Biomedicine & Pharmacotherapy 133 (2021) 111049

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Original article

Unveiling six potent and highly selective antileishmanial agents via the open source compound collection 'Pathogen Box' against antimony-sensitive and -resistant *Leishmania braziliensis*

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ARTICLE INFO

Keywords:
Leishmania braziliensis
Pathogen box
MMV
CRK3
CYP450
PKA

ABSTRACT

Despite all efforts to provide new chemical entities to tackle leishmaniases, we are still dependent on a the limited drug arsenal, together with drawbacks like toxicity and drug-resistant parasites. Collaborative drug discovery emerged as an option to speed up the way to find alternative antileishmanial agents. This is the case of Medicines for Malaria Ventures - MMV, that promotes an open source drug discovery initiative to fight diseases worldwide. Here, we screened 400 compounds from 'Pathogen Box' (PBox) collection against *Leishmania braziliensis*, the main etiological agent of cutaneous leishmaniasis in Brazil. Twenty-three compounds were able to inhibit $\geq$ 80 % *L. braziliensis* growth at 5 μM. Six out of the PBox selected 23 compounds were found to be highly selective against *L. braziliensis* intracellular amastigotes with selectivity index varying from $>$ 104 to $>$ 746 and IC_{50}s ranging from 47 to 480 nM. The compounds were also active against antimony-resistant *L. braziliensis* isolated from the field or laboratory selected mutants, revealing the potential on treating patients infected with drug resistant parasites. Most of the selected compounds were known to be active against kinetoplastids, however, two compounds (MMV688703 and MMV676477) were part of toxoplasmosis and tuberculosis 'PBox' disease set, reinforcing the potential of phenotyping screening to unveil drug repurposing. Here we applied a computational prediction of pharmacokinetic properties using the ADMET predictor pkCSM (http://biosig.unimelb.edu.au/pkcsm/). The tool offered clues on potential drug development needs and can support further *in vivo* studies. Molecular docking analysis identified CRK3 (*LbrM.35.0660*), CYP450 (*LbrM.30.3580*) and PKA (*LbrM.18.1180*) as *L. braziliensis* targets for MMV676604, MMV688372 and MMV688703, respectively. Compounds from 'Pathogen Box' thus represents a new hope for novel (or repurposed) small molecules source to tackle leishmaniases.

1. Introduction

Leishmaniasis is a neglected disease caused by more than 20 species of *Leishmania* protozoan parasites. It is transmitted by the bite of different species of phlebotominae sandfly. More than 1 billion people are at risk of contracting the disease and 94 % new cases reported to WHO occurred in seven countries: Brazil, India, Ethiopia, Kenya, South Sudan, Somalia and Sudan [1]. The available treatments for leishmaniasis are limited and have significant drawbacks. Pentavalent antimony, the first line drug for most of the affected countries is widely used for its low cost; however, it is toxic and has become less effective due to parasitic resistance. Liposomal amphotericin B is less toxic but the full treatment has high cost. Miltefosine is the first and only oral administered drug registered in India, it is expensive and teratogenic [2,3].

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https://doi.org/10.1016/j.biopha.2020.111049
Received 30 September 2020; Received in revised form 15 November 2020; Accepted 19 November 2020
Available online 4 December 2020

Thus, developing new options for treating leishmaniasis is highly needed [4].

Open-source drug discovery offers a potential for developing new and inexpensive drugs to fight diseases that affect mostly poor people in developing countries [5]. Following this demand for collaborative research to discover and develop new treatments for neglected diseases, the 'Pathogen Box' (PBox) [6] offers a cost free compound collection provided by Medicine for Malaria Venture – MMV [7]. It contains 400 compounds that demonstrates activity against a variety of pathogens, mainly *Plasmodium*, *Mycobacterium* and kinetoplastid parasites (*T. cruzi*, *Leishmania spp* and *T. brucei*) [8]. All compounds are categorized into subsets of which class of parasite they have been tested against (disease set) by partners and the MMV group. The compounds have also been tested for cytotoxicity in the human HepG2 cell line and were considered acceptable for an initial drug discovery program, being 5-fold less potent against the human cell line than the pathogen [6]. The library also contains a set of 26 reference compounds with biological activity associated with at least one pathogen. Successful screenings of the PBox have already been published, such as the identification of Tolfenpyrad (MMV688934), a pyrazole-5-carboxamide based insecticide that was demonstrated to be active against the helminth barber's pole worm [9] and a new *Candida albicans* biofilm inhibitor, MMV688768, which was originally indicated against schistosomiasis [10]. Recent advances on PBox compounds against *Leishmania* parasites were reported [11–14] including some target elucidation on MMV676477 *Leishmania* tubulin dynamics [15] supporting the wide-spectrum drug action of a previously known as anti-tuberculousis agent [16].

To contribute to the effort on finding new antileishmanial drug candidates, we screened the compounds provided by MMV PBox, selected the 27 most active (including four reference compounds among them), evaluated their toxicity to host cells, their predictive pharmacokinetic properties and the susceptibility of antimony resistant strains. We found 6 highly selective hits, with good potential drugability. Based on molecular docking computational analysis, three of them: MMV676604, MMV688372 and MMV688703, are strong candidates to bind respectively to the CRK3, CYP450 and PKG from *L. braziliensis* as part of their mechanisms of action against the parasite.

The data presented here, reinforces previous work and open new avenues on drug repurposing and will endorse open-source drug discovery providing new chemical starting points for experimental chemotherapy to tackle leishmaniases, including alternatives for antimony-resistant strains.

2. Materials and methods

2.1. MMV Pathogen Box compound library

An unit of the *Pathogen Box* was gifted/shared with our group from Medicine for Malaria Venture organization (https://www.mmv.org/). All compounds were supplied by the MMV group in 5 × 96 well plates containing 10 μL of the samples at 10 mM in dimethyl sulfoxide (DMSO) stock solutions each.

2.2. Leishmania braziliensis culture

Leishmania (Viannia) braziliensis (MHOM/BR/1994/H3227) and (MHOM/BR/1975/M2904) promastigotes were maintained in minimum essential medium (α - MEM) at pH 7.0 supplemented with 10 % (v/v) heat inactivated fetal bovine serum and incubated at 25 °C. A *Leishmania braziliensis* antimony (Sb^{III})-resistant strain (SbR) selected *in vitro* was maintained by adding 30 μM of Sb^{III} (15 μL at 10 mM in 5 mL) to the culture. Another *L. braziliensis* Sb^{III}-resistant strain (MHOM/BR/2008/330) was obtained from a human cutaneous lesions [17,18] and maintained without any Sb^{III} addition.

2.3. Culture and cytotoxicity using macrophages

Differentiated macrophages derived from human immortalized monocytic lineage - THP-1, were maintained in RPMI 1640 culture medium supplemented with 10 % fetal bovine serum, 2 mM Glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The percentage of cell viability was given by the differential cell count in the presence of trypan blue. The cytotoxic concentration (CC_{50}) was determined using the classical colorimetric method of cell viability using the MTT reagent [19]. Macrophages were seeded (2×10^5/well) in 96-well cell culture plates containing 0.2 mL of RPMI medium. Different concentrations of the compounds were added to the culture and the plates were incubated for 72 h at 37 °C in a humid atmosphere containing 5 % CO_2. After incubation, the medium was replaced with RPMI containing 0.5 mg/mL MTT and the plates were incubated for an additional 4 h. The supernatants were removed and the formazan crystals were solubilized in DMSO. The absorbance was measured at 570 nm in a microplate reader (Spectramax 340, Molecular Devices) to obtain the cellular viability profile.

2.4. Antileishmanial phenotypical screening

Promastigote forms of *L. braziliensis* were previously transfected by electroporation with the pSP1.2_LUC_αHYGα vector containing the firefly luciferase (LUC) reporter gene and the gene coding for the hygromycin phosphotransferase protein as a selection marker [20,21]. THP-1 macrophages were infected in 96 well plates for 3 h with *Leishmania* expressing LUC. The experimentally infected macrophages were kept in a humid atmosphere containing 5 % CO_2 at 37 °C. After 72 h of incubation with the compounds, the anti-amastigote activity was measured indirectly by the evaluation of luciferase activity, measured by luminometry [21]. Initially, intracellular amastigote susceptibility profile was evaluated in the presence of 5 μM of each compound. Compounds with activities above 80 % growth inhibition at 5 μM had the CC_{50} and IC_{50} established.

2.5. Computational prediction of pharmacokinetic properties

Selected compounds had their SMILES used as input to the pkCSM predictor [22] which is able to predict small-molecule pharmacokinetic properties using graph-based signatures (http://biosig.unimelb.edu.au/pkcsm/). The tool is dynamic and curated using experimental data to predict concomitantly several properties related to ADMET - Absorption, Distribution, Metabolism, Excretion, and Toxicity. Quantitative data returned by pkCSM were converted to binary data according to the thresholds previously defined [23]. These binary vectors describing the ADMET properties of each compound were combined in a matrix used as input to GenePattern to perform hierarchical clustering parameterized with Euclidean distance and average linkage. Here we also created a pkCSM score based on 29 features, including for example, Caco2 cell permeability and hepatotoxicity. For each match of positive feature – for example, intestinal permeability is a characteristic that favors druggability and is desirable – we added one point to the score. The same was true when an undesirable feature was not reached by the small molecules. However, if the pkCSM prediction was positive for an unwanted (no advantageous for druggability) the score was reduced in one point as a penalty. The sum of points determined the score of the compounds. Here the pkCSM score varied from 12 to 19.

2.6. Susceptibility profiles in Sb^{III}-resistant Leishmania strains

Three strains were used as follows: a wild type *Leishmania braziliensis* (MHOM/BR/75/M2904), a SbR strain that was previously selected *in vitro* for Sb^{III} resistance and a natural resistant strain (MHOM/BR/2008/330) that was isolated from a human cutaneous lesion [17,18]. Log phase promastigotes (1×10^6 parasites/mL) were seeded in 24-well cell

culture plates containing 1.5 mL of α-MEM and incubated while shaking at 25 °C for 72 h in the presence of several concentrations of selected PBox compounds. Nontreated parasites were established for growth comparison. For drug susceptibility assays, *Leishmania* growth curves were constructed by measuring absorbance at 600 nm on a Spectramax M5 (Molecular Devices). The anti-leishmanial activity is expressed as IC_{50}/72 h, which is the concentration that reduces cell growth by 50 % compared to the untreated control (growth inhibition). Antimony-resistant *Leishmania* spp. were previously selected by *in vitro* stepwise selection, where promastigote forms are exposed to an increased drug concentration until they achieved a growth pattern similar to the wild-type counterparts. Resistance indexes were then obtained on the basis of drug sensitivity profiling curves.

2.7. Computational prediction of MMV676604, MMV688703 and MMV688372 targets in L. braziliensis

Structural similarity search of phenotypic hits through ChEMBL identified that three of the 14 selected compounds have previously experimentally characterized biological mechanisms of actions. Compound MMV676604 is a human cyclin-dependent kinase inhibitor, MMV688703 inhibits cGMP-dependent protein kinases from *Plasmodium falciparum* (PfPKG) and *Eimeria tenella* (EtPKG), and MMV688372 inhibits human CYP450 3A4. We performed protein sequence similarity searches across the entire *L. braziliensis* proteome to identify homologous proteins and conserved binding sites. One homologous CDK protein was identified in *L. braziliensis* (*LbrM.35.0660*), two homologous PKG proteins (*LbrM.18.1180* and *LbrM.34.3990),* and two homologous CYP450 proteins (*LbrM.27.0100* and *LbrM.30.3580*). Homology models of the known targets, and of the *L. braziliensis* homologues, were built using ensembles of experimental crystal structures in Modeller. *LbrM.35.0660* was modelled using experimental structures of homologous phosphotransferases (PDB ID's:1UNL, 1OB3, 1GZ8 and 4YCG; 50–60 % sequence identity); and *LbrM.18.1180* and *LbrM.34.3990* were modeled using experimental homologous kinases (PDB ID's: 1FOT, 1XH6, 1RDQ, 3PFQ, 4WNK, 4WB7, 5 × 3F and 5DYL; 35–55 % sequence identity). *LbrM.27.0100* and *LbrM.30.3580*, were modeled using the highest identity experimental structures (PDB ID's: 2IAG, 2RCH, 3NA0, 3DAN, 2 × 2N and 4LXJ; 15–20 % sequence identity). The models were then minimized using the MMF94 s forcefield in Sybyl-X 2.1.1. (Certara L.P.). MMV676604, MMV688703, and MMV688372 were docked blindly into the homology models of the experimentally validated targets and proposed *L. braziliensis* targets using AutoDock 4.2.6 [24]. Where more than one docking pose was suggested, poses were evaluated by CSM-Lig [25] and the top solution analyzed. Intra-molecular interactions were calculated and visualized using Arpeggio [26], and their relative strength analyzed by mCSM-Lig [25]. Pocket analysis was performed using Ghecom [27]. Some pharmacokinetics, drug-likeness and medicinal chemistry friendliness features were evaluated based on swiss ADME (http://www.swissadme.ch/) [28]. Target prediction were also screened using swiss target prediction tool (http://www.swisstargetprediction.ch/) [29].

3. Results

3.1. 'Pathogen Box' screening resulted in 27 hit compounds as a source of drug repurposing

The drug screening using a fixed concentration of 5 μM revealed that 27 out of 400 compounds – approximately 7 % of PBox collection – presented anti-leishmanial activity with, at least, 80 % growth inhibition against *L. braziliensis* intracellular amastigotes (Fig. 1A). Among them, 4 were identified as reference compounds: pentamidine (MMV000062), amphotericin B (MMV689000); delamanid (MMV688262) and buparvaquone (MMV689480); resulting in 23 out of 400 potential new small molecules with distinguished anti-leishmanial activity (Fig. 1A). When evaluated according to disease set in PBox the compounds were related with, most of the anti-leishmanial compounds (32 %) identified in this first phenotypical drug screening were previously known to be active against kinetoplastids; followed by compounds with anti-*Plasmodium* (28.6 %) anti-*Mycobacterium tuberculosis* (25 %) and anti-*Toxoplasma* (Fig. 1B). The identification of anti-leishmanial activity from compounds from different disease set, represents a source of molecules for further investigations based on drug repurposing strategy

3.2. Six of the selected anti-leishmanial compounds demonstrated extraordinary selectivity index

The 27 pre-selected PBox compounds were further investigated and tested in a concentration range to establish their IC_{50} or the drug concentration able to inhibit 50 % parasite growth. Six out of 23 compounds (without considering the reference compounds) where shown to be extremely selective against *L. braziliensis* intracellular amastigotes with selectivity indexes (SI) reaching more than 740 fold when considering the THP-1 host cell CC_{50} (Table 1 and Fig. 2). Three compounds MMV676358, MMV659004 and MMV011903 presented good selectivity with SI from 38.5 to > 67 fold (Table 1), two of them classified as anti-*Plasmodium* molecules (malaria disease set). On the other hand, we were not able to calculate the IC_{50} of six other compounds once it was greater than 10 μM, the maximal tested concentration. We thus attribute this discrepancy to thaw-freezing process resulting in loss of activity. The only compound we did not calculate IC_{50} was MMV690102, since it was

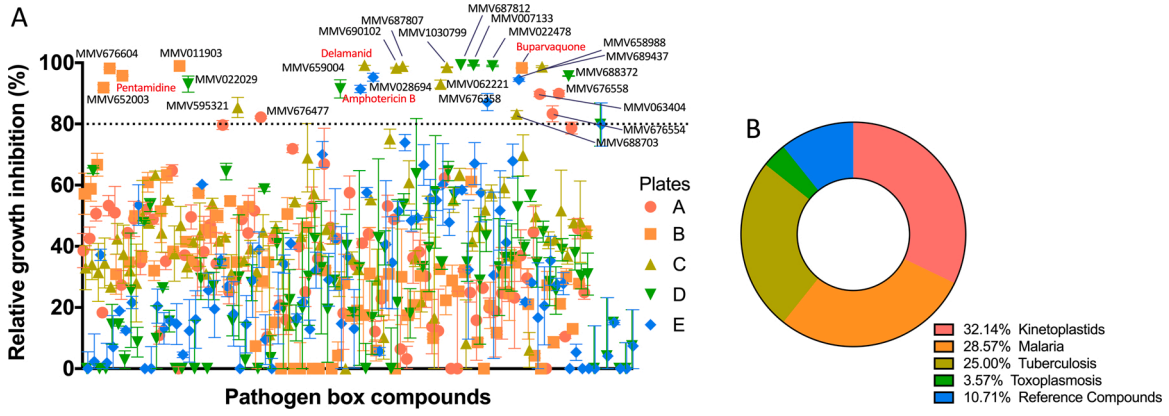


Fig. 1. Antileishmanial phenotypic drug screening revealed 27 out 400 active compounds from PBox against *L. braziliensis* intracellular amastigotes. A) Each compound was tested at fixed 5 μM concentration and 80 % of growth inhibition was the cut-off to select the compounds for the further assay of IC_{50} measurement. Graph represents the mean with its standard error of, at least, two assays performed in duplicate. The A, B, C, D and E color code represents each PBox plate.

Table 1
Antileishmanial, cytotoxic activity, selectivity index and predictive *in vivo* toxicity of 27 compounds from the open source small molecule collection Pathogen Box.

'Pathogen Box' Compounds	CC_{50} (μM) (95% CI) Macrophages	IC_{50} (μM) (95% CI) *L. braziliensis* Intracell. amast.	SI	pkCSM score	'Pathogen Box' Disease set
MMV688703	> 50	0.067 (0.02 - 0.22)	> 746	14	Toxoplasmosis
MMV688372	29.64 (24.38–36.04)	0.047 (0.03 - 0.06)	630	13	Kinetoplastids
MMV676604	> 50	0.083 (0.06 - 0.10)	> 602	13	Kinetoplastids
MMV689480 (Buparvaquone)	12.03*	0.02 (0.01 - 0.04)	601	19	Ref. Compound
MMV676477	> 30	0.05 (0.02 - 0.09)	> 600	14	Tuberculosis
MMV689437	23.63 (17.88–31.23)	0.08 (0.04 - 0.16)	295	14	Kinetoplastids
MMV652003	> 50	0.48 (0.30 - 0.74)[#]	> 104	14	Kinetoplastids
MMV689000 (Amphotericin B)	12 (11.43–12.59)**	0.12 (0.10 - 0.15)	100	16	Ref. Compound
MMV007133	19.75 (14.72–26.51)	> 10	1.97	14	Malaria
MMV028694	1.18 (0.81–1.73)	1.42 (0.74–2.73)	0.83	18	Malaria
MMV688262 (Delamanid)	1.09 (0.83–1.42)	0.015 (.008 - 0.02)	73	18	Ref. Compound
MMV676358	> 50	0.75 (0.25–2.23)	> 67	17	Malaria
MMV022478	2.12 (1.85–2.42)	3.5 (2.4–5.23)	0.60	16	Malaria
MMV687807	4.77 (3.02–7.52)	0.56 (0.35 - 0.88)	8.51	15	Tuberculosis
MMV000062 (Pentamidine)	> 50	1.15 (0.65–2.03)[†]	> 43	16	Ref. Compound
MMV659004	20.4 (14.5–28.71)	0.53 (0.3 - 0.98)	38.5	15	Kinetoplastids
MMV011903	3.89 (1.56–9.68)	0.09 (0.050 - 0.18)[a]	43	14	Malaria
MMV022029	3.44 (2.58–4.58)	2.61 (1.75–3.9)	1.31	16	Malaria
MMV595321	0.62 (0.4 - 0.94)	0.05 (0.03 - 0.1)	12.4	19	Kinetoplastids
MMV687812	1.68 (0.92–3.07)	0.35 (0.12–1)	4.8	19	Tuberculosis
MMV690102	ND	ND	–	14	Kinetoplastids
MMV1030799	> 50	> 10	–	12	Malaria
MMV062221	> 50	> 10	–	13	Malaria
MMV658988	> 50	> 10	–	13	Kinetoplastids
MMV676558	> 50	> 10	–	13	Tuberculosis
MMV063404	> 50	> 10	–	16	Tuberculosis
MMV676554	> 50	> 10	–	14	Tuberculosis

IC_{50}: inhibitory concentration of 50 % parasite growth or the drug concentration enough to inhibit 50 % a given effect. CC_{50}: cytotoxic concentration of 50 % or the drug concentration enough to be toxic to 50 % of the cells; SI: selective index (CC_{50}/IC_{50}); *Buparvaquone: Ref. [30]; **Amphotericin B: Ref: [31]; ND: not determined; Ref. compounds: commercially available know active compounds present among PBox small molecule collection; 95 % CI: confidence interval of 95 %; pkCSM score: is a score based on multiple ADMET features from the pharmacokinetic predictor pkCSM. Here it varied from 12 to 19, as an evidence of *in vivo* toxicity. The higher, the safer it will be in an *in vivo* context (please see Fig. 2). Compound MMV6882626 is not listed as a reference compound in the original Pathogen Box biological activity table, however, delamanid is already in the market as a drug used to treat tuberculosis, and for this reason we considered it as reference compound. Six highly selective compounds: MMV688703, MMV688372, MMV676604, MMV676477, MMV689437, MMV652003; Compounds presenting good selectivity: MMV676358, MMV659004, MMV01193; Reference compounds: MMV689480 (Bupavarquone), MMV689000 (Amphotericin B), MMV688626 (Delamanid), MMV000062 (Pentamidine). [#]MMV652003 anti-leishmanial activity differs from the other five compounds as revealed by ANOVA followed by Dunnet's multiple comparison test with p varying from 0.01 to 0.0003, respectively against MMV676604 and MMV688372, respectively. However it is statistically similar to the [†]Pentamidine antileishmanial activity, that differ from the other five compounds ($p = 0.004$ to $p = 0.0002$). [a]Compound MMV011903 presented similar potency to the five first compounds but MMV652003, and is more active than Pentamidine and MMV659004 ($p < 0.005$). Statistical analysis were performed using Graphpad Prism software version 7 (Graphpad Software Inc, San Diego, USA).

one of the compounds used to establish drug screening optimization, however, anti-promastigote assay indicated it had low anti-leishmanial activity (Table 2).

We also calculated the predicted *in vivo* ADMET properties using a computational tool, the pkCSM score (see details in methods section). All the above mentioned six highly selective anti-leishmanial compounds presented a low pkCSM score of 13 or 14 (in a scale varying from 12 to 19) (Table 1 and Fig. 2). It can be indicative that these hit compounds should pass drug development improvements in order to fit with bioavailability and toxicological criteria. It is worthy of note that reference compounds presented higher pkCSM scores (Table 1). In this regard, we also highlight compound MMV676358 with SI > 67 fold and pkCSM score of 17, the highest presented by selective compounds (Table 1), which is an example of anti-malarial molecule that deserves more attention as anti-leishmanial drug.

Considering the six most potent and selective anti-leishmanial PBox compounds, we clearly see that MMV688372 is clustered together with amphotericin B, glucantime and delamanid (MMV688262), while MMV676604, MMV652003 and MMV689437 shared a branch close to miltefosine (Fig. 2). The others MMV688703 and MMV676477 seems to be classified apart in a different cluster, which could indicate they present distinct mode of action (Fig. 2).

All selected compounds do not violate any of Lipinski's rule of five, as revealed by screening using Swiss ADME (http://www.swissadme.ch/) [28] – the Ro5 evaluate druglikeness based on key pharmacokinetic and physical properties parameters that favors activity when administered by oral route. The small molecules must present: molecular weight (MW) ≤ 500 Da; mlog$P$ ≤ 4.15; N or O ≤ 10; NH or OH ≤ 5 [32] (Fig. 3). Following medicinal chemistry friendliness, none of the six anti-leishmanial selected PBox presented PAINS (pan-assay interference compounds) alert and only MMV652003 fitted leadlikeness, with 250 ≤ MW ≤ 350; xlog$P$ ≤ 3.5 and number of rotatable bonds ≤ 7 [33] (Fig. 3).

3.3. None of the selected compounds presented cross-resistance to Sb^{III}

A good way to infer the anti-leishmanial mode of action of a given small molecule is to compare its sensitivity profile using drug-resistant parasites. Here we applied this strategy using laboratory-selected and field isolate antimony-resistant *L. braziliensis* mutants in which drug transport is modulated [17,18]. None of the tested compounds presented antimony-resistance or the resistance index was below 2 fold (Table 2). It indicates that these small molecules target different biochemical pathways and are of great interest for further investigations including antimony-resistant parasites. The anti-malarial small molecule MMV676358 did not present the same potency as it does for anti-amastigote activity. In fact it can be clearly observed that *L. braziliensis* amastigotes are more sensitive to the compounds when compared with promastigotes (Tables 1 and 2). Reinforcing the importance of using the clinically relevant stage for drug screening purpose.

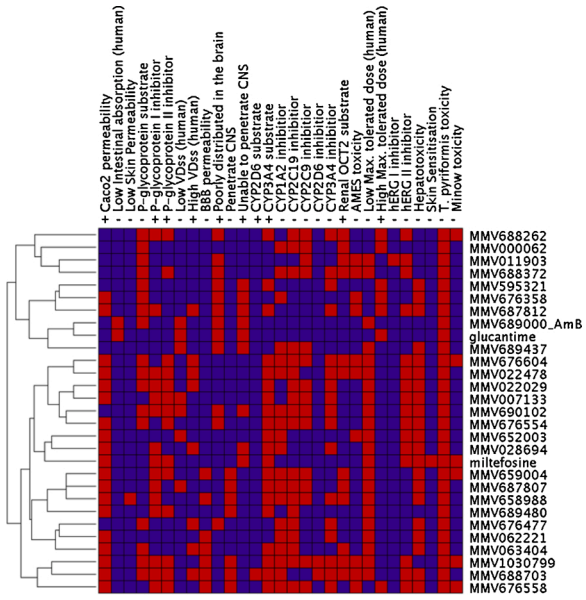


Fig. 2. Hierarchical clustering of compounds based on their ADMET properties predicted with pkCSM. The matrix contain pkCSM [22] features on the top line against the selected Pathogen Box compounds. The red squares indicates a positive output (presence or 1) while dark blue indicates lack of the respective feature. Druggability favorable desired and undesired features are indicated by + and - signs. Antileishmanial drugs glucantime, miltefosine and amphotericin B were included as reference molecules. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

3.4. MMV676604, MMV688703 and MMV688372 have potential targets in L. braziliensis

Computational analysis revealed that the targets previously described for compounds MMV676604, MMV688703 and MMV688372 in other organisms are also putative candidates in *L. braziliensis*.

MMV676604 docked into the ATP binding pockets of human cyclin dependent kinases: CDK1, CDK2, CDK4 and *LbrM.35.0660* (CRK3 – cdc-related kinase 3). Comparison of MMV676604 docked into *LbrM.35.0660* with experimental structures of other ATP inhibitors co-crystallised with CDK2 (PDB ID's: 2WO6, 2XMY, 1OIT, 1URW) showed MMV676604 adopted a very similar conformation, and made similar local interactions (Fig. 4). This provided additional confidence that MMV676604 acts through inhibition of *LbrM.35.0660*. The binding pocket where MMV676604 docked into *LbrM.35.0660* is a large hydrophobic pocket, where it makes extensive use of pi-interactions to the surrounding residues (Fig. 4B). Looking at the pockets binding potential (hot and warm spots), we can see additional regions of binding potential that would allow for extension from the imidazolidine ring (Fig. 4C). This additional binding potential is not present in the human CDK structures (Fig. 4D), suggesting that this could be used to introduce binding specificity.

A search through ChEMBL revealed that MMV688703 had previously been shown to inhibit EtPKG and PfPKG. Docking into models of these known targets, in addition to the *L. braziliensis* homologs *LbrM.18.1180* (Protein Kinase A catalytic subunit 3) and *LbrM.34.3990* (Protein Kinase A catalytic subunit isoform 1), indicated that MMV688703 was likely to bind to the ATP-binding pockets. The top poses for both *L. braziliensis* proteins were consistent and overlaid nicely with the docked poses for PfPKG and EtPKG (Fig. 5A). Interactions performed by the compound for both proteins were also consistent, composed mainly of a dense network of pi and hydrophobic interactions, with the addition of a persistent hydrogen bond (Fig. 5B). Further analysis of the binding pockets in *LbrM.18.1180* and *LbrM.34.3990* revealed the pocket was largely apolar, but in *LbrM.18.1180* in particular there is extra binding potential not satisfied by MMV688703 that could be explored as part of future molecule elaboration (Fig. 5C).

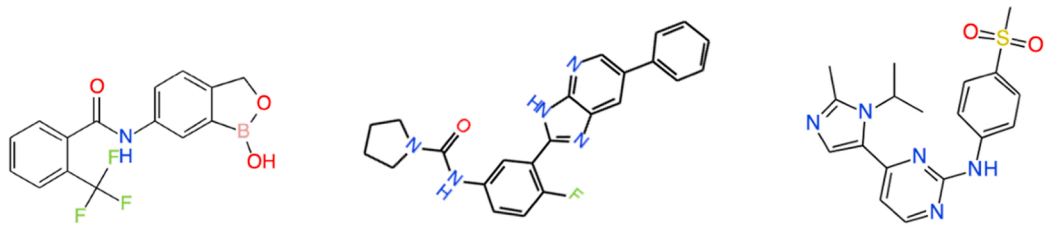
The top docking solutions for MMV688372 into human CYP450 3A4, *LbrM.27.0100* and *LbrM.30.3580* – both cytochrome p-450-like protein – were all similar, making extensive pi and hydrophobic interactions within the large active site apolar pocket and to the heme group (Fig. 6).

Table 2
Antileishmanial activity of selected Pathogen Box compounds against antimony-sensitive (M2904 WT) and antimony-resistant (M2904 SbR or 330 SbR) *L.braziliensis.*

'Pathogen Box' Compounds	IC_{50} (μM) (CI 95%) *L. braziliensis* promastigotes			Resistance Index	
	M2904 WT	**M2904 SbR LabSel**	**330 SbR Field**	**M2904 SbR**	**330 SbR**
MMV652003	1.85 (1.11–3.08)	3.32 (2.7–4.08)	2.05 (1.5–2.82)	1.8	1.1
MMV689437	1.84 (1.55–2.20)	1.42 (1.15–1.76)	1.42 (1–2)	0.8	0.8
MMV687807	0.91 (0.73–1.14)	1.55 (1.22–1.98)	1.8 (1.4–2.82)	1.7	2
MMV022029	2 (1.65–2.44)	1.34 (1.15–1.54)	0.61 (0.51 - 0.72)	0.7	0.3
MMV028694	3.31 (2.45–4.47)	2.4 (1.8–3.21)	3.30 (2.67–4.08)	0.7	1
MMV676477	1.54 (1.38–1.72)	1.06 (0.91–1.22)	ND	0.7	–
MMV659004	2.58 (2.06–3.22)	1.31 (1.06–1.62)	1.64 (1.36–1.99)	0.5	0.6
MMV676358	> 10	5.58 (3.72–8.37)	> 10	< 0.5	–
MMV687812	4.14 (3.07–5.6)	1.56 (1.35–1.80)	1.95 (1.60–2.36)	0.4	0.5
MMV063404	> 10	3.47 (2.5–4.84)	> 10	< 0.4	–
MMV007133	> 10	3.87 (2.21–6.8)	> 10	< 0.4	–
MMV688703	1.78 (1.55–2.04)	0.32 (0.30 - 0.35)	ND	0.2	–
MMV022478	> 10	< 0.3	< 0.3	–	–
MMV595321	ND	1.77 (1.36–2.30)	1.82 (1.22–2.72)	–	–
MMV011903	ND	1.09 (0.93–1.3)	0.75 (0.63 - 0.9)	–	–
MMV688262 (Delamanid)	ND	0.38 (0.33 - 0.43)	0.16 (0.10 - 0.25)	–	–
MMV690102	ND	4.65 (4.18–5.19)	3.95 (3.02–5.17)	–	–
MMV689480 (Buparvaquone)	ND	0.16 (0.1 - 0.27)	0.25 (0.2 - 0.32)	–	–
MMV688372	0.47 (0.43 - 0.51)	< 0.3	ND	–	–

IC_{50}: inhibitory concentration of 50 % parasite growth or the drug concentration enough to inhibit 50 % a given effect; CI: confidence interval of 95 %; M2904 WT: wild-type *L. braziliensis* strain (MHOM/1975/BR/M2904); M2904 SbR: laboratory-selected antimony-resistant *L. braziliensis* mutant derived from M904 WT; 330 SbR: antimony-resistant *L. braziliensis* mutant isolated from a patient with cutaneous leishmaniasis [17]; LabSel: laboratory-selected; Field: field isolated parasite; SbR: antimony-resistant.2.

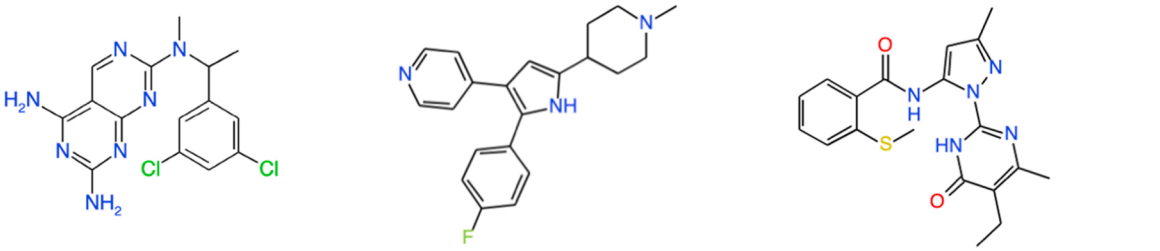
Highly selective compounds: MMV652003, MMV689437, MMV676477, MMV688703, MMV688372. Compounds with good selectivity: MMV659004, MMV676358, MMV011903. Reference compounds: MMV688262 (Delamanid) and MMV689480 (Buparvaquone).



MMV652003
IC_{50}: 480 nM
IS: > 104
Disease set: Kinetoplastids
Synthetic acessibility: 2.91
Molecular weight: 321.06 g/mol

MMV688372
IC_{50}: 47 nM
IS: 630
Disease set: Kinetoplastids
Synthetic acessibility: 3.06
Molecular weight: 401.44 g/mol

MMV646604
IC_{50}: 83 nM
IS: > 602
Disease set: Kinetoplastids
Synthetic acessibility: 3.17
Molecular weight: 371.46 g/mol



MMV689437
IC_{50}: 80 nM
IS: 295
Disease set: Kinetoplastids
Synthetic acessibility: 2.88
Molecular weight: 364.23 g/mol

MMV688703
IC_{50}: 67 nM
IS: > 746
Disease set: Toxoplasmosis
Synthetic acessibility: 2.99
Molecular weight: 335.42 g/mol

MMV676477
IC_{50}: 50 nM
IS: > 600
Disease set: Tuberculosis
Synthetic acessibility: 3.24
Molecular weight: 383.47 g/mol

Fig. 3. Molecular structure of the six most potent and selective Pathogen Box compounds against *L. braziliensis* intracellular amastigotes. Potency varies from 47 to 480 nM with selectivity index (IC_{50}) ranging from > 104 to > 746. Most of them were already known to present anti-kinetoplastid activity, however, MMV688703 and MMV676477, respectively classified within toxoplasmosis and tuberculosis PBox disease set. Synthetic accessibility score varies from 1 (easy to make) to 10 (very difficult to make) based on 1024 fragmental contributions (FP2) modulated by size and complexity penalties, trained 12.782.590 molecules and tested on 40 external molecules [34].

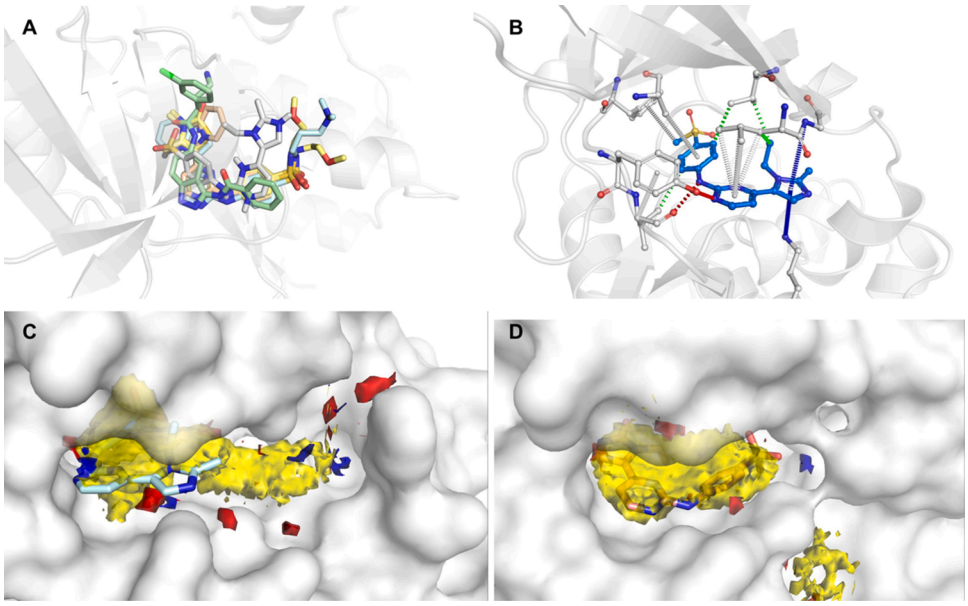


Fig. 4. Characterisation of MMV676604 binding *L. braziliensis* CRK3. A) The top ranked docking pose of MMV676604 binds to *LbrM.35.0660* (CRK3 – cdc-related kinase 3) in the ATP binding pocket in a similar conformation to experimental structures of related CDK inhibitors. B) Within this pocket, MMV676604 makes extensive pi interactions (grey and blue dashed lines) with *LbrM.35.0660*. C) Mapping the binding hot-spot potential of *LbrM.35.0660* reveals a large apolar pocket (yellow), with potential to accommodate donor (blue) and acceptor (red) moieties. Compared to the hot-spot binding potential of CDK2, which only just accommodates the docked MMV676604 shown in pale cyan (D), the pocket of *LbrM.35.0660* is larger and would allow extension of the hit to improve binding affinity and specificity. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

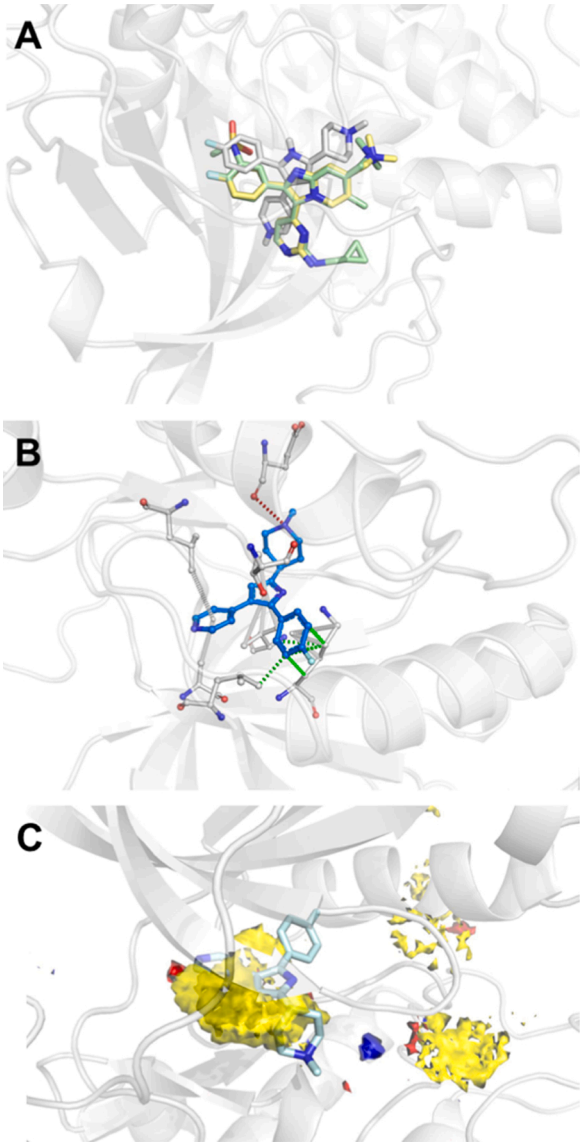


Fig. 5. Characterisation of MMV688703 binding. A) MMV688703 docked to the ATP-binding pocket of *Plasmodium falciparum* PKG (shown in green sticks), *Eimeria tenella* PKG (shown in yellow sticks) and *LbrM.18.1180* (shown in grey sticks). B) MMV688703 made extensive pi interactions to *LbrM.18.1180* and *LbrM.34.3990*, in addition to a strong hydrogen bond and some hydrophobic interactions. (C) Mapping the binding hot-spot potential of *LbrM.18.1180* shows MMV688703 bound within an apolar pocket (yellow), but with additional nearby binding hot-spot potential, which could be further explored to improve compound binding affinity. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

While the overall sequence identity between human CYP450 3A4 and *LbrM.27.0100* and *LbrM.30.3580* is low (21 % sequence identity), the proposed binding site is more strongly conserved, which would be consistent with a conserved mode of inhibition between the proteins.

4. Discussion

The Pathogen Box collection demonstrated to be a valuable source of anti-leishmanial compounds, revealing around 7 % (27 compounds) of its small molecule content as being active against *L. braziliensis* intracellular amastigotes by reducing at least 80 % parasite growth at 5 μM. It represents a high percentage compared to other chemical library screens towards *Leishmania* spp. [35–38]. Previous reports using the MMV open access box revealed 14 active hits against *L. major* intracellular amastigotes [39], and none of them match with the compounds selected here against *L. braziliensis*. Recent studies have found potential anti-leishmanial compounds against *L. donovani* intracellular amastigotes; where nine PBox compounds presented > 70 % growth inhibition with IC_{50} of 53–704 nM [11]; and five leads presented SI up to 453 fold [12]. Pathogen Box screened by Ullah and cols. (2020) yielded six lead compounds presenting IC_{50} varying from 50 to 480 nM against *L. amazonensis* intracellular amastigotes [15], including MMV676477, an anti-parasitic small molecule which they revealed to promote *Leishmania* microtubule polymerization [15]. In a similar way, Berry and cols. (2018) published six anti-leishmanial leads from PBox screening against *L. mexicana* intracellular amastigotes, however, with higher IC_{50} (0.1–5.3 μM) than recent findings [13].

Here, among the 23 selected hit compounds (excluding 4 reference compounds) we highlighted 14 molecules presenting SI above 10 fold (3.5 %) and filtered 6 highly selective anti-leishmanial leads: MMV688703, MMV688372, MMV676604, MMV676477, MMV689437 and MMV652003; against *L. braziliensis* intracellular amastigotes with SI reaching >746 and IC_{50} ranging from 47 to 480 nM. As above mentioned, independent studies were able to identify similar anti-leishmanial compounds. Three out of six selected anti-leishmanial compounds by Ullah and cols. [15] (MMV652003, MMV688372 and MMV676477) matched with our findings here. However, while they found IC_{50}s of 220, 160 and 79 nM, respectively for MMV652003, MMV688372 and MMV676477, we found 480, 47 and 50 nM for the same compounds. We also observed overlaps among anti-leishmanial compounds selected by Berry and cols. [13] and Tadele and cols. [11]. Delamanid (MMV688262), an anti-tuberculosis agent had IC_{50}s of 1.78 μM [13] and 0.249 μM [11], while we found 15 nM for the same compound. Four out of the six selected compounds highly selective against *L. braziliensis* – MMV688372, MMV676604, MMV689437 and MMV652003 – were previously reported as showing *in vitro* anti-leishmanial activity against *L. infantum* (PBox biological activity sheet) and *L. donovani* intracellular amastigotes [8]. However, they were less active reaching IC_{50}s of 1.4 and 5.73 μM, respectively for *L. infantum* and *L. donovani* intracellular amastigotes [8]; compared to 0.047 - 0.48 μM IC_{50}s for *L. braziliensis*, described here. Similar results were found in PBox biological activity data against *L. mexicana*, where MMV676604 IC_{50} was 3.2 μM against 0.08 μM for *L. braziliensis*, presented in this study. Even considering the experimental and laboratorial conditions/variations, the main lesson here is the importance of species-specific phenotypic drug screening, since it could point out potential different targets and involved pathways in distinct parasites. In general, it seems that *L. braziliensis* are more sensitive than other *Leishmania* spp. to PBox compounds, however, additional confirmations are needed to explore this possibility. Indeed, previous studies filtered PBox compounds that were not selected under analysis. In addition, some of the selected compounds were not classified as kinetoplastid disease set within PBox, reinforcing the use of this collection as insight and source of drug repurposing strategy. Nine compounds (compounds with high or good selectivity in Table 1) were selective against *L. braziliensis* in which 4 of them: MMV688703, MMV646766, MMV676358 and MMV011903 were respectively classified as anti--*Toxoplasma*, anti-*Mycobacterium tuberculosis* and anti-malarial agents.

Our screening yielded 6 hits with extraordinary selectivity index, which could be chemical starting points for lead optimization programs.

MMV688703 is in the class of pyrole-piridin and has demonstrated activity against *Toxoplasma gondii*, *Plasmodium* and *Eimeria tenella* [8, 40–42]. This chemical class is shown to target cGMP-dependent protein kinase (PKG) in these parasites. This compound was also able to inhibit infection by *P. falciparum* [43] and the parasite growth of *Babesia bovis* [44], *L. major* [45] and *L. donovani* [11]. Molecular docking of MMV688703 reveal the chance of its binding to *L. braziliensis* PKA homolog (*LbrM.18.1180* and *LbrM.34.3990*) revealed that this is a putative target in *Leishmania*.

MMV688372 is an oxazolopyridine, a class of compounds known to

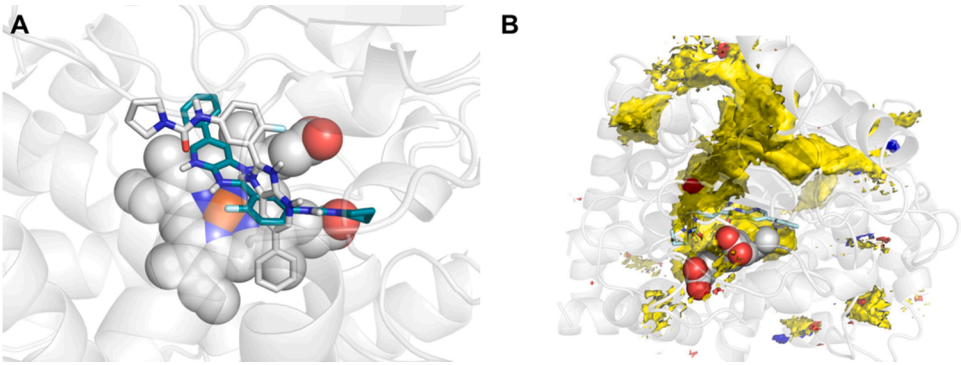


Fig. 6. Characterisation of MMV688372 binding at *L. braziliensis* CYP450-like protein. A) MMV688372 bound to *LbrM.30.3580* (shown in grey sticks) and human CYP450 3A4 (shown in blue sticks). In both structures, MMV688372 made extensive hydrophobic and pi interactions within the pocket and to the heme group. (B) Mapping the binding hot-spot potential of the structures revealed a large apolar pocket, consistent with many CYP450's. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

be active against *T. brucei* by targeting the sphingolipid metabolism [46]. Lipid metabolism is a described drug target in *Leishmania* [47] still, oxazolopyridine compounds are yet to be explored in anti-leishmania drug discovery, however, MMV688372 was find to inhibit *L. amazonensis* axenic amastigotes growth [15]. Our molecular docking study indicated that MMV688372 may be able to inhibit human CYP450 3A4 as well as its *L. braziliensis* homolog. Since MMV688372 SI is 630, it is not considered toxic to human cells in the concentration used against *L. braziliensis*, indicating that the inhibition of this enzyme is not critical for the survival of mammalian cells but is for *Leishmania.* In fact, CYP5122A1 - a *L. donovani* CYP450 - was demonstrated to be essential for the parasites survival, playing a role in cell growth, infection and ergosterol biosynthesis [48].

MMV676604 is an 2-aminopyramidine (also known as AZD438) was initially conceived as human cyclin-dependent kinase inhibitor [49]. This compound has recently demonstrated activity against *Toxoplasma gondii* [40] and other homologs were also studied for different human diseases, such as cancer and dermatological disorders [50]. It also demonstrates anti-trypanosomal activity by inhibiting TbERK8 [51]. The different CDKs have highly conserved active sites, therefore developing selective CDK inhibitors is a difficult task [50]. However, the molecular docking results presented here suggest that MMV676604 have additional binding sites in *L. braziliensis* CRK3 (*LbrM.35.0660)* that are not present in the human CDK, explaining its high SI value found here. When screened using swiss target prediction tool based on human targets MMV676604 returned 26.7 % chance to have kinase as top 15 target classes with high probability of cyclin-dependent kinase as main target. Since it presented high selectivity towards the parasite, it is an evidence of target confirmation and deserves to be further investigated.

MMV676477 is a pyrimidinone-pyrazole was reported to be active against *Mycobacterium tuberculosis* [16] and more recently against the apicomplexan *Neospora caninum* [52]. This compound has a benzamide group that was shown to be a potent inhibitor of *Trypanossoma brucei* [53]. Although its analog is a human tyrosine kinase TrkB inhibitor [54], in *Leishmania amazonensis* MMV676477 affected cell division and was shown to selectively disrupt *Leishmania* tubulin dynamics [15]. It is supported by another study that found MMV676477 as active against *L. mexicana* intracellular amastigotes [13].

MMV689437 belongs to pyrimido[4,5-d]pyrimidine-2,4,7-triamine chemotype and has demonstrated activity against *L. donovani* and *T. cruzi*. This class of compounds has been identified to target DHFR (dihydrofolate reductase) a known drug target for *Leishmania* and a good treatment strategy for antimicrobial infections [55]. This compound represents an excellent choice for progression into hit-to-lead projects of drug discovery for leishmaniases, since its supposed target and pathway have already been carefully studied [8]. Among the 27 anti-leishmanial hits highlighted here, we also selected MMV690102, which is a MMV689437 analog, however, anti-promastigote sensitivity profile revealed a reduced potency when compared to the most prominent candidates.

MMV652003 is a benzoxaborole, a class of compounds known to be active against bacterial, fungal and protozoan pathogens. Boron-containing molecules have been reported to be active against *T. brucei* [56], demonstrating cure in murine model and excellent pharmacokinetic behavior; also targeting PfCPSF3 *P. falciparum* [57], which encodes a homologue of mammalian cleavage and polyadenylation specificity factor subunit 3 (CPSF-73 or CPSF3). MMV652003 analogs, which had improved pharmacokinetics properties and good performance *in vivo* against human African trypanosomiasis [58]. *In vitro* anti-leishmanial activity of MMV652003 has been reported against *L. amazonensis* [15]; *L. mexicana* [13] and *L. donovani* [8].

In order to further explore the mode of action of selected PBox compounds we tested their activity against antimony-resistant *L. braziliensis* parasites, either laboratory-selected strain or field isolated mutants. All tested compound were also active against these mutants which are not cross-resistant to the compounds but sometimes more sensitive. It represents a hope on treating patients infected with drug-resistant parasites.

Another advance here was the power of computational *in vivo* drug toxicity prediction based on pkCSM, a tool that predicts pharmacokinetics and toxicity properties of a given small molecule based on experimental data. In this regard, ADMET features together can be used to guide hit-to-lead drug selection and prioritize drug candidates either for drug development optimization and *in vivo* further experimental efficacy and safety evaluation. This strategy is also currently being applied to study anti-*Schistosoma* drug discovery (Sandra Gava, Instituto René Rachou - Fiocruz Minas, personal communication).

Here, we demonstrate that PBox is an important source of compounds for hit-to-lead development and target identification studies for anti-leishmanial drugs discovery. Our pkCSM study revealed several hit compounds with desirable pharmacokinetics properties, such as Caco2 permeability and no CYP inhibition, which represents potential oral administration and low side effects. The high selectivity indexes of the 6 selected compounds, combined with the fact that they do not present cross resistance to Sb^{III}make these hits promising candidates for drug development for leishmaniasis resistant to antimony treatment.

5. Conclusions

This is the first study reporting activity of Pathogen Box compounds against *Leishmania braziliensis*. We were able to filter 6 highly selective compounds that are promising anti-leishmanial drug candidates. All of them do not shown antimony cross-resistance indicating it can be an alternative for drug-resistant leishmaniasis chemotherapy. We provided several clues on *in vivo* drug toxicity prediction and putative *L. braziliensis* drug molecular drug targets that deserves validation and efficacy studies in animal models. Mode of action assays will provide additional support for the finding here, some of them confirmed by independent research groups with different *Leishmania* spp. Some of the promising anti-leishmanial compounds reported here can be considered

for further drug repurposing studies since they were previously reported as anti-malarial, anti-toxoplasma and anti-*Mycobacterium tuberculosis* activities. The Pathogen Box, is a valuable open source of selective anti-leishmanial agents an provides a useful collaborative drug discovery and development to faster advance on drug alternatives to tackle leishmaniasis.

Declaration of Competing Interest

The authors declare that there are no conflict of interest.

Acknowledgements

The authors thank the Medicines and Malaria Ventures (MMV) for providing the Pathogen Box and promoting collaborative drug discovery worldwide. We also thanks Dr. Marina Mourão – Instituto René Rachou, Fundação Oswaldo Cruz, for sharing some extra quantities of PBox compounds. We thank Dr. Heveline Silva, Department of Chemistry – Universidade Federal de Minas Gerais, for her help on data mining from PBox compounds. JASS. received a scientific initiation (undergraduate) fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, Brasil. This work was financed in part by the Coordenacão de Aperfeiçoamento de Pessoal de Nível Superior−Brasil (Capes) with an award to LGT for a Ph.D. (Proap-Capes) scholarship. DBA was funded by an Investigator Grant from the National Health and Medical Research Council (NHMRC) of Australia [GNT1174405] and supported in part by the Victorian Government's OIS Pro-gram. RLMN is a CNPq Research Fellow (#310640/2017-2).

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