Drug Screening using Shape-based Virtual Screening and In Vitro Experimental Models of Cutaneous Leishmaniasis

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RUNNING TITLE: Leishmanicidal activity of Oxazolo[4,5-b] pyridine derivative and benzimidazole derivative.

Synopsis

Cutaneous leishmaniasis (CL) is one of the most disregarded tropical neglected disease with the occurrence of self-limiting ulcers and triggering mucosal damage and stigmatizing scars, leading to huge public health problems and social negative impacts. Pentavalent antimonials are the first-line drug for CL treatment for over 70 years and present several drawbacks in terms of safety and efficacy. Thus, there is an urgent need to search for non-invasive, non-toxic, and potent drug candidates for CL. In this sense, we have implemented a shape-based virtual screening approach and identified a set of 32 hit compounds. *In vitro* phenotypic screenings were conducted using these hit compounds to check their potential leishmanicidal effect towards *Leishmania amazonensis*. The findings showed that two (Cp1 and Cp2) out of the 32 compounds revealed promising antiparasitic activities, exhibiting considerable potency against intracellular amastigotes present in peritoneal macrophages (IC_{50} values of 9.35 and 7.25 μM, respectively). Also, a sterile cidality profile was reached at 20 μM after 48 hours of incubation, besides a reasonable selectivity (≈8), quite similarly to pentamidine, an aromatic diamidine still in use clinically for leishmaniasis. Cp1 with an Oxazolo[4,5-b]pyridine scaffold and Cp2 with a benzimidazole scaffold could be developed further by lead optimization studies to enhance their leishmanicidal potency.

KEY WORDS: Cutaneous leishmaniasis, *Leishmania amazonensis*, shape-based virtual screening, *in vitro* experimental chemotherapy.

Introduction

Cutaneous leishmaniasis (CL) is a vector-borne tropical neglected disease caused by over 20 different species of kinetoplastid parasites of the genus *Leishmania*. This disfiguring and stigmatizing disease occurs through the injection of promastigote forms into the mammalians by infected female sandflies, triggering ulcers and permanent scars at skin and/or oral and nasal mucosa injuries, thus contributing to high social stigmatization and public health issue (WHO, 2020). Although about 1,2 million of new cases occur annually, CL does not have adequate treatment that are mostly based on old and highly toxic drugs besides the occurrence of high number of parasite species with drug resistance profile (Bailey *et al*., 2019, Alvar *et al*., 2012, de Vries *et al.,* 2015, Van Bocxlaer *et al.,* 2019).

First line treatments include the clinical use of pentavalent antimonials drugs developed 70 years ago that present several drawbacks in terms of efficacy, safety and require long painful periods of administration (Eiras *et al.,* 2015, DNDi 2018). In the case of antimonial resistance, the second-choice therapy includes pentamidine and amphotericin B (deoxycholate), which also share the previous reported concerns and limitations (Croft 2006, de Vries *et al.,* 2015). Up to now, the only oral and less toxic alternative drug – Milteforan – is unavailable in many developing poorest countries (Bilgic Temel 2019). Besides, the safer liposomal formulation of amphotericin B is highly costly and still under evaluation for effectiveness against CL (Shirzadi 2019). Last clinical trials for CL were mostly based on drug repurposing and/or combination but unfortunately the overall findings were not very successful to demonstrate an improvement of therapeutic efficacy, such as the combination of Pentavalent antimonial with imiquimoid (Miranda-Verastequi *et al*., 2009) and the topical use of 3% amphotericin B (Lopez *et al*., 2018). A phase II clinical trial regarding a shorter course of oral miltefosine administrated in combination with thermotherapy,

conducted in Peru and Colombia, ended in 2019 but the outcomes were not published yet (Valencia *et al*., 2013, https://www.dndi.org/diseases-projects/portfolio/new-cl-combos).

Computer aided drug design is an efficient strategy to identity active compounds. Shape-based screening has been successfully employed for the development of anti-fungal and anti-bacterial agents (Swinney & Anthony, 2011). To employ this approach, 3D structure of the target protein/receptor is not required. However, an established active compound (defined as a query compound) against a target is a starting point for this approach. The purpose of the shape-based screening is to identify chemically diverse compounds that show similar biological activity as the query compound. This is based on the principle that diverse structures that share similar shape and electrostatic potential surface, or topology will have highest probability to bind to the same pocket and consequently share the similar activity (Kumar & Zhang, 2018). Due to the limited information on target proteins of *Leishmania amazonensis* and the non-availability of quality 3D structures of target proteins, we have carried out ligand-based shape screening approach using an established active compound GNF5343 that is reported to display broad spectral anti-parasitic activity (Khare *et al*., 2016). We have identified a set of 32 hit compounds from this study. Thus, the urgent need for safer and selective potent drugs associated with promising aspects of identified diverse hit compounds encouraged us to perform *in vitro* phenotypic screening of these compounds upon amastigotes of *Leishmania amazonensis*, which is one of the main agents of CL in the Americas (Martins 2014, de Vries *et al.*, 2015).

Methods

Compounds: All 32 identified hit compounds (Chart 1 and Figure 1) that were purchased from Asinex commercial vendor and the reference drug, Pentamidine (Pt), were dissolved in DMSO (stock solutions at 20 mM) and fresh dilutions prepared extemporaneously, with the final concentration never exceeding 0.6% for *in vitro* experiments, which does not induce host cell toxicity (Santos *et al.,* 2019).

Parasite strain and mammalian host cell cultures: *Leishmania amazonensis* (strain *LTB0016*) was used throughout the study. Male BALB/c mice were infected (10^6 amastigotes/20 µL culture medium, via subcutaneous) at their foot paws, using a BD ultrafine^{TM} 6 mm syringe (15/64") x 31 G, following previous reported protocol, with minor modifications (Van Bocklaer *et al*., 2019). After 30 days post infection, the animal skin lesions were aseptically removed, and the parasites obtained by mechanic dissociation (pipetting). The purified amastigotes were then assayed directly with the studied compounds to check the activity upon free amastigote forms (FA), or used to infect primary cultures of peritoneal macrophages (PMM) to investigate their potency against intracellular forms (IA) (Feitosa LM *et al*., 2019). Swiss male mice (18-20 g) were inoculated with 3% thioglycolate, and after 4 days, PMM collected by rinsing the animals' peritoneum with RPMI 1640. Mammalian cells were seeded at 24 ($3x10^5$ cells/well) and 96-wells ($5x10^4$ cells/well) plates and used for *in vitro* infection and host cell cytotoxicity analysis, respectively. The cultures were sustained at 37°C with 5% $C0_2$ atmosphere in RPMI 1-640 medium (pH 7.2 to 7.4) without phenol red (Gibco BRL) but supplemented with 1% L-glutamine, 1% PEN-STR, 10% fetal bovine serum (FBS). Assays using FA were also maintained at 32ºC using the same RPMI culture medium but adding 5% FBS instead of 10%.

126 *Cytotoxicity upon mammalian host cells and leishmanicidal analysis*: For cytotoxicity
127 analysis, PMM were incubated for 48h with increasing concentrations of the tested
128 compounds (up to 500 µM). Cellular viability was evaluated by AlamarBlue tests
129 (Invitrogen) following the manufacturer's instructions (Da Silva *et al.,* 2007,
130 Romanha *et al.,* 2010). The leishmanicidal activity was explored in two steps: in the
131 first set of assays, amastigotes (10^6 parasites per well in 0.2mL) purified from animal
132 lesions (free amastigotes -FA) were exposed for 48 h using a fixed concentration (10
133 µM), and then, drug activity assessed by AlamarBlue tests (Mikus J & Sterverding D,
134 2000). Then, in a second set of phenotypic screenings, the activity of the compounds
135 was further validated upon intracellular amastigotes (IA). In these assays, PMM
136 ($3x10^5$) were infected with amastigotes ($9x10^5$ amastigotes) using MOI 3:1 (Van
137 Bocxlaer *et al*., 2019). After 48 h of compound incubation (0-20 µM), infected PMM
138 were rinsed with saline buffered with phosphate (PBS), fixed with Bouin and stained
139 with Giemsa solution for light microscopy analysis (Santos *et al.,* 2019). Then, the
140 percentage of infected host cells and the number of parasites per infected cells were
141 scored for determination of the corresponding infection index (II) that represents the
142 multiplication factor of both parameters. Only characteristic parasite nuclei and
143 kinetoplasts were counted as surviving parasites since irregular structures could mean
144 parasites undergoing death. The results were expressed as % of reduction of the
145 parasite burden and the IC_{50} and IC_{90} calculated (Santos *et al.,* 2019). All assays were
146 run in at least twice in three independent repeats.

147 *Compound database preparation*: Using the LigPrep module of the Schrödinger drug
148 design software, a database (Asinex gold) of commercially available compounds was
149 prepared by performing 2D to 3D conversion, addition of hydrogens, generation of
150 ionization states, tautomeric states, stereoisomers and ring conformations at the

physiological pH 7.0 ± 2.0. Further, energy minimization of all the compounds was carried out using the molecular mechanics OPLS3 force field.

Results

Shape-based virtual screening: Compound GNF5343, an Oxazolo[4,5-b]pyridine derivative (Figure 1) was reported to display activity against *Leishmania donovani*, *Trypanosoma cruzi* and *Trypanosoma brucei* (Khare *et al*., 2016). This compound was used as a query compound to perform the virtual screening of a set of 60,000 chemically diverse compounds from the Asinex database. These compounds were selected by applying the Lipinski filter (Mol.Wt ≤ 500; cLogP ≤ 5; HBA ≤ 10; HBD ≤ 5), removing the compounds with nitro groups and reactive functional groups (QikProp, Schrödinger release 2017-2). Using the shape-based virtual screening approach, each conformer of the molecule from the database was aligned to the query compound and phase sim score was computed based on the maximum overlapping characteristics. Compounds with shape sim score between 0.85 to 0.6 were visually inspected to assess the structural diversity and synthetic accessibility. Based on these criteria, a subset of 31 compounds (Chart 1) was selected and Hierarchical clustering was performed, in which 2D fingerprints and atom pairs were used as metrics to quantify the chemical diversity (Canvas, Schrödinger release 2017-2). A total of 11 clusters were identified, of which five were singletons with diverse chemical structures such as Quinoxaline derivative (cluster 4), Imidazo[2,1-b]thiazole derivative (cluster 6), benzoxazole derivative (cluster 9), 2-oxo-2H-chromene derivative (cluster 10) and 3-imidazo[1,2-a] pyridine derivative (cluster 11). As the query compound GNF5343 was not available for purchase, we have selected one of its close analogue Cp1 (Figure 1) for the comparative studies along with the selected set of 31 hit compounds. Cp1 differs from GNF5343 in having a thiophenyl ring

substitution instead of furyl ring. All 32 compounds were purchased from Asinex and assayed using different protocols *in vitro*. A fixed concentration (10 µM) was first assessed on free amastigotes (FA) and findings demonstrated that two (Cp1 and Cp2) out of the 32 compounds reduced (≥ 50 %) the number of live parasites. Analogues of Cp2 from cluster 1 (Chart 1: 2a and 2b) displayed weak inhibition. Compounds from other clusters did not show any activity. While Cp1 is a close analogue of query compound, Cp2 is structurally diverse from GNF5343 in having benzimidazole scaffold. Both GNF5343 and Cp2 displayed good alignment and maximum volume overlap (Figure 1) indicating that these two compounds are having similar topology to form similar van der Waals surface interactions at the same region. It is interesting to notice the varied distribution of hydrogen bond donors (HBD), hydrogen bond acceptors (HBA) and hydrophobicity between the basic scaffolds (Oxazolo[4,5-b]pyridine Vs benzimidazole) of GNF5343 and Cp2. These active compounds (Cp1 and Cp2) and the reference drug Pentamidine (Pt) were further analyzed against FA using increasing concentrations of the tested compounds and Pt. The findings showed moderate leishmanicidal effect, with IC_{50} values of 14.93 and 15.86 µM for Cp1 and Cp2 respectively, being less potent than Pt (0.71 µM) (Table 1). In the second round of assays, the compounds were further evaluated against intracellular amastigotes present in the cytoplasm of PPM, which represent the gold models for *in vitro* screening of leishmanicidal agents (DNDi, 2018). Our data showed that both compounds were active upon IA, exhibiting IC_{50} values of 7 and 8.50 µM, respectively, while Pt gave 1.94 µM (Table 1). Against the IA, Cp1 and Cp2 reached low IC_{90} values (17.25 ± 0.21 µM and 18.54±0.96 µM), exhibiting a leishmanicidal profile since both drastically dropped the number of parasites per cells as well as the percentage of infected PMM (Figure 2). Regarding the mammalian host cell toxicity,

we found that after 48 h of exposure, Cp1 and Cp2 were about 3-fold less toxic as compared to Pt, giving IC_{50} of 62.75±0.27 and 65.39±0.61, with selectivity indexes of 6 and 9, respectively, in similar range than the reference drug (SI = 8, Table 1). *In silico* assessment of drug likeness and DMPK properties is most effective way in reducing time, expenses and maximize the success in drug discovery process (Lombardo *et al.,* 2017). Therefore, drug likeness and DMPK properties were predicted for both Cp1 and Cp2 using the QikProp module (QikProp, Schrödinger release 2017-2). Recommended compliance scores are given in Table 2. The predicted properties of Cp1 and Cp2 showed compliance with "Lipinski rule of five". According to "Jorgensen rule of three" any compound that meet the recommended criteria (Table 2) are more likely to be orally available. The predicted properties of both Cp1 and Cp2 displayed compliance with "Jorgensen rule of three". Hence these compounds could show good permeability and solubility properties. Both these properties are crucial for good oral bioavailability. QPPMDCK values are the prediction of MDCK cell permeability (nm/s) which is a good mimic for blood brain permeation. According to this, both compounds displayed good blood brain permeation. The efficiency of a drug may be affected by the extent at which it binds to human plasma protein. If compounds show high binding affinity to serum albumin this could lead to poor efficacy. Hence it is very crucial to understand the binding characteristics of Cp1 and Cp2. The predicted human serum albumin binding values (Table 2) for both Cp1 and Cp2 are within the permissible range indicating a lower binding affinity to the serum albumin protein.

Discussion

The entire process of drug discovery is extremely costly and takes at least one decade of pre-clinical and clinical studies: 1 out of 10,000 drug candidates succeeds in this long flowchart, and finally reaches successfully into the market (Van Norman 2016). In this context, more reliable and reproductible experimental models (*in vitro* and *in vivo*) are needed to find better translation among pre-clinical and clinical outcomes of novel antiparasitic drugs (Chatelain & Konar 2015, Katsuno 2015). Presently, our analysis was performed using primary cultures of peritoneal macrophages infected with *L. amazonensis* since professional phagocytes are the main source of host cells for those obligate intracellular parasites (Walker *et al.,* 2015). This *in vitro* standardized experimental model for CL is claimed to closely reproduce *in vivo* conditions (Stacey 2006), therefore contributing to novel drug candidate screenings for this neglected illness (Chatelain & Ioset 2011, Caridha *et al.,* 2019). Also, although in Asia and Africa continents CL is mainly caused by *L. major* and *L. tropica*; in the Americas, the disease is trigged by a higher number of species including *L. amazonensis,* justifying the present use of this parasite species (Martins 2014, de Vries *et al.*, 2015). *L. amazonensis* is a relevant species in Brazil closely related to a wide spectrum of CL pathologies, including highly severe diffuse cutaneous leishmaniasis (Lainson, 1994). These data corroborate the choice of our *in vitro* model to explore the potential effect of novel anti-CL compounds. Another interesting point to be addressed is the use of protocols that enable the identification of antiparasitic drugs that induces rapid parasite lysis (Da Silva *et al*., 2011).This is especial characteristic as most of the CL patients live in very poor areas with difficult access to public health assistance and then, frequently display advanced pathologies, demanding a fast *killer* drug (Ruoti *et al.,* 2013, Okwor & Uzonna 2016). Aiming to

fulfill this demand, we established a period of 48 h of drug exposure while testing the parasites and host mammalian cells, a shorter period of incubation as compared others reported in the current literature for CL *in vitro* models (Van Bocklaer, 2019).

Our present study explored the leishmanicidal effect of Cp1 (an analogue of GNF5343) and Cp2 (a benzimidazole analogue) identified using shape-based virtual screening approach. Both Cp1 and Cp2 achieved quite relevant potency against amastigotes, especially those lodge inside macrophages, reaching IC_{50} values below 10 μM, a considerable characteristic preconized for a hit compound with anti-leishmania effect (Katsuno, 2015). Remarkably, clearance on *L. amazonensis* infection was found at 20 μM in the infected PMM, a relevant feature to mitigate the possible occurrence of parasite drug resistance and relapses after ceasing the drug administration (Cal *et al*., 2016). The Cp1 and Cp2 were less toxic than the reference drug (pentamidine) still in use for Leishmaniasis but their selectivity indexes (>10) discouraged to move them for *in vivo* proof of concept. However, their chemical optimization for a wider therapeutic window and promotion of potency is largely desirable to continue further studies using these compounds. It is also important to state that the first-line drug pentamidine – used to treat the first stage of Sleeping Sickness and CL-caused by *L. guyanensis* by over seven decades – also doesn't have a desirable selectiveness *in vitro* due to high toxic profile, although display an outstanding potency against these parasites. Currently, we are performing structural modifications of Cp2 aiming to improve its potency, selectivity and satisfactory pharmacological profile, favoring future phenotypic studies in order to move its derivatives forward to new *in vitro* screening and *in vivo* evaluation, aiming to contribute for drug discovery process of new therapeutic approaches for cutaneous

leishmaniasis. These analogues will also be tested against other parasites to assess their broad-spectrum antiparasitic activity.

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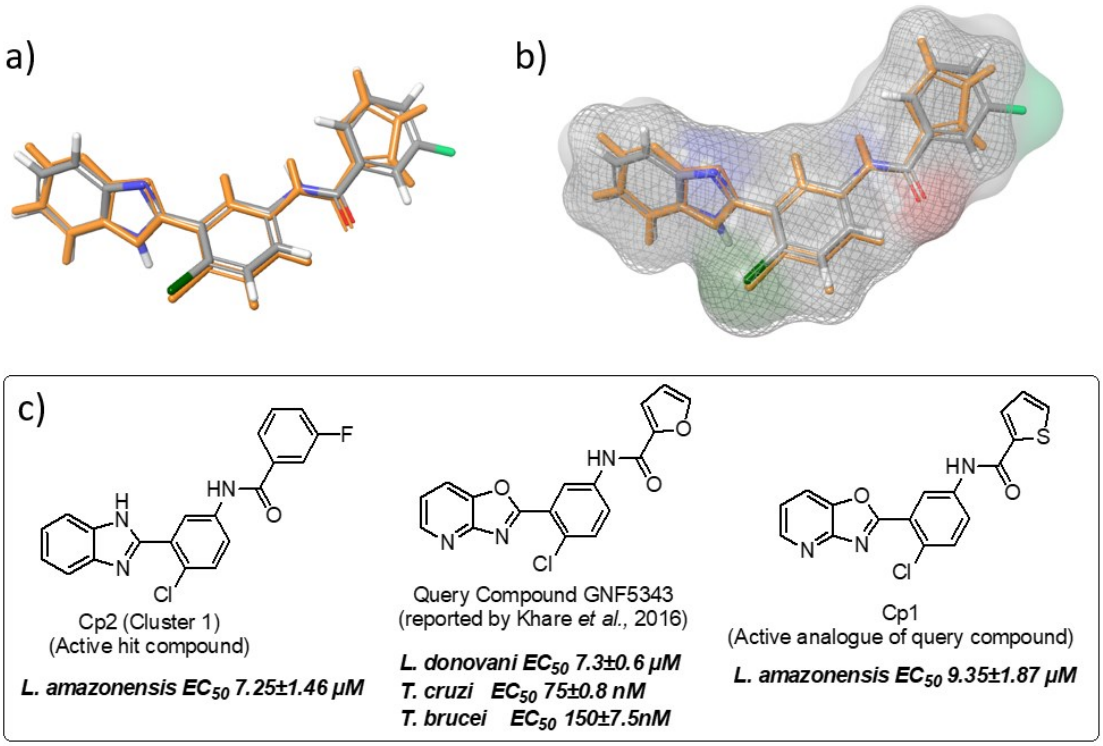
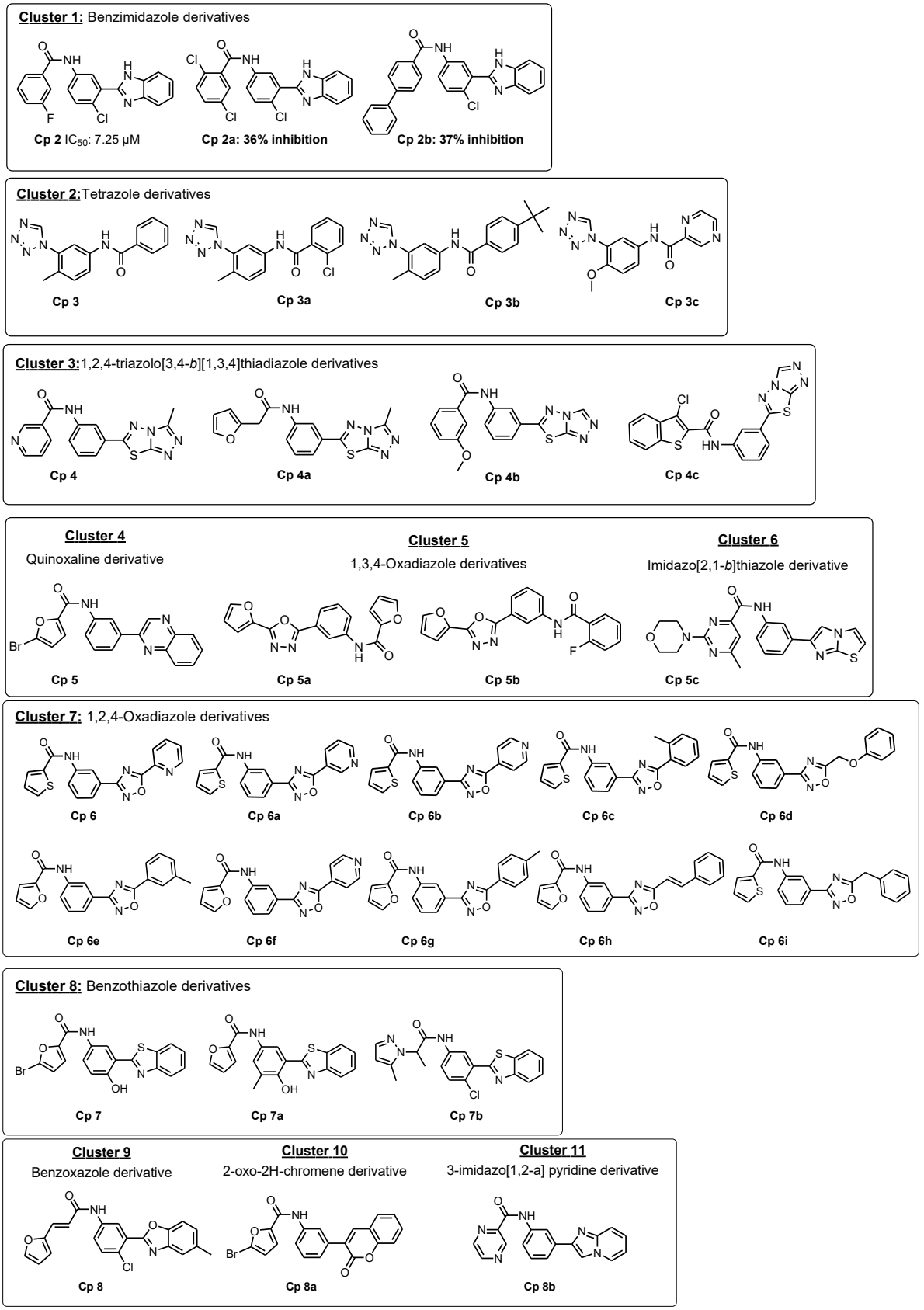


Figure 1. Shape-based virtual screening results. a) Illustrating the good alignment of query compound GNF5343 (orange sticks) with compound **2** (atom type coloured sticks). b) Displaying maximum volume overlap that indicates good shape complementarity between the query compound (represented with mesh with an area of 300 Å^2) and the compound **2** (represented with van der Waals molecular surface area of 326.5 Å^2). c) Molecular structures and their associated activity data. 1a & 1b Images are generated using Phase-Schrödinger drug design software. (Reddy, please correct "brucei" in figure 1c)

Chart 1. Clusters of hit compounds identified by shape-based virtual screening strategy



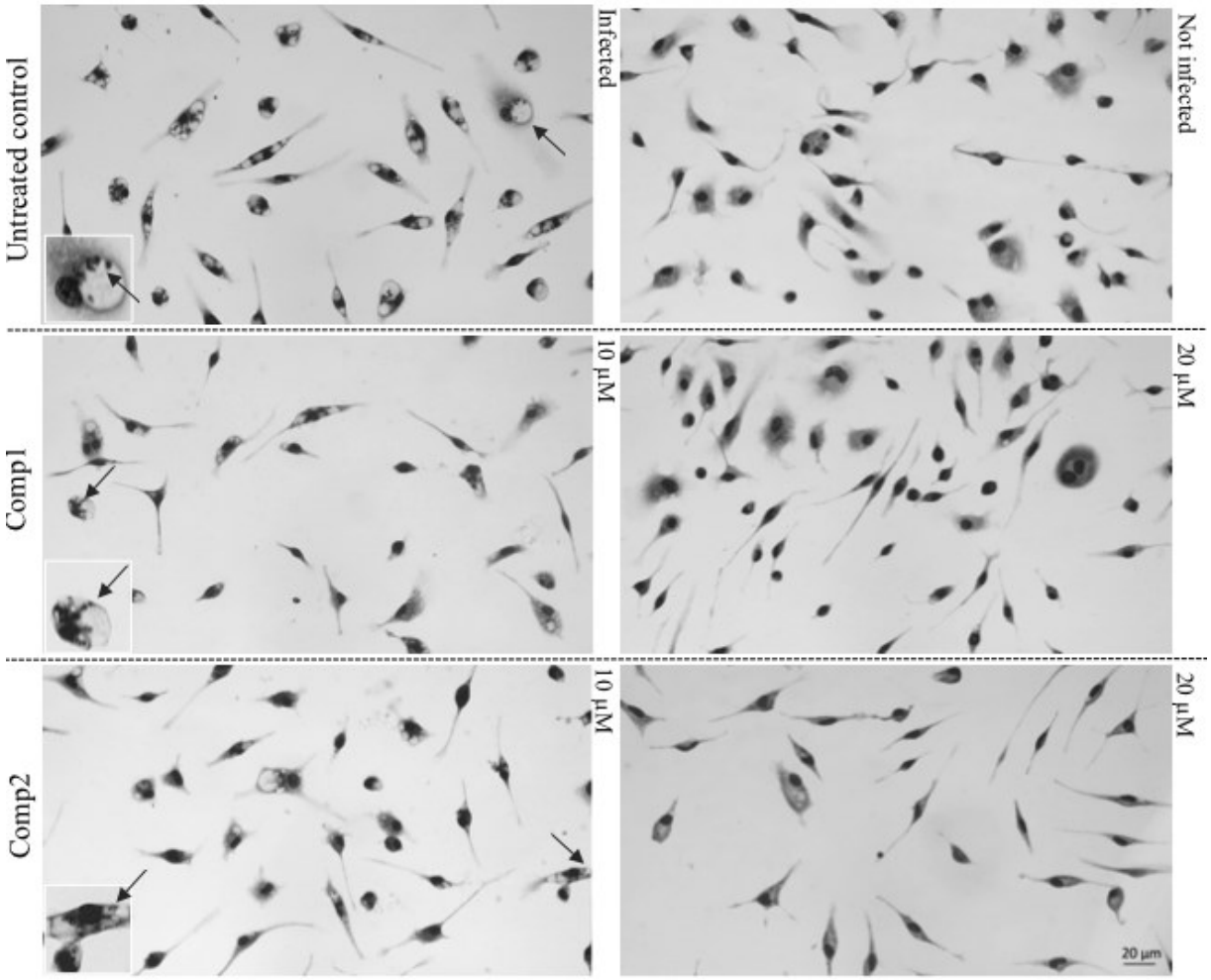


Figure 2. Light microscopy images of Giemsa-stained uninfected and infected PMM submitted or not (untreated) to 10 and 20 µM of Cp1 and Cp2, demonstrating parasite sterilization at 20 µM. Arrows: intracellular parasites.

Table 1. The leishmanicidal activity and cytotoxicity effect (IC_{50} - mean and SD) on peritoneal macrophage (PMM) of Cp1 and Cp2. The compounds were tested (48 hours of incubation) upon amastigote forms purified from mice lesions (FA) and on intracellular amastigote forms (IA) hosted in PMM. SI: selective indexes.

Compound	IC_{50} (μM) $_{PMM}$	IC_{50} (μM) $_{FA}$	IC_{50} (μM) $_{IA}$	SI_{FA}	SI_{IA}
Cp 1	62.75±0.27	13.03±2.69	9.35±1.87	4.82	6.71
Cp 2	65.39±0.61	14.09±2.24	7.25±1.46	4.64	9.02
Pentamidine	15.88±0.59	0.71±0.05	1.94±0.50	22.37	8.19

Table 2. *In-silico* assessment of drug likeness and DMPK properties of Cp1 and Cp2

		Recommended compliance score (range for 95% of drugs)	Compound ID	
			Cp1	Cp2
Lipinski Rule of five	Mol.Wt	≤ 500	355.80	365.794
	HBD	≤ 5	1	2
	HBA	≤ 10	4	2
	cLogP	≤5	3.36	4.59
Jorgensen Rule of three	QPlogS	–6.5 to 0.5	-5.380	-6.289
	QPCaco	<25 poor, >500 great	1403	1721
	Primary metabolites	<7	3	0
% Human oral absorption	-	>80% is high, <25% is low	100	100
QPPMDCK	-	>500 great, <25 poor	2650	3285
QPlogKhsa	-	–1.5 to 1.5	0.243	0.616

Mol.Wt: Molecular Weight; HBD: hydrogen bond donor; HBA: hydrogen bond acceptor; cLogP: calculated logarithm of partition coefficient; QPlogS: the logarithm of aqueous solubility; QPCaco: Caco-2 cell permeability in nm/sec, model for the gut-blood barrier; QPPMDCK: Madin-Darby canine kidney (MDCK) cell permeability in nm/sec, model for the blood-brain barrier; QPlogKhsa: the logarithm of predicted binding constant to human serum albumin.