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ORIGINAL ARTICLE

Systematic search for benzimidazole compounds and derivatives with antileishmanial effects

Juan Carlos Sánchez-Salgado[1] · Pablo Bilbao-Ramos[2] · María Auxiliadora Dea-Ayuela[3] · Francisco Hernández-Luis[4] · Francisco Bolás-Fernández[2] · José L. Medina-Franco[4] · Yareli Rojas-Aguirre[4,5,6]

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Abstract

Leishmaniasis is a neglected tropical disease that currently affects 12 million people, and over 1 billion people are at risk of infection. Current chemotherapeutic approaches used to treat this disease are unsatisfactory, and the limitations of these drugs highlight the necessity to develop treatments with improved efficacy and safety. To inform the rational design and development of more efficient therapies, the present study reports a chemoinformatic approach using the ChEMBL database to retrieve benzimidazole as a target scaffold. Our analysis revealed that a limited number of studies had investigated the antileishmanial effects of benzimidazoles. Among this limited number, *L. major* was the species most commonly used to evaluate the antileishmanial effects of these compounds, whereas *L. amazonensis* and *L. braziliensis* were used least often in the reported studies. The antileishmanial activities of benzimidazole derivatives were notably variable, a fact that may depend on the substitution pattern of the scaffold. In addition, we investigated the effects of a benzimidazole derivative on promastigotes and amastigotes of *L. infantum* and *L. amazonensis* using a novel fluorometric method. Significant antileishmanial effects were observed on both species, with *L. amazonensis* being the most sensitive. To the best of our knowledge, this chemoinformatic analysis represents the first attempt to determine the relevance of benzimidazole scaffolds for antileishmanial drug discovery using the ChEMBL database. The present findings will provide relevant information for future structure–activity relationship studies and for the investigation of benzimidazole-derived drugs as potential treatments for leishmaniasis.

Keywords Benzimidazoles · Chemoinformatics · *Leishmania* · Molecular scaffold

Introduction

Leishmania protozoan hemoflagellate parasites are transmitted via the bite of the infected female phlebotomine sandfly and cause a group of neglected tropical diseases that are collectively known as leishmaniasis. These include a wide range of clinical manifestations that are determined by the infecting species and the immune response of the human host [1]. Leishmaniasis is therefore classified into three groups: (a) visceral leishmaniasis, (b) mucocutaneous leishmaniasis, and (c) cutaneous leishmaniasis [2]. This group of diseases affects approximately 12 million people in about 98 countries. It is estimated that over 1 billion people are at risk, indicating that this is one of the neglected tropical diseases with the highest disease burden [3–5].

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✉ Yareli Rojas-Aguirre
yarelirojas@gmail.com

1 Instituto de Medicina Molecular y Ciencias Avanzadas, Rayo 11 Jardines del Pedregal, Álvaro Obregón, 01900 Mexico City, Mexico

2 Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza Ramón y Cajal s/n, 28040 Madrid, Spain

3 Departamento de Farmacia, Universidad CEU Cardenal Herrera, s/n, 46113 Moncada, Valencia, Spain

4 Facultad de Química, Departamento de Farmacia, Universidad Nacional Autónoma de México, 04510 Mexico City, Mexico

5 Cátedra Conacyt-Laboratorio Nacional de Manufactura Aditiva y Tomografía Computarizada, Centro de Ciencias Aplicadas y Desarrollo Tecnológico, Universidad Nacional Autónoma de México, 04510 Mexico City, Mexico

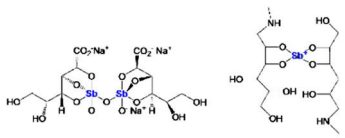
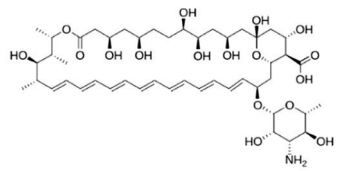
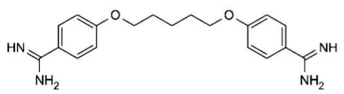
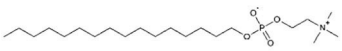
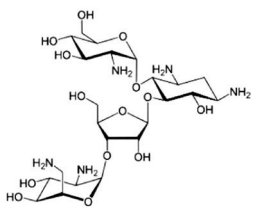
6 Present Address: Instituto de Investigaciones en Materiales, Universidad Nacional Autónoma de México, 04510 Mexico City, Mexico

No vaccine exists for the prevention of leishmaniasis, and current treatments rely on chemotherapeutics. Although several drugs are used to treat the infection, these treatments are limited by their severe toxicity, poor or variable efficacy, and emerging drug resistance; some drugs are also expensive and this limits their availability in some neglected geographical regions [2,6,7].

The development of new antileishmanial agents is challenging because of the complex biology of the *Leishmania* parasite. First, drug efficacy varies depending on the parasite species, the clinical symptoms, and even the geographical region [7,8]. Second, the parasite life cycle is a factor because *Leishmania* alternates between the extracellular promastigote found in the sandfly vector and the intracellular amastigote, which is present in macrophages and causes the clinical symptoms [9]. Therefore, an antileishmanial compound needs to enter the host macrophage and eliminate the parasites, without damaging the host macrophage. As shown in Table 1, current antileishmanial drugs have a broad diversity of chemical core scaffolds, which are associated with different mechanisms of action.

Sangshetti et al. [11] reviewed the progress of antileishmanial drug discovery, focusing on the chemical compositions of new classes of synthetic and naturally occurring antileishmanial agents. This analysis identified molecules belonging to a wide variety of chemical families. This diversity means

Table 1 Current treatments for leishmaniasis

Name	Chemical structure	Mechanism of action	Uses	Advantages	Limitations
Pentavalent antimonials: sodium stibogluconate, meglumine antimoniate		Not clearly elucidated.	VL and CL	Low cost. Can be used in combination with amphotericin B	Efficacy ranges from 35-95%. Drug resistance. Severe kidney and liver toxicity; pancreatitis; tachycardia, fibrillation, and cardiac arrhythmias
Amphotericin B		Forms complexes with ergosterol (a major parasite membrane component). Consequently, membrane permeability increases, aqueous pores are formed, the ionic balance is altered, and the parasite dies	VL, CL, and MCL	Efficacy of 95-100% Effective when resistance to pentavalent antimonials is observed	Requires hospitalization because of renal toxicity, myocarditis, and reactions at the infusion site. Liposomal form: renal toxicity and high cost
Pentamidine		The primary mode of action remains unknown. Possible binding with	VL	Efficacy of 70-80% Could be used in combination with other drugs	Drug resistance Renal toxicity, myocarditis, fever, hypotension, irreversible insulin-dependent diabetes mellitus, death
Miltefosine		Inhibition of phosphatidylcholine biosynthesis and alteration of phospholipid and sterol composition	VL and variable efficacy in CL	Efficacy of 94-97% Highly potent Administered orally	Renal and hepatic toxicity Teratogenicity
Paromomycin		Possible alteration of membrane fluidity, interaction with ribosomes, interference with mitochondrial membrane potential, and inhibition of respiration	VL and CL. More effective for CL	Low cost	Highly hepatotoxic and ototoxic; abdominal cramps, diarrhea

The information was extracted from the following Refs. [2,8,10]
VL visceral leishmaniasis, *CL* cutaneous leishmaniasis, *MCL* mucocutaneous leishmaniasis

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that it is difficult to establish an overall relationship between chemical structure and antileishmanial activity.

Chemoinformatic approaches can be used to analyze and integrate information related to the physicochemical properties of compounds and their biological activities, in order to identify novel drug candidates [12–14]. Chemoinformatics has been applied to aid antiparasitic drug discovery [15–17], including the search for potential antileishmanial compounds through distinct approaches [18–20].

The ChEMBL bioactivity database is administered by the European Molecular Biology Laboratory in the UK (http://www.ebi.ac.uk/chembl/). This free public online repository provides large amounts of information on the relationships between the physicochemical properties of molecules and their molecular targets [21,22]. The inclusion of user feedback has allowed ChEMBL to expand over time, and it has recently included the direct deposition of data relating to neglected diseases [23].

The present study aimed to identify potential antileishmanial scaffolds by mining the ChEMBL database. We focused on the benzimidazole scaffold because this is a major documented pharmacophore in drug discovery [24] and benzimidazole derivatives have been widely used as antiparasitic drugs in the treatment of helminthic and protozoan infections, where they primarily act by inhibiting tubulin polymerization [25,26]. The α-, β-, and γ-tubulins are essential for microtubule formation and play a key role in the growth and differentiation of kinetoplastid protozoa [27–30]. For this reason, tubulin is a significant target in the antiparasitic and antileishmanial drug discovery fields [31–33].

There has been increasing interest in exploring the antileishmanial potential of benzimidazoles [34–37]. For this reason, it is important to start analyzing the benzimidazole scaffold content to identify representative compounds displaying activities against particular *Leishmania* species or all the species to contribute to further structure–activity relationship studies.

To ascertain the antileishmanial potential of benzimidazole derivatives, we mined the ChEMBL database systematically to identify benzimidazole scaffolds with activities against diverse species of *Leishmania* and their relevance for these species. In addition, we synthesized and determined the activities of a benzimidazole derivative, namely 5,6-dichloro-2-(trifluoromethyl)-1*H*-benzimidazole (G2), against the promastigotes and intra-macrophage amastigotes of two *Leishmania* species.

Materials and methods

Chemical informatics

A range of methods can be employed to derive the scaffold of a molecule in a systematic manner, as described previously [38]. The present study based the scaffold analysis and its distribution in the database on specific molecular chemotypes, an approach previously described by Medina-Franco et al. [39]. Hence, in the present work the molecular chemotypes, also called cyclic systems, were defined as a set of rings plus the chains of atoms that linked them to one another. To isolate the scaffold, all the substituent groups, with the exception of endocyclic carbonyls and imines, were removed from the rings and linkers. Heteroatoms were retained, whereas all hydrogen atoms attached to them were considered side chains and therefore deleted. The Molecular Equivalence Indices (MEQI) program (version 2.41) [40] was used to obtain the specific cyclic system, which had a five-character alphanumeric chemotype identifier that was present on each compound in the subsets obtained from ChEMBL.

Biological assays

Model benzimidazole compound

We used 5,6-dichloro-2-(trifluoromethyl)-1*H*-benzimidazole (G2) as a model compound with a benzimidazole scaffold to assess antileishmanial activity. This compound was previously designed and synthesized by our research group as part of a 2-(trifluoromethyl)-1*H*-benzimidazole derivatives series. G2 showed significant activity when evaluated against *L. mexicana* promastigotes. Although it was not the most active among that series, we selected it because of the simplicity of its structure, in comparison with the other active compounds that contained bulky substituents at the 5 and 6 positions. We therefore considered G2 as a starting point [34].

Parasites

Two *Leishmania* species were used for the following experiments: *L. amazonensis* (MHOM/BR/79/Maria) was kindly provided by Dr. Alfredo Toraño (Instituto de Salud Carlos III, Madrid, Spain), and an autochthonous strain of *L. infantum* (MCAN/ES/92/BCN83), isolated from an asymptomatic dog in the Priorat region (Catalonia, Spain), was generously provided by Professor Montserrat Portús (Universidad de Barcelona). Promastigotes of both species were cultured at 26 °C in 25-mL culture flasks containing Schneider's insect medium (Sigma) supplemented with 20% heat-inactivated fetal bovine serum (Sigma), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma).

Effect of G2 on Leishmania promastigotes

The effect of G2 on promastigotes was analyzed using the procedure reported by Bilbao-Ramos et al., with minor mod-

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ifications [41]. Briefly, serial dilutions of G2 (40, 20, 6, 5, 2.5, 1.2, 0.6, 0.3 μM) were prepared using fresh culture medium. The parasites were seeded in 96-well microplates (2.5×10^5 promastigotes/well) prior to adding the indicated concentration of G2 and incubating at 26 °C for 48 h. Resazurin dye solution (20 μL of 2.5 mM) was then added, and the plates were incubated for 3 h. Fluorescence intensity was then measured using an Infinite 200 (Tecan i-Control) fluorometer at an excitation wavelength of 535 nm and an emission wavelength of 590 nm. These readings were used to calculate the growth inhibition rate, which was expressed as the 50% inhibitory concentration (IC_{50}) and calculated using SPSS 17.0 Statistics Software. Miltefosine (Sigma) was used as reference drug, and all tests were performed in triplicate.

Effect of G2 on Leishmania amastigotes

The experiments involving *L. amazonensis* were performed using the fluorometric method reported by Bilbao-Ramos et al. [42], with some modifications. The experiments involving *L. infantum* amastigotes were conducted for the first time, although these experiments were also based on the protocol reported by Bilbao-Ramos et al. [42]. Briefly, J774 macrophages were grown in RPMI-1640 medium. These cells (5×10^4 cells/well) were seeded in 96-well microplates and then infected with 5×10^5 promastigotes/well. The macrophages infected with *L. infantum* were incubated for 48 h at 37 °C, whereas those infected with *L. amazonensis* were incubated at 33 °C for 24 h. The temperature of both cultures was then adjusted to 37 °C. After 24 h, the culture medium was removed and the cells were washed with RPMI-HEPES to eliminate the non-internalized promastigotes. Thereafter, the infected cells were exposed to 100 μL of G2 at different concentrations (200, 100, 50, 25, 12.5, 6, 3, 1.5 μM) in RPMI-1640 for 48 h at 37 °C. The culture medium was then removed by centrifugation at 3500 rpm for 5 min (Centrifuge 5403, Eppendorf), and a lysis solution (0.01% sodium dodecyl sulfate in RPMI-HEPES) was added to the pellet. After 20 min, the treated cells were centrifuged (3500 rpm, 5 min, 4 °C) and the supernatants were replaced by 200 μL of Schneider's insect medium. Finally, the plates were incubated at 26 °C for 3 days to allow the transformation of viable amastigotes to promastigotes. The proliferation and viability of *Leishmania* promastigotes, expressed as the IC_{50}, was then determined using the resazurin method described above. Miltefosine was used as a reference, and all experiments were performed three times.

Microscopy studies

Confocal laser fluorescence microscopy

Promastigotes of *L. infantum* (10×10^6 parasites) were incubated in Schneider's insect medium containing G2 at its IC_{50} of 8.6 μM for 48 h. The culture medium was then removed, and the promastigotes were washed with phosphate-buffered saline (PBS) containing 0.25 mM $MgCl_2$ and 0.35 mM $CaCl_2$ [43]. Thereafter, the samples were incubated with a rabbit anti-β-tubulin primary antibody (Thermo Fisher Scientific) (1:100 dilution in PBS containing $MgCl_2$ and $CaCl_2$) overnight at 4 °C. Subsequently, the cells were washed three times with PBS–Mg^{2+}–Ca^{2+} prior to exposure to a secondary antibody (goat anti-rabbit IgG FITC-conjugated, 1:100 in PBS–Mg^{2+}–Ca^{2+}). Cells were maintained at 37 °C for 1 h and then washed with PBS–Mg^{2+}–Ca^{2+}. Slides were air-dried in darkness at room temperature and then observed under a Leica DM-IRE2 inverted microscope. Confocal microscopy images were acquired using LCS 2.6 software.

Light microscopy

These studies were performed in 8-well Lab-Tek chamber slides (Nunc) using *L. amazonensis* and *L. infantum*. Macrophage infection was developed as described above, and the infected macrophages were exposed to G2 at 10 μg/mL for 48 h. The cells were then fixed with methanol and stained with 10% Giemsa (200 μL/well). After 15 min, the samples were washed and air-dried at room temperature. Before the microscopic analysis, the upper structure of the chamber was removed, and the coverslips were placed and sealed with DPX.

Results and discussion

Chemoinformatic analysis

The workflow used to systematically retrieve benzimidazole scaffolds from the ChEMBL database is shown in Fig. 1.

Antileishmanial data retrieval

We conducted an initial exploratory search for molecules in ChEMBL that had been reported to show antileishmanial activity. This also allowed us to identify the *Leishmania* species associated with the highest number of records. An initial dataset including 6903 chemical entities with activity against ten *Leishmania* species was retrieved from the database (Supplementary File 1, Table S1). *L. donovani*, *L. infantum*, and *L. amazonensis* were the most commonly studied species, with 3472, 1681, and 716 active compounds,

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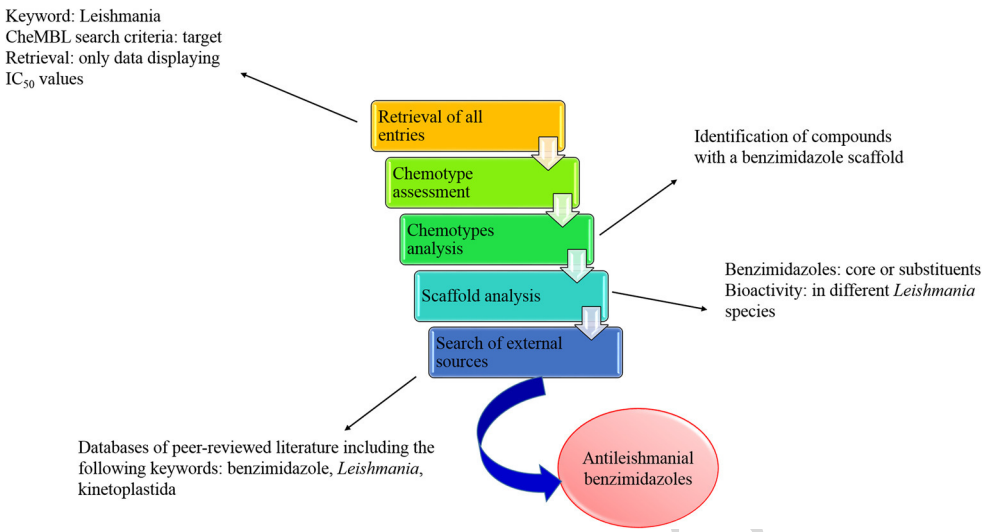

Fig. 1 Workflow used for identification of benzimidazole scaffolds

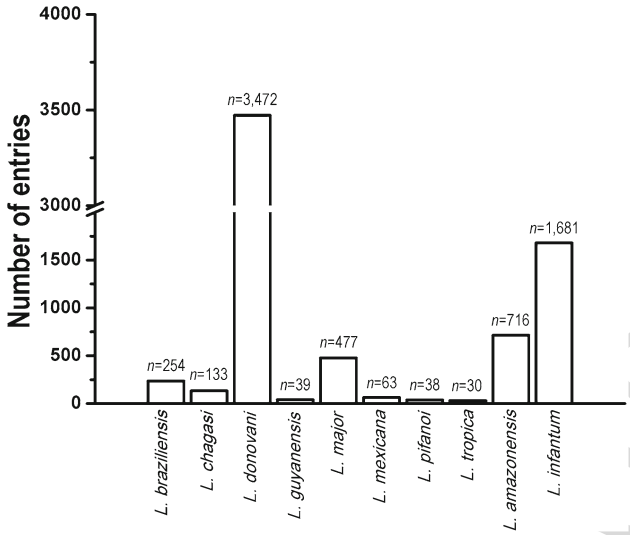

Fig. 2 ChEMBL database entries associated with effects on the indicated *Leishmania* species

respectively (Fig. 2). These values reflected the fact that *L. donovani* and *L. infantum,* which cause lethal visceral leishmaniasis, have been prioritized by the scientific research community.

This initial dataset was then searched to identify compounds with a benzimidazole scaffold.

Chemotype assessment

The total number of cyclic chemotypes was analyzed for each species using MEQI, which has previously been used successfully to classify several compound collections [44–46]. As shown in Fig. 3a, the 1681 entries associated with *L. infantum* included more chemotypes (1105) than the 716 chemical entries relating to *L. amazonensis* (249 chemotypes). The scaffolds for *L. infantum* were thus more diverse (58.56%) than those for *L. amazonensis* (28.39%) (Fig. 3b).

Chemotype analysis

This analysis revealed that single aromatic ring compounds were the most prevalent in entries associated with both *L.amazonensis* and*L.infantum*. Regarding *L. amazonensis*, it was found that the bioactive molecules mostly contained cyclic systems with 2–5 aromatic or heterocyclic rings. Some of these were linked by a long unsaturated carbon chain, resembling the structure of pentamidine (Supplementary File 2, Fig S1). However, there were no benzimidazoles within this dataset.

With respect to *L. infantum*, the active compounds had different structural features and were more complex (Supplementary File 2, Fig. S2). These scaffolds included shorter chemotypes with either heterocyclic or aromatic systems, with a considerable number of acyclic compounds (1.72%). Chemotypes including single cyclic or bridged alkanes, corresponding to cyclohexane or adamantane structures, respectively, were present; a cyclic chemotype containing selenium atoms was also identified. Interestingly, a cyclic system with a benzimidazole moiety (chemotype ID NM7HR) was identified within this group with a frequency of 0.65%. Four additional cyclic systems containing the benzimidazole moiety, albeit at lower frequencies were detected. Therefore, five cyclic chemotypes containing benzimidazole were found in the *L. infantum* dataset (Fig. 4). Interestingly, these were all *N*-ferrocenylmethyl, *N*'-methyl-2-substituted benzimidazoles and benzimidazolium iodide salts belonging to a chemical series that included 26 compounds [47].

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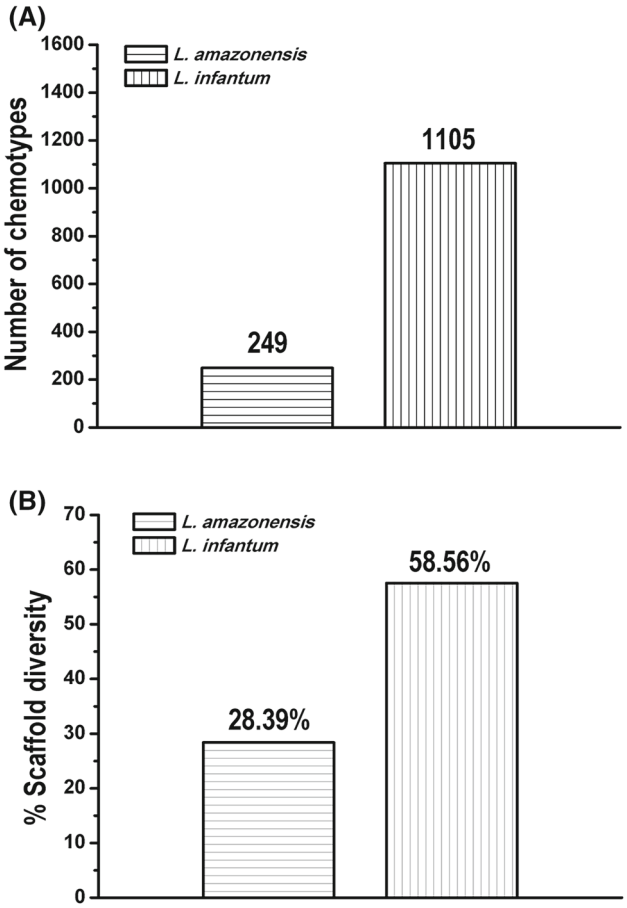

Fig. 3 Chemotypes and scaffold diversity determined by MEQI. **a** Total number of chemotypes and **b** scaffold diversity associated with *L. amazonensis* and *L. infantum*

Analysis of benzimidazole chemotypes

The analysis described above identified five chemotypes containing benzimidazoles in the *L. infantum* dataset. We were curious about the low frequency of these scaffolds and therefore modified our search criteria to find cases where benzimidazole was present as the main ring or as a substituent. This approach identified 13 additional cyclic chemotypes containing benzimidazoles (corresponding to 30 derivatives). Eight chemotypes with benzimidazole as the main system were identified. Within this group, two bis-benzimidazole systems were found, whereas the rest of the scaffolds were 1,2-disubstituted benzimidazoles. Five of the chemotypes did not include benzimidazole as the main scaffold; these were all 2-aryl substituted benzimidazoles (Supplementary File 2, Fig. S3). In this manner, 56 benzimidazole derivatives were identified as being active against *L. infantum*. This approach yielded no results for *L. amazonensis*, but its application to the datasets relating to the other eight *Leishmania* species recorded in ChEMBL identified a total of 99 benzimidazole entries; these were associated with *L. donovani* ($n = 40$), *L. major* ($n = 39$), and *L. mexicana* ($n = 20$).

In the case of *L. donovani*, eight scaffolds with benzimidazole as the main system were identified. Interestingly, the structural features of this group mostly corresponded to bis-benzimidazole systems linked by a long chain of carbon atoms, resembling the structure of pentamidine (Supplementary File 2, Fig. S4a). In contrast, five scaffolds corresponding to benzofuran, benzothiazole, or 7-chloroquinoline main heterocyclic systems were found to contain benzimidazole as a substituent (Supplementary File 2, Fig. S4b). The benzimidazole heterocycle substituted at the 1, 2, 5, and 6 positions was the scaffold detected for both *L. major* and *L. mexicana*. For the latter species, an additional scaffold comprising a hybrid system from benzimidazole and pentamidine was also identified (Supplementary File 2, Fig. S5).

Search of external sources

Although ChEMBL is a high-quality public database, it can contain gaps that can be addressed by additional literature searching [48]. To this end, databases of the peer-reviewed literature were searched for publications reporting benzimidazoles, beyond those already identified using ChEMBL. Three publications were found that reported the effects of benzimidazole analogs on *Leishmania* species. One of these publications reported the synthesis of gold complexes using benzimidazole moieties as ligands. These compounds (4 entries) showed significant activities against promastigotes and amastigotes of *L. major*, *L. amazonensis*, and *L. braziliensis* (Supplementary File 2, Fig. S6) [49]. Thirty-one entries relating to*L. donovani* were identified; these reported a series of 2,3-dihydroimidazo[1,2-α]benzimidazole analogs, some of which showed promising antiparasitic activity against the two biological stages of this parasite with IC_{50} values of 3.05–5.29 μM (Supplementary File 2, Fig. S6b) [50]. In addition, 2-aryl- and 5-nitro-2-arylbenzimidazole derivatives (31 entries) were effective against *L. major* promastigotes (Supplementary File 2, Fig. S6c) [51].

We combined these additional findings with those identified in ChEMBL. Figure 5 shows the total number of benzimidazole-containing molecules identified, and the *Leishmania* species in which they were evaluated. These findings demonstrate that benzimidazole scaffold bioactivity has mostly been studied using *L. major*, followed by *L. donovani*; these species cause the cutaneous and visceral forms of the disease, respectively. In contrast, the effects of benzimidazoles were seldom investigated using *L. amazonensis* and *L. braziliensis*.

In total, 235 benzimidazole scaffolds evaluated against *Leishmania* species were retrieved. A total of 127 structures (54%) showed activity against visceral leishmaniasis

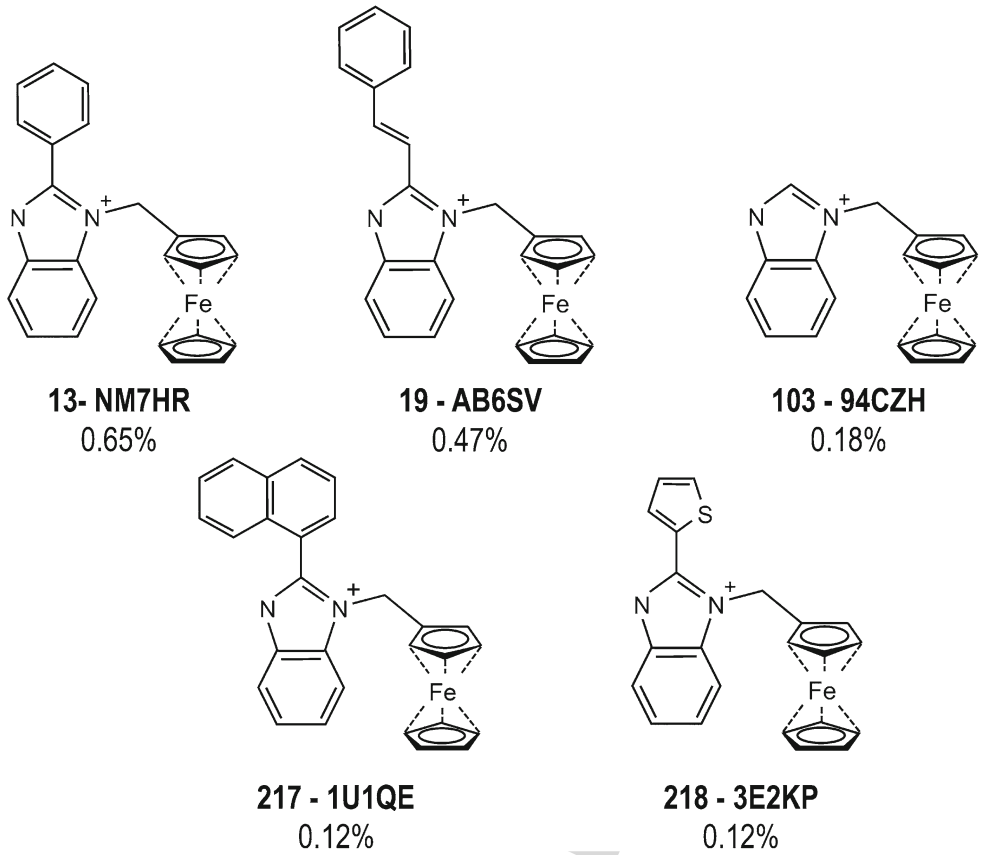

Fig. 4 Benzimidazole scaffold-containing chemotypes identified for *L. infantum*

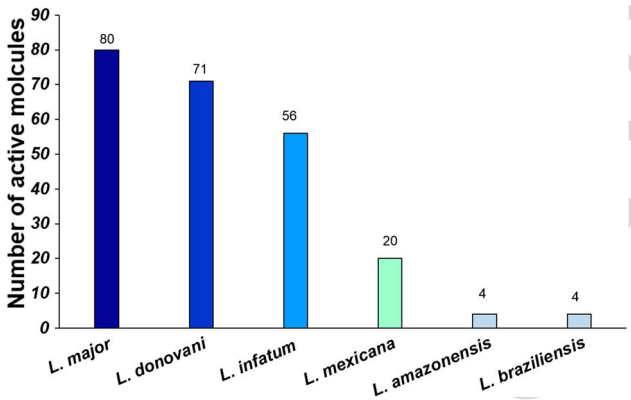

Fig. 5 Frequency of benzimidazole scaffolds for each *Leishmania* species

(*L. donovani*, *L. infantum*), 104 molecules (44.3%) were studied in species associated with cutaneous leishmaniasis (*L. major*, *L. mexicana*, *L. amazonensis*), and only 4 benzimidazole derivatives (1.7%) were evaluated against mucocutaneous leishmaniasis (*L. braziliensis*). These results reflect the fact that interest in discovering novel chemotherapeutics has focused on the visceral form of the disease.

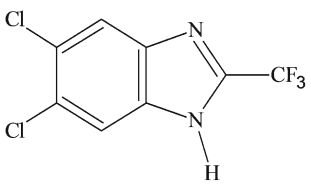

Fig. 6 Chemical structure of G2

Benzimidazole antileishmanial activity

To explore benzimidazole bioactivity directly, we performed biological assays focusing on *L. amazonensis* (cutaneous leishmaniasis) and *L. infantum* (visceral leishmaniasis). Our research group previously synthesized a number of benzimidazole compounds and found some activity against a broad spectrum of parasites, ranging from helminths to protozoans. Within these molecules, G2 (Fig. 6) showed activity against *L. mexicana* promastigotes [34], and we therefore investigated its effects on other *Leishmania* species.

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Table 2 Antileishmanial activity of G2

Compound	IC_{50} (μM)			
	Promastigotes		Amastigotes	
	L. amazonensis	*L. infantum*	*L. amazonensis*	*L. infantum*
G2	4.11	8.63	9.00	15.79
Miltefosine	9.56	7.42	3.21	4.6

Activity against promastigotes and amastigotes

In *L. amazonensis* promastigotes, G2 showed greater antileishmanial activity than miltefosine. In *L. infantum* promastigotes, the activity of G2 was comparable to that of miltefosine (Table 2).

Although *Leishmania* promastigote screening provides valuable preliminary information, this assay could generate false positives. The amastigote is more clinically relevant because it infects the mammalian host. Previous studies have identified a poor correlation between the biological activities of compounds in these two stages [52,53], and evaluation of amastigote effects is therefore essential. The method used to identify activity against amastigotes is worth careful consideration. Axenic amastigote cultures have provided a useful tool for high-throughput screening studies. Nevertheless, this method has some limitations because it does not reflect many aspects of intracellular parasite development [54]. In the present study, we employed a novel fluorometric assay of intra-macrophage amastigotes to evaluate the antileishmanial effects of G2. This method was initially developed for *L. amazonensis*; however, we successfully adapted it for *L. infantum* by making the appropriate modifications. As shown in Table 2, G2 showed antileishmanial effects on the intracellular amastigotes of both species, with *L. amazonensis* being the most sensitive. This was the same trend as that observed for the promastigotes. Although the anti-amastigote activities were lower than those of the reference drug, the IC_{50} values were below an arbitrary threshold of 25 μM for both species [55], demonstrating that this benzimidazole derivative had the potential to act as an antileishmanial compound.

To date, only four compounds containing a benzimidazole moiety have been reported as active against *L. amazonensis*. These gold-based complexes with benzimidazole moieties as the ligands [49] showed antileishmanial antiparasitic activity, with IC_{50} values of 5.18–42.19 μM for promastigotes and 5.77–25.95 μM for amastigotes. Interestingly, the benzimidazole moieties *per se* did not show this activity. However, the IC_{50} values for G2 on *L. amazonensis* promastigotes and amastigotes were within the ranges reported by Mota et al. for the gold complexes. A comparison of the chemical structures of the benzimidazole ligands employed (Supplementary file 2, Fig. S6) and G2 indicated that the substitution of the benzimidazole scaffold at positions 1 and 2 was important. In particular, a bulky group in position 1 might influence antileishmanial activity, and positions 5 and 6 could also play important roles. These findings provide a starting point for further studies of the relationship between benzimidazole structure and antileishmanial activity. These preliminary results could inform further exploration of the benzimidazole scaffold in the design of novel compounds, or the repositioning of existing benzimidazole-derived drugs, for the treatment of *L. amazonensis* infections.

Howarth and Hanlon [47] reported the synthesis of 26 benzimidazolium derivatives that were evaluated against *L. infantum*; however, they did not indicate whether the assays were performed on promastigotes and no technical details were provided. It is therefore difficult to compare their work with our results directly, although our findings showed that G2 significantly affected *L. infantum* promastigotes and amastigotes, with IC_{50} values below 25 μM.

Microscopy studies

Figure 7a depicts a promastigote of *L. infantum* that was incubated with an anti-β-tubulin antibody. The image shows the normal distribution pattern of this protein, which is principally concentrated in the subpellicular region of the protozoa [56]. In contrast, marked morphological changes were observed when the parasite was exposed to G2 (Fig. 7b), with the circular shape indicating a benzimidazole-induced redistribution of β-tubulin. This indicated an alteration of the tubulin-microtubule equilibrium and suggested that tubulin could be a target for G2.

Benzimidazoles are well known to target the β-tubulin subunit of some pathogens [57]. Although the β-tubulin structures in both helminths and protozoa have not been resolved, significant efforts have been made to elucidate the benzimidazole binding site using homology modeling studies [58,59]. In these reports, binding sites have been proposed and a series of benzimidazole carbamates has been docked into the models. Based on these studies, it could be hypothesized that the structural features of G2 could promote a hydrogen bonding interaction with the Glu 198 residue at the β-tubulin binding site. More detailed docking and molecular dynamics studies will be required to develop a deeper insight into the interaction between G2 and β-tubulin. Furthermore,

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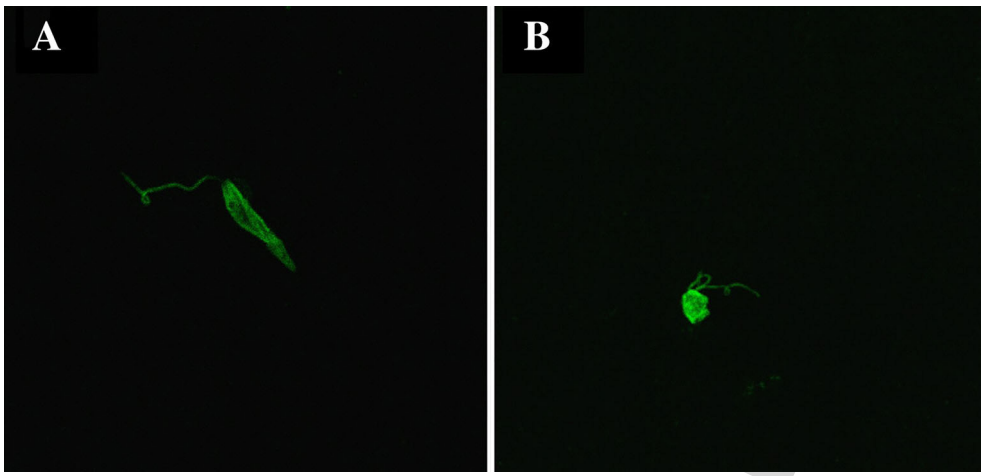

Fig. 7 Confocal microscopy of *L. infantum* promastigotes incubated with anti-β-tubulin without G2 treatment (**a**) and treated with G2 (**b**)

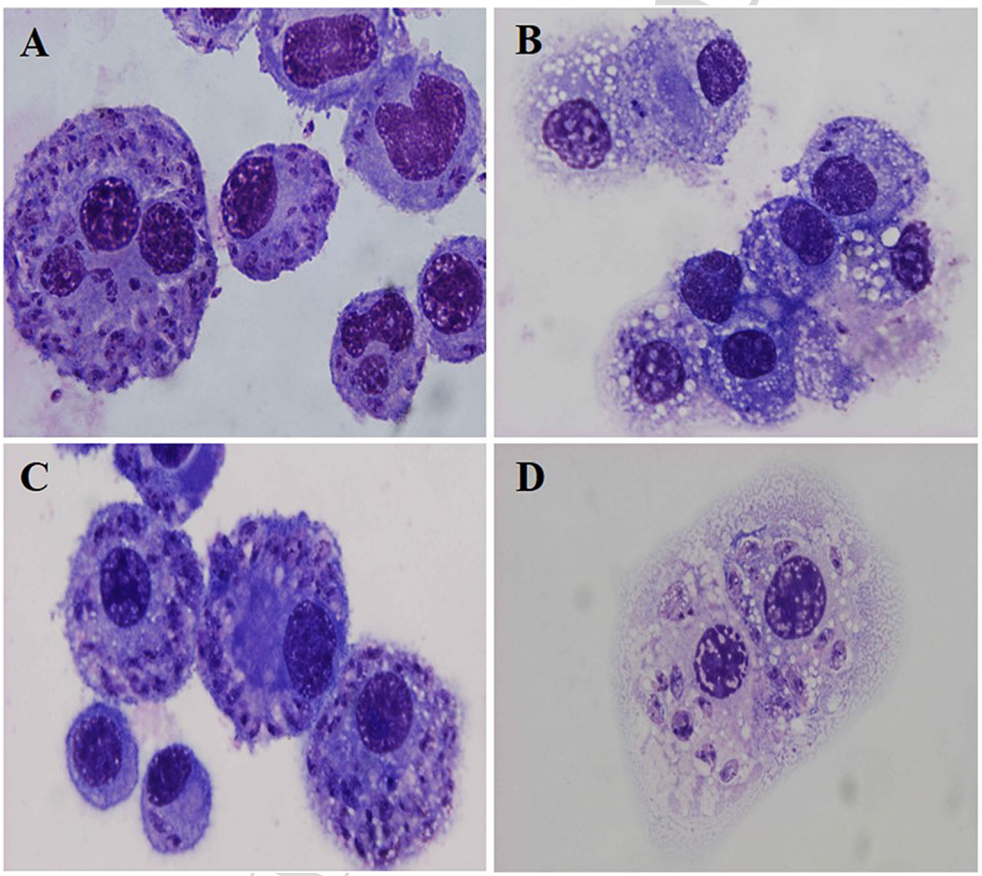

Fig. 8 Macrophages infected with *L. amazonensis* (**a** and **b**) or *L. infantum* (**c** and **d**) were left untreated (**a** and **c**) or treated with G2 (**b** and **d**)

in silico studies with other targets will provide a valuable source of information related to alternative mechanisms of action. For example, effects on *Leishmania* arginase have been explored by Méndez-Cuesta et al. and Nieto-Meneses et al. [60,61]. The bioactivities of benzimidazoles may also include disruption of metabolism, glucose uptake, and inhibition of mitochondrial dehydrogenase activity [62]. Thus, future research should consider all of these as potential mechanisms of action.

The effect of G2 on intracellular amastigotes was observed using light microscopy. For both species examined, the infected macrophages treated with G2 showed a marked decrease in parasite load (Fig. 8). A vacuolated appearance was observed in the G2-treated cells (Fig. 8b, d), and this

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was particularly apparent in those infected with *L. amazonensis*. Weakly basic compounds were previously reported to cause cytoplasmic vacuolation in macrophages [63]. Therefore, these observations indicate that this benzimidazole was internalized by the host cell, an essential feature of an antileishmanial compound.

Conclusions

This study was performed to investigate benzimidazole scaffolds as potential antileishmanial agents. We searched for target scaffolds in the ChEMBL database, combined with additional literature searches. Benzimidazole derivatives were found to be active against 6 *Leishmania* species, with a frequency ranking of *L. major* > *L. donovani* > *L. infantum* > *L. mexicana* > *L. amazonensis* = *L. braziliensis*. The biological activities of benzimidazole derivatives varied greatly and may depend on the substitution pattern of the scaffold, with the 1, 2, 5, and 6 positions identified as relevant for future structure–activity relationship studies. In addition, the approach used could be applied to other chemoinformatic studies of different structural features.

The benzimidazole derivative G2 demonstrated antileishmanial activity against both *L. amazonensis* and *L. infantum*. This finding indicates that deeper investigation is warranted into the potential treatment of cutaneous leishmaniasis using benzimidazoles. Furthermore, our results provide a rationale for elucidation of the interaction between benzimidazoles and tubulin, as well as other possible biological targets. These studies can be initiated using computational techniques.

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