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2-Aryl-quinazolin-4(3*H*)-ones as an inhibitor of leishmania folate pathway: *In vitro* biological evaluation, mechanism studies and molecular docking

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ABSTRACT

To identify new agents for the American Cutaneous Leishmaniasis treatment, a series of 2-aryl-quinazolin-4(3*H*)-ones were tested against *L. mexicana, L. braziliensis* and *L. amazonensis* parasites as potential inhibitor of folic metabolism pathway. In general, the *L. braziliensis* and *L. mexicana* promastigote parasites were more sensitive to the action of the quinazolinones than *L. amazonensis*. The most active derivatives showed low-micromolar EC_{50} ranging from 4 to 10 μM, being 1.3 to 4 fold more potent than glucantime reference drug. A complete *in vitro* evaluation on intracellular amastigote, axenic amastigote and murine peritoneal macrophage were performed for the most active derivatives. The compounds **2j, 2h, 2t** and **2u** displayed acceptable responses against intracellular amastigote compared to reference drug, excellent antileishmanial activities against axenic amastigote (LD_{50} ranging from 1 to 4 μM) and relative low toxicities on peritoneal macrophages. To validate the efficacy of these four derivatives, an *in vitro* evaluation was performed against an antimony-resistant amastigote strain; identifying to **2h** and **2u** as promising antileishmanial leads for further pharmacokinetics and *in vivo* studies. Experimental mechanism assays putted in evidences that the most active compounds act as folate inhibitor. A tentative molecular docking on pteridine reductase 1 (PTR1) enzyme showed that the most active quinazolinones **2j** and **2t** are located in almost identical place compared with methotrexate reference into active site.

1. Introduction

Leishmaniasis is a neglected tropical disease caused by a protozoan parasite of the genus *Leishmania* [1–3]. There are mainly three clinical forms of the disease, with different immunopathologies and degrees of morbidity and mortality: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and muco-cutaneous leishmaniasis (MCL) [4]. CL is the most common clinical manifestation of the disease in Latin-America and, it is caused mainly by *L. braziliensis, L. mexicana, L. amazonensis and L. panamens*is species [5]. *Leishmania braziliensis* is responsible by about 90% of all CL cases annually and the absence or incomplete treatment can induce to the subsequent development of muco-cutaneous leishmaniasis [6]. *Leishmania amazonensis* causes a rare manifestation of CL in human such as diffuse CL. Leishmaniasis is prevalent in about 88 countries with 350 million people at risk of acquiring this disease [7]. World Health Organization (WHO) has estimated that between 0.6 and 1.0 million new cases of cutaneous leishmaniasis occur worldwide annually [7,8]. In addition, the number of leishmaniasis cases is increasing globally by *Leishmania*/HIV co-infection [9], international travels and migration [10,11].

Pentavalent antimonials (Sb^{v}) such as Pentostam (sodium stibogluconate) and glucantime (*N*-methylglucamine antimonate) are currently the first line of drugs for the leishmaniasis treatment in their different clinical manifestations. Alternatively, pentamidine, amphotericin B and miltefosine are used as a second line of antileishmanial drugs [12]. Miltefosine was developed as the first oral drug against visceral and cutaneous leishmaniasis. Nevertheless, these drugs are potentially toxic, highly cost, low effective and the administration via results unpractical for the patient [13]. Moreover, recent reports have revealed that pentavalent antimonials have developed resistance in endemic areas [14]. Thus, it is an urgent need the design and synthesis of new potent, nontoxic and selective antileishmanial drugs for the treatment of leishmaniasis in their different forms as well as *Leishmania*-HIV co-infection. In recent decades, an enormous variety of new potent and active heterocycles based on quinoline [15,16], quinazolin [17–22] and phthalazine [23–24] scaffolds have emerged as potential antileishmanial candidates. To identify novel antileishmanial agents, it is important to take account the essential metabolic pathways of the parasite. For example, folates are essential for a number of important biochemical reactions in the parasite metabolism, being the enzymes

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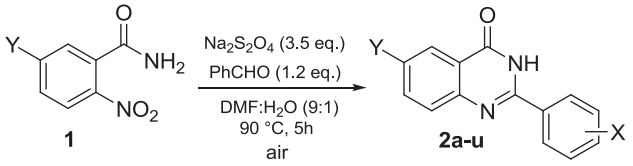
associated to folate pathways an important key to design antifolate agents [25]. Dihydrofolate reductase (DHFR) is an important enzymes involving in folate metabolism and it has widely used as a drug target. DHFR acts in conjunction with thymidylate-synthase (TS) enzyme, constituting a bifunctional DHFR-TS enzyme [26]. DHFR leads the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF), which is an important co-factor that the TS enzyme uses to catalyze the conversion from dUMP to dTMP. The dTMP is a vital metabolite in the DNA synthesis and its low production affects replication processes, leading to cell death [25a]. It is well documented that the enzymatic machinery of the dihydrofolate reductase-thymidylate synthase (DHFR-TS) is modulated by the pteridine reductase 1 (PTR1) enzyme, acting as a metabolic bypass of DHFR inhibition [26b]. Thus, folates are reduced either by the bifunctional DHFR (dihydrofolate reductase)-TS (thymidylate synthase) as well as by the PTR1 enzyme within the parasite. In addition, PTR1 also has the ability to reduce biopterin into biological system [26c–26e]. Then, the inhibition of the bifunctional DHFR- TS or pteridine reductase 1(PTR1) represents important therapeutic targets to design new antileishmanial drugs based on antifolate mechanism. Traditionally, pteridin and quinazolin scaffolds have been used as plataforms to design new antifolate agents and their derivatives have displayed significant antileismanial activities with good inhibitory responses on DHFR enzymes [18–22,25a]. Methotrexate, a folic acid analog based on pteridin core, is the most popular antifolate agent, which has resulted to be a potent antileishmanial agent and selective DHFR inhibitor [25a]. Quinazolin-4(3*H*)-ones containing attachments at 2- and 3-position of quinazoline core have recently received extended attention as antileishamanial agent [27], and some of their derivatives such as 1,2-susbtituted quinazolin-4(3*H*)-ones and 2-aryldihydroquinozalinones have exhibited from discrete to excellent antileishmanial activities with one digit micromolar IC_{50} on proliferative promastigote or amastigote of *Leishmania* parasite [27]. Based on the important role of quinazoline scaffold in the design of novel and potent antileishmanial agents, we reported herein the antileishmanial activity of a series of 2-(substituted-aryl)-quinazolin-4(*3H*)-ones, no containing substitution at 3-position, against different *Leishmania* species responsible of Americam cutaneous leishmaniasis: *L. braziliensis L. mexicana* and *L. amazonensis*. Toxicity assays on peritoneal macrophage, the natural host of *Leishmania* parasite, as well as mechanistic and molecular docking studies based on a folate metabolism also were carried out in the present investigation. Some of the tested 2-aryl-quinazolin-4(*3H*)-ones have demonstrated to be potent and selective agents against a panel of human cancer cells [28a].

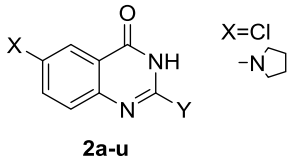
2. Results and discussion

The target 2-aryl-quinazolin-4(*3H*)-ones **2a-u** were previously prepared by our group [28b] from commercially available starting materials: 2-nitrobenzamide, benzaldehydes and sodium dithionite (see Scheme 1). The procedure involved three successive reactions: (i) the first consists in the reduction of the nitro group of the 2-nitrobenzamide by the action of sodium dithionite, (ii) the amine-intermediaries are cyclised with aryl-aldehydes, and (iii) the resulting dihydro 2-aryl-quinazolin-(3*H*)-4-ones through a redox process to give the desired heterocyclic compounds **2a-u** in good yields ranging from 60 to 83%.

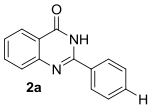
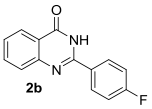
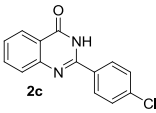
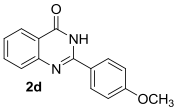
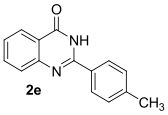
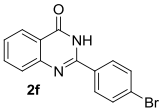
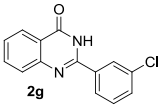
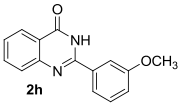
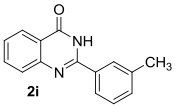
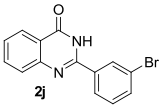
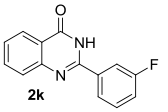
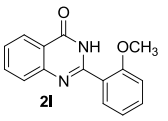


Scheme 1. Synthesis of 2-aryl-quinazolin-4(*3H*)ones **2a-u**. Conditions: $Na_2S_2O_4$ (3.5 equiv.), aryl/heteroarylaldehyde (1.2 equiv.), DMF/H_2O (9:1), 90 °C, 5 h.

Table 1
Antileishmanial activity of the 2-aryl-quinazolin-4(3*H*)-ones **2a–u** against *L. braziliensis, L. mexicana* and *L. amazonensis* promastigote stage.

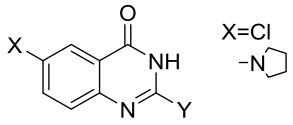


2a-u

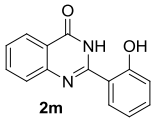
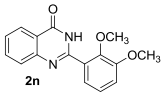
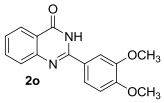
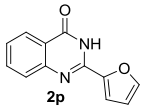
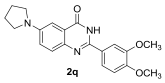
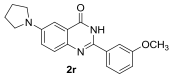
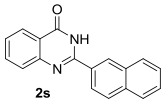
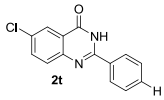
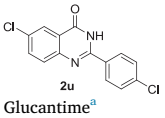
Entries	Compounds (R)	EC_{50} (μM)		
		L.braziliensis	*L.mexicana*	*L.amazonensis*
1	 **2a**	**8.68**	**9.24**	> 50.0
2	 **2b**	**8.53**	**6.75**	55.92
3	 **2c**	**12.36**	**15.34**	32.48
4	 **2d**	**14.26**	19.14	41.41
5	 **2e**	16.64	19.39	36.24
6	 **2f**	> 50.0	54.55	> 50.0
7	 **2g**	> 50.0	44.79	> 50.0
8	 **2h**	**4.89**	**5.29**	**9.14**
9	 **2i**	> 50.0	> 50.0	> 50.0
10	 **2j**	> 50.0	> 50.0	> 50.0
11	 **2k**	N.D	31.59	> 50.0
12	 **2l**	**7.54**	**10.11**	> 50.0

(*continued on next page*)

Table 1 (*continued*)



2a-u

Entries	Compounds (R)	EC_{50} (μM)		
		L.braziliensis	*L.mexicana*	*L.amazonensis*
13	 **2m**	**8.06**	37.58	**15.44**
14	 **2n**	> 50.0	> 50.0	> 50.0
15	 **2o**	≤50.0	≥50.0	> 50.0
16	 **2p**	**9.17**	**10.87**	> 50.0
17	 **2q**	≤50.0	> 50.0	35.13
18	 **2r**	> 50.0	> 50.0	> 50.0
19	 **2s**	> 50.0	> 50.0	> 50.0
20	 **2t**	**12.20**	**11.49**	**8.97**
21	 **2u**	**6.09**	**7.22**	**7.87**
22	Glucantime[a]	21.60	17.34	> 50.0

[a] Reference drug for Leishmaniasis treatment. Note: marked in bold parameters indicated a pronounced antileishmanial activity. N.D.: Not determined. Results are the mean of three independent experiments with a SD less than 5% for all cases.

The mechanistic reaction, experimental procedure details and spectroscopic data of all synthesized compounds can be found in our previous report [28b].

2.1. Antileishmanial activity

***In vitro* antileishmanial efficacy of 2-aryl-quinazolin-4(3*H*)ones 2a-u.** We focused our attention in the identification of potential agents to treat American Cutaneous Leishmaniasis [29]. Then, *L. braziliensis, L. mexicana* and *L. amazonensis* parasites were used for our *in vitro* study. Initially, all target compounds were tested on the promastigote proliferation of these three *Leishmania* parasites. Glucantime was used as reference drug. Concentration response data for each compound was fitted by a nonlinear regression model, and the concentration that induces 50% inhibition was calculated as the effective concentration EC_{50} on the three *Leishmania* species. Results are summarized in Table 1. In general, *L. amazonensis* parasite was less susceptible to the action of the 2-aryl-quinazolin-4(3*H*)ones than *L. mexicana* and *L. braziliensis* parasites, which these last two strains showed comparable activity trends. In particular, from 8 to 10 compounds showed good antileishmanial activities against *L. mexicana* and *L. braziliensis,* respectively, with EC_{50} values ranging from 4.89 to 15.44 μM; while only four derivatives exhibited acceptable antileishmanial activities in the mentioned range of EC_{50} against *L. amazonensis.* Compounds **2h, 2t** and **2u** were considered as the first line of active compounds, displaying EC_{50} values by no more than 12 μM against all three *Leishmania* species. The **2a, 2b, 2l** and **2p** derivatives can be selected as a second line of active 2-arylquinazolin-4(3*H*)ones, with EC_{50} values ranging from 5 to 10 μM against *L. mexicana* and *L. braziliensis*. Interestingly, these seven compounds resulted to be from 1.3 to 4.0 fold more potent than glucantime reference, the first antileishmanial drug commercially available for treatment in humans [12]. Finally, other compounds such as **2c, 2d, 2e**, and **2m** displayed EC_{50} values by no more than 20 μM for at least two species of *Leishmania*.

To identify the pharmacophores responsible of antileishmanial activity of the studied 2-arylquinazolin-4(3*H*)ones, a structure-activity relationship study was performed. Our analysis is focused on the influence of the 2- and 6-attachments in the antileishmanial response of this type of quinazolin system (see structures in Table 1). In general, the chlorine substitution at the position "6" on the benzenoid ring seems to be essential in the antileishmanial activity of the studied quinazolinones due to that their compounds **2t** and **2u** displayed the best biological response against tested *Leishmania* species, while the pyrrolidino substitution (a donor group) at the 6 position suppress the antileishmanial response for their corresponding quinazolinones, **2q** and **2r**. With regards to the Y substitution (2-attachment), the methoxy group seems to be the most important pharmacophore on the aryl ring and, the potency of their derivatives increases following the order: 3-methoxy > 2-methoxy > > 4-methoxy. The 4-fluorophenyl (**2b**), phenyl (**2a**) and furyl (**2p**) as Y substitutions generated compounds with acceptable antileishmanial responses, displaying IC_{50} responses between 5 and 9 μM against *L. mexicana* and *L. braziliensis*. The incorporation of bromide or dimethoxy substitutions on Y aryl ring causes a decrease in the antileishmanial activity of our compounds against the three species of *Leishmania*. The 4-methylphenyl (**2e)** and 4-chlorinephenyl (**2c**) substitutions on the Y aryl ring generated quinazolin derivatives with modest biological activity, while their corresponding 3-methylphenyl (**2g**) and 3-chlorinephenyl (**2i**) derivatives showed a weak or no inhibition at 50 μM of compound.

Activity against *L. braziliensis, L. mexicana and L. amazonensis* amastigotes stages. To evaluate the potential of the tested quinazolines, an *in vitro* study of the most active derivatives against **2a–e, 2h, 2l, 2m, 2p, 2t** and **2u** was performed against intracellular amastigote stage of *L. braziliensis, L. mexicana* and *L. amazonensis* parasites. The intracellular amastigote model is one of the most important assays for the *in vitro* leishmanial evaluation, because of the amastigote stage is found in mammalian host and its infection causes the clinical manifestations of leishmaniasis [30a]. Traditionally, the intracellular amastigote are generated *in situ* from macrophage putted in presence of promastigote parasite under specific conditions (pH 5.5 at 37 °C). Nevertheless, this lab adapted strain could be more susceptible to the action of drug than isolate strain due to that the promastigote are found in axenic culture for many passages. Then, to obtain closer information to the reality, we decided to use intracellular amastigote isolates, which were directly extracted from lesion footpad in mouse infected with reference strains of *L. braziliensis, L. mexicana* and *L. amazonensis.* The murine amastigote isolates were maintained in culture for 48 h under specific conditions (pH 5.5 at 37 °C). This protocol was previously reported by our group [30b]. The therapeutic effect of the selected compounds was evaluated at 48 h post-treatment and, the results are expressed in terms of LD_{50} in Table 2. It should be noted that the

Table 2
Antileishmanial activity of the most active 2-aryl-quinazolin-4(*3H*)one derivatives against *L.braziliensis, L.mexicana* and *L.amazonensis* amastigotes stages.

Entries	Compounds	LD_{50} (μM)		
		L. mexicana	*L. braziliensis*	*L. amazonensis*
1	**2a**	36.93	**19.41**	44.52
2	**2b**	**16.90**	**20.75**	46.94
3	**2c**	34.77	28.38	> 50.00
4	**2d**	45.21	26.28	**21.32**
5	**2e**	27.67	24.38	> 50.00
6	**2f**	> 50.00	> 50.00	> 50.00
7	**2g**	39.98	> 50.00	> 50.00
8	**2h**	**11.04**	22.53	**29.34**
9	**2l**	**7.48**	**13.71**	**23.67**
10	**2p**	23.37	**18.93**	**29.68**
11	**2t**	**16.84**	**17.24**	N.D.
12	**2u**	**10.23**	**12.47**	N.D.
13	**Glt**[a]	**12.30**[b]	**14.38**	**22.71**

[a] Glucantime (Glt) was used as reference drug.
[b] Taken from Ref. [30b]. Note: marked in bold parameters indicated a pronounced antileishmanial activity. N.D.: Not determined. Results are the mean of three independent experiments with a SD less than 5% for all cases.

amastigote strains were less susceptible to the therapeutic action of the tested derivatives than promastigote strains, requiring higher concentration to suppress the half population. This fact may be associated to that: (i) the murine isolate possesses more sophisticated defence machinery than lab strain of promastigote, or (ii) the promastigote form is more susceptible to the action of these 2-arylquinazolin-4(3*H*)ones than amastigote form, which is common for many drugs or active molecules. Similarly to promastigote evaluation, *L. braziliensis* and *L. mexicana* parasites specie were more susceptible to drug action than *L. amazonensis* parasite. In general, the compounds **2d, 2h, 2l** and **2p** showed the best antiamastigote response against *L. amazonensis*. Compounds **2t** and **2u** were not evaluated against *L. amazonensis*. With regards to *L. braziliensis* and *L. mexicana,* the derivatives **2b, 2h, 2l, 2t** and **2u** displayed good antileishmanial responses, with LD_{50} values from 7.48 and 22.53 μM, respectively. Derivatives **2a** and **2p** only showed an acceptable activity against *L. braziliensis*, with LD_{50} of 19.41 and 10.43 μM, respectively. The rest of tested compounds showed a modest or poor antiamastigote response against the three *Leishmania* species. The antiamastigote response of the tested compounds showed a similar structure-activity relationship compared to the antipromastigote activity. In particular, compounds **2l** and **2u** showed similar trends to glucantime reference drug. From the results, compounds **2h, 2l, 2t** and **2u** were selected for further *in vitro* evaluations.

To validate the potential of these 2-arylquinazolin-4(3*H*)ones, we evaluated the effect of the most active derivatives **2h, 2l , 2t** and **2u** against antimony-resistant amastigote, clinical amastigote isolate and axenic amastigote, all them corresponded to *L. braziliensis* parasite specie. The antimony-resistant strain, called MHOM/VE/2017/GltR, was isolated from a mice not responding to glucantime therapy (see details in Supplementary material). The clinical human isolate, called MHOM/VE/2017/MR, was identified as a *L. braziliensis* specie and it was obtained from a Venezuelan cutaneous leishmaniasis patient (see details in Supplementary material). Axenic amastigote was obtained from promastigote under specific conditions of pH = 5.5 at 35 °C for 48 h. Then, all these amastigote strains were independently maintained in culture and the therapeutic effect of the derivative **2h, 2l, 2t** and **2u** was evaluated at 48 h post-treatment. Results are listed in Table 3. In general, the clinical amastigote strain was significantly less susceptible to the action of the 2-aryl-quinazolin-4(3*H*)ones than the glucantime-resistant and reference murine isolate. Specifically, clinical isolate showed an appreciable response at 50 μM for the most active quinazolinone. This fact putted in evidence that the clinical isolate can have a greater fitness and strong defence machinery than the reference and

Table 3
Activities of the most active 2-aryl-quinazolin-4(*3H*)one derivatives against *L. braziliensis* clinical isolate and glucantime-resistant strains.

Entries	Compds	Amastigote of *L. braziliensis* LD_{50} (μM)				RR[b]
		Axenic	Reference[a]	Antimony-resistant	Clinical isolate	
1	**2h**	**4.09**	22.53	44.13	> 50.00	1.96
2	**2l**	**2.73**	**11.48**	N.D.	N.D	N.D.
3	**2t**	**1.09**	**17.24**	**27.63**	> 50.00	1.60
4	**2u**	**1.28**	**12.47**	36.89	> 50.00	2.96
5	**Glt**	**N.D.**	**14.38**	> 50.00	> 50.00	N.D.

[a] Intracellular amastigote from mice infected with reference *L. braziliensis*.
[b] Ratio of resistance: LD_{50} of antimony-resitant amastigote/LD_{50} of reference amastigote.. Note: marked in bold parameters indicated a pronounced antileishmanial activity. N.D.: Not determined. Results are the mean of three independent experiments with a SD less than 5% for all cases.

antimony-resistant strain, which is completely expected due to that human amastigote isolate came from stronger and complex host as the humans. A direct comparison between the antimony-resistant amastigote and reference amastigote strains showed that reference amastigote are from 1.60 to 2.96-fold more susceptible to the action of 2-arylquinazolin-4(3*H*)one than antimony-resistant amastigote, being **2t** the most active derivative. Finally, it should be noted that axenic amastigote were significant more sensitive to the action of the most active compounds than reference, antimony-resistant and clinical amastigote isolates, which putted in evidence the susceptibility of this adapted lab amastigote strain compared to amastigote isolate strains. This last highlighted the importance of isolate strains to estimate the real potential of new agents against *leishmania* parasites. In general, the tested compounds displayed antiamastigote responses ranging from 1.09 to 4.09 μM against axenic amastigote, being the compounds **2t** and **2u** more potent than compounds **2h** and **2l**.

2.2. Toxicity assays on peritoneal macrophage and selectivity indexes

A promising antileishmanial candidate must follow two important conditions: (i) possess a high therapeutic effect on amastigote strains, and (ii) exhibit a low toxicological effect on macrophage host. Then, to evaluate the potential of our derivatives, an *in vitro* toxicological study on peritoneal macrophages derived from narrow mouse was performed for the most active compounds **2h, 2l, 2t** and **2u**. Toxicity results (CC_{50}) are listed in Table 4 together with their respective selective indexes related to promastigote and amastigote evaluations. In particular, the compounds **2h** and **2u** showed relatively low *in vitro* toxicities with CC_{50} values of 82 and 157 μM, respectively. Specifically, **2h** emerged as a potential antileishmanial candidate due to its good antipromastigote and antiamastigote activities, low toxicity and good selectivity indexes against promastigote and amastigote stages (S.I > 5) (see Table 4); although its weak activity against clinical amastigote strain may limit its projection toward the development of a new antileishmanial drug, but 2-arylquinanolin-4(3*H*)one can be used as platform to design alternative potent antileishmanial agents. More specific assays on human isolate as well as an *in vivo* evolution are needed to estimate the real potential of the compound **2h**. Compounds **2l** and **2u** were considered as a second line group of potential 2-aryl-quinazolin-4(3*H*)ones as antileishmanicidal, whereas **2t** was discarded by its relative high toxicity on peritoneal macrophages (CC_{50} = 49.56 μM).

2.3. Mechanism of action studies

In this section was investigated the capacity of our 2-arylquinzaolin-4(3*H*)ones to act as antifolate agent, which has been widely reported for quinazoline derivatives and their quinazolinone analogues [25,26]. The study was performed using *L. amazonensis* promastigote, which were

Table 4
Cytoxicity evaluation (CC_{50}) of the most active 2-aryl-quinazolin-4(*3H*)one derivatives against peritoneal macrophages and selectivity indexes.

Entries	Compds	*S.I.*[a]						*Macrophages* CC_{50} (μM)
		L. braziliensis		*L. mexicana*		*L. amazonensis*		
		P	*A*	*P*	*A*	*P*	*A*	
1	2h	**32.1**	**7.0**	**29.7**	**14.2**	**17.2**	**5.3**	156.90
2	2l	**7.7**	**2.7**	**5.7**	**7.8**	~1.0	**2.0**	58.12
3	2t	4.1	2.9	4.3	2.9	8.3	N.D.	49.56
4	2u	**13.4**	**6.6**	**11.4**	**6.0**	**10.4**	N.D.	82.12

[a] Selectivity index: CC_{50} of macrophages/LD_{50} of promastigote or amastigote of *Leishmania*. Note: P: promastigote, A: amastigote. Note: marked in bold parameters indicated a pronounced antileishmanial activity. N.D.: Not determined.

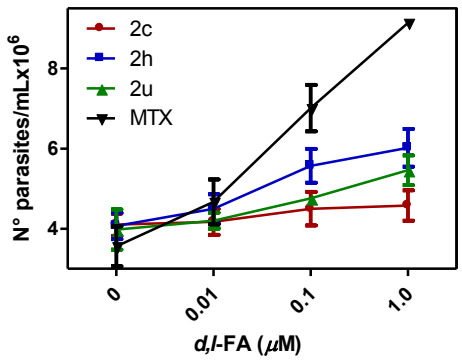


Fig. 1. *In vitro* efficacy of quinazolinones **2c, 2h** and **2t**, and methotrexate (MTX) for promastigotes of *L. amazonensis* in the presence or absence of *d,l*-folic acid (FA). Results are presented as number of parasites ($\times 10^6$) (in Y axis) in the presence of increasing concentration of FA (μM) (in X axis).

adapted to grow in media deficient in folic acid prior to susceptibility testing. The effect of the most active compounds on the proliferative growth of *L. amazonensis* promastigote was assessed in presence or absence of folic acid, following reported procedure [17]. In addition, susceptibility of antifolate drug methotrexate as positive control was estimated in the presence and absence of folic acid. Concentrations of 0.01, 0.1 and 1.0 μM were used for the folic acid. The effect of the compounds was measured at 72 h through a comparison between the resulting population after treatment with the corresponding quinazolinone or reference methotrexate. The assay was performed using the two most active derivatives **2h** and **2u** and a compound **2c** with a modest antileishmanial response. The EC_{50} of the compounds **2c, 2h** and **2u** against *L. amazonensis* was used as standard concentration for the antifolate assay. The results are shown graphically in Fig. 1. It is readily clear that the increase of folic acid concentration dramatically increased the cell growth promastigote, in particular under the action of the compounds **2h** and **2u**, which is corresponded with their respective biological activities (compare Fig. 1 and Table 1). For example, the compound **2c**, with a modest response against *L. amazonensis* (EC_{50} = 32.48 μM), exhibited a discrete increase of *Leishmania* population with the increase of folid acid in culture medium; while the most active derivatives **2h** (EC_{50} = 9.14 μM) and **2u** (EC_{50} = 7.87 μM) showed an appreciable antifolate effect. The methotrexate has a significant antifolate effect compared with the most active quinazolinones **2h** and **2u,** due to that MTX has a low EC_{50} value by about 0.2 μM [25a]. In summary, the synergistic behaviour found for the most active 2-arylquinazolin-4(3*H*)ones suggested that they can interfere with folate metabolism in *L. amazonensis* parasite, and probably may also affect the growth cellular in *L. braziliensis* and *L. mexicana* parasites either under their promastigote or amastigote stage.

In order to obtain more information about the antifolate action of the most active 2-arylquinazolin-4(3*H*)one **2h** and **2u** and confirm the experimental mechanistic findings, a tentative molecular docking study was performed on pteridine reductase 1 (PTR1) enzyme, unique antifolate enzyme of *Leishmania* available in Protein data Bank (PDB). This protein represents a pteridine reductase 1 (PTR1) and it is a good template to understand molecular bases of antifolate response. It is well documented that pteridine reductase 1 enzyme play an important role in the modulation of the dihydrofolate reductase-thymidylate synthase (DHFR-TS) enzymatic machinery and it can reduce the amount of folates into parasite [26c–26e]. Crystal structure of the ligand-PTR1 complex is available in the Protein Data Bank under code 1E7W for *Leishmania major* [31a]. Methotrexate (MTX) is co-crystallized a competitive inhibitor into crystal PTR1 protein. As was mentioned in the introduction, methotrexate is the most popular antifolate agent [25a,31b] and a comparative study about the interactions and location of methotrexate and our quinazolinones into active site permitted to understand the molecular bases involving in the possible inhibitory effect of the studied compounds. PTR1 enzyme has been used to study the nature of ligand-PTR1 complex of some potential antifolate agents [20]. Arguslab 4.0 software [32a] was used to perform the molecular docking of the most active 2-arylquinazolinones **2h** and **2u** on the mentioned enzyme. The geometry of the derivatives **2h** and **2u** was optimized using the AM1 semiempirical method [32b]. Docking data are listed in Table 5 and a representation of DHFR-ligand complex into active site is illustrated in Fig. 2. PTR1 enzyme possesses two active site pockets with similar residue composition and similar box size (see details in Supplementary material). Our molecular docking study was focused into ligand-protein derived from active site of chain A. The molecular docking showed that the most active 2-arylquinazolin-4(3*H*) ones **2h** and **2u** exhibited better binding energies (−8.67 and −-8.72 kcal/mol, respectively) into active site than MTX reference inhibitor (−8.37 kcal/mol). A detailed analysis of the ligand-enzyme complex into PTR1 active site permitted to appreciate that the two studied 2-arylquinazolin-4(3*H*)ones are confined almost in identical place into active site compared to methotrexate inhibitor, which suggested that our quinazolinones can act as a competitive inhibitor, although an enzymatic experimental assay on the PTR1 enzyme would help to elucidate the type of inhibition. It is important to mention that the fused-aromatic ring of the derivatives **2h** and **2u** are oriented in similar

Table 5
Binding energies (kcal/mol) of affinity quinazolinone-protein into binding site (chain A) of PTR1 enzyme.

Entries	Compds	*E* (kcal/mol)	*H*-interactions[b]	Hydrophobic interactions
1	**2h**	−8.67	No *H*-interactions	Phe-113, Leu-229, Pro-115, Met-233, Leu-188, Leu-189, Lys-198, Arg-17 and Asp-232.
2	**2u**	−8.72	Arg-17 (3.00 A°) Tyr-194 (2.95A°), Ser-111 (2.78A° and 2.89 A°)	
3	**MTX**[a]	−8.37		

[a] Reference substrate of methotrexate (MTX) taken from pteridine reductase 1 protein (PDB code: 1E7W).
[b] *H*-interactions (in A°) in PTR1 active site.

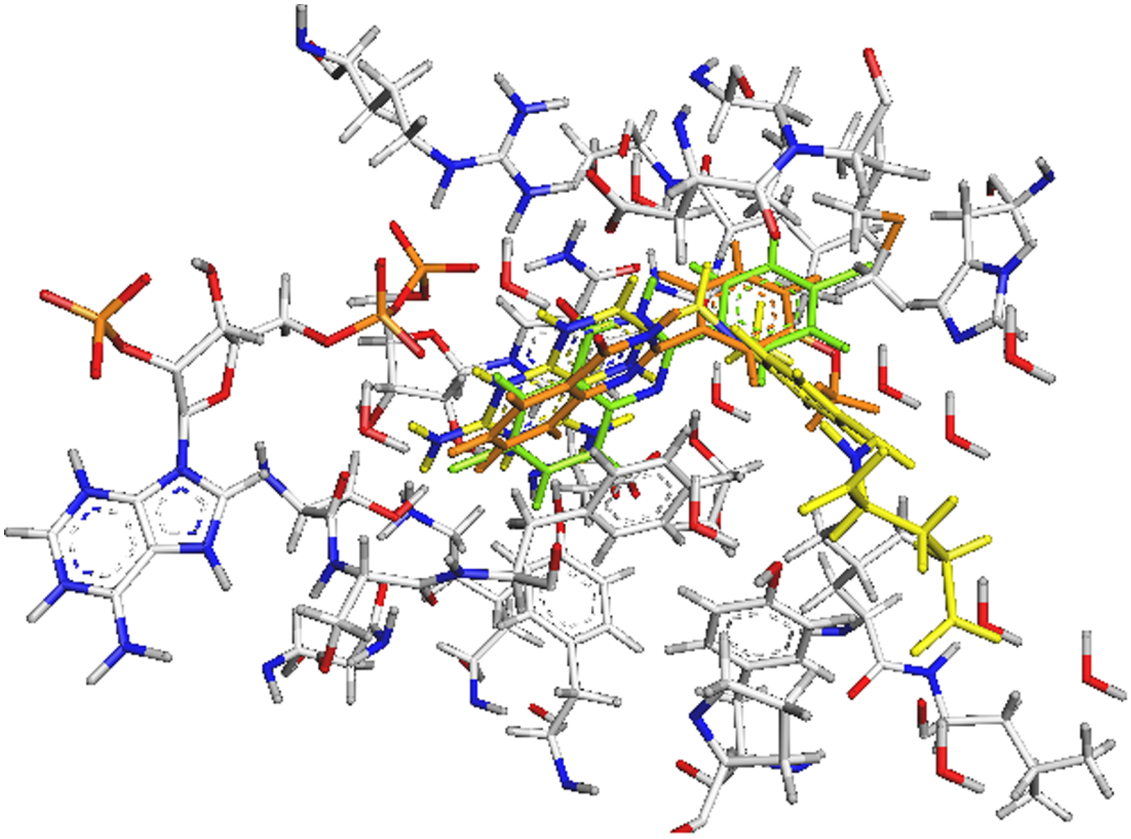


Fig. 2. Location of MTX reference drug (yellow stick) and the most active compounds **2h** (orange stick) and **2u** (green stick) into pteridine reductase 1 active site.

disposition compared to fused-ring of MTX with common hydrophobic and other intermolecular interactions such as the π-π interactions found between the phenyl ring of Phe-113 and fused-ring of tested 2-arylquinazolinones and MTX (see details in Supplementary Material). In other words, the two tested 2-arylquinazolinones are perfectly superimposed with MTX molecule into active site. This last suggested that the orientation of the quinazolin or pteridin cores into active site seem to be crucial in the antifolate response of any chemical systems based on the mentioned scaffolds.

Despite the tested 2-arylquinazolinone and methotrexate shared similar disposition into active site, different hydrogen bonding with essential residues into active site were detected for each one of them. Methotrexate exhibited hydrogen bonding with essential residues of active site such as Try-194 and Ser-111, while a hydrogen interaction with the Arg-17 residue were found for the derivative **2u** through the oxygen attached at 4-position of quinazolinone core. No hydrogen interaction with any residue was found for compound **2h**. With regards to the hydrophobic interactions, either derivatives **2h** and **2u** or MTX exhibited similar hydrophobic interactions with relevant residues into active site pocket such as Phe-113, Leu-229, Pro-115, Met-233, Leu-188, Leu-189, Lys-198, Arg-17 and Asp-232. In summary, experimental assay and molecular docking putted in evidence that the most active quinazolinones act as an antifolate agent and, further enzymatic experiments are needed to elucidate the specific inhibitory action of the quinazolinone due to that the folate pathway is mainly modulated by two enzyme such as pteridin reductase 1 (PTR1) and dihydrofolate reductase (DHFR).

In order to explore an alternative mechanism to understand the antiamastigote response of the studied 2-arylquinazolin-4(3*H*)ones, we decide to study its capacity to induce the generation of nitric oxide (NO) in intracellular amastigote model. It is well documented that the protein oxidation and nitration can alter functions in many metabolic enzymes in mitochondrial electron-transport chain, including nicotinamide adenine dinucleotide dehydrogenase, cytochorme *c* and adenosine triphosphate synthase [33a]. A direct correlation has been identified between the generation of nitric oxide by macrophage and intracellular parasite damage, reflecting the cytotoxic nature of the NO molecule [33b–33c]. Based on this principle, we verified whether the antiamastigote response found for our most active derivatives **2h** and **2u** is associated to the generation of nitric oxide from macrophage. The intracellular amastigote culture was used to perform this mechanistic protocol. The strains were exposed to increasing concentration of the compounds **2h** and **2u** and at 72 h post-treatment was estimated the amount of nitric oxide generated by the action of the compounds. Results are listed in Fig. 3. In general, a modest amount of nitric oxide was observed for the parasite treated with the most active derivatives compared to the untreated parasite, observing a barely increase of the

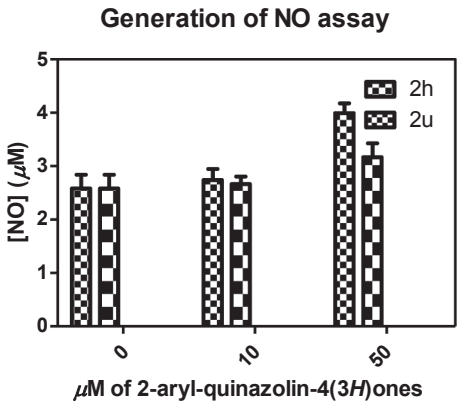


Fig. 3. Effect of the 2-arylquinazolin-4(3*H*)-ones **2h** and **2u** at increasing concentration 0, 10 and 50 μM on the production of nitrite (nitric oxide metabolite) for the macrophages infected with *L. braziliensis*.

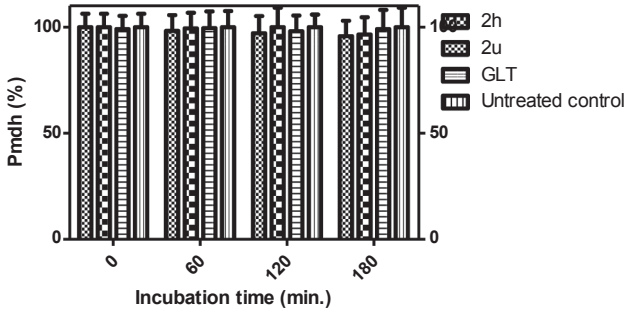


Fig. 4. Percentage of mitochondrial dehydrogenase (Pmdh) (%) (Y axis) of the 2-arylquinazolin-4(3*H*)-ones **2h** and **2u** as function of time (minutes, X axis).

production of nitric oxide to increasing concentration of the 2-arylquinozalin-4(3*H*)one. Thus, it possible suggests that these compounds induce to a small production of nitric oxide into macrophage that can affect the amastigote cell; however, the therapeutic effect of these 2-arylquinazolin-4(3*H*)ones is associated mainly to an antifolate mechanism as was previously demonstrated in the present report.

On the other hand, to discard that the antileishmanial activity of our compounds may be associated to the generation of any radical oxidative (RO) or nitrosative (RN) species by metabolized quinazolinones into parasite, we evaluated the mitochondrial dehydrogenase activity of the most active compounds at short times from 0 to 180 min. It is well documented that oxidative or nitrosative species affect the mitochondrial dehydrogenase activity and its measurement at short time can permit us to estimate if an oxidative mechanism can be operating into parasite culture. The generation of these radical species can damage essential macromolecules such as DNA, protein and lipids within mitochondria [34]. With this in mind, the percentage of mitochondrial dehydrogenase activities (Pmdh) with respect to untreated control and reference drug was assessed using the colorimetric MTT assay at very short times, from 30 min. and 180 min. of incubation, according to standard protocols reported for *Leishmania* parasite [24a,34f]. Results are shown in Fig. 4. The EC_{50} against *L. braziliensis* was used as standard concentration of the compounds **2h** and **2u**. The Pdmh (percent of mitochondrial dehydrogenase activity) was taken from resulting population as function of time. It should be noted that neither **2h** nor **2u** affected the mitochondrial dehydrogenase activity at short time, which suggests that they do not lead the generation of any radical into parasite. In summary, the mode of action of the most active 2-arylquinazolin-4(3*H*)ones seems to be mainly associated to an antifolate mechanism; however, their antileismanial response may also be caused by other alternative target modulation.

As last topic, an *in silico* drug-likeness study was performed on the tested compounds using Chemdraw Ultra suite software [35a]. Then, the parameters of Lipinskís rules, which can predict oral bioavailability and membrane permeability of synthetic drugs [35b], were calculated for the 21 derivatives. Results are summarized in Table 6. In general, all quinazolinone derivatives displayed good bioavailability properties, with a molecular weight < 500 g/mol, acceptor and donor hydrogen bond interactions below 10 and 5. Nevertheless, the calculated partition coefficient values (Log *P*) are closer to the optimum Lipinski value of 5 in most of the studied cases. This last indicates that all tested quinazolinone have a low water solubility character, which may compromise the absorption properties of these compounds [36]. For example, the weakest antileishmanial responses found for the compounds **2f** (4Br), **2j** (3Br), **2q, 2r, 2s** can be associated with their corresponding higher Log *P* compared to the rest of compounds. Other compounds with relatively high Log *P* such as chlorine (**2c, 2g**) or methyl derivatives (**2e, 2i**) showed poor biological activity in most of studied compounds, confirming the importance of this parameter in the biological effect. To complement our *in silico* evaluation, we calculated other molecular descriptors such as the topological polar surface area and the absorption percentage (% Abs). This study reflected the potential intestinal absorption, bioavailability and hematoencephalic barrier permeation of our compounds according to Veber's rules (TPSA < 140 A^2) [37], with TPSA ranging from 41.46 to 63.16 A^2 and % absorption between 87.72 and 94.70%.

3. Conclusion

The antileishmanial activity of a series of 2-aryl-quinazolin-4(3*H*)-ones **2a-u** was carried out on a group of *leishmania* responsible of cutaneous leishmaniasis such as *L. braziliensis, L. mexicana and L. amazonensis*. From the biological evaluation, the derivatives **2h, 2l** and **2u** displayed a good *in vitro* activity against promastigote and reference intracellular amastigote isolate of the three *Leishmania* species, with low-micromolar and moderate IC_{50} responses, low toxicities by about 82–157 μM, moderate response against antimony-resistant amastigote

Table 6
Calculated theoretical parameters of the Lipinskís ruler for the 2-arylquinazolin-4(3*H*)-ones **2a-u.**

Entries	Compds	M.W. (g/mol)	% Absorption	Log *P*	TPSA	HA	HD	ROTB	Lipinskís rule violations
1	**2a**	222.08	94.70	2.87	41.46	3	1	1	0
2	**2b**	240.07	94.70	3.03	41.46	4	1	1	0
3	**2c**	256.04	94.70	3.43	41.46	3	1	1	0
4	**2d**	252.09	91.51	2.75	50.69	4	1	3	0
5	**2e**	236.09	94.70	3.36	41.46	3	1	2	0
6	**2f**	299.99	94.70	3.70	41.46	3	1	1	0
7	**2g**	256.04	94.70	3.43	41.46	3	1	1	0
8	**2h**	252.09	91.51	2.75	50.69	4	1	3	0
9	**2i**	236.09	94.70	3.36	41.46	3	1	2	0
10	**2j**	299.99	94.70	3.70	41.46	3	1	1	0
11	**2k**	240.07	94.70	3.03	41.46	3	1	1	0
12	**2l**	252.09	91.51	2.75	50.69	4	1	3	0
13	**2m**	238.07	87.72	2.48	61.69	4	2	2	0
14	**2n**	282.10	88.33	2.62	59.92	5	1	4	0
15	**2o**	282.10	88.33	2.62	59.92	5	1	4	0
16	**2p**	212.06	91.51	1.49	50.69	4	1	1	0
17	**2q**	351.16	87.21	3.22	63.16	6	1	10	0
18	**2r**	321.15	90.39	3.35	53.93	5	1	9	0
19	**2s**	272.09	94.70	3.87	41.46	3	1	1	0
20	**2t**	256.04	94.70	3.43	41.46	3	1	1	0
21	**2u**	290.00	94.70	3.99	41.46	3	1	1	0

Percentage of absorption is calculated by % Absorption = 109 − (0.345 × TPSA). TPSA, topological polar surface area, Log *P* (partition coefficient) calculated by Chemdraw program package, HA hydrogen bond acceptors, HD hydrogen bond donors, ROTB number of rotatable bonds.

and acceptable selectivity indexes between 5 and 10. Specifically, compound **2h** emerged as a promising antileishmanial agent for further *in vivo* and pharmacokinetic assays as well as further structural optimization. A structure-activity relationship study suggests that the chlorine atom at position 6 of quinazolin fused ring as well as the methoxy substitution at position 2 or 3 on Y aryl ring play an important role in the biological activity of these 2-arylquinazolin-4(3*H*)ones. All active compounds followed the Lipinskís parameters, having a good theoretical bioavailability. Experimental mechanistic and molecular dockings studies permitted to suggest that the active 2-arylquinazolin-4(3*H*)one act mainly through an antifolate mechanism. The antileishmanial efficacy of the most active 2-aryl-quinazolin-4(3*H*)-ones as well as its ease synthetic procedure indicate that 2-arylquinazolin core is a promising system for the development of new potent antileishmanial agents.

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Conflict of interest

The author reports no conflicts of interest. The author is responsible for the content and writing of this article.

Appendix A. Supplementary material

Biological procedures and the supplementary data associated to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.10.028 as an Supplementary material document. An additional pdb document with molecular docking details is deposited at www.sciencedirect.com.

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