro-Favorski reaction of 22a–c with KOH under reflux in toluene generated the terminal acetylenes 25a–c with 75% to 79% yield [12,28–30]. Ethynyl-1,2,3-trimethoxybenzene 25d was synthesized by the Corey-Fuchs method (Scheme 1) [12,31].

![Scheme 1. Synthesis of aryl acetylenes 25a–d [12]. Reagents and reaction: (a) 20a–c (75 mmol), NBS (75 mmol), TsOH (10 mmol), SiO₂ (37 g), CH₂Cl₂ (210 mL), room temperature, 3 h, 21a = 79%, 21b = 82%, 21c = 85%; (b) 21a–c (50 mmol), 2-methyl-3-butyn-2-ol (183 mmol), PdCl₂(PPh₃)₂ (2.5 mmol), CuI (5 mmol), Et₃N (250 mL), reflux, 24 h, 22a = 81%, 22b = 82%, 22c = 86%; (c) 22a–c (30 mmol), KOH (90 mmol), toluene (225 mL), reflux, 24 h, 25a = 75%, 25b = 78%, 25c = 79%; (d) CCB₄ (100 mmol), PPh₃ (200 mmol), CH₂Cl₂ (100 mL), 0 °C, then 23 (50 mmol), 0 °C–room temperature, 5 h, 24 = 87%; (e) 24 (10 mmol), THF (50 mL), n-BuLi 2.5 M in hexanes (22 mmol), −25 °C—room temperature, 1 h, 25d = 83%.](image)

Next, aromatic azides 27a–d were prepared by the reaction of aromatic amines 26a–d with t-BuONO/TMSN₃ using the protocol reported by Moses et al. (Scheme 2) [12,32].
The 1,3-dipolar cycloaddition occurred when terminal acetylenes 25a–d reacted with aryl azides 27a–d using CuSO₄·H₂O, sodium ascorbate and CH₂Cl₂/H₂O 1:1 as solvents, yielding the compounds 4–19 in 78% to 92% yield [12,33].

3.2. General Procedure for the Synthesis of Triazoles 4–19

To a solution of terminal acetylenes 25a–d (2 mmol, 1.0 equiv) and azides 27a–d (2 mmol, 1.0 equiv) in dichloromethane (2 mL) and water (2 mL), were added CuSO₄·5H₂O (0.128 mmol, 0.064 equiv) and sodium ascorbate (0.352 mmol, 0.176 equiv). The reaction mixture was stirred for 24 h. Then it was added a saturated solution of NH₄Cl (30 mL) and the product was extracted with dichloromethane (3 × 20 mL). The organic phase was dried over anhydrous MgSO₄, and the solvent was evaporated under reduced pressure. The products were purified by recrystallization from ethyl acetate.

3.3. ClogP

In order to estimate the molecular hydrophobicity of triazole derivatives 4–19, theoretical values of logP (ClogP) were calculated with ChemAxon software. The program estimated octanol/water partition coefficient based on a modified version of the method of Viswanadhan et al. [34].
3.4. Antileishmanial Activity

3.4.1. Parasites and Peritoneal Macrophages

In vitro antileishmanial activity was evaluated on peritoneal macrophages infected with *L. (L.) amazonensis* intracellular amastigotes. Parasites (IFLA/BR/1967/PH8 strain) were routinely isolated from BALB/c mice and maintained as promastigotes at 25 °C in Schneider’s Insect Medium (Sigma-Aldrich®, St. Louis, MO, USA) supplemented with 20% fetal calf serum (FCS-Cultilab® Campinas, Brazil) and 140 µg·mL⁻¹ gentamicin (Sigma-Aldrich®). Macrophages were obtained from peritoneal wash of BALB/c mice after euthanasia. 10 mL of RPMI 1640 (Sigma-Aldrich®) supplemented with 2% L-glutamine, 2.8% bicarbonate buffer, 100 U·mL⁻¹ penicillin and 100 mg·mL⁻¹ streptomycin were injected into the peritoneal cavity. After massage area, liquid was aspirated and transferred to tubes on ice. Peritoneal cells were quantified in Neubauer chamber after cellular exclusion with Trypan Blue staining (Sigma-Aldrich®).

3.4.2. In Vitro Antileishmanial Activity on Intracellular Amastigotes

Peritoneal cells (1 × 10⁵ cells/well) were added to 24-well plates containing circular coverslips. Plates were incubated for one hour at 37 °C/5% CO₂ to allow cell adhesion and then 1 × 10⁶ *L. (L.) amazonensis* promastigotes were added to each well. Plates were incubated at 35 °C/5% CO₂ for four hours and then cells were treated for 24 h with synthetic compounds 4–19 (6.25–50 µg·mL⁻¹). Amphotericin B (Sigma-Aldrich®) was used as the reference drug (0.25 to 2 µg·mL⁻¹) and untreated cells were used as negative control. Coverslips were processed as described by Rizk et al. [35]. The overall number of amastigotes was determined by counting 100 cells in six replicates. The half maximal inhibitory concentration (IC₅₀) was calculated using a nonlinear regression curve. Infection index was obtained as described by Paladi et al. [36].

3.5. Nitric Oxide Production

To evaluate the nitric oxide production (NO) by infected and treated peritoneal cells, 50 µL of culture supernatant from the antileishmanial assay were collected and incubated with equal volume of Griess reagent (1% sulfanilamide /0.1% naphthalene diamine in 5% phosphoric acid) for 10 min at room temperature. According to Ding et al. [37], the absorbance was determined at 540 nm and converted to NO₂⁻ (µM) by comparing to a standard curve of known concentrations of sodium nitrite (1–10 µM) in RPMI medium (Sigma-Aldrich®).

3.6. Cytotoxicity Assay

Murine macrophages (J774.A1, Rio de Janeiro Cell Bank, Brazil) were seeded in 96-well plates (1 × 10⁵ cells·mL⁻¹) and incubated with compounds at 37 °C/5% CO₂ for 48 h at concentrations of 0.25–250 µg·mL⁻¹ to estimate IC₅₀. Amphotericin B (Sigma-Aldrich®) was used as the reference drug (0.025 to 25 µg·mL⁻¹). Cell growth was evaluated according to Skehan et al. [38] using the sulforhodamine B assay. Dimethyl sulfoxide (DMSO, Vetec®, Rio de Janeiro, Brazil) was used as negative control at the concentration used to solubilize the highest concentration of compounds. IC₅₀ was calculated by nonlinear regression curve. Selectivity index was calculated according to Medeiros et al. [39].

3.7. Ethical Aspects

This study received approval from the local Animal Experimentation Ethical Committee (CEUA/UFMS) under protocol 503/2013.
4. Conclusions

Among 16 synthetic derivatives of tetrahydrofuran neolignans veraguensin 1, grandisin 2 and machilin G 3, 15 showed high or moderate antileishmanial activity. Compounds 14 and 19, containing a trimethoxy substituent on ring B, were the most active against intracellular amastigotes, with low cytotoxicity on mammalian cells. These compounds induced nitric oxide production by the host macrophage cells, which could be suggested as the mechanism involved in the intracellular killing of parasites. These results would be useful for the planning of new derivatives with higher antileishmanial activities.

Acknowledgments: We gratefully acknowledge financial support received from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Protocol 563977/2010-8), Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT, Protocol 23/200.137/2010), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Author Contributions: C.C.P.A., A.C.M.B., M.F.C.M., and M.C.T.K. conceived and designed the experiments; E.C.C., L.S.S.B., M.O., and T.V.X. performed the experiments; E.C.C. analyzed the data; T.B.C. and D.B.C. contributed analysis tools; E.C.C., C.C.P.A, and A.C.M.B wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

- C.L. Cutaneous Leishmaniasis
- FCS Fetal Calf Serum
- RPMI Roswell Park Memorial Institute
- IC_{50} Half maximal inhibitory concentration
- DMSO Dimethyl sulfoxide
- S.I. Selectivity Index

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**Sample Availability:** Samples of the compounds 4–19 are available from the authors.

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