The disease *P. vivax*, *Plasmodium* morbidity and economic loss in the world nowadays [3]. Human global health problem and represents the main cause of high rates of mortality and pose an additional socioeconomic burden [1,2].

In this scenario, it would be of great interest to develop syndromes caused by the agents that act based on new targets, with novel modes of action.

Since there is no vaccine and the resistance to drugs, specially virulent species and is responsible for 90% of global malaria cases [5], have increased, even to artemisinin [6], there is an urgent need for new antimalarial agents [7,8]. In this scenario, it would be of great interest to develop agents that act based on new targets, with novel modes of action.

The term leishmaniasis refers to a wide variety of clinical syndromes caused by the *Leishmania* species [9–11]. The disease can be classified into three main forms: cutaneous, mucocutaneous and visceral leishmaniasis, which is fatal if untreated. Recently, the visceralization by dermatropic species of this parasite has also been reported as a complication in HIV co-infected persons [9,10,12]. The primary chemotherapy of leishmaniasis has been based on the use of pentavalent antimonial drugs [12]. Other medications, such as pentamidine, amphotericin B and paromycin are used as secondary options in resistant cases, despite their high toxicity to the host [9–11]. However, these drugs are not orally active, requiring long-term parenteral administration and displaying serious side effects [9,10,12].

Protozoan parasites are unable to synthesize purines de novo and this fact represents potential alternatives for drug design in the treatment of parasitic disease [13,14]. The 6-thiopurine interfere with nucleic acid synthesis and are commonly used to treat cancer or as immunossupressors. Some of these derivates containing an acetic acid part showed biological activity [15,17]. Various 1,2,3-triazole derivates are reported in the literature to exhibit several biological activities such as analgesic, antibacterial, fungicial, anti-inflammatory, antihypertensive, antiviral and antitumoral [18]. 1,2,3-triazole moieties are attractive connecting units, since they are stable to metabolic degradation and capable of hydrogen bonding, which can be favorable in binding of biomolecular targets and for solubility [19].

Steroid transporters have been shown to accept and carry a variety of drugs [20]. Cholic acid is the most common form of the.
steroid [21] and they are known to exhibit antimicrobial activities [22]. Its derivates are reported to improve permeability of membranes, including the bacterial cell wall [23]. Moreover, steroids are amphiphilic molecules [24] which may represent alternatives for chemotherapeutical agents by acting synergistically with antibiotics as membrane permeabilizers [25].

Prompted by these biological properties, we reported herein the synthesis and the in vivo antimalarial and in vitro antileishmanial evaluation of a series of 6-thiopurine derivates containing 1,2,3-triazole, and steroid derivatives. 1,3-dipolar cycle addition of terminal acetylene and azides has been a method of choice for the synthesis of 1,2,3-triazoles [26]. To verify the activity of the steroid, the acetic acid was used in the cycle addition to 6-thiopurine.

2. Experimental

2.1. Chemistry

Melting points were determined using a MQAPF-301-Microquimica digital apparatus and are uncorrected. The infrared spectra (wave numbers in cm⁻¹) were taken on a BOMEM–FTIR MB–102 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a BRUKER AVANCE DRX300 apparatus operating at 300 MHz (¹H) and 75 MHz (¹³C), using standard Bruker software; the dioxane (δ 67.30) was used as reference for ¹³C NMR. Column chromatography was performed on Merck silica gel (70–230 mesh). Electron impact mass spectra were measured with an AEI MS 50 mass spectrometer. Compounds 2, 6, 8 and 9 have been described in the literature [24,27,28].

2.1.1. Synthesis of 9-(prop-2-ynyl)-6-(prop-2-ynylthio)-9H-purine (3), and 7-(prop-2-ynyl)-6-(prop-2-ynylthio)-7H-purine (4)

Propargyl bromide (1.67 g, 14.0 mmol) was added to a solution of sodium 6-thiopurine, generated from the reaction of 6-thiopurine (1.0 g, 7.0 mmol) with NaH (0.34 g, 14.0 mmol) in EtOH (30 mL) at 0°C for 1 hour. The reaction mixture was stirred for an additional 48 hours at 25°C. The solvent was evaporated in vacuum until dryness. The crude reaction product was purified by flash chromatography (eluent: CH₂Cl₂: MeOH 9.7:0.3) producing 3 (0.70 g, 3.07 mmol) in 56% yield as a white solid, m.p. 170–172°C, and 4 (0.40 g, 1.75 mmol) in 32% yield as a brown solid, m.p. 132–135°C.

Compound 3: IR, νmax (KBr): 3279, 3225, 2129, 1568, 634. ¹H NMR (300 MHz, CDCl₃): δ 8.78 (s, 1H, H-2); 8.19 (s, 1H, H-8); 5.03 (s, 2H, H-1₀'); 4.20 (s, 2H, H-1₀); 2.55 (s, 1H, H-3₀'); 2.22 (s, 1H, H-3₀). ¹³C NMR (75 MHz, CDCl₃): δ 159.5 (C-6); 152.3 (C-2); 148.5 (C-4); 142.3 (C-8); 131.1 (C-5); 75.7 (C-2₀, C-2₀'); 71.3 (C-3₀, C-3₀'); 33.5 (C-1₀'); 17.4 (C-1₀) (Fig. 1).

2.1.2. General procedure for cycloaddition (10, 12 and 13)

The alkyne 2 or 3 (1 equivalent) and the azide 6 or 9 (1.3 equivalent) were dissolved in DMSO/H₂O 4:1 (5 mL). To this solution, CuSO₄·5H₂O (0.05 equivalent) and sodium ascorbate (0.40 equivalent) were added. The reaction mixture was stirred for 59–60 hours, then it was extracted with DCM and washed with water. The organic phase was dried over Na₂SO₄, filtered and concentrated to yield the crude product. The crude product was purified by flash chromatography (eluent: CH₂Cl₂: MeOH 9.7:0.3) producing 10 (0.95 g, 3.46 mmol) in 55% yield as a white solid, m.p. 150–152°C; 11 (0.60 g, 2.52 mmol) in 36% yield as a yellow solid, m.p. 140–142°C; and 12 (0.15 g, 0.54 mmol) in 22% yield as a brown solid, m.p. 120–122°C. ¹H NMR (400 MHz, CDCl₃): δ 8.65 (s, 1H, H-2); 8.18 (s, 1H, H-8); 5.01 (s, 2H, H-1₀'); 4.18 (s, 2H, H-1₀); 2.53 (s, 1H, H-3₀'); 2.16 (s, 1H, H-3₀). ¹³C NMR (100 MHz, CDCl₃): δ 159.5 (C-6); 152.3 (C-2); 148.5 (C-4); 142.3 (C-8); 131.1 (C-5); 75.7 (C-2₀, C-2₀'); 71.3 (C-3₀, C-3₀'); 33.5 (C-1₀'); 17.4 (C-1₀) (Fig. 1).

Fig. 1. Reagents and reaction conditions: (a) NaH, EtOH, 0°C, 1 hour, propargyl bromide, 0°C, 72 hours; (b) NaH, EtOH, 0°C, 1 hour, propargyl bromide, 0–25°C, 48 hours; (c) Na,NH₄, H₂O, 0–25°C, 72 hours; (d) 1-HCl, MeOH, 25°C, 24 hours; 2-MsCl, Et₃N, CH₂Cl₂, 0°C, 2 hours; (e) Na,NH₂, DMF, 120°C, 24 hours.

96 hours at 25°C. Solvents were evaporated under reduced pressure and crude product was purified by column chromatography on silica gel using 8% MeOH/CH₂Cl₂ system to obtain 6-thiopurine/acetic acid and 6-thiopurine/steroid conjugates or linked with 1,4-disubstituted 1,2,3-triazole (Fig. 2).

2.1.3. Synthesis of 2-(4-((9H-purin-6-ylthio)-1H-1,2,3-triazol-1-yl)acetic acid (10)

Brown oil (0.08 g, 0.27 mmol), yield 40%; ¹H NMR (300 MHz, D₂O): δ 8.33 (s, 1H, H-2); 8.10 (s, 1H, H-8); 7.91 (s, 1H, H-3); 4.96 (s, 2H, H-4); 4.45 (s, 2H, H-1). ¹³C NMR (75 MHz, D₂O): δ 173.7 (C-5); 158.2 (C-6); 152.1 (C-2); 150.0 (C-4); 144.4 (C-8, C-2'); 128.0 (C-5); 126.4 (C-3'); 53.9 (C-4'); 23.6 (C-1').

2.1.4. Synthesis of 6,9-(2-(4-methyl)-1H-1,2,3-triazol-1-yl) acetic acid-purine (12)

Black solid (0.10 g, 0.23 mmol), m.p. 268-272°C, yield 27%; ¹H NMR (300 MHz, D₂O): δ 8.57 (s, 1H, H-2); 8.37 (s, 1H, H-8); 5.58 (s, 2H, H-1'); 5.05-5.00 (m, 4H, H-4', H-4'); 4.57 (s, 2H, H-1'). ¹³C NMR (75 MHz, D₂O): δ 173.4

2.2. Pharmacology

2.2.1. In vivo antimalarial assay

2.2.1.1. Animals. For the in vivo tests, Swiss albino female mice, 6–8 weeks of age and weighting 20–22 g, were inoculated with 1 × 10⁶ P. berghei (NK65 strain) infected red blood cells by the intraperitoneal route, randomly distributed in groups of four mice each. These animals were obtained from CBR/Federal University of Juiz de Fora (process in Ethic Committee number 063/2007-CEEA).

2.2.1.2. Antimalarial activity evaluation. The animals were inoculated on day 1. The drugs were diluted in dimethyl sulfoxide 5% plus water, providing an aqueous suspension. Using the 4-day suppressive test [29], the compounds 10, 11, 12 and 14 were tested at 10 mg/kg. Treatment was performed daily by oral route (0.1 mL for each mouse) for 4 consecutive days, starting on the day of infection. Two control groups were used, one receiving the standard antimalarial drug chloroquine at curative dosage and one was treated with water. Mice were also treated with chloroquine at 10 mg/kg in order to compare the results obtained from these compounds with the standard antimalarial drug. Giemsa stained blood smears were made on day 5, 7, 9 and 12 after infection and then examined by microscopy to assess the percentage parasitaemia.

2.2.1.3. Inhibition of parasite multiplication. The antimalarial activity of the drugs was established on the basis of average parasitaemia of each group. The percent inhibition of parasite multiplication was calculated using treated group compared with untreated group, by means of the following formula [30]: average parasitaemia in the control (untreated) group minus parasitaemia in the test group, divided by parasitaemia in the control (untreated) group X100. Results were expressed as a percentage of inhibition of parasite multiplication.

2.2.1.4. Statistical analysis. The data of in vivo antimalarial tests were analysed using SPSS for Windows (v.13) (SPSS Inc. SPSS 13.0 for Windows, Chicago, IL: SPSS Inc.; 2004) using Non-parametric Test Mann-Whitney – since our data were not normally distributed – for comparison between two independent samples. P values lower than 0.05 were considered statistically significant.

2.2.2. In vitro antileishmanial activity evaluation

2.2.2.1. Cytotoxicity to macrophages. Mouse peritoneal macrophages were plated at 1 × 10⁶ cells/mL in 96-well culture plates and incubated for 48 hours at 37°C in RPMI-1640 medium containing 10% fetal bovine serum and different concentrations of the drugs. After days of incubation, drug treatment was performed for 48 h. The results are expressed as the percentages of inhibition of parasite growth calculated using treated group compared with untreated group, by means of the following formula [31]: 100 × (1 – (percent of inhibition of parasite growth))/100.

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test compounds in 0.5% DMSO. The viability of the macrophages was determined with the MTT assay, as described in section 2.2.2, and was confirmed by comparing the morphology of the control group via light microscopy. Dose response curves were plotted (values expressed as percentage of control optical density) and CC50 values (50% cytotoxicity concentration) were obtained by using GraFit version 5 software (Erithacus Software Ltd., Horley, UK).

3. Results and discussion

3.1. Chemistry

This work describes the synthesis of a series of 6-thiopurine derivates containing 1,2,3-triazole, and steroid derivatives. 1,3-Dipolar cycle addition of terminal acetylene and azides has been a method of choice for the synthesis of 1,2,3-triazoles. The intermediates terminal alkynes 2, 3 and 4 were synthesized by reaction of propargyl which 6-thiopurine 1 using propargyl bromide (Fig. 1). Compounds intermediates 2, 6 and 9, have been described in the literature [24,26,27]. The 6-thiopurine/acetic acid (10 and 12) and 6-thiopurine/steroid 13 conjugates (Fig. 2) were obtained via 1,3-dipolar cycloaddition of terminal alkynes 2 to 3 or to an azide group attached to acetic acid or steroid (cholic acid), respectively in CuSO4·5H2O, sodium ascorbate, DMSO/H2O at 25 °C for 96 hours in 40–50% yield. The compound 11 was obtained by esterification reaction of the acid 10. Finally, the product 14 was synthesized in 60% yield, adding the mesyl compound 8 to a solution of sodium 6-thiopurine in DMF at 100 °C for 24 hours (Fig. 2).

3.2. Pharmacology

3.2.1. Antiprotozoal and cytotoxicity screening

P. berghei-infected mice model was used as a biological model for in vivo antimalarial tests as suggested by the World Health Organization and others [30,32]. The antimalarial activity was expressed as percentage of inhibition of parasite multiplication, summarized in Table 1. All the compounds tested displayed antimalarial activity. Compounds 10 and 11 were more effective than chloroquine at 10 mg/kg on day 9, showing suppression of 82 and 72% (p < 0.05), respectively. Compound 14 showed a percentage of inhibition relatively similar to chloroquine at the same dosage on days 7 and 9, as well as compound 12 on day 9. The analysis was extended until day 12 when all the compounds were more active than chloroquine at the same dosage. All the four compounds tested showed increased antimalarial activity over time, whereas chloroquine at dosage of 10 mg/kg had reduced its activity from day 5 to day 12 (Fig. 3).

![Fig. 3. Inhibition of parasite multiplication (suppression) percentage of compounds 10, 11, 12 and 14 at 10 mg/kg, compared with chloroquine at 10 mg/kg on days 5, 7, 9 and 12 post-infection.](image-url)

Concerning structure relationship, it can be seen that the 6-thiopurine derivate that has 1,2,3-triazole and acetic acid (compound 10) present the highest suppression values, notably on day 9, whereas it is similar to the other compounds on day 12 (except for compound 12 and chloroquine at 10 mg/kg, whose inhibition of parasite multiplication values are lower). Moreover, as for 12, it seems that having 6,9-di substituted-purine does not contribute to the antimalarial activity, since its inhibition of parasite multiplication is lower than compound 10, a 6-substituted-purine, and it is also the lowest one, if we consider the other compounds on day 9.

As the compounds tested herein are composed by mercaptopurine and triazoles or steroid, their antimalarial activity might be based on its reported modes of action, as mentioned before. Mercaptopurine action may be based on competitive inhibition of hypoxanthine-guanine phosphoribosyltransferase, which is the only parasite enzyme capable of salvaging hypoxanthine – the main purine used by the parasite [33]. Alternatively, the activity of these purine analogs can be attributed to interactions with heme molecules that play a crucial role in Plasmodium metabolism, as suggested for adenosine analogs [34]. Triazole action may be based on inhibition of glycolytic or Krebs cycle enzymes [18], blockade of some lipids biosynthesis [35], or inhibition of tubulin polymerization [36]. As for steroid (cholic acid) action, its action may be based on improving permeability of membranes, what, for its part, facilitates the penetration of the compound [37].

For antileishmanial activity, the results are summarized in Table 2. Among the five compounds tested only one, 6-thiopurine/stereoid conjugates 14 showed activity against promastigotes of Leishmania species. Interestingly, despite the biochemical differences existing between the species of this parasite, this compound showed activity against the three species of Leishmania tested with IC50 of 22.8, 13.9 and 17.3 μM for L. amazonensis, L. braziliensis and L. major, respectively. All protozoan parasites studied to date are incapable of synthesizing purines de novo and nucleoside transporter has been identified, in several parasitic protozoa, including L. braziliensis and L. major [13,14,38] (Table 2).

Table 1. Inhibition of parasite multiplication (% on days)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dosage (mg/kg)</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated (control)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>64</td>
<td>31</td>
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<td>58</td>
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<tr>
<td>11</td>
<td>10</td>
<td>0</td>
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<tr>
<td>12</td>
<td>10</td>
<td>51</td>
<td>0</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
<td>50</td>
<td>0</td>
<td>54</td>
<td>65</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>10</td>
<td>89</td>
<td>50</td>
<td>43</td>
<td>31</td>
</tr>
</tbody>
</table>

Significant differences in relation to non-treated controls by Mann-Whitney Test.

a p < 0.021.

b p = 0.034.

c p = 0.034.

d Statistically identical to chloroquine at the same dosage.

e Statistically different from chloroquine at the same dosage.
Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antileishmanial activity IC₅₀ (µM) L. amazonensis</th>
<th>Macrophages CC₅₀ (µM) L. major</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>&gt;87.0</td>
<td>&gt;87.0</td>
</tr>
<tr>
<td>11</td>
<td>&gt;87.0</td>
<td>&gt;87.0</td>
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<tr>
<td>12</td>
<td>&gt;87.0</td>
<td>&gt;87.0</td>
</tr>
<tr>
<td>13</td>
<td>&gt;87.0</td>
<td>&gt;87.0</td>
</tr>
<tr>
<td>14</td>
<td>22.8 (±0.86)</td>
<td>13.9 (±0.20)</td>
</tr>
</tbody>
</table>

Amphotericin B 0.4 (±0.05) 0.3 (±0.002) 0.3 (±0.04) –

Values represent the mean of triplicate samples. NC: not cytotoxic (maximum concentration tested: 100 µM). Amphotericin B: reference drug for antileishmanial tests. IC₅₀ values (concentrations inhibiting parasite growth by 50%). CC₅₀ values (50% cytotoxicity concentration).

Table 2: Cytotoxicity of compounds on promastigote forms of Leishmania and murine peritoneal macrophages.

4. Conclusion

In summary, syntheses of 6-thiopurine derivates containing 1,2,3-triazole have been described. The compounds have exhibited promising antimalarial and antileishmanial activities. The compounds 10, 11, 12 and 14 were found to be equipotent when compared to reference standard chloroquine at same dosage. The compound 14 showed activity against the three species of Leishmania tested.

4.1 Uncited references

[16].

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References
