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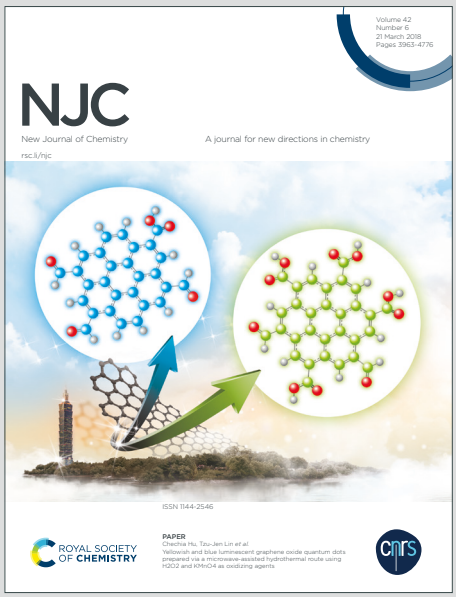

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Optimization of phthalazin-based aryl/heteroarylhydrazones to design new promising antileishmanicidal agents: Synthesis and biological evaluation of 3-aryl-6-piperazin-1,2,4-triazolo[3,4-a]phthalazines

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Abstract: 1-Monosubstituted and 1,4-substituted phthalazin-based on aryl/heteroarylhydrazinyl have demonstrated attractive antileishmanial profiles against amastigote forms of *Leishmania braziliensis* parasite. More optimization on mentioned acyclic scaffold motivated us to design a series of 3-aryl-1,2,4-triazolo[3,4-*a*]phthalazine, a cyclic version of the phthalazin-based on aryl/heteroarylhydrazinyl which has not been evaluated against *Leishmania* parasites yet. In order to compare to phthalazine-based aryl/heteroarylhydrazones, five essential 3-aryl-6-piperazin-1,2,4-triazolo[3,4-*a*]phthalazine were efficiently prepared in excellent yields (73-83%) through a facile one-pot procedure from 4-chloro-1-phthalazinyl-arylhydrazones via a C-H dehydrogenative cyclization using silver(I) salt. From *in vitro* antileismanial evaluation, compound **2d**, a nitro derivative, was identified as the most promising agent with a good anti-amastigote response (IC_{50} = 9.37 μM) and low relative toxicity against peritoneal macrophage (LD_{50}=123.93 μM). A moderate response was found against clinical amastigote isolates of *L. braziliensis,* although superior compared to reference glucantime. A comparison with their phthalazin-based on aryl/heteroarylhydrazinyl analogues put in evidence that the efficacy of each chemical system is determined by the nature of the functionalization in turn to aryl moiety, which suggests that different mechanism of action are involved for each chemical system. The cyclized form led an enhancement on the antileismanial activity compared to acyclic form, but the nitroderivatives seemed to be highly more toxic than parent non-cyclized compounds. From the three compared phthalazine groups, the 4-chloro-1-phthalazine-(5-nitrofuryl)hydrazinil with nanomolar antileishmanial response was identified as promising lead for further optimization, whereas the compound **2d** emerges as prominent hit platform to prepare a group of derivatives based on phthalazine-1,2,4-triazolo bearing 3-nitro-phenyl at 3-position.

***Keywords*:** *Leishmania braziliensis*, phthalazine, 1,2,4-triazolo[3,4-*a*]phthalazine, promastigote, amastigote, cutaneous leishmaniasis.

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1. Introduction

Leishmaniasis is a neglected tropical disease resulting from infection of macrophages by obligate intracellular parasites of the genus *Leishmania* spp., which is transmitted to human by insects [1,2]. There are mainly three clinical manifestations for the leishmaniasis including: (i) mucocutaneous leishmaniasis (ML), cutaneous leishmaniasis (CL), and visceral leishmaniasis (VL), which are caused by different *Leishmania* species depending on geographic region [3]. Leishmaniasis is prevalent in 88 countries with 350 million people at risk of acquiring the disease. World Health Organization revealed that between 0.6 and 1.0 million cases of CL are registered annually [4]. *Leishmania braziliensis* is responsible for nearly 90% of all CL cases annually in South America, and the absence or incomplete treatment can facilitate the subsequent development of ML [5,6]. Treatments against cutaneous leishmaniasis is severely limited due to that only two approved drugs (e.*g.* pentavalent antimonials such as pentostam and glucantime) are currently used, have more than 60 years in the market and, they are highly toxic (affecting heart, liver and kidneys), highly expensive, low effective and unpractical administration for patient [7]. A second line of current drugs such as pentamidine, amphotericin B and miltefosine also are employed when the first line failed. Moreover, resistance reports have been found for pentavalent antimonials in endemic areas [8].

The discovery of new potential drugs against neglected tropical disease, in this case against Leishmaniasis, prevail as a challenge for the science due to the complexity of parasite can cause diverse pathologies depending on the type of *Leishmania*, and its high adaptability that permits to develop resistance to drugs in short time [9a]. Diverse criteria have been established in order to identify potential antileishmanial agents. DNDi (Drug for Neglected Disease initiative) described four stages for the final discovery of a medication against neglected tropical disease: (i) the discovery stage that implies initial screening, hit to lead and lead optimization; (ii) the translation stage that implies pre-clinical, phase I and phase IIa steps, (iii) the development stage that consists of two steps, phase IIb/III and registration, and (iv) the fourth stage that involves the implementation for the treatment access to public [9b]. To access to stages II, III and IV, it is important to overcome the different criteria established for the hit step of stage I [9c], which included: (i) IC_{50} below 10 μM against form clinically relevant of any parasite (*T. cruzi, Leishmania, T. brucei*, etc), (ii) selectivity index superior to 10 related to mammalian cell lines [9c], and (iii) good drug-likeness parameters. Whereas significant exigencies are included for the

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step of hit to lead: (i) activities *in vitro* against relevant stage of parasite from 10 to 20-fold more than hit stage; (ii) selectivity index superior to 50-fold more than hit stage, (iii) significant reduction of parasitaemia (> 70%) and/or increase of life-span of animal *in vivo* assay, (iv) good solubility by about 0.01 mg/mL at pH 7.4; (v) plasma concentration in the range of active concentration after administration; (vi) relevant further toxicity assays such as genotoxicity (*e.g.* Ames test) and cardiotoxicity (*e.g.* hEGR assay) are needed. A large number of new heterocycles based on quinolin [10a-e], quinazoline [10f-i] and phthalazine [11] have been identified as promising hit as leishmanicidal agent. Recently, our group identified to the 4-chloro-phthalazine **1** functionalized with highly conjugated nitroheteroarylhydrazones at 1-position of phthalazine core as a potential lead for further improvements (Figure 1). Further optimization motivated the design, synthesis and evaluation of disubstituted phthalazine-arylhydrazinyl **2**, identifying compounds with good anti-amastigote response against *L. braziliensis* parasite [11a-d]. A isosteric form of these compounds of phthalzin-arylhydrazinyl is the 1,2,4-triazolo[3,4-a]phthalazine, a cyclic version, which has not been proved against trypanosomal parasites yet. The 1,2,4-triazolo[3,4-a]phthalazine system is a known multi-target agents that possess a wide spectrum of pharmacological activities, including anti-inflammatory agents [12], anticancer drugs [13], bromo-domain inhibitors[14], GABA receptors [15], anticonvulsants [16] and antimicrobials [17]. Specifically, 3-substituted-6-alkylamino-1,2,4-triazolo[3,4-*a*]phthalazine **1** system (Figure 1) is present in the structure of some of these bioactive triazolo agents. From our previous results, it was suggested that the high conjugation from phthalazine core to nitro-aryl/heteroaryl through hydrazine linker is crucially important in the antileishmanial response of the monosubstituted phthalazine 4-chlorophthalazin-arylhydrazones, while the disubstitution in the 1,4-disusbtituted-phthalazine are essential to enhance the biological activity [11a-d]. Therefore, it results very interesting to study the biological relevance of the hydrazine linker for the mentioned system compared to triazolo linker of the phthalazine-1,2,4-triazolo system (Figure 1). Then, based on the above mentioned, we evaluated leishmanicidal activity of a series of 1,2,4-triazolo[3,4-*a*]phthalazines (as lead core) containing a *N*-methylpiperazine at 6-position and a substituted aryl/heteroaryl R_1 group at 3-position of the 1,2,4-triazolo core. We focused our attention in the evaluation of phthalazine-1,2,4-triazolo bearing relevant non-toxic and active moieties detected from active previous phthalazine-arylhydrazones mono-substituted and di-substituted including: 4-nitrophenyl, furyl, phenyl, 4-fluorophenyl and 3-nitrophenyl (Figure 1).

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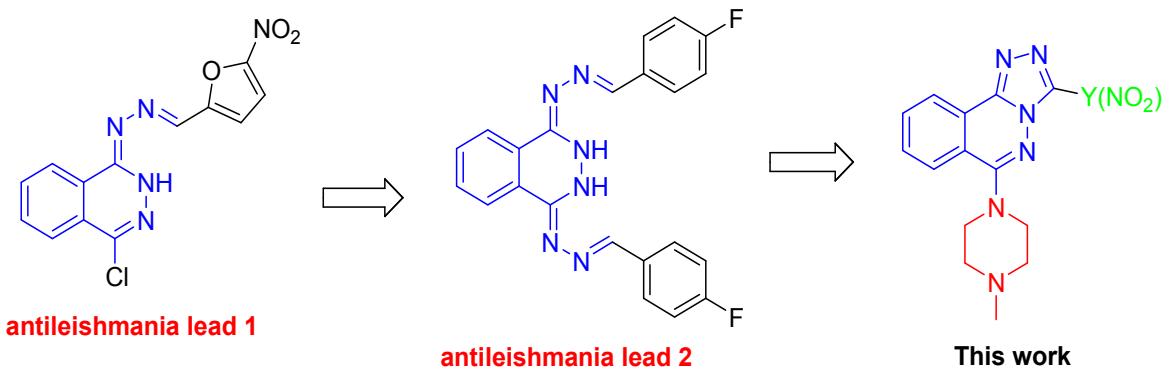

Figure 1. General structure from antileishmania lead (**1** and **2**) to current design 6-(*N*-piperazin)-3-aryl-1,2,4-triazolo[3,4-*a*]phthalazine lead.

A number of specific derivatives were prepared by a facile and efficient protocol based on the intramolecular cyclization via C-H activation mediated by coinage transition metal such as cooper (I) and silver (I). In the last decades, the metal-catalyzed intramolecular cyclization has been employed as a competitive strategy to obtain a variety of fused-heterocyclic systems [18-25]. Specifically, strategies based on dehydrogenation of vicinal C-H ylidenic or C-H aldehyde bonds as well as vicinal C_{sp3}-H bonds in *N,N*-disubstituted hydrazones has permitted the synthesis of five-membered heterocycles via the formation of C-N or C-O bonds [25]. Recently, our group reported an one-pot protocol for the synthesis of 6-(*N*-piperazin)-3-aryl-1,2,4-triazolo[3,4-*a*]phthalazine via a copper-catalyzed dehydrogenation of ylidenic hydrogen from 4-chloro-1-phthalazinyl-arylhydrazones [26]. The majority of the mentioned strategies are catalyzed through redox action of transition metals such as copper(I), copper(II), palladium (II) and iron (II and III) and, the reactions in general require of temperature above 80 ºC, prolonged time more than 24 h and the use of a base. In last decades, a large number of silver-catalyzed C_{sp^2}-*H* functionalization have been reported using silver(I)/$S_2O_8^{2-}$ system under mild conditions in absence of base and, the reactions proceeded under a Ag(I)/Ag(II) catalytic cycle [27]. Other way for the silver-mediated C_{sp^2}-*H* functionalization is to use silver (I) salts in equimolar ranges under hard conditions and prolonged time and, the reaction is determined by a non-catalytic redox Ag(I)/Ag(0) process [27]. This last strategy in general involves re-aromatization process in heteroaromatic systems. From these evidences, we developed an intramolecular dehydrogenative cyclization mediated by a silver (I) to obtain a series of 3-aryl-6-(*N*-methylpiperazin)-1,2,4-triazole[3,4-*a*]phthalazines **2** from 4-chloro-1-phthalazinyl-arylhydrazones **1** (Table 1).

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2. Chemistry

Initially, the reaction conditions were optimized using 4-chloro-1-phthalazinyl-arylhydrazone **1a** and *N*-methylpiperazine as model substrates. The initial reaction conditions were extracted from silver-protocol reported by H.J. Xu [28] for a standard Ag(I)/Ag(II) catalytic process, a silver-strategy reported by S. Chang [29] for a Ag(I)/(Ag(0) mediation and our cooper based-protocol [26] with a few modifications including reaction time of 48 h and temperatures from 40 to 100ºC). Silver(I) nitrate was used as standard silver catalyst or mediator and, the results were compared with cooper (I) catalysis [26]. Optimization conditions are summarized in Table 1. In general, a poor reaction yield was found in absence of catalyst (entries 1-2, Table 1). In presence of copper (I)-*L*-proline system as catalyst and reaction conditions including 100ºC of temperature and reaction time of 48 h, the following remarks are extracted: (i) the selection of solvent plays an important role in the progression of the reaction, finding the best results for DMF or *N*-methylpiperazine as solvents, whereas a decrease in reaction yield was found under toluene environment (entries 3-5, Table 1); and (ii) the oxidizing is mandatory, finding good results under aerobic conditions (entries 3-5, Table 1) and, contrary poor results under argon environment (entry 6, Table 1). The oxygen is crucial to keep the catalytic cycle. Regarding to silver (I) catalyst, we found that the system $Ag^{+}/S_2O_8^{2-}$ was not compatible for our chemical system under different oxidizing (*e.g.* $S_2O_8^{2-}$, TBHP), solvents (*e.g.* THF/H_2O, acetone/H_2O, CH_2Cl_2/H_2O, DMF/H_2O) and temperature (40-80 ºC) conditions (entries 7-12, Table 1). Probably, the potential radical species formed on hydrazinil moiety in our system during Ag(I)/Ag(II) process is not sufficient stable compared to phenyl radical formed in reported protocol, which is a strong limitation in the progress of the reaction. Discarded the applicability of Ag(I)/Ag(II) process in our system, we decided to study the potential of Ag(I)/Ag(0) mediation according to reported protocol [29]. As expected, the strategy based on Ag(I)/Ag(0) process using non-stoichiometric amount of silver (I) salt displayed poor reaction yields (11-35%) (entries 13-14, Table 1), whereas a significant increase in reaction yields was found for stoichiometric quantities of silver (I) (entries 15-16, Table 1), being 2.5 equivalent of silver(I) nitrate the most convenient amount to afford an optimal reaction. The selection of solvent also was an important point. The best results were obtained under DMF milieu with a reaction yield of 83% (entry 16, Table 1); whereas modest results were found under THF or toluene milieu (entries 17-18, Table 1). A decrease of temperature to 80ºC reduced the reaction yield from 83%

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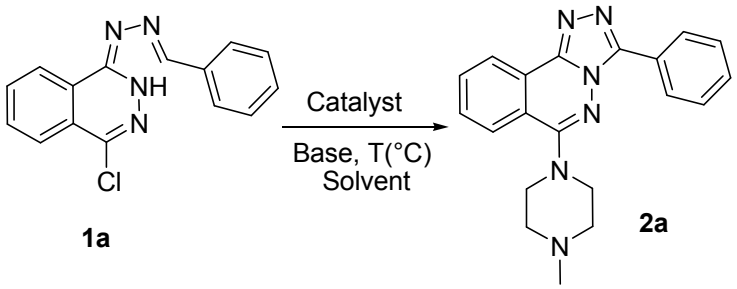
to 44% (compare entries 16 and 18 in Table 1). K_3PO_4 was selected as the most convenient base compared to K_2CO_3 (compare entries 16 and 20, Table 1). Finally, the reaction operates well under air environment for 48 h.

Table 1. Reaction optimization conditions



Entries	Catalysts	Solvent[a]	Oxidizing	T(ºC)	Base or additive	Y (%)[c]
1	No Catalyst	NMPz	None	100	K_3PO_4 (3 equiv.)	11
2	No Catalyst	DMF	none	100	K_3PO_4 (3 equiv.)	10
3	**CuI/L-proline (15%/30%)**	**NMPz**	**air**	**100**	K_3PO_4 (3 equiv.)	**73**
4	CuI/L-proline (15%/30%)	DMF	air	100	K_3PO_4 (3 equiv.)	70
5	CuI/L-proline (15%/30%)	Toluene	air	100	K_3PO_4 (3 equiv.)	46
6	CuI/L-proline (15%/30%)	NMPz	none[b]	100	K_3PO_4 (3 equiv.)	9
7	$AgNO_3$ (20 %)	THF/ H_2O (1:1)	$K_2S_2O_8$ (3 eq.)	40	KOAc (1.1 eq.)	0
8	$AgNO_3$ (20 %)	Acetone/H_2O (1:1)	$K_2S_2O_8$ (3 eq.)	40	KOAc (1.1 eq.)	0
9	$AgNO_3$ (20 %)	DMF/H_2O (1:1)	$K_2S_2O_8$ (3 eq.)	40	KOAc (1.1 eq.)	0
10	$AgNO_3$ (20 %)	CH_2Cl_2/H_2O (1:1)	$K_2S_2O_8$ (3 eq.)	40	KOAc (1.1 eq.)	0
11	$AgNO_3$ (20%)	CH_2Cl_2/H_2O (1:1)	TBHP (3 eq.)	40	KOAc (1.1 eq.)	0
12	$AgNO_3$ (20%)	THF/H_2O (1:1)	$K_2S_2O_8$ (3 eq.)	80	KOAc (1.1 eq.)	0
13	$AgNO_3$ (15%)	DMF	air	100	K_3PO_4 (3 equiv.)	11
14	$AgNO_3$ (50%)	DMF	air	100	K_3PO_4 (3 equiv.)	35
15	$AgNO_3$ (1.0 eq.)	DMF	air	100	K_3PO_4 (3 equiv.)	52
16	**$AgNO_3$ (2.5 eq.)**	**DMF**	**air**	**100**	**K_3PO_4** (3 equiv.)	**83**
17	$AgNO_3$ (2.5 eq.)	THF	air	100	K_3PO_4 (3 equiv.)	31
18	$AgNO_3$ (2.5 eq.)	Toluene	air	100	K_3PO_4 (3 equiv.)	38
19	$AgNO_3$ (2.5 eq.)	DMF	air	80	K_3PO_4 (3 equiv.)	44
20	$AgNO_3$ (2.5 eq.)	DMF	air	100	K_2CO_3 (3 equiv.)	74

Reaction conditions: **1a** (1eq., 1 mmol), catalyst (CuI/*L*-proline) or $AgNO_3$, base (3 eq., 3 mmol) or additive (3 eq., 3 mmol), *N*-methylpiperazine (5 eq., 5 mmol), solvent (5 mL). All reactions were carried out in presence of air for 48 h. [a]NMPz (*N*-methylpiperazine). [b]Reaction under argon. [c]Isolated yield of pure product after purification.

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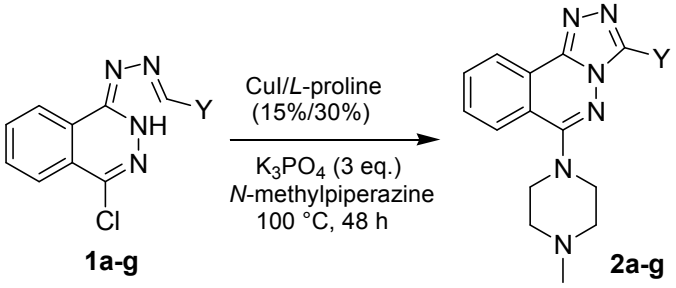
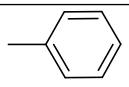
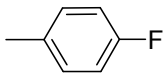
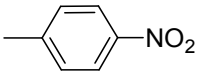
With optimized reaction conditions in hand (entry 2, Table 1), different 3-aryl-6-piperazin-1,2,4-triazolo[3,4-*a*]phthalazines **1a-g** were prepared from the corresponding phthalazinyl-arylhydrazone **2a-g (**Table 2), focusing our attention on relevant functionalization found in previous active mono- and di-substituted phthalazines [11a-b]. These starting phthalazine-hydrazones were prepared following the reported procedure [11a]. As shown in Table 2, the reaction mediated by silver (I) afforded better reaction yields for most of studied cases compared to copper (I) catalyst, with a good group tolerance in general. Unfortunately, substrates containing electron-deficient heteroaryl moieties such as 5-nitrofuryl or 5-nitrothiophene generated a complex mixture and products containing amine moiety by nitro group was presumably formed during the reaction according to ^{1}H-NMR evidence. There are many reported about the high susceptibility of nitro-substitution in aromatic systems highly electron-deficient to suffer nucleophilic aromatic substitution in presence of aliphatic amines [30]. Moreover, collateral products derived from ring opening may be generated from the catalyzed-reaction for high electron-deficient ring such as 5-nitrofuryl and 5-nitrothiophene in substrates **2f** and **2g**, respectively. Some recent reports have demonstrated that five membered rings such as furyl and thiophene are susceptible to ring opening reaction under the presence of metallic-catalyst (Co, Cu, Sc or Ir) [31-32].

Table 2. Synthesis of 3-aryl-6-(*N*-methylpiperazin)-1,2,4-triazolo[3,4-*a*]phthalazines **2a-g.**



Entries	Y	Compound codes	%Yield[a,c]	%Yield[b,c]
1		2a	73	83
2		2b	62	79
3		2c	83	73

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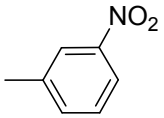
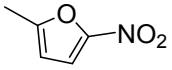
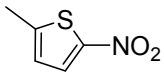
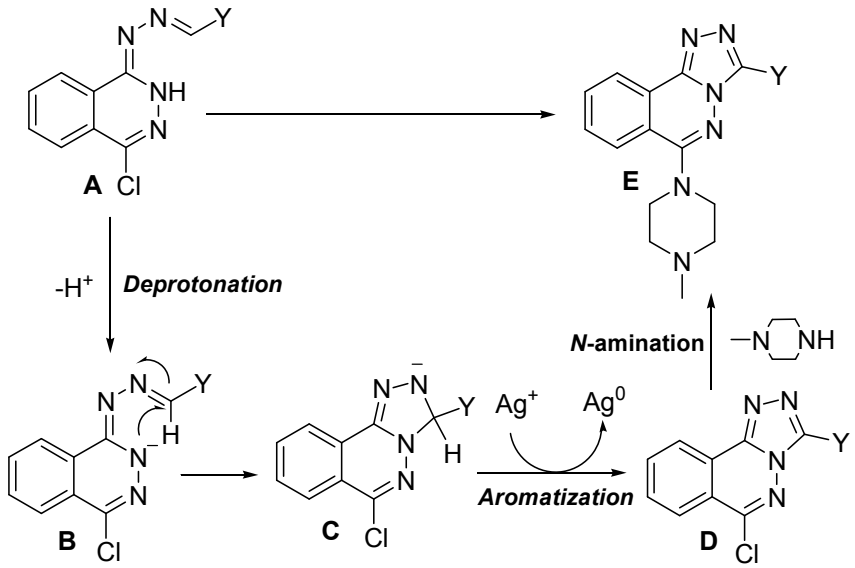
4		**2d**	70	76
5		**2e**	79	81
6		**2f**	traces	traces
7		**2g**	traces	traces

[a]Reaction conditions: **1a-g** (1.0 eq., 1 mmol), CuI (0.15 eq., 0.15 mmol), *L*-proline (0.30 eq., 0.3 mmol), K_3PO_4 (3.0 eq., 3 mmol), *N*-methylpiperazine (5.0 eq., 5 mmol). [b]Reaction conditions: **1a-g** (1.0 eq., 1 mmol), $AgNO_3$ (2.5 equiv., 2.5 mmol), K_3PO_4 (3 eq., 3 mmol), *N*-methylpiperazine (5 eq., 5 mmol), solvent (5 mL). [c]Isolated yield of pure product after purification. N.I.: not isolated.

A putative and simplified mechanism of reaction for the formation of the product **2** (or E) from the starting material **1** (or **A**) was proposed based on our previous copper protocol [27] and reported examples of C-H functionalization mediated by Ag(I)/Ag(0) redox [29] (Scheme 1). Initially, starting 4-chloro-1-phthalazin-aryl/heteroarylhydrazone **A** under basic conditions give the intermediate ion **B** possessing a nitrogen at position 3 highly nucleophilic, which attacks to adjacent ylidenic carbon via radical nucleophilic intamolecular addition to form the intermediate ion **C. C** aromatized via dehydrogenation of the "*pseudo ylidenic*" C-H bond to lead the intermediate **D**, directed by the reductive action of silver (I) releasing silver (0) metal. Finally, the intermediate **D** suffers a *N*-amination on the C-Cl bond via SNAr to give the desired product **E**.



Scheme 1. Plausible mechanism.

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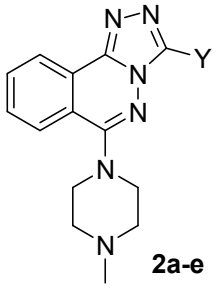
3. Antileishmanial evaluation

We have focused our research in the identification of any potential molecule to treat the American CL. In this sense, all compounds were tested against *L. braziliensis* parasite, the main responsible of the mentioned disease in Latin-America (90% of all CL cases) [5]. Initially, we evaluated the therapeutic effect of the five synthesized compounds on proliferative culture of the promastigote stage. Results are shown in Table 3. It should be noted that four derivatives showed excellent activities with low-micromolar IC_{50} values, ranging from 1.60 to 4.95 μM, being 5-13.5 fold more potent than glucantime (reference drug). It should be noted that the Y substitution on 1,2,4-triazolo ring has an important effect in the biological response of these phthalazine-triazolos, where the 4-nitrophenyl and 4-fluorphenyl Y substituent led to an increase in the antileishmanial activity, with low micromolar IC_{50} of 2.46 and 1.60 μM, respectively. The 3-nitrophenyl and furyl substitutions generated a second line of active derivatives against promastigote proliferation of *L. braziliensis* with good IC_{50} values of 4.73 and 4.86 μM, respectively. The derivative containing an unsubstituted phenyl (**2a**) as Y group displayed a weak antileishmanial activity at 50 μM. Therefore, we can conclude that the potency of tested compounds based on the Y substitution increased in the following order: 4-fluorophenyl > 4-nitrophenyl > 3-nitrophenyl > furyl > phenyl. These results demonstrated that 1,2,4-triazolo ring fused with phthalazine ring generated active compounds against *Leishmania* parasite, reflecting that the phthalazine ring play an important role in the activity of these compounds because previous compounds based on 1,2,4-triazolo have demonstrated from a moderate to weak antileishmanial activity [35].

Table 3. *In vitro* growth inhibitory effects of the compounds **2a-e** against promastigote and amastigote forms of *L. braziliensis*.



2a-e

Ent.	Compd	Promastigote IC_{50}	Amastigote LD_{50} (μM)	Toxicity CC_{50} (μM)

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		(μM)	Axenic[a]	Intracellular[b]	Human isolate[c]	Macrophage[d]
1	2a	> 20.0	N.D	> 50.0	N.D	N.D.
2	2b	**1.60 ± 0.08 (65)**	**5.69±0.32 (18)**	28.93±1.21 (4)	> 50.00 (< 2)	**103.34± 5.12**
3	2c	**2.46 ± 0.19 (16)**	**0.83 ±0.04 (47)**	**9.01±0.81 (4)**	48.18±2.12 (1)	**38.89±2.15**
4	2d	**4.73 ± 0.32 (26)**	**2.58 ±0.12 (48)**	**9.37±0.72 (13)**	43.24±2.23 (<3)	**123.93±7.89**
5	2e	**4.89 ± 0.21**	N.D.	32.02±1.56	N.D.	N.D.
6	Glt[e]	32.00± 1.78 (4.3)	N.D.	12.21± 2.12 (12)	>50.0 (<3)	138.22 ± 10.11

[a]Axenic amastigote of *L. braziliensis*. [b]Reference murine intracellular amastigote isolate of *L .braziliensis*. [c]Clinical intracellular amastigote isolate of *L. braziliensis*.[d]Murine peritoneal macrophage. [e]Glucantime reference drug. The results are the mean of three independent experiments with a SD less than 5 % in all cases. Note: marked in bold parameters indicated a pronounced antileishmania activity. N.D.: Not determined. Glt: glucantime. IC_{50}: inhibitory concentration to inhibit 50% of cell proliferation; LD_{50}: lethal doses to kill the haft cell population (50%); CC_{50}: 50% cytotoxicity concentration. Selectivity indexes are reported in parenthesis.

To validate the therapeutic effect of the most active compounds, an *in vitro* evaluation at different compound concentration was performed against axenic and intracellular amastigote isolates. Firstly, promastigote of *L. braziliensis* was transformed in axenic amastigote under specific conditions of pH=5.5 at 35 ºC for 48 hours. Then, amastigote was maintained in culture and the therapeutic effect of the derivative **2b**, **2c** and **2d** was evaluated at 48 h. Results are shown in Table 3. Interestingly, compounds **2c** and **2d** were by about 2-fold more selective toward axenic amastigote than toward promastigote form with low IC_{50} values of 0.83 and 2.58 μM, respectively. While compound **2b** was significantly more active against promastigote than axenic amastigote strain. To complement the anti-amastigote evaluation, the effect of the mentioned compounds was assessed on reference intracellular amastigote isolates. The intracellular amastigote model is one of the most important assays for the *in vitro* antileishmanial evaluation because of the amastigote stage, that is found in mammalian host, is the main responsible of the clinical manifestations of leishmaniasis. Traditionally, the intracellular amastigote are produced *in situ* from macrophage in presence of promastigote parasite under specific conditions (pH 5.5 at 37ºC). Nevertheless, this lab adapted strain could be more susceptible to the action of drug compared to amastigote isolate because the machinery defence is less activated in axenic culture as consequence of numerous passages. Then, we decided to work with amastigote isolates from murine footpad lesion to obtain more approximated information to the reality. This reference isolate was directly extracted from murine lesion footpad, and parasite cells was exposed in culture media at specific conditions (pH 5.5 at 37 °C) and, treated with the

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selected compounds **2b, 2c** and **2d.** This protocol was previously reported by our group [9a, 10a, 36]. The effect of the compounds was evaluated at 48 h post-treatment and, the results are shown in Table 3. As was expected, it should be noted that the amastigote intracellular isolate was from 5- to 9-fold more resistant than axenic amastigote for the tested compounds. Specifically, nitro-compounds **2c** and **2d** were identified as the most potent derivatives, confirming that the nitro-substitution has a specific role in the biological responses of these compounds. On the other hand, compounds **2b** and **2e** exhibited a moderate response against amastigote intracellular isolate, while the compound **2a** no responding at 50 μM. It is important to mention that the compound **2d** was comparable in therapeutic potency against amastigote intracellular isolate and selectivity to glucantime drug.

To evaluate the real potentiality of the most active derivatives **2b, 2c** and **2d**, we performed an *in vitro* evaluation using clinical amastigote intracellular isolate. The clinical human strain, called MHOM/VE/2018/M2903/LR, was identified as *L. braziliensis* specie and, it was isolated from a Venezuelan CL patient [10f]. Experimental details can be found in supplementary material. Results are listed in Table 3. The clinical amastigote isolate was 4-fold more resistant than reference amastigote strain for the two most active derivatives **2c** and **2d**, whereas not appreciable response was observed for the compound **2b** at 50 μM. Compounds **2c** and **2d** were more potent than reference drug against human amastigote isolate. Despite the decreasing antileishmanial activity of the compounds **2c** and **2d** against human amastigote isolate, compound **2d,** with a better activity/toxicity profile than reference drug against promastigote, axenic amastigote and amastigote isolates, was identified as a hit platform from initial screening step for further structural improvements to identify an antileishmanial lead based on phthalazine-1,2,4-triazolo according to criteria of drug discovery for neglected tropical disease [9c].

To quantify the selectivity of the most active derivatives, it is important to know its effect on macrophage cells, which are the traditional host cell of *Leishmania* parasite. The toxicity of the selected compounds **2b, 2c** and **2d** was evaluated on peritoneal macrophages derived from peritoneal cavity of Balb/c mice. Toxicity results (expressed in terms of CC_{50} parameter) are summarized in Table 3 together with their respective selectivity indexes related to anti-promastigote and anti-amastigote activities. In particular, the mentioned compounds showed moderate *in vitro* toxicities with CC_{50} values from 38 to 123 μM. Specifically, compound **2d** emerged as the most promising antileishmanial agents for further *in vivo* assays, with selectivity

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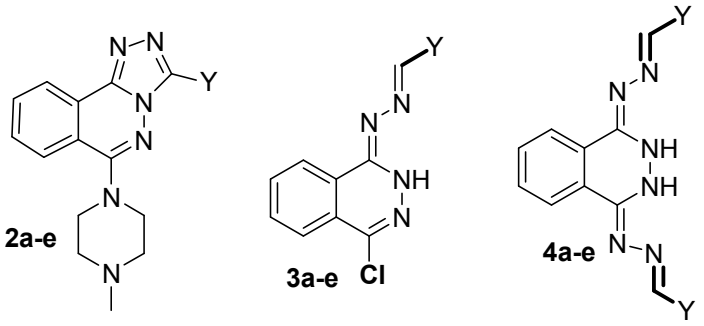
indexes by about 48 and 13 against promastigote or against amastigote stages of *L. braziliensis*, respectively, indicating that **2d** is significantly more destructive on the parasite form than on the host cells. Compound **2c** is one of the most active derivatives, but its moderate toxicity (LD_{50}=38.89 μM) compromises its selectivity (S.I.~4 against amastigote reference strain) as antileishmanial agent. This cytotoxic effect associated to the 4-nitrophenyl suggests that other compounds bearing higher electron-deficient nitro-groups such as the compound **2f** and **2g** may be highly toxic against host mammalian cell.

Table 4. Comparison between anti-promastigote and anti-amastigote activities for the compounds **2a-e**, **3a-e** and **4a-e.**



Ent.	Y	Promastigote IC_{50} (μM)			Amastigote IC_{50} (μM)		
		2a-e	3a-e[a]	4a-e[b]	2a-e	3a-e[c]	4a-e[b]
1	**C_6H_5**	> 25.0	>25.0	>25.0	N.D.	N.D.	>50.0
2	**4F- C_6H_5**	**1.60**	> 25.0	**2.37**	28.93	N.D.	**4.71**
3	**4NO_2- C_6H_5**	**2.46**	14.16	>25.0	**9.01**	**31.31**	>50.0
4	**3NO_2- C_6H_5**	**4.73**	> 25.0	**23.70**	**9.37**	**N.D.**	**9.23**
5	**C_5H_4O**	**4.89**	> 25.0	> 25.0	> 50.0	>50.0	>50.0

[a]Biological data for **3a-e** was extracted from Ref. 11b. [b]Biological data for 3a-e was extracted from Ref. 11d.

[c]Antiamastigote biological data for 3a-e was performed in the present investigation.

A comparison between the activity of these five compounds **2a-e** and their corresponding acyclic version, arylhydrazone-phthalazinyl **3a-e** and disubstituted phthalazines **4a-e** (see Table 4), put in evidence that the cyclic version **2a-e** (IC_{50} ranging from 1.6 to 4.89 μM) was in general more active than corresponding acyclic analogues **3a-e** (IC_{50} > 14 μM) and **4a-e** (IC_{50} > 8 μM for the majority of compound with exception of **4b** with an IC_{50} value of 2.37 μM) (see comparative details in Table 4). We think that all these three phthalazine systems act under different mode of action due to that specific and different functionalizations are essential for each system (Figure

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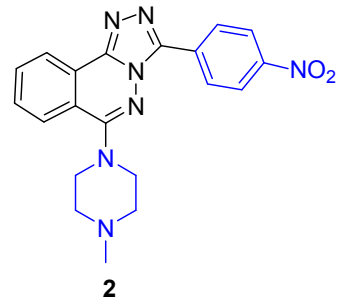
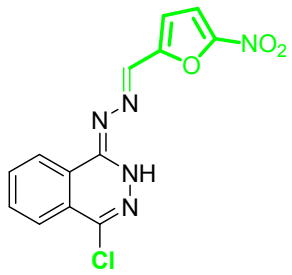
2): (i) the 5-nitrofuryl for the monosubstituted phthalazines **3a-e**, (ii) the 4-fluorophenyl for the disubstituted phthalazines **4a-e** and, (iii) the 3-nitrophenyl for the 1,2,4-triazolo[3,4-*a*]phthalazine **2a-e**. Figure 2 showed the most prominent candidate for each phthalazine group, and from this data it should be noted that the 4-chloro-1-phthalazine-(5-nitrofuryl)hydrazinil **3** was identified as a promising lead for further chemical modifications according to criteria established for the identification of lead in the stage I of drug discovery for neglected tropical disease [9c], in particular to improve or regulate its toxicity level. The chemical modifications may be directed to incorporate specific substitutions, for example, on 1-position of phthalazine core by replacement of chlorine atom. Meanwhile phthalazine-1,2-4-triazolo **2** and disubstituted phthalazine **3,** with a more moderate profile compared to phthalazine **3**, emerge as promising hit for further improvements in order to identify an antileishmanial lead.



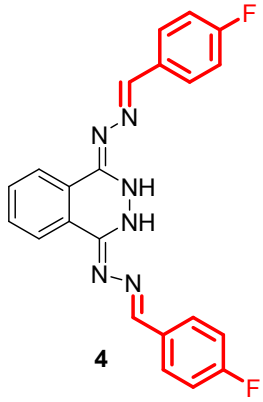
2

IC_{50} = 4.73 μM (promastigote)
LD_{50} = 2.58 μM (axenic amastigote)
LD_{50} = 9.37 μM (intracellular amastigote)
LD_{50} = 43.24 μM (clinical amastigote)
CC_{50} = 123.93 μM (peritoneal macrophage)



3

EC_{50} = 0.12 μM (promastigote)
EC_{50} = 0.50 μM (intracellular amastigote)
CC_{50} = 15.0 μM (macrophage BMDM)



4

IC_{50} = 2.37 μM (promastigote)
LD_{50} = 4.71 μM (intracellular amastigote)
LD_{50} = 19.62 μM (resistant amastigote)
LD_{50} = 35.30 μM (clinical amastigote)
CC_{50} = 30.00 μM (macrophage BMDM)

Figure 2. Comparison between the most active compounds of different phthalazine-based derivatives **2, 3** and **4**. Antileismanial biological data for phthalazine-1,2,4-triazolo **2** (in blue color), monosusbtituted-phthalazine **3** (in green color) and disusbtituted-phthalazine **4** (in red color). Biological information for **3** and **4** were extracted from ref. 11b and 11d, respectively.

In summary, an one-pot catalyzed strategy was developed for the synthesis of a series of 3-aryl-6-(*N*-methylpiperazine)-1,2,4-triazolo[3,4-*a*]phthalazines from phthalazin-based on aryl/heteroarylhydrazinyl derivatives using silver(I) as mediator. Compared to the traditional synthetic strategies, this protocol represents an attractive and competitive strategy by its simplicity, economy and one-pot reaction procedure. From biological evaluation, two derivatives

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2b and **2d** displayed a good *in vitro* activity against the promastigote and axenic amastigote forms of *Leishmania braziliensis* with low-micromolar IC_{50} values (ranging from 1 to 4 μM), low toxicities by about 103-125 μM and acceptable selectivity indexes. Compound **2d** showed better activity/toxicity profiles than glucantime drug against the tested promastigote and amastigote strains. A structure-activity relationship study suggests that the formation of fused triazolo-phthalazine and nitro-substitution are crucial pharmacophores in the biological activity of this kind of compounds, although the electron-deficiency nature of that nitro-substitution must be moderate such as a 3-nitrophenyl. Finally, the results demonstrated that compound **2d** represents a prominent antileishmanial hit platform for further chemical modifications, whereas the 4-chloro-1-phthalazine-(5-nitrofuryl)hydrazinil was identified as a promising lead for further biological experiments including genotoxicity evaluation, drug-likeness parameters, and *in vivo* assays to estimate its real therapeutic potential within the stage I of drug discovery for neglected tropical disease.

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Conflict of Interest

Author declared not conflict of interest related to present report.

Supplementary material

Full chemistry and biological experimental details, ^{1}H-NMR and ^{13}C-NMR data, IR and elemental analysis information associated with the compounds obtained herein can be found in the online version of the Supplementary material.

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