

## Synthesis and antileishmanial activity of naphthoquinone-based hybrids

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### SUMMARY

**Introduction:** leishmaniasis is a disease caused by protozoa of the genus *Leishmania* and is considered endemic in 98 countries. Treatment with pentavalent antimonials has a high toxicity, which motivates the search for effective and less toxic drugs.  $\alpha$ - and  $\beta$ -lapachones have shown different biological activities, including antiprotozoa. In recent studies, the isonicotinoylhydrazone and phthalazinylhydrazone groups were considered innovative in the development of antileishmania drugs. Molecular hybridization is a strategy for the rational development of new prototypes, where the main compound is produced through the appropriate binding of pharmacophoric subunits. **Aims:** to synthesize four hybrids of  $\alpha$ - and  $\beta$ -lapachones, together with the isonicotinoylhydrazone and phthalazinylhydrazone groups and to determine the antileishmania activity against the promastigotic forms of *L. amazonensis*, *L. infantum* and *L. major*. **Results:**  $\beta$ -lapachone derivatives were more active against all tested leishmania species.  $\beta$ ACIL (IC<sub>50</sub> 0.044 $\mu$ M) and  $\beta$ HDZ (IC<sub>50</sub> 0.023 $\mu$ M) showed 15-fold higher activity than amphotericin B. The high selectivity index exhibited by the compounds indicates greater safety for vertebrate host cells. **Conclusion:** the results of this work show that the hybrids  $\beta$ ACIL and  $\beta$ HDZ are promising molecules for the development of new antileishmania drugs.

**Keywords:**  $\beta$ -lapachone,  $\alpha$ -lapachone, molecular hybridization, hydralazine, isoniazid, hydrazone.

## RESUMEN

### Síntesis y actividad antileishmania de híbridos naftoquinónicos

**Introducción:** leishmaniasis es una enfermedad causada por protozoos del género *Leishmania* y se considera endémica en 98 países. El tratamiento con antimoniales pentavalentes tiene una alta toxicidad, lo que motiva la búsqueda de fármacos eficaces y menos tóxico.  $\alpha$ - y  $\beta$ -lapachones han mostrado diferentes actividades biológicas, incluido los antiprotozoarios. En estudios recientes, los grupos isonicotinoilhidrazona y ftalazinilhidrazona se consideraron innovadores en el desarrollo de fármacos antileishmania. La hibridación molecular es una estrategia para el desarrollo racional de nuevos prototipos, donde el compuesto principal se produce a través de la unión apropiada de subunidades farmacofóricas. **Objetivos:** sintetizar cuatro híbridos de  $\alpha$ - y  $\beta$ -lapachones, junto con los grupos isonicotinoilhidrazona y ftalazinilhidrazona y determinar la actividad antileishmania frente a las formas promastigotas de *L. amazonensis*, *L. infantum* y *L. major*. **Resultados:** los derivados de  $\beta$ -lapachone fueron más activos contra todas las especies de leishmania probadas. La  $\beta$ ACIL ( $CI_{50}$  0,044 $\mu$ M) y  $\beta$ HDZ ( $CI_{50}$  0,023 $\mu$ M) mostraron actividad 15 veces mayor que la anfotericina B. El alto índice de selectividad que presentan los compuestos indica una mayor seguridad para las células huésped del vertebrado. **Conclusión:** los resultados de este trabajo demuestran que los híbridos  $\beta$ ACIL y  $\beta$ HDZ son moléculas prometedoras para el desarrollo de nuevos fármacos antileishmania.

**Palabras clave:**  $\beta$ -lapachone,  $\alpha$ -lapachone, hibridación molecular, hidralazina, isoniazida, hidrazona.

## RESUMO

### Síntese e atividade antileishmania de híbridos naftoquinônicos

**Introdução:** a leishmaniose é uma doença causada por protozoários do gênero *Leishmania* e é considerada endêmica em 98 países. O tratamento com antimoniais pentavalentes apresenta alta toxicidade, o que motiva a pesquisa por medicamentos eficazes e menos tóxicos.  $\alpha$ - e  $\beta$ -lapachones têm mostrado diferentes atividades biológicas, incluindo antiprotozoários. Em estudos recentes, os grupos isonicotinoilhidrazona e ftalazinilhidrazona foram considerados inovadores no desenvolvimento de drogas antileishmania. A hibridização molecular é uma estratégia para o desenvolvimento racional de novos protótipos, onde o composto principal é produzido através da ligação apropriada de subunidades farmacofóricas. **Objetivos:** sintetizar

quatro híbridos de  $\alpha$ - e  $\beta$ -lapachones, juntamente com os grupos isonicotinoil-hidrazona e ftalazinilhidrazona e determinar a atividade antileishmania contra as formas promastigóticas de *L. amazonensis*, *L. infantum* e *L. major*. **Resultados:** os derivados de  $\beta$ -lapachona foram mais ativos contra todas as espécies de leishmania testadas. BACIL (IC<sub>50</sub> 0,044  $\mu$ M) e  $\beta$ HDZ (IC<sub>50</sub> 0,023  $\mu$ M) apresentaram atividade 15 vezes maior do que a anfotericina B. O alto índice de seletividade dos compostos indica maior segurança para células hospedeiras de vertebrados. **Conclusão:** os resultados deste trabalho mostram que os híbridos  $\beta$ ACIL e  $\beta$ HDZ são moléculas promissoras para o desenvolvimento de novos fármacos antileishmania.

**Palavras-chave:**  $\beta$ -lapachona,  $\alpha$ -lapachona, hibridização molecular, hidralazina, isoniazida, hidrazona.

## INTRODUCTION

Leishmaniasis are neglected parasitic diseases caused by more than 20 protozoan species of the genus *Leishmania*, considered endemic in 98 countries [1]. Transmission to humans occurs during the blood repast of Phlebotomines from the Phlebotomus (Old World) and Lutzomyia (New World) families. Only the hematophagous females are responsible for the inoculation of promastigote forms in the host's skin [2]. The species *Leishmania amazonensis* is associated with the development of cutaneous leishmaniasis with the formation of necrotic ulcers, and is capable of causing the disseminated form of the disease [3].

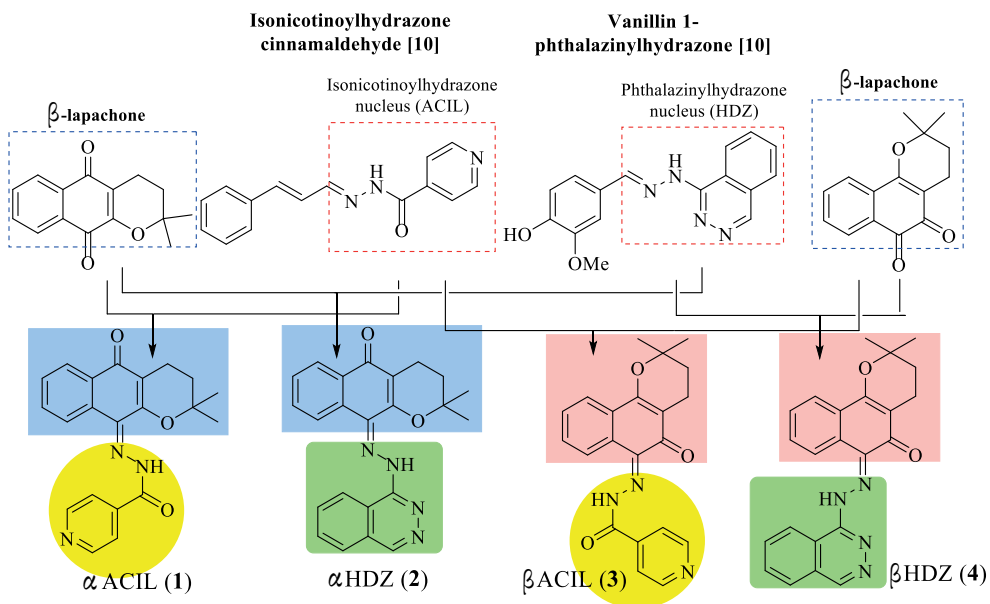
Pharmacological therapy with pentavalent antimonials (*N*-methylglucamine antimoniate) is the first choice treatment, and amphotericin B and pentamidine are the second choice [4]. And there are successful reports of the use of miltefosine in cases of antimonium-resistant protozoa [5, 6]. However, the treatment of leishmaniasis is still a difficulty, considering that the available pharmacological therapies present limitations in terms of efficacy and safety, prolonging the treatment; therefore, a range of adverse reactions, the need for parenteral administration, in addition to the possible emergence of resistance, lead to low adherence to treatment by patients.

Several efforts search for bioactive natural compounds that can be used in the treatment of parasitic diseases [7]. Lapachol,  $\alpha$ - and  $\beta$ -lapachones are promising natural naphthoquinones for Medicinal Chemistry due to their structural properties. Goulart *et al.* described the leishmanicidal activity of lapachol and some derivatives, showing the pharmacological potential of these substances [8]. In 2013, Guimarães *et al.* presented the

activity of lapachol,  $\alpha$ - and  $\beta$ -lapachones against the promastigote forms of four species of the genus *Leishmania*, ratifying the relevance of these naphthoquinones in the development of new leishmanicides [9].

In a study published this year, Souza *et al.* (2020) pointed out the relevance of the presence of isonicotinoylhydrazone and phthalazinylhydrazone nuclei in the structures of compounds for the antileishmanial activity. In this work, the authors synthesized five hydrazones and evaluated *in vitro* the activity against the promastigote form of *L. amazonensis* [10].

The development of new substances with therapeutic potential is a complex task involving multi and interdisciplinary efforts. Several strategies are used by Medicinal Chemistry uses to make the process of developing new drugs more effective. Molecular planning is one of the crucial steps in the process, and Molecular Hybridization (MH) is an effective alternative for the rational design of molecular structures of new prototype compounds. In this proposal, hybrid compounds are the result of joining molecular structures of distinct bioactive compounds [11, 12].



**Figure 1.** Scheme of the designer of hybrid compounds  $\alpha$ ACIL (1),  $\alpha$ HDZ (2),  $\beta$ ACIL (3) and  $\beta$ HDZ (4).

Considering the activity against protozoa of the genus *Leishmania* presented by the  $\alpha$ -,  $\beta$ -lapachones and compounds containing the isonicotinoylhydrazone (ACIL) and

phthalazinylhydrazone (HDZ) nuclei, four compounds were designed through the of MH strategy employing the molecular structures of the naphthoquinones and the ACIL and HDZ nuclei, as shown in figure 1. Subsequently, the hybrids were prepared and had their activities evaluated against the promastigote forms of *L. amazonensis*, *L. infantum* and *L. major*, besides the cytotoxic evaluation in murine macrophages.

## MATERIALS AND METHODS

For the synthesis, all the reagents used were obtained from commercial sources and used without prior purification, with the exception of hydralazine hydrochloride, which was obtained from Apresolina® pills (Anovis Industrial Farmacêutica Ltda) [10, 13]. The synthesized substances had its melting temperatures determined in triplicate using analog fusimeter, model PFM-II (MS Tecnopon® instrumentation). The chromatographic profile of the substances was determined by Analytical Thin Layer Chromatography (TLC), using 2x4 cm aluminum/silica gel 60 plates with UV<sub>254</sub> fluorescence indicator, revealed in ultraviolet (UV) or with iodine (I<sub>2</sub>). The purification in chromatographic column used silica gel 60 (70 - 230 mesh) and ethyl acetate/hexane (AcOEt/Hex) mixture with increasing polarity, as a mobile phase. After preparation and purification all, the synthesis products were stored under refrigeration and protected from light.

The structural elucidation of naphthhydrazones  $\alpha$ ACIL (**1**),  $\alpha$ HDZ (**2**),  $\beta$ ACIL (**3**) e  $\beta$ HDZ (**4**) was performed using <sup>1</sup>H and <sup>13</sup>C Nuclear Magnetic Resonance techniques (<sup>1</sup>H and <sup>13</sup>C NMR) [13]. The NMR spectra have been registered in a Bruker Ascend TM 400 device, which operates at 400 MHz for the <sup>1</sup>H nuclei and at 100 MHz for the <sup>13</sup>C nuclei. The chemical displacements ( $\delta$ ) were given in ppm using tetramethylsilane solvent (TMS) as internal standard. All samples were solubilized in deuterated solvent (CDCl<sub>3</sub> or DMSO-d<sub>6</sub>).

Infrared (IR) absorption spectra were obtained using PerkinElmer® (Spectrum 400) equipment with Attenuated Total Reflectance (ATR) device with zinc selenide crystal, from 4000 to 650 cm<sup>-1</sup>, and 4 cm resolution<sup>-1</sup>.

### Extraction, purification, and characterization of lapachol

Lapachol was extracted from the stalk of the ipe (*Tabebuia sp.*). The splinters of the heartwood were submerged in an aqueous solution of sodium hydroxide (NaOH) at 1% (m·v<sup>-1</sup>) for about 24 h. The filtrate was then acidified with a solution hydrochloric acid 6 M (HCl), and the lapachol was precipitated in the aqueous medium as a yellow solid. The solid was filtered by vacuum filtration and dried at room temperature.

Lapachol was purified by recrystallization from ethanol/water. Yellow crystalline solid, yield of 1.5 % (m/m) and melting point (m.p.): 138.33-140.33 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>, δ in ppm): 8.12 (1H, m, H5); 8.07 (1H, m, H8); 7.75 (1H, td, *J* = 7.6, 1.4 Hz, H6); 7.67 (1H, td, *J* = 7.5, 1.4 Hz, H7); 7.33 (1H, s, -OH); 5.21 (1H, m, H10); 3.31 (2H, d, *J* = 7.4 Hz, -CH<sub>2</sub>); 1.79 (3H, s, -CH<sub>3</sub>); 1.69 (3H, s, -CH<sub>3</sub>). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>, δ in ppm): 184.59 (C4); 181.70 (C1); 152.67 (C2); 134.87 (C6); 133.87 (C4 and C11); 132.88 (C7); 129.41 (C8); 126.77 (C5); 126.06 (C8); 123.45 (C3); 119.62 (C10); 25.77 (-CH<sub>3</sub>); 22.61 (C9); 17.90 (-CH<sub>3</sub>).

#### Synthesis, purification, and characterization of α-lapachone

Lapachol (1 mmol; 242 mg) was dissolved in a solution of glacial acetic acid (AcOH; 240 μL) and concentrated HCl (630 μL). It was heated to 100 °C for 1.5 h, and then cooled to room temperature. The reaction mixture was poured into a beaker containing cold distilled water, the precipitated pale yellow solid was filtered and dried at room temperature. The obtained solid was purified by simple recrystallization using ethanol. Yellow crystals, yield of 71 % and m.p.: 116.33-117.66 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>, δ in ppm): 8.08 (2H, m, H6 and H9); 7.68 (2H, m, H7 and H8); 2.63 (2H, t, *J* = 6.6 Hz, H4); 1.83 (2H, t, *J* = 6.6 Hz, H3); 1.44 (6H, s, 2-CH<sub>3</sub>). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>, δ in ppm): 184.42 (C5); 180.03 (C10); 154.62 (C1); 133.87 (C7); 132.93 (C8); 132.07 (C5↔C9); 131.16 (C9↔C5); 126.33 (C6); 125.97 (C9); 120.15 (C4); 78.17 (C2); 31.41 (C3); 26.51 (2-CH<sub>3</sub>); 16.74 (C4).

#### Synthesis, purification, and characterization of β-lapachone

Cooled concentrated sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) (730 μL) was added to a reaction vessel (25 mL) containing lapachol (1 mmol; 242 mg) and immersed in an ice bath at 0 °C. The reaction was stirred for 20 min. Then, the reaction mixture was poured into a beaker containing cold distilled water, the precipitated orange solid was filtered and dried at room temperature. β-lapachone was purified by column chromatography. Reddish-orange crystals, yield of 69% and m.p.: 154.66-158.0 °C. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>, δ in ppm): 7.91 (1H, d, *J* = 7.6 Hz, H7); 7.77 (2H, m, H9 and H10); 7.61 (1H, m, H8); 2.40 (2H, t, *J* = 6.6 Hz, H4); 1.82 (2H, t, *J* = 6.6 Hz, H3); 1.43 (6H, s, 2-CH<sub>3</sub>). <sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>, δ in ppm): 179.06 (C6); 177.83 (C5); 160.65 (C1); 135.02 (C9); 132.10 (C10); 130.84 (C8); 129.96 (C6); 127.83 (C7); 123.70 (C10); 112.51 (C4); 79.07 (C2); 30.81 (C3); 26.33 (2-CH<sub>3</sub>); 15.97 (C4).

#### General procedure for the preparation of the compounds αACIL (1) and βACIL (3)

To a methanolic solution (5 mL) containing isoniazid (90 mg, 0.6 mmol) and the appropriate naphthoquinone (121 mg, 0.5 mmol) (α- or β-lapachone) was added a

drop of concentrated HCl (37%). The reaction mixture was stirred until complete consumption of naphthoquinone. The crystals formed were filtered, washed with methanol and distilled water and dried at room temperature. The product was purified by column chromatography [13].

#### General procedure for the preparation of compounds $\alpha$ HDZ (2) and $\beta$ HDZ (4)

In a solution containing hydralazine hydrochloride (1.5 mmol) in methanol (11 mL), H<sub>2</sub>SO<sub>4</sub> concentrate (approximately 700  $\mu$ L) was slowly added, followed by appropriate naphthoquinone (121 mg, 0.5 mmol) ( $\alpha$ - or  $\beta$ -lapachone). After the end of the reaction, the mixture was neutralized with 5% (m·v<sup>-1</sup>) NaHCO<sub>3</sub> solution. The precipitate formed was filtered, washed with distilled water and dried at room temperature. The product was purified by column chromatography [13].

#### Data from $\alpha$ ACIL (1): (Z)-N<sup>2</sup>-(2,2-dimethyl-5-oxo-3,4-dihydro-2H-benzo[g]chromen-10(5H)-ylidene)isonicotinohydrazide

The reaction mixture remained under agitation at room temperature for 72 h [13]. Greenish-yellow solid (figure 2), yield of 11.3% and m.p.: 226.50-227.83 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>,  $\delta$  in ppm): 12.80 (1H, s, N-H); 8.86 (1H, m, H16 and H18); 8.52 (1H, d,  $J$  = 7.9 Hz, H9); 8.11 (1H, m, H6); 7.74 (2H, dd,  $J$  = 4.4; 1.6 Hz, H15 and H19); 7.64 (1H, dd,  $J$  = 12.9; 4.6 Hz, H8); 7.54 (1H, t,  $J$  = 7.5 Hz, H7); 2.69 (2H, t,  $J$  = 6.1 Hz, H4); 1.93 (2H, t,  $J$  = 6.3 Hz, H3); 1.53 (6H, s, 2 -CH<sub>3</sub>). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>,  $\delta$  in ppm): 183.43 (C5); 161.99 (C13); 154.51 (C1a); 150.94 (C16 and C18); 140.39 (C14); 134.93 (C10); 133.22 (C9a); 132.45 (C8); 129.84 (C7); 129.61 (C5a); 125.70 (C6); 124.93 (C9); 120.96 (C15 and C19); 117.33 (C4a); 79.91 (C2); 31.14 (C3); 26.97 (2 -CH<sub>3</sub>); 16.88 (C4). IR (ATR,  $\nu$  in cm<sup>-1</sup>): 3330 (N-H); 1697 (C=O; N-C=O); 1633 (C=N); 1605-1455 (C=C<sub>Ar</sub>); 1265-1229 and 874 (C-O); 907-688 (C-H<sub>Ar</sub>). HRMS (ES<sup>+</sup>) calculated for C<sub>21</sub>H<sub>19</sub>N<sub>3</sub>O<sub>3</sub> [M+H]<sup>+</sup>: 362.1504. Found: 362.1469.

#### Data from $\alpha$ HDZ (2): (Z)-2,2-dimethyl-10-(2-(phthalazin-1-yl)hydrazono)-3,4-dihydro-2H-benzo[g]chromen-5(10H)-one

The reaction mixture remained under stirring and reflux for 6 h [13]. Red crystalline solid (figure 2), yield of 10.1% and m.p.: 238.83-240.50 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>,  $\delta$  in ppm): 11.11 (1H, s, N-H); 9.75 (1H, d,  $J$  = 8.1 Hz, H9); 8.56 (1H, dd,  $J$  = 6.1; 3.2 Hz, H15); 8.27 (1H, dd,  $J$  = 7.8; 1.4 Hz, H6); 8.10 (1H, s, H20); 7.80 (2H, m, H16 and H17); 7.66 (2H, m, H8 and H18); 7.53 (1H, m, H7); 2.66 (2H, t,  $J$  = 6.7 Hz, H4); 1.84 (2H, t,  $J$  = 6.7 Hz, H3); 1.46 (6H, s, 2 -CH<sub>3</sub>). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>,  $\delta$  in ppm): 184.37 (C5); 159.37 (C1a); 150.96 (C13); 141.73 (C10); 140.13

(C20); 133.06 (C17); 132.39 (C16); 131.59 (C8); 130.90 (C9); 130.83 (C5a); 129.33 (C7); 128.89 (C9a); 127.82 (C19↔C14); 126.71 (C14↔C19); 126.41 (C18); 126.11 (C6); 125.34 (C15); 113.51 (C4a); 77.36 (C2); 31.75 (C3); 26.74 (2 -CH<sub>3</sub>); 17.09 (C4). IR (ATR,  $\nu$  in cm<sup>-1</sup>): 3346 (N-H); 1622 (C=O; C=N); 1598 and 1572 (C=N; -C=N-N=C-); 1488, 1448 and 1396 (C=C<sub>Ar</sub>); 1368 and 1350 (C-N); 1246-1231 and 865 (C-O); 907-691 (C-H<sub>Ar</sub>). HRMS (ES<sup>+</sup>) calculated for C<sub>23</sub>H<sub>20</sub>N<sub>4</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 385.1664. Found: 385.1657.

**Data from  $\beta$ ACIL (3): (E)-N'-(2,2-dimethyl-5-oxo-3,4-dihydro-2H-benzo[h]chromen-6(5H)-ylidene)isonicotinohydrazide**

The reaction mixture was stirred at room temperature for 10 minutes [13]. Yellow-orange solid (figure 2), yield of 66 % and m.p.: 237.66-238.50 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>,  $\delta$  in ppm): 8.86 (2H, d,  $J$  = 5.8 Hz, H16 and H18); 8.46 (1H, s, -NH); 7.89 (4H, m, 7H7, H10, H15 and H19); 7.50 (2H, m, H8 and H9); 2.61 (2H, t,  $J$  = 6.6 Hz, H4); 1.90 (2H, t,  $J$  = 6.7 Hz, H3); 1.49 (6H, s, 2 -CH<sub>3</sub>). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>,  $\delta$  in ppm): 182.29 (C5); 163.38 (C1a and C13); 150.76 (C16 and C18); 139.67 (C14); 131.40 (C6 and C6a↔C10a); 130.51 (C8); 129.45 (C9); 127.00 (C10a↔C6a); 124.52 (C7); 123.42 (C10); 121.53 (C15 and C19); 111.24 (C4a); 79.09 (C2); 31.60 (C3); 26.77 (2 -CH<sub>3</sub>); 15.99 (C4). IR (ATR,  $\nu$  in cm<sup>-1</sup>): 3350 (N-H); 1703 (C=O; N-C=O); 1597 (C=N); 1558, 1511, 1493 and 1394 (C=C<sub>Ar</sub>); 1230 (C-O); 918-693 (C-H<sub>Ar</sub>). HRMS (ES<sup>+</sup>) calculated for C<sub>21</sub>H<sub>19</sub>N<sub>3</sub>O<sub>3</sub> [M+H]<sup>+</sup>: 362.1504. Found: 362.1486.

**Data from  $\beta$ HDZ (4): (E)-2,2-dimethyl-6-(2-(phthalazin-1-yl)hydrazone)-3,4-dihydro-2H-benzo[h]chromen-5(6H)-one**

The reaction mixture remained at room temperature for 78 h [13]. Orange crystalline solid (figure 2), yield of 22.4 % and m.p.: 237.16-239.00 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>,  $\delta$  in ppm): 9.30 (1H, s, H20); 8.56 (2H, dd,  $J$  = 7.6; 3.8 Hz, H7 and H15); 7.95 (4H, m, H10, H16, H17 and H18); 7.55 (1H, m, H8); 7.45 (1H, m, 1H, H9); 2.70 (2H, t,  $J$  = 6.7 Hz, H4); 1.92 (2H, t,  $J$  = 6.7 Hz, H3); 1.49 (6H, s, 2 -CH<sub>3</sub>). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>,  $\delta$  in ppm): 181.42 (C5); 161.87 (C1a); 148.40 (C20); 132.69 (C17); 132.47 (C6 and C13); 132.40 (C16); 132.29 (C6a); 129.92 (C8); 128.17 (C19↔C14); 127.74 (C9); 126.84 (C18); 125.75 (C10a); 123.51 (C7); 122.99 (C10); 122.35 (C15); 118.78 (C14↔C19); 111.15 (C4a); 78.21 (C2); 31.78 (C3); 26.80 (2 -CH<sub>3</sub>); 16.19 (C4). IR (ATR,  $\nu$  in cm<sup>-1</sup>): 1612 (C=O; C=N; -C=N-N=C-); 1588 (C=N; -C=N-N=C-); 1501, 1437 and 1395 (C=C<sub>Ar</sub>); 1225 and 862 (C-O); 900-691 (C-H<sub>Ar</sub>). HRMS (ES<sup>+</sup>) calculated for C<sub>23</sub>H<sub>20</sub>N<sub>4</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 385.1664. Found: 385.1686.



### Determination of the mean inhibitory concentration (IC<sub>50</sub>) of naphthhydrazones on the promastigotic forms of *Leishmania amazonensis*, *Leishmania major* and *Leishmania infantum*

The promastigote forms of *Leishmania* were kept cryopreserved in liquid nitrogen and in Schneider's<sup>o</sup> medium (Sigma, Chemical - USA) supplemented with Fetal Bovine Serum (FBS), 100 IU·mL<sup>-1</sup> penicillin-streptomycin (Sigma) and glycerol as cryopreservative. To perform the assays, the parasites were thawed and kept in the same medium, without cryopreserver, at 26 ± 1 °C in a biological oxygen demand oven (Eletrolab EL202, São Paulo, Brazil). The promastigotic forms of *Leishmania amazonensis* (IFLA/BR/67/PH8), *Leishmania major* (MHOM/IL/80/Friendli) and *Leishmania infantum* (MHOM/5745) in stationary growth phase were washed in 0.9% sterile saline solution, counted in Neubauer chamber and the volume adjusted to the desired concentration. The substances  $\alpha$ ACIL (1),  $\alpha$ HDZ (2),  $\beta$ ACIL (3) and  $\beta$ HDZ (4) were added to the microplate wells for cell culture, in triplicate, and serial dilutions were performed, reaching twelve concentration ranges (0.0097 to 20  $\mu$ g·mL<sup>-1</sup>). Soon after, the parasites were sown in the amount of 1 × 10<sup>6</sup> leishmanias/100  $\mu$ L of supplemented medium. The plate was then incubated in an oven at a temperature of 26 °C for 48h and, 6h remaining for the end of this period, 20  $\mu$ L of 1 × 10<sup>-3</sup> mol·L<sup>-1</sup> resazurin was added, and the plate was incubated again. After the incubation period, the reading was performed on a 550 nm wavelength absorption plate reader (Biosystems model ELx800, Curitiba, PR, Brazil). For positive control, amphotericin B was used at a concentration of 2  $\mu$ g·mL<sup>-1</sup>, diluted in a supplemented Schneider's medium. The negative control was equivalent to Schneider's medium containing 1 × 10<sup>6</sup> promastigotes per well and, in this case, the viability was 100% for the parasite. The reading of white, for each concentration and for the controls was necessary to disregard the absorbance resulting from the medium itself with interference or not of the substances studied. From these absorbances the concentration able to inhibit the growth in 50% of the parasites was calculated (IC<sub>50</sub>) [14, 15].

### Determination of the mean cytotoxic concentration (CC<sub>50</sub>) of synthetic naphthoquinone on RAW macrophages

The assessment of macrophage cytotoxicity was performed using the MTT assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide). In the plates of 96 wells, 2 × 10<sup>5</sup> macrophages of the RAW 264.7 strain were incubated per well in 100  $\mu$ L of RPMI 1640 medium (supplemented with 10% SFB, 10 000 IU penicillin and 1000 IU streptomycin) in an oven at 37 °C and 5% CO<sub>2</sub> for 4 h for cell adhesion. The supernatant was then withdrawn to remove the non-adhered cells. The

DMSO solubilized naphthahydrazonic derivatives were diluted in RPMI supplemented medium, added to the plate containing the macrophages in serial concentrations reaching twelve ranges of final concentrations, starting from  $100 \mu\text{g}\cdot\text{mL}^{-1}$ , and incubated at  $37^\circ\text{C}$  and 5 % of  $\text{CO}_2$  for 48 h. After this period, the cytotoxicity was evaluated by adding 10 % MTT at a concentration of  $5 \text{ mg}\cdot\text{mL}^{-1}$ , diluted in  $100 \mu\text{L}$  of RPMI medium, and the plate was incubated again for 4 h at  $37^\circ\text{C}$  and 5 % of  $\text{CO}_2$ . At the end of this period, the supernatant was discarded and the formazan crystals were dissolved by adding  $100 \mu\text{L}$  DMSO. Finally, the absorbance (550 nm) was measured using a Biotek plate reader (ELx800) [16]. The mean cytotoxic concentration  $\text{CC}_{50}$  ( $\mu\text{M}$ ) was determined from the linear portion of the curve, calculating the concentration of the compound that reduced the absorbance in treated macrophages by 50 % compared to negative control cells. The selectivity index (SI) was calculated by the ratio between  $\text{CC}_{50}$  and  $\text{IC}_{50}$  [17].

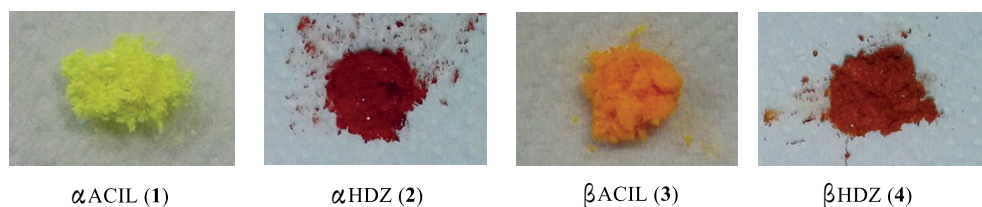
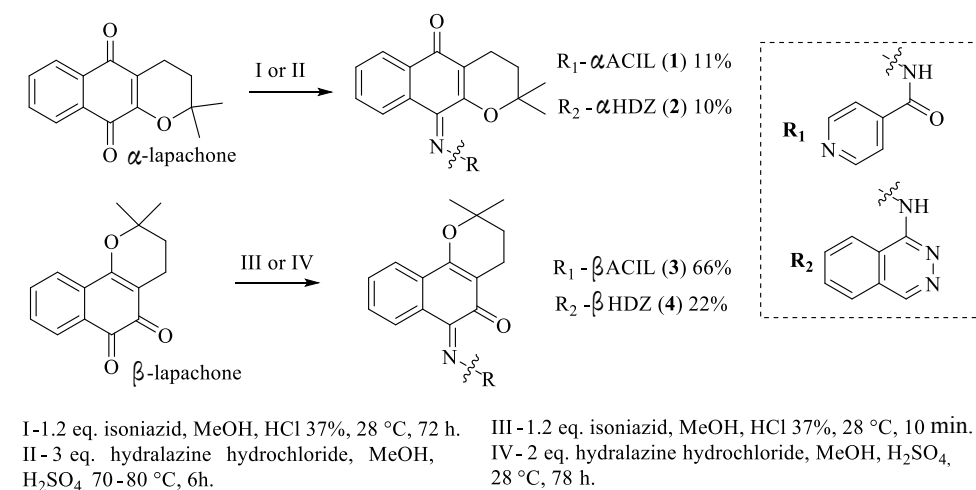
### Statistical analysis

All the biological trials were performed in three independent experiments. The mean inhibitory concentration ( $\text{IC}_{50}$ ) and the mean cytotoxic concentration ( $\text{CC}_{50}$ ) with 95 % confidence limit were calculated using probit regression. Analysis of variance ANOVA followed by Bonferroni's test was performed taking  $p < 0.05$  as the maximum level of statistical significance.

## RESULTS AND DISCUSSION

### Synthesis of the naphthohydrazones $\alpha\text{ACIL}$ (1), $\alpha\text{HDZ}$ (2), $\beta\text{ACIL}$ (3) and $\beta\text{HDZ}$ (4)

The hybrid compounds designed from  $\alpha$ - and  $\beta$ -lapachones, with the isonicotinoylhydrazone and phthalazinylhydrazone nuclei were called  $\alpha\text{ACIL}$  (1),  $\alpha\text{HDZ}$  (2),  $\beta\text{ACIL}$  (3) and  $\beta\text{HDZ}$  (4) (figure 1). The prefixes  $\alpha$ - and  $\beta$ - refer to lapachol derivatives,  $\alpha$ - and  $\beta$ -lapachones, and ACIL and HDZ refer to the isonicotinoylhydrazone and phthalazinylhydrazone nuclei, respectively. The hybrids were synthesized by condensation reaction between  $\alpha$ - and  $\beta$ -lapachones with isoniazid and hydralazine through acid catalysis, as described by Guimarães *et al.* (2020) [13]. The reaction yields varied between 10 % and 66 %, and the compounds had their molecular structures established by NMR, IR and MS techniques (figure 2).



**Figure 2.** Scheme of the synthesis reaction of the designed compounds  $\alpha$ ACIL (1),  $\alpha$ HDZ (2),  $\beta$ ACIL (3) and  $\beta$ HDZ (4).

### Antileishmanial activity of naphthohydrazones

The performance of *in vitro* tests of antileishmanial activity only in promastigote forms has been used as a screening test [16-20]. The *in vitro* evaluation of the antileishmanial activity of naphthohydrazones  $\alpha$ ACIL (1),  $\alpha$ HDZ (2),  $\beta$ ACIL (3) and  $\beta$ HDZ (4) has shown that these four hybrids were effective against promastigotic forms of *L. amazonensis*, *L. infantum* and *L. major*, this action being dependent of the concentration of the substance in test and the species of the parasite. When comparing the results obtained with the antileishmanial capacity of the precursor naphthoquinones ( $\alpha$ - and  $\beta$ -lapachones) it was observed that the majority of the hybrids were more active.

Among the hybrids of  $\alpha$ -lapachone both compounds,  $\alpha$ ACIL (1) and  $\alpha$ HDZ (2), showed high antileishmanial activity in at least one of the strains tested in the experimental time of 48 h. However,  $\alpha$ HDZ (2) was more potent against *L. amazonensis* and *L. major*, with IC<sub>50</sub> of 0.156 and 0.572  $\mu$ M, respectively (table 1). Naphthohydrazone 2 showed higher activity than its precursor,  $\alpha$ -lapachone (IC<sub>50</sub> 16.08  $\mu$ M in 72 h of experiment) [9] for *L. amazonensis*, exhibiting 103 times higher activity in a shorter

experimental time (48 h). For *L. infantum*,  $\alpha$ ACIL (1) and  $\alpha$ HDZ (2) presented activity 24 and 4 times higher, respectively, than  $\alpha$ -lapachone (IC<sub>50</sub> 13.88  $\mu$ M in 72 h of experiment) [9], in an experimental time of 48 h.

The most active compounds against all the promastigotic forms of leishmanias tested were those that have the framework of phenanthrene, as occurs in  $\beta$ -lapachone and its hybrids  $\beta$ ACIL (3) and  $\beta$ HDZ (4). While 3 had the lowest IC<sub>50</sub> (0.318  $\mu$ M) against *L. infantum*, compound 4 was the most potent against *L. amazonensis*, with a IC<sub>50</sub> of 0.023  $\mu$ M. Both hybrids, 3 and 4, were more active than the precursor compound,  $\beta$ -lapachone, on all tested strains of leishmanias.  $\beta$ ACIL (3) was 65 times more active than  $\beta$ -lapachone (IC<sub>50</sub> 2.90  $\mu$ M in 72 h of experiment) [9] for *L. amazonensis*; whereas,  $\beta$ HDZ (4) was 126 times more active than the precursor orthonaphthoquinone in *L. amazonensis*. Compared to *L. infantum*, hybrids 3 and 4 were 2 times more active than  $\beta$ -lapachone (table 1).

When comparing the IC<sub>50</sub> of the synthesized naphthohydrazones with the amphotericin B, it can be affirmed that after exposure to *L. amazonensis* and *L. major*, the majority of the naphthohydrazones presented activity superior to the positive control. Highlight to  $\beta$ HDZ (4) over *L. amazonensis* and  $\beta$ ACIL (3) compared to *L. major*, both with action 15 times greater than amphotericin B. Regarding *L. infantum*, the compounds 3 and 4 presented activity similar to the standard drug (table 1).

**Table 1.** *In vitro* antileishmanial activity in promastigotic forms of *L. Amazonensis*, *L. major* and *L. infantum* of synthetic naphthohydrazones, in 48 h of experimental time.

	<i>L. amazonensis</i> <sup>a</sup>		<i>L. infantum</i> <sup>a</sup>		<i>L. major</i>		RAW 264.7
	IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>	SI <sup>c</sup>	IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>	SI <sup>c</sup>	IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>	SI <sup>c</sup>	CC <sub>50</sub> <sup>b</sup>
$\alpha$ ACIL (1)	>5.534	< 0.840	0.578±0.028	8.043	1.356±0.111	3.428	4.649±0.055
$\alpha$ HDZ (2)	0.156±0.052	24.179	3.408±0.026	1.107	0.572±0.156	6.594	4.649±0.055
$\beta$ ACIL (3)	0.0443±0.028	52.460	0.318±0.055	7.308	0.044±0.083	52.818	2.324±0.055
$\beta$ HDZ (4)	0.023±0.026	42.956	0.318±0.05	8.821	1.145±0.107	0.863	0.988±0.078
$\alpha$ -Lap	16.08 <sup>d</sup>	- <sup>f</sup>	13.88 <sup>d</sup>	- <sup>f</sup>	- <sup>f</sup>	- <sup>f</sup>	- <sup>f</sup>
$\beta$ -Lap	2.90 <sup>d</sup>	- <sup>f</sup>	0.67 <sup>d</sup>	- <sup>f</sup>	>104 <sup>e</sup>	- <sup>f</sup>	- <sup>f</sup>
AnfB	0.35±0.3 <sup>g</sup>	0.8	0.3±0.1	0.93	0.68±0.151	0.41	0.28±0.09

<sup>a</sup>IC<sub>50</sub>: concentration of the compound that causes 50 % mortality.

<sup>b</sup>CC<sub>50</sub>: concentration of the compound that causes 50 % of macrophage mortality.

<sup>c</sup>SI – CC<sub>50</sub>/IC<sub>50</sub> when SI >1 means higher toxicity to the parasite.

<sup>d</sup>IC<sub>50</sub> ( $\mu$ M) in 72 h (Guimarães *et al.*, 2013) [9].

<sup>e</sup>Tanga 2013 [21].

<sup>f</sup>Non-existent Result.

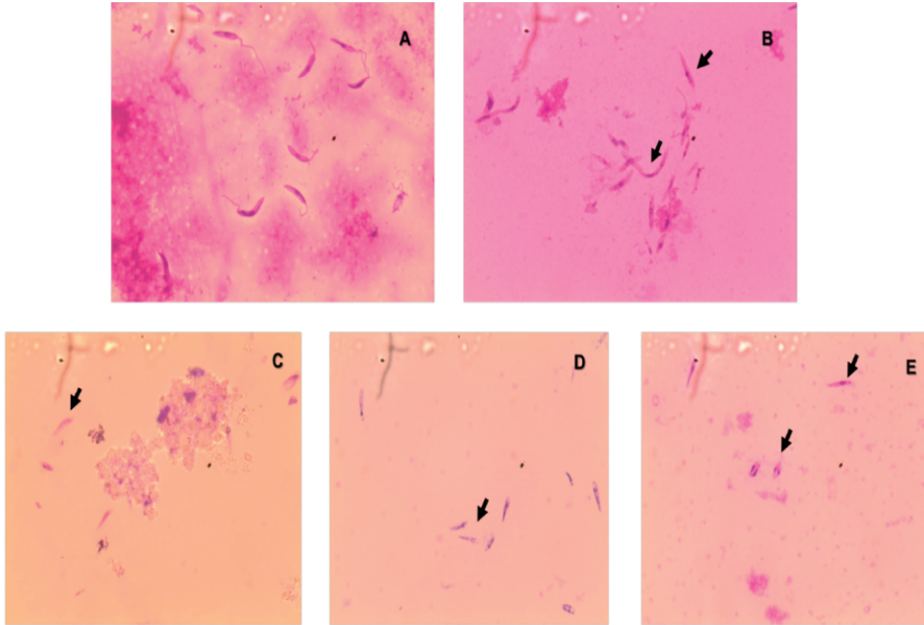
<sup>g</sup>Dias *et al.*, 2020 [22].

Leishmaniasis are intracellular macrophage parasites in vertebrate hosts; therefore, assessing toxicity to these cells is essential when planning a drug for the treatment of visceral or tegumentary leishmaniasis [23]. The factor used to measure this safety was the selectivity index (SI), which is calculated by the ratio between the Cytotoxic Concentration for 50% of macrophages ( $CC_{50}$ ) and  $IC_{50}$ . The molecules that present  $SI > 1$  and  $SI > 20$  are classified, respectively, as good and high safety, and those of high safety are qualified for further studies in infected macrophages and *in vivo* models [24, 25].

The tested naphthohydrazones showed good safety for use as antileishmanial, highlighting the hybrid  $\beta$ ACIL (3), 52 times more selective for *L. amazonensis* and *L. major* than for the host cell. The compounds that have the HDZ nucleus,  $\alpha$ HDZ (2) and  $\beta$ HDZ (4), also showed high safety for mammalian cells with SI of 42,956 and 24,179, respectively, when used on promastigotes of *L. amazonensis*. These results qualify the naphthohydrazones  $\alpha$ HDZ (2),  $\beta$ ACIL (3) and  $\beta$ HDZ (4) for the trials on infected macrophages and *in vivo* models. Among the compounds tested,  $\alpha$ ACIL (1) and  $\beta$ HDZ (4) were the most selective for *L. infantum* in relation to macrophages, presenting SI of 8.821 and 8.043, respectively.

Although the mechanism of action of naphthohydrazones on leishmanias is not known, the results of this work show that the compounds promoted damage in the morphological structure of these parasites (figure 3). Among these changes, the variation in parasite size, scourge and shape stand out. According to Rodrigues *et al.* (2014) [26] and Gadelha *et al.* (2013) [27], such changes may be caused by the destabilization of the tubulin-dependent cytoskeleton, since both the parasite's body shape and the scourge's integrity are highly dependent on the stability of the microtubules.

Although naphthohydrazones have relatively complex molecular structures, they were prepared using accessible compounds, the lapachol, a natural product from a renewable source; and two low-cost drugs, with known toxicity and pharmacokinetics, the isoniazid and hydralazine. In addition, classic reactions in mild conditions were used, and accessible synthetic routes are desired by the pharmaceutical industry, especially when it comes to drugs for the treatment of neglected diseases, such as leishmaniasis. The promising antileishmania activity presented by naphthohydrazones, in association with the synthetic advantages of the route used, make the compounds  $\alpha$ HDZ (2),  $\beta$ ACIL (3) and  $\beta$ HDZ (4) suitable for the later stages of drug development.



**Figure 3.** *Leishmania amazonensis* treated with naphthohydrazones in the concentration corresponding to its IC<sub>50</sub>. (A) without treatment; (B)  $\alpha$ HDZ (2); (C)  $\beta$ HDZ (4); (D)  $\alpha$ ACIL (1); (E)  $\beta$ ACIL (3). 100x magnification. Arrows represent promastigotes with altered morphology.

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## CONFLICT OF INTEREST

All authors report that they do not have any conflicts of interest.

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