Experimental Parasitology xxx (2017) 1–8

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

In vitro activity of new *N*-benzyl-1*H*-benzimidazol-2-amine derivatives against cutaneous, mucocutaneous and visceral *Leishmania* species

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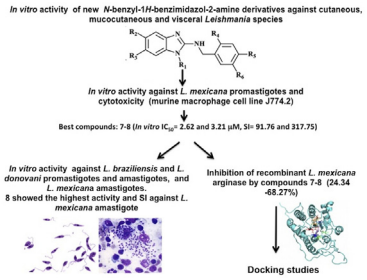
HIGHLIGHTS

- A series of 28 *N*-benzyl-1*H*-benzimidazol-2-amine derivatives were synthesized.
- Compounds **7** and **8** were very active against *L. mexicana* promastigotes and amastigotes.
- Compound **8** had high selectivity index against *L. mexicana* and *L. braziliensis*.
- Compound **8** inhibited 68.27% the activity of recombinant *Leishmania mexicana* Arginase.
- Compound **8** is a good scaffold for the development of new antileishmanial agents.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 5 July 2017
Received in revised form
20 October 2017
Accepted 25 November 2017
Available online xxx

Keywords:
Benzimidazole derivatives
Antileishmanial activity

ABSTRACT

The identification of specific therapeutic targets and the development of new drugs against leishmaniasis are urgently needed, since chemotherapy currently available for its treatment has several problems including many adverse side effects. In an effort to develop new antileishmanial drugs, in the present study a series of 28 *N*-benzyl-1*H*-benzimidazol-2-amine derivatives was synthesized and evaluated *in vitro* against *Leishmania mexicana* promastigotes. Compounds **7** and **8** with the highest antileishmanial activity (micromolar) and lower cytotoxicity than miltefosine and amphotericin B were selected to evaluate their activity against *L. braziliensis* 9and *L. donovani,* species causative of mucocutaneous and visceral leishmaniasis, respectively. Compound **7** showed significantly higher activity against *L. braziliensis* promastigotes than compound **8** and slightly lower than miltefosine. Compounds **7** and **8** had IC_{50} values in the micromolar range against the amastigote of *L. mexicana and L. braziliensis.*

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https://doi.org/10.1016/j.exppara.2017.11.009
0014-4894/

Selectivity Index
Arginase

However, both compounds did not show better activity against *L. donovani* than miltefosine. Compound **8** showed the highest SI against both parasite stages of *L. mexicana*. In addition, compound **8** inhibited 68.27% the activity of recombinant *L. mexicana* arginase (LmARG), a therapeutic target for the treatment of leishmaniasis. Docking studies were also performed in order to establish the possible mechanism of action by which this compound exerts its inhibitory effect. Compound **8** shows promising potential for the development of more potent antileishmanial benzimidazole derivatives.

1. Introduction

Leishmaniasis is a disease caused by several species of the genus *Leishmania,* having a wide range of hosts, including man. It is considered a neglected tropical disease (NTDs), affecting mainly developing countries. The World Health Organization (WHO) estimates that approximately 350 million people are living in areas characterized by active transmission of *Leishmania,* with 14 million people directly affected by the disease. There are three main types of leishmaniasis: cutaneous (CL), the most common form of the disease, causes ulcers, leading to disfigurement, permanent scars and in some cases disability; mucocutaneous (MCL), the most destructive form of the disease, causes partial or total mutilation of mucous membranes in the nose, mouth and throat; and visceral kala–azar (VL), the most severe form of the disease, fatal if left untreated. It is estimated that there are 300,000 cases of VL and more than 20,000 to 40,000 annual deaths from this form of the disease; in the case of CL, it has been reported over one million cases around the world in the last 5 years (WHO, 2016).

The main drugs available for the treatment of leishmaniasis are the pentavalent antimonials (SbV), Sodium Stiboglucanate (Pentostam), Meglumine Antimoniate (Glucantime), and Amphotericin B (Fungizone). Amphotericin B is the first drug of choice for visceral leishmaniasis in regions with high resistance to treatment with SbV. Miltefosine is the most recent antileishmanial drug in the market and the first effective oral treatment against VL, being recommended as first line drug for childhood VL (Freitas-Junior et al., 2012). Chemotherapy currently available for leishmaniasis treatment is far from satisfactory and has several problems including many adverse side effects, high costs and toxicity (Savoia, 2015; Varela-M et al., 2012). Furthermore, drug resistance to all known antileishmanial drugs has been reported (Bhattacharya et al., 2016; Fernandes et al., 2016: Mondelaers et al., 2016; Shaw et al., 2016; Coelho et al., 2014; Kumar et al., 2014). Therefore, the identification of specific therapeutic targets and the development of new drugs are urgently needed.

In this regard, benzimidazole derivatives are of wide interest because of their biological activities and clinical applications (Alhtar et al., 2016). Their use as antibacterial, antifungal, antimalarial, antileishmanial as well as anti-inflammatory and anticancer agents was reviewed by Keri et al. (2015). Our research group has demonstrated the antiprotozoal activity of benzimidazole derivatives against *Giardia intestinalis*, *Entamoeba histolytica*, *Trichomonas vaginalis*. *L. mexicana* and *Trypanosoma cruzi* (Melchor-Doncel de la Torre et al., 2017; Velázquez-López et al., 2016; Díaz-Chiguer et al., 2012; Hernández-Luis et al., 2010; Navarrete-Vázquez et al., 2001; Valdez et al., 2002). Among them, 2-(trifluoromethyl)-1*H*-benzimidazole derivatives showed promising *in vitro* activity against *L. mexicana* with IC_{50} values in the range of 4–24 μM (Hernández-Luis et al., 2010). The antileishmanial activity of benzimidazole derivatives has also been demonstrated in other studies (Méndez-Cuesta et al., 2016; Mota et al., 2014; Oh et al., 2014). Recently, 2-arylbenzimidazole derivatives have proven to be good candidates as antileishmanial agents (Keurulainen et al., 2015); however, this kind of compounds have high carboaromatic rings and rigid systems, these properties are associated with low solubility and pharmacokinetics/pharmacodynamics problems. Besides, the target of these derivatives on *Leishmania* was not characterized.

Among druggable targets of the parasite, important for parasite survival and proliferation, is the enzyme arginase (ARG) that participates in the polyamine pathway (Balaña-Fouce et al., 2012). L-ornithine, the amino acid from which polyamines are generated, is produced from the hydrolysis of L-arginine by ARG. Inhibition of ARG by *N*-hydroxyarginine (NOHA) reduces polyamine levels in *Leishmania* amastigotes and parasite load (Iniesta et al., 2001). *Leishmania* ARG shares 39–43% identity with human ARG (Ilari et al., 2015); therefore, it is considered a therapeutic target for the treatment of leishmaniasis.

Previously, we performed a virtual screening study of ZINC database on the LmARG in order to find inhibitors. From this study, compounds with *N*-benzyl-1*H*-benzimidazole-2-amine scaffold were identified as potential LmARG inhibitors (Méndez-Cuesta et al., 2012). Inspired in these results and continuing our search for benzimidazole derivatives with antileishmanial activity, a new series of *N*-benzyl-1*H*-benzimidazol-2-amine derivatives was synthesized. The main features in these compounds are the substituent at position 1 of the benzimidazole nucleus, from C_4- alkyl groups to hydrogen; the substitution in the benzenoid ring, with or without chlorine; and a substituted benzyl group on the 2-amino to increase the flexibility of the compounds. Nine compounds are not substituted at positions 5 and/or 6 of the benzimidazole nucleus, but the others have one or two chlorine atoms at positions 5 and 6. These compounds will give information about how the activity is affected with the different substitutions, especially, when positions 5 and 6 of the benzimidazole nucleus are substituted, since it is known that these positions undergo the first step metabolism, which could increase the half-life of a possible drug.

The biological activity of the new compounds was initially evaluated against promastigotes of *L. mexicana* and those with the highest antileishmanial activity and lower cytotoxicity than miltefosine and amphotericin B were further tested against the promastigote and amastigote of *L. braziliensis*, and *L. donovani* as well as *L. mexicana* amastigotes. In addition, the effect of compounds **7** and **8** on LmARG activity was also evaluated, and in order to know how these benzimidazole derivatives inhibit the LmARG activity, additional *in silico* docking study was performed.

2. Materials and methods

2.1. Chemistry

All *N*-benzyl-1*H*-benzimidazol-2-amine derivatives **1**–**28** were synthesized by our research group thought a reductive amination method between 1*H*-benzimidazol-2-amines **29**–**39** and aldehydes **40**–**47**. The structure of target compounds is shown in

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Scheme 1. The final compounds were purified by recrystallization and their structures were confirmed by ^{1}H NMR and ^{13}C NMR spectroscopy and elemental analyses (see Supplementary Information).

2.2. Parasites

Leishmania species used: *L. mexicana* strain MNYC/BZ/62/M379, *L. braziliensis strain* MHOM/BR/75/M2903 and *L. donovani* strain MHOM/IN/80/DD. Promastigotes were grown in Schneider's Insect Medium (Sigma-Aldrich) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and 100 U/mL of penicillin plus 100 μg/mL of streptomycin (Sigma-Aldrich), in 25 mL culture flasks. Parasites grown for 4 days and 7 days were used to evaluate the activity of the synthesized compounds against promastigotes and intracellular amastigotes, respectively.

2.3. Activity evaluation of benzimidazole derivatives against promastigotes of Leishmania spp

The activity of all compounds was initially evaluated against promastigotes of *L. mexicana* and those with the highest antileishmanial activity and lower cytotoxicity than miltefosine and amphotericin B were further tested against promastigotes of *L. braziliensis* and *L. donovani* using the resazurin method previously described by Bilbao-Ramos et al. (2012). For this, 2.5×10^6 parasites/well were cultured in 96-well microliter plates, compounds were dissolved in DMSO and diluted in the culture medium at concentrations ranging from 100 to 0.8 μg/mL in a final volume of 200 μL. After incubation for 48 h at 26 °C, 20 μL of 2.5 mM resazurin solution were added to each well, and incubated for 3 h. The fluorescence intensity (535 nm −excitation wavelength and 590 nm −emission wavelength) was measured in a fluorometer (Infinite 200, Tecan Group Ltd, Männedorf, Switzerland). All assays were carried out in triplicate. The half maximal inhibitory concentration (IC_{50}) was determined by a Probit analysis.

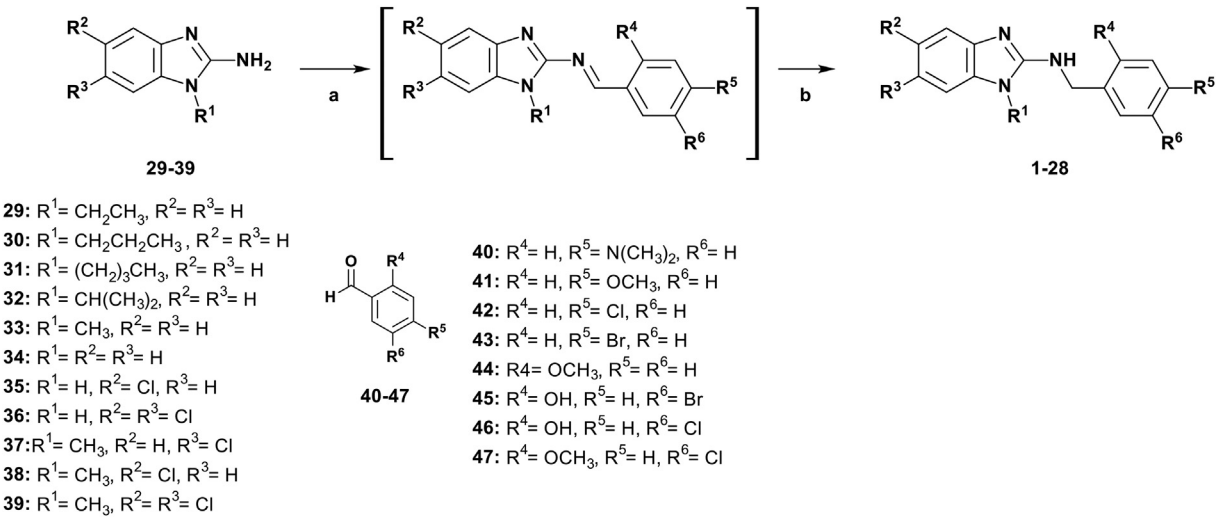
2.4. Macrophage cytotoxicity assays

The cytotoxicity of all compounds was tested using the murine macrophage cell line J774.2 (ATCC®TIB-67). For this, macrophages were seeded (5×10^4 cells/well) in 96-well flat-bottomed microplates and allowed to adhere for 24 h at 37 °C in 5% CO_2. The culture medium was then replaced with different concentrations of the compounds (100−0.8 μg/mL), followed by incubation for another 24 h. Miltefosine and amphotericin B were included as reference drugs. All assays were carried out in triplicate. Thereafter, 20 μL of a 2.5 mM resazurin solution were added to each well and the plates were incubated for another 3 h. The fluorescence emission was measured as described above. All assays were carried out in triplicate. The half maximal cytotoxicity concentration (CC_{50}) was determined by a Probit analysis.

2.5. Activity evaluation of compounds 7 and 8 against amastigotes of Leishmania spp

The activity of compounds **7** and **8** was evaluated against amastigotes of *L. mexicana, L. braziliensis and L. donovani,* species representative of the three main clinical manifestations of leishmaniasis.

The activity of compounds **7** and **8** was evaluated using the resazurin method (Bilbao-Ramos et al., 2012). Briefly, 5×10^4 macrophages and stationary promastigotes at a 1:10 ratio in 200 μL/well of culture medium were seeded and the plates were incubated for 24 h at 33 °C, 5% CO_2 in humidity chamber. The temperature was then increased to 37 °C for another 24 h. The cells were then washed several times to remove free non-infective promastigotes, and the final washing medium was replaced with 200 μL/well of culture medium containing 2-fold serial dilutions of the benzimidazole compound ranging from 100 to 0.8 μg/mL. In these assays, miltefosine was used as reference drug since it is used for the treatment of all three forms of leishmaniasis (Vakil et al., 2015). Amphotericin B was also used as reference drug for *L. mexicana* amastigote. After incubation of the plates for 48 h at 37 °C in 5% CO_2, the culture medium was replaced with an equal volume of lysis solution (Schneider's with 0.048% HEPES and 0.01% SDS) and maintained at room temperature for 20 min. The lysis solution was then replaced with Schneider's medium followed by incubation at 26 °C for another 3 days to allow transformation of viable amastigotes into promastigotes and their subsequent proliferation. Aliquots of 20 μL of 2.5 mM resazurin were added to each well and the plates were incubated for 3 h. Finally, fluorescence emission was measured as described above. All assays were carried out in triplicate. The half maximal inhibitory concentration (IC_{50}) was



Scheme 1. Synthesis and chemical structure of *N*-benzyl-1*H*-benzimidazol-2-amines (**1**–**28**). Reagents and conditions: (a) substituted benzaldehyde **40**–**47**, toluene, reflux, (b) $NaBH_4$, MeOH, 0 °C to room temperature.

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determined by a Probit analysis.

2.6. Determination of the inhibitory effect of compounds 7 and 8 on recombinant LmARG activity

The effect of compounds **7** and **8** on LmARG activity was determined using the QuantiChom™ Arginase Assay Kit (DARG-100). Recombinant LmARG was incubated with compounds at 40 μM for 1 h at 25 °C, as recommended by Riley et al. (2011). Non-treated enzyme was included as negative control and the ARG specific inhibitor Nor-NOHA (40 μM) was used as positive control. In order to evaluate the effect of **7** and **8** on human ARG (HsARG), total extracts were obtained from THP-1 human differentiated macrophages treated with **7** and **8** at 40 μM for 24 h at 37 °C. Total extracts from non-treated macrophages were included as negative control and the inhibitor Nor-NOHA (40 μM) was used as positive control. Recombinant LmARG and total extracts were incubated with L-arginine and urea production was quantified as indicative of ARG activity, following the supplier's instructions. The arginase activity (units per litter of sample (U/L) was determined by measuring absorbance at 430 nm in an ELISA reader and calculated following the supplier's instructions. Experiments were carried out in duplicate and the percentage of inhibition in relation to control was calculated.

To determine the IC_{50} of **8** against recombinant LmARG the QuantiChom™ Arginase Assay Kit was used. For this, recombinant LmARG was incubated with **8** at different concentrations and the arginase activity determined as mentioned above. Experiments were carried out in duplicate. The percentage of inhibition was calculated and the IC_{50} value was determined by a Probit analysis.

2.7. Molecular docking

Compounds **7** and **8** were built and optimized through an energy minimization in Spartan'10 (Wavefunction Inc, 2010) using PM6 semi-empirical method. Polar hydrogens, rotatable bonds and Gasteiger-Marsilli atomic charges were computed utilizing MGLTools 1.5.6 (Sanner, 1999). The tridimensional structure of LmARG was obtained from the Protein Data Bank (PDB ID: 5HJA) (Hai and Christianson, 2016). During protein preparation, water molecules and the co-crystal ligand were deleted from the protein, while the manganese atoms (Mn^{2+}) were conserved for the calculations. The structure of LmARG was subjected to an energy minimization employing the AMBER99SB force field by 1000 steepest-descent minimization steps implemented in GROMACS 4.5.5 software. Then, MGLTools 1.5.6 was used to merge all non-polar hydrogens and to assign Gasteiger charges for each atom of the macromolecule. Docking calculations were performed using the AutoDock 4.2 software (Morris et al., 2009; Huey et al., 2007). A grid box of 50 × 50 × 50 points with a grid spacing of 0.375 Å was calculated for the proper atom types and centered in the catalytic site. The Lamarckian genetic algorithm was used as a search method with a total of 20 runs being undertaken with a maximum number of 5,000,000 energy evaluations and initial populations of 150 conformers. The clusters were ranked by the lowest energy representative of each cluster. All molecular graphics were prepared with PyMOL 0.99 version.

2.8. Multiple-sequence alignment of ARG amino acid sequence of Leishmania spp

Alignments of the amino acid sequence of ARG from *L. mexicana* (UniProt: **Q6TUJ5**), *L. braziliensis* (UniProt: **A4HMH0**), and *L. donovani* (UniProt: **A0A0M4CX5**) were performed with the Clustal-W program server (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Larkin et al., 2007).

2.9. Statistical analysis

All the data was expressed as mean ± standard deviation. ANOVA and T3 Dunnett's Post hoc test was performed using IBP SPSS Statistics, in order to determine differences between groups by analyzing statistical significance. Differences were considered significant at 0.05 level of confidence.

3. Results and discussion

Compounds **1**–**28** were synthesized by straightforward reductive amination method with moderate to good yields. The synthesized compounds were purified by crystallization from the adequate solvent or mixture of solvents. Spectrometric and spectroscopic data obtained were consistent with the expected structures.

The activity of compounds **1**–**28** was evaluated against *L. mexicana* promastigotes. Results are presented in Table 1. It is seen that compounds **7**, **8** and **19** were more active than miltefosine, while **4**, **9** and **11** had similar IC_{50} values. Compounds **1**–**28** were less active than amphotericin B. Compounds **7** and **8** showed the highest antileishmanial activity with IC_{50} values of 2.62 μM and 3.21 μM, respectively, being 5.8 and 4.8 times significantly more active than miltefosine ($P \leq 0.05$). It is important to mention that the IC_{50} value of miltefosine obtained in this study against the promastigote of *L. mexicana* (15.34 μM) is in agreement with values reported in other studies (15.7 and 13.45 μM) (Wijnant et al., 2017; Enciso et al., 2016). In relation to the IC_{50} value of amphotericin B against *L. mexicana* promastigote reported herein (0.95 μM), it is in the range of the IC_{50} values reported by Al-Abdely et al. (1998) against two different *L. mexicana* isolates (0.5 and 1.0 μM). However, Varela-M et al. (2012) obtained an IC_{50} of 2.7 μM for miltefosine and in the case of amphotericin B Escobar et al. (2002) reported an IC_{50} in the range of 0.22–0.35 μM. Difference in IC_{50} values can be attributed to different factors such as the parasite strain, growth medium and method used for IC_{50} determination in the assays.

Compounds **7** and **8**, with no substituent in the benzenoid ring, have a methyl group at position 1 of the benzimidazole nucleus and an oxygen at position 2 of the benzyl moiety, whether as a hydroxyl (**7**) or as a methoxide group (**8**). Modification of this substitution pattern leads to a detriment in the activity, as seen in compounds **1**–**5** with an alkyl group from C2 to C4 and no oxygen at position 2 of the benzyl moiety. In general, all compounds with chlorine atoms in the benzenoid ring, regardless of having a 1-methyl and an oxygen at position 2 of the benzyl moiety, were less active than the reference drug, except **19** (5-chloro-*N*-(5-chloro-2-hydroxybenzyl)-1-methyl-1*H*-benzimidazol-2-amine). The same applies for compounds with 1-H, except **11** (5-chloro-*N*-(2-methoxybenzyl)-1*H*-benzimidazol-2-amine) that is as active as miltefosine.

The cytotoxicity of all compounds was tested using the murine macrophage cell line J774.2; results are presented in Table 1. CC_{50} values for the reference drugs, amphotericin B and miltefosine were of 6.19 μM and 157.03 μM, respectively. All compounds were less cytotoxic than amphotericin B. On the other hand, compounds **2**, **7**–**10**, **13**, **16**, **19**, **25**–**28** were less cytotoxic than miltefosine. CC_{50} value obtained for the reference drug, miltefosine, is agreement with the value reported by Dea-Ayuela et al. (2016) using murine macrophage cell line J774.2 (136.4 μM). It is important to mention that other authors using peritoneal macrophages obtained CC_{50} values for miltefosine in the range of 87.30 μM–241.4 μM (Stroppa et al., 2017; Tempone et al., 2017; Bezerra-Souza et al., 2016;

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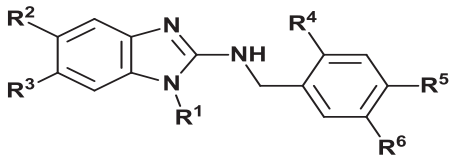
Table 1
Inhibition of proliferation of *Leishmania mexicana* promastigote, cytotoxicity and selectivity index of compounds **1**–**28**, miltefosine and amphotericin B.



Compound	R^1	R^2	R^3	R^4	R^5	R^6	[a]IC_{50} (μM)± SD	[b]CC_{50} (μM)± SD	[c]SI
1	CH_3CH_2	H	H	H	$N(CH_3)_2$	H	107.09 ± 7.75	84.17 ± 0.49	0.78
2	$CH_3CH_2CH_2$	H	H	H	OCH_3	H	78.69 ± 3.74	302.55 ± 4.42	3.84
3	$CH_3(CH_2)_2CH_2$	H	H	H	Cl	H	32.19 ± 1.50	61.39 ± 0.49	1.91
4	$(CH_3)_2CH$	H	H	H	Br	H	19.60 ± 1.87	68.09 ± 0.54	3.47
5	CH_3CH_2	H	H	H	OCH_3	H	83.15 ± 2.17	70.67 ± 0.65	0.85
6	CH_3	H	H	H	Br	H	32.06 ± 2.18	100.13 ± 1.33	3.12
7	CH_3	H	H	OH	H	Br	2.62 ± 0.79	240.43 ± 2.27	91.76
8	CH_3	H	H	OCH_3	H	H	3.21 ± 0.89	1020 ± 8.5	317.75
9	CH_3	H	H	OH	H	Cl	13.98 ± 1.83	196.44 ± 3.34	14.05
10	H	H	H	OCH_3	H	H	200.47 ± 6.16	978.80 ± 5.18	4.88
11	H	Cl	H	OCH_3	H	H	14.53 ± 1.54	65.59 ± 4.17	4.51
12	H	Cl	Cl	OCH_3	H	H	406.56 ± 9.55	75.11 ± 1.36	0.18
13	CH_3	Cl	H	OCH_3	H	H	324.45 ± 6.14	288.35 ± 4.05	0.89
14	CH_3	H	Cl	OCH_3	H	H	225.14 ± 4.06	65.29 ± 1.31	0.29
15	CH_3	Cl	Cl	OCH_3	H	H	438.57 ± 10.62	87.13 ± 1.92	0.19
16	H	H	H	OH	H	Cl	431.49 ± 24.76	169.09 ± 2.05	0.39
17	H	Cl	H	OH	H	Cl	351.03 ± 13.57	30.18 ± 1.94	0.09
18	H	Cl	Cl	OH	H	Cl	187.10 ± 10.52	40.42 ± 2.81	0.22
19	CH_3	Cl	H	OH	H	Cl	8.01 ± 1.38	318.94 ± 3.83	39.84
20	CH_3	H	Cl	OH	H	Cl	217.87 ± 15.55	47.90 ± 2.41	0.22
21	CH_3	Cl	Cl	OH	H	Cl	62.45 ± 6.60	34.02 ± 2.38	0.55
22	H	H	H	OCH_3	H	Cl	106.06 ± 10.01	39.96 ± 2.05	0.38
23	H	Cl	H	OCH_3	H	Cl	164.78 ± 9.57	50.61 ± 1.86	0.31
24	H	Cl	Cl	OCH_3	H	Cl	89.14 ± 3.45	26.81 ± 2.08	0.30
25	CH_3	H	H	OCH_3	H	Cl	129.34 ± 6.35	352.08 ± 9.45	2.72
26	CH_3	Cl	H	OCH_3	H	Cl	129.95 ± 5.99	197.43 ± 5.73	1.52
27	CH_3	H	Cl	OCH_3	H	Cl	145.95 ± 5.47	267.56 ± 4.64	1.83
28	CH_3	Cl	Cl	OCH_3	H	Cl	47.48 ± 1.73	318.35 ± 5.98	6.71
miltefosine	–						15.34 ± 0.36	157.03 ± 2.86	10.23
amphotericin B	–						0.95 ± 0.0598	6.19 ± 1.614	6.5

SD: Standard deviation of three independent determinations.
[a] IC_{50}: Compound concentration that produced a 50% reduction in parasites.
[b] CC_{50}: Compound concentration that produced a 50% reduction in J774.2 Cell.
[c] SI: Selectivity Index = CC_{50}/IC_{50}.

Coimbra et al., 2016; Dea-Ayuela et al., 2016). In relation to CC_{50} value for amphotericin B, Garcia et al. (2017) obtained a CC_{50} of 8.4 μM using murine macrophage cell line J774, meanwhile Ribeiro et al. (2014) using murine peritoneal macrophages reported a CC_{50} of 1.08 μM. Different CC_{50} values can be due to the cell type and determination method used in the assays.

Importantly, compounds **7**, **8**, **9** and **19** showed higher Selectivity Index (SI) (CC_{50}/IC_{50}) than the reference drugs, miltefosine and amphotericin B. Compounds **7** and **8** had the highest SI (91.76 and 317.75, respectively) towards *L. mexicana* promastigotes in comparison to miltefosine and amphotericin B (10.23 and 6.5, respectively).

Taking into account their high antileishmanial activity and lower cytotoxicity in comparison to miltefosine and amphotericin B, compounds **7** and **8** were selected to search for their wide range antileishmanial activity against the promastigote and amastigote of different *Leishmania* species, responsible for the different clinical manifestations of the disease. Compound **7** showed significantly higher activity against *L. braziliensis* promastigotes than compound **8** ($P \leq 0.05$) and slightly lower than miltefosine ($P \geq 0.05$). In addition, **7** and **8** did not show better activity against *L. donovani* than miltefosine ($P \leq 0.05$) (Table 2).

In relation to the *in vitro* efficacy of compounds **7** and **8** against the intracellular amastigote of *L. mexicana,* they showed IC_{50} values of 0.28 and 0.26 μM, respectively, being significantly different with respect to miltefosine (IC_{50} = 0.51 μM) and amphotericin B (IC_{50} = 0.87 μM) ($P \leq 0.05$) (Table 2). Other authors have reported IC_{50} values for miltefosine and amphotericin B of 0.96 μM and 0.51 μM (Bilbao-Ramos et al., 2017; Na et al., 2004). However, Stroppa et al. (2017) reported for miltefosine an IC_{50} value of 4.2 μM and Bilbao-Ramos et al. (2017) and Fortin et al. (2012), reported IC_{50} values for amphotericin of 0.24 μM, and 0.07–0.09 μM, respectively. Difference in IC_{50} values can also be attributed to the parasite strain, growth medium and method for determination used in the assays.

Compounds **7** and **8** had SI values of 942.8 and 3939.2 against *L. mexicana* amastigote (Table 3). Compound **8** was 12.8 and 554 times more selective than miltefosine and amphotericin B, respectively. In the case of *L. braziliensis*, **7** and **8** showed slightly lower antileishmanial activity than the reference drug, miltefosine, although it was not significantly different ($P \geq 0.05$). In addition, both compounds were significantly less active against *L. donovani* amastigote than miltefosine ($P \leq 0.05$) (Table 2). It is worth noticing that compound **8** showed the highest SI against *L. mexicana* promastigote and amastigote (SI = 317.75 and 3939.2, respectively) (Table 3).

In order to know more about possible enzyme targets that may explain the high activity of **7** and **8** against *L. mexicana* promastigote and amastigote, it was our interest to analyze their ability to

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Table 2
Inhibition of proliferation of different *Leishmania* spp by compounds **7** and **8**, miltefosine and amphotericin B.

Parasite stage	IC_{50}(μM) ± SD			
Promastigotes	**7**	**8**	miltefosine	amphotericin B
L. mexicana	2.62 ± 0.79	3.21 ± 0.89	15.34 ± 0.36	0.95 ± 0.06
L. braziliensis	54.05 ± 10.02	127.7 ± 21.6	48.37 ± 11.37	–
L. donovani	198.47 ± 4.86	223.8 ± 8.17	114.68 ± 11.37	–
Amastigotes	**7**	**8**	**miltefosine**	**amphotericin B**
L. mexicana	0.28 ± 0.02	0.26 ± 0.04	0.51 ± 0.08	0.87 ± 0.03
L. braziliensis	34.22 ± 1.12	33.78 ± 2.47	22.67 ± 6.03	–
L. donovani	222.23 ± 4.8	218.24 ± 8.46	66.74 ± 1.05	–

IC_{50}: Compound concentration that produced a 50% reduction in parasites.
SD: Standard deviation of three independent determinations.

Table 3
Selectivity Index (SI) of compounds **7**, **8**, miltefosine and amphotericin B.

Parasite stage	SI (Selectivity Index = CC_{50}/IC_{50})			
Promastigotes	**7**	**8**	miltefosine	amphotericin B
L. mexicana	91.76	317.75	10.23	6.5
L. braziliensis	4.88	9.61	3.25	–
L. donovani	1.33	5.48	1.37	–
Amastigotes	**SI (Selectivity Index = CC_{50}/IC_{50})**			
L. mexicana	942.8	3939.2	307.8	7.11
L. braziliensis	7.71	30.32	6.92	–
L. donovani	1.18	4.69	2.35	–

inhibit LmARG activity. LmARG is considered a potential drug target for the treatment of leishmaniasis since it is essential, not only for parasite growth and survival, but also in the evasion of the host immune response (Balaña-Fouce et al., 2012; D'Antonio et al., 2013; Iniesta et al., 2001).

The inhibitory effect of compounds **7** and **8** on recombinant LmARG activity was experimentally tested using the QuantiChom™ Arginase Assay Kit. Nor-NOHA, considered a specific inhibitor of LmARG (D'Antonio et al., 2013; Riley et al., 2011) was included as positive control. Compounds **7** and **8** inhibited the activity of recombinant LmARG, 24.34% and 68.27%, respectively. Compound **8** was more active than Nor-NOHA (32% inhibition) and had an IC_{50} value of 35.9 μM. It is worth noticing that compound **8** did not inhibit the activity of HsARG, on the contrary, it stimulated the enzyme activity by 24.21%.

To elucidate how compounds **7** and **8** inhibited LmARG activity, an *in silico* docking study was performed. Compound **8** was the best ranked with -5.5 kcal/mol followed by **7** with -4.46 kcal/mol. As seen in Fig. 1, both compounds do not show interactions with residues of the catalytic site and do not show electrostatic interaction with the Mn^{2+} atoms. However, these compounds block the cavity of the catalytic site and are mainly stabilized by hydrogen bond (H-bond) interactions. Compound **7** shows an H-bond interaction with residue His139 and hydrophobic interactions with residues Thr257 and Val259. In the case of compound **8**, it adopts a conformation where the benzyl moiety is closer to the catalytic pocket and shows H-interactions with residues Asn143, Thr148, Ser150 and Asn152. In addition, the physicochemical descriptors were calculated for compounds **7** and **8** (Supplementary Material), and they do not violate the optimal requirements for druggability.

The activity of **8** against *L. mexicana* can be explained in part by its inhibitory interactions on LmARG demonstrated both by molecular docking studies and experimental assays. In an attempt to

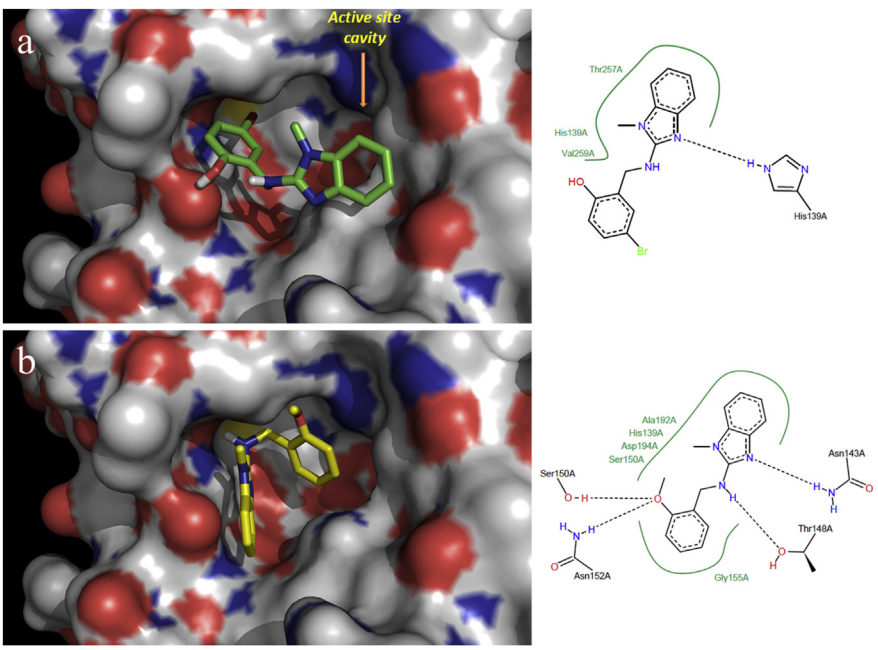

Fig. 1. Predicted binding mode and interactions of compounds **7** (a) and **8** (b) with LmARG. 2D interactions were obtained from the PoseView server (http://proteinsplus.zbh.uni-hamburg.de/#poseview).

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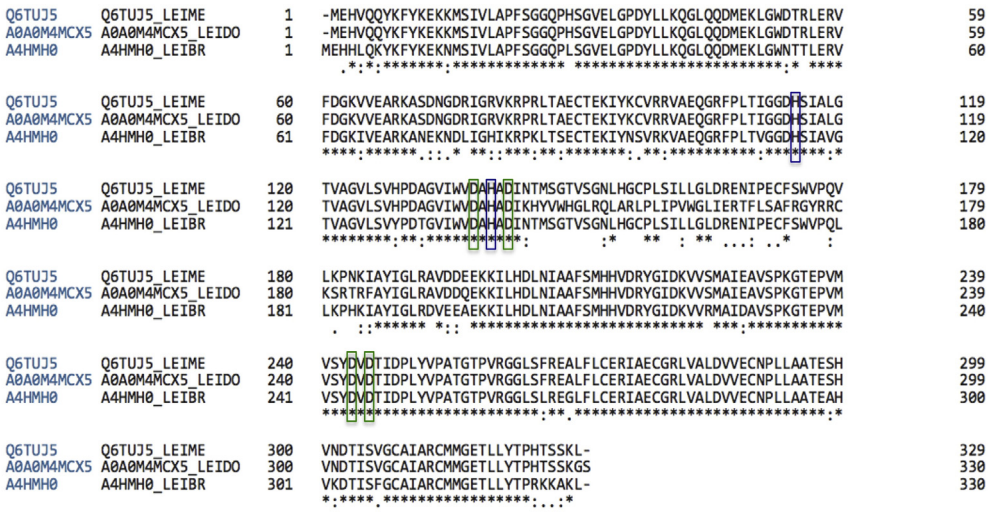

Fig. 2. Alignment among ARG sequences of *L. braziliensis, and L. donovani,* with the amino acid sequence of *L. mexicana* ARG. Multiple sequence alignment using ClustalW2-Clustal Omega. In blue the active site and green the metal binding region. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

explain the lower activity of **8** against visceral species, a sequence alignment of ARG from the different *Leishmania* species was performed (Fig. 2). No differences were detected around the active site (blue) and the metal binding region (green), among cutaneous, mucocutaneous and visceral species, sharing 75.8% identity among species. It may be that amino acid differences outside the active site of *L. donovani* ARG could influence the affinity binding of compound **8.** Further docking studies with the different ARGs will give some light about their interaction with compound **8**.

Another explanation of compound **8** activity against different *Leishmania* species could be based on a different L-arginine metabolism in cutaneous and visceral *Leishmania* species. In fact, Westrop et al. (2015) performed the metabolomics analysis of the promastigotes of *L.* mexicana, *L. major* and *L. donovani*, demonstrating important differences in the amino acid metabolism of these species, especially tryptophan, aspartate, L-arginine and L-proline. Regarding L-arginine, authors found that *L. mexicana* and *L. major* internalize this amino acid in greater extent than *L. donovani*, detecting more L-ornithine. In addition, it was recently demonstrated that in *L. donovani*, although ARG is essential for L-ornithine and polyamine synthesis, ornithine descarboxylase appeared to be the rate-limiting enzyme for polyamine production (Boitz et al., 2016). Therefore, these evidences could explain the poor activity of compound **8** against visceral species of *Leishmania*.

4. Conclusions

In this study, a series of 28 benzimidazole derivatives were tested for their activity against different *Leishmania* species, responsible for the different clinical manifestations of the disease. Compound **8** exhibited the highest potency and SI against both parasite stages of *L. mexicana.* Compound **8** showed inhibitory effect on LmARG activity that indeed contributes to its antileishmanial activity, even though its effect on other parasite molecules cannot be ruled out. Compound **8** is a promising scaffold for the development of more potent antileishmanial benzimidazole derivatives.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

Nieto-Meneses, as a PhD student from the Programa de Doctorado en Ciencias Quimicobiológicas, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional was supported by a PhD fellowship from the IMSS (Grant 062-2013) and CONACyT-México (Grant 246636). We acknowledge to the UNAM, Posgrado en Ciencias Químicas and CONACyT for grants to AMR (299651), JBMR (308208) and PTS (409406/318775). Authors also acknowledge USAII (Maricela Gutierrez-Franco, Rosa Isela del Villar-Morales, Nayeli López-Balbiaux, Víctor Lemus-Neri) for the determination of analyses and spectra and Dr. Antonio Miranda-Duarte for the statistical analyses. This work was supported by FIS/IMSS/PROT/G12/1130 and CONACYT (Grant 251726).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.exppara.2017.11.009.

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Please cite this article in press as: Nieto-Meneses, R., et al., *In vitro* activity of new *N*-benzyl-1*H*-benzimidazol-2-amine derivatives against cutaneous, mucocutaneous and visceral *Leishmania* species, Experimental Parasitology (2017), https://doi.org/10.1016/j.exppara.2017.11.009