Anti-parasitic effect of novel amidines against *Trypanosoma cruzi:* phenotypic and *in silico* absorption, distribution, metabolism, excretion and toxicity analysis

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SUMMARY

New more selective and potent drugs are urgently need to treat Chagas disease (CD). Among the many synthetic compounds evaluated against *Trypanosoma cruzi*, aromatic amidines (AAs) and especially arylimidamides (AIAs) have potent activity against this parasite. Presently, the effect of four mono-amidines (DB2228, DB2229, DB2292 and DB2294), four diamidines (DB2232, DB2235, DB2251 and DB2253) and one AIA (DB2255) was screened *in vitro* against different forms (bloodstream trypomastigotes – BT and intracellular forms) and strains from discrete typing unit (DTU) I and VI of *T. cruzi* and their cytotoxic profile on mammalian host cells. Except for DB2253, all molecules were as active as benznidazole (Bz), resulting in 50% of reduction in the number of alive BT, with EC_{50} ranging from 2·7 to 10·1 μM after 24 h of incubation. DB2255 was also the most potent against amastigotes (Tulahuen strain) showing similar activity to that of Bz (3 μM). *In silico* absorption, distribution, metabolism, excretion and toxicity analysis demonstrated probability of human intestinal adsorption, while mutagenicity and inhibition of hERG1 were not predicted, besides giving acceptable predicted volumes of distribution. Our findings contribute for better knowledge regarding the biological effect of this class of aromatic molecules against *T. cruzi* aiming to identify novel promising agent for CD therapy.

Key words: aromatic amidines, Chagas disease, experimental chemotherapy, toxicity, selectivity, *in silico* ADMET analysis.

INTRODUCTION

Over 100 years ago, Carlos Chagas, a Brazilian researcher discovered a new disease, American trypanosimiasis or Chagas disease (CD) caused by a flagellated protozoan *Trypanosoma cruzi*. CD is endemic to 21 countries of Latin American, affecting more than 6 million individuals (WHO, 2016).

CD is currently emerging in non-endemic areas, such as North America, Europe and Oceania, mostly associated with the migration of infected carriers (Albajar-Viñas and Dias, 2014). CD presents in two stages: an acute and a later chronic phase, which after years or decades about 30–40% of patients progress to symptomatic forms, causing heart disease and/or digestive and neurological disorders (Teixeira *et al*. 2006; Marin-Neto *et al*. 2008; Coura and Dias, 2015). The two drugs currently available for clinical treatment are the nitroderivates, nifurtimox (Nif) and benznidazole (Bz), were introduced about four decades ago into clinical use and up to now remain the only treatment options (Patterson and Wyllie, 2014). The major limitations of these compounds include the need for long-time administration and their considerable side-effects that in some cases leads to the discontinuation of treatment, therapeutic failure at the later chronic phase and exhibition of limited effectiveness against naturally resistant strains (Wilkinson *et al*. 2008). A novel candidate for CD therapy should present as drug characteristics: (i) efficacy upon the two phases of the disease, especially the later chronic stage; (ii) potency on different parasite discrete typing units (DTUs; I, II, V and VI) and forms relevant for human infection (trypomastigotes and amastigotes); (iii) low toxicity and absence of genotoxicity, mutagenicity and cardiotoxicity; (iv) be orally administrated; (v) with good stability (3–5 years in climatic zone) with (vi) low costs (Chatelain and Konar, 2015; DNDi, 2016).

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A recent clinical trial, which included a 5-year follow-up, seeking the benefits of the trypanocidal therapy using Bz in patients with established Chagas' cardiomyopathy showed that although there was a significant reduction in total parasite load, this drug was not able to impair cardiac clinical deterioration (Morillo *et al*. 2015). These findings corroborate the need to find alternative therapies for CD. Aromatic amidines (AA) are dicationic molecules with many of them such as pentamidine (Pt) presenting DNA minor groove-binding characteristics (Soeiro *et al*. 2013). The anti-parasitic action of Pt has been known since 1937 (King *et al*. 1937) and in the ensuing years many analogues and derivatives have been synthetized and screened against parasitic organisms. Several of these molecules have demonstrated a wide spectrum of activity against human and veterinary pathogens such as leishmaniasis, human African trypanosomes and *T. cruzi* (De Souza *et al*. 2004; Soeiro *et al*. 2008, 2013; De Araújo *et al*. 2014). Among novel amidine molecules, the arylimidamides (AIAs) have shown very promising profiles and potent activity against intracellular parasites like *Neospora caninum, Leishmania* sp and *T. cruzi* (Soeiro *et al*. 2013). The present study investigates the anti-*T. cruzi* activity of additional novel amidines (four mono-amidines, four diamidines and one AIA) through phenotypic studies *in vitro* by assessing different forms and parasite strains besides determining their toxicity towards different host cell types (as L929 cell lines and primary cultures of cardiac cells) and their absorption, distribution, metabolism, excretion and toxicity (ADMET) properties from *in silico* predictions.

MATERIAL AND METHODS

Compounds

The synthesis of the four studied mono-amidines (2 – (5 – (4 – ((1 (quinolin - yl-1-1,2,3-triazol-4-yl) methoxy) phenyl) thiophen-2-yl)-1H-benzo[d]imidazole-6-carboximidamide hydrochloride (DB2228), 2-(5-(4-((1-(2-(naphthalen-1-yl) ethyl) – 1H-1,2,3–triazol-4-yl) methoxy) phenyl) thiophen-2-yl)-1H-benzo[d]imidazole–6-carboximidamide hydrochloride (DB2229), 2-(5–4-((1-(2-(2-(naphthalene–2-yloxy) ethoxy) ethyl)-1H-1,2,3-triazol-4-yl) methoxy) phenyl) thiophen- 2- yl-1H-benzo[d]imidazole – 6 - carboximidamide hydrochloride (DB2292) and 2-(5-(4-((1- (2-(2-(2-(naphthalene–2-yloxy) ethoxy) ethoxy) ethyl)-1H-1,2,3- triazol - 4-yl) methoxy) phenyl) thiophen-2-yl)-1H-benzo[d]imidazole–6-carboximidamide hydrochloride (DB2294)) has been previously described (Green, 2014). The synthetic route of the four diamidines (2,2′-((propane-1,3-diylbis (oxy)) bis (4,1-phenylene)) bis (1H-benzo [d]imidazole–6-carboximidamide) dihydrochloride (DB2232), 4,4′-(1-phenyl-1H-pyrrole-2,5-diyl) dibenzimidamide dihydrochloride (DB2235), 2,2′-((1-phenyl-1 H-pyrrole-2,5-diyl) bis (4,1-phenylene)) bis (4,5-dihydro-1H-imidazole) dihydrochloride (DB2251), 2,2′-((1-phenyl-1H-pyrrole-2,5-diyl) bis (4,1-phenylene)) bis (1, 4, 5, 6-tetrahydropyrimidine) dihydrochloride (DB2253)) was also conducted using a methodology previously reported (Ismail *et al*. 2004; Farahat *et al*. 2011). The synthesis of the bis-AIA N, N″-((2-oxoimidazolidine-1,3-diyl) bis (3–isopropoxy-4,1- phenylene)) dipicolinimidamide dihydrochloride (DB2255) was previously reported (Stephens *et al*. 2001) (Fig. 1). All compounds have been fully characterized by spectral methods (nuclear magnetic resonance [NMR] and mass spectrometry [MS]) and by satisfactory C, H, N analysis. Bz (2-nitroimidazole; Laboratório Farmacêutico do Estado de Pernambuco [LAFEPE], Brazil) was used as reference drug. Stock solutions were prepared in dimethyl sulfoxide (DMSO) with the final concentration of the solvent never exceeding 0·6% DMSO, which is not toxic to the parasite and mammalian cells.

Parasites

Bloodstream trypomastigote (BT) forms of the Y strain were obtained from the blood samples of infected albino Swiss mice at the peak of parasitaemia. The purified parasites were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) as reported previously (Batista *et al*. 2010). Trypomastigotes of Tulahuen strain expressing the *Escherichia coli* β-galactosidase gene (Buckner *et al*. 1996) were collected from the supernatant of infected cell cultures (L929 culture) as reported (Romanha *et al*. 2010).

Cell cultures

For the toxicity assays on mammalian cells, primary cultures of cardiac cells were obtained from mice embryos plated onto coverslips in 96 well plates previously coated with 0·01% gelatin (Meirelles *et al*. 1986). L929 cell lineages were obtained as described and maintained in RPMI-1640 medium (pH 7·2–7·4) without phenol red (Gibco BRL) supplemented with 10% FBS and 2 mM glutamine (RPMI), as reported previously (Romanha *et al*. 2010).

Cytotoxicity in vitro tests

The cardiac cells were incubated for 24 h at 37 °C with different concentrations of each compound diluted in RPMI and then, the morphology, cell density and spontaneous contractibility evaluated by light microscopy and their cellular viability determined by the Presto Blue test as reported

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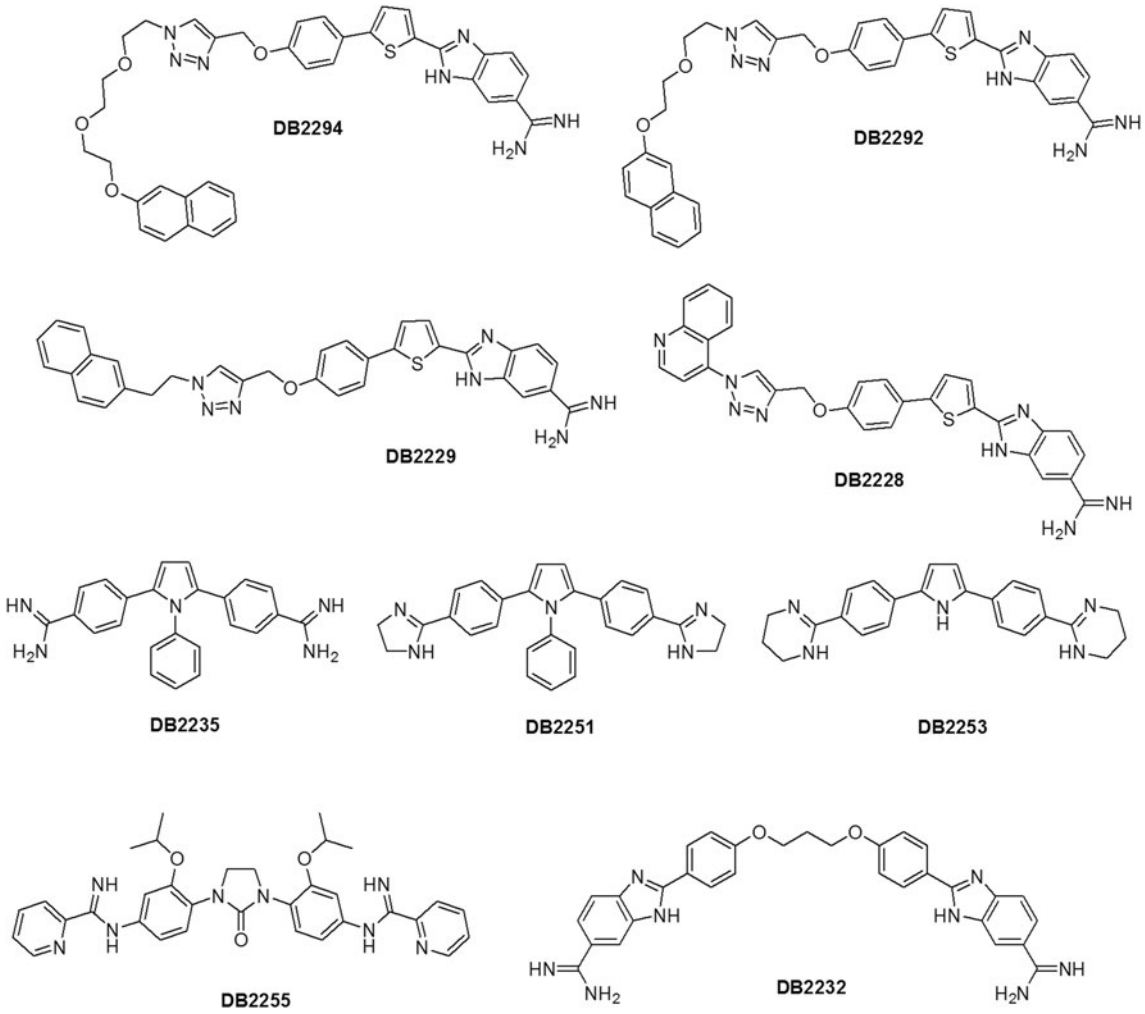

Fig. 1. Chemical structure of the nine selected amidines assayed in this work.

(Timm *et al*. 2014). L929 cell lineages incubated for 24 and 96 h at 37 °C, with different concentrations of each compound diluted in RPMI and their cellular viability determined by the AlamarBlue test as reported (Timm *et al*. 2014). The maximum concentration of each compound was 96 μM due to molecule precipitation. The results expressed by following the manufacturer instructions and the value of CC_{50} that corresponds to the concentration that reduces in 50% the cellular viability, determined. Selective index (SI) expressed by ratio between the values obtained for CC_{50} over the host cells and the EC_{50} obtained over the parasites.

Trypanocidal activity

Bloodstream trypomastigotes (BT) of the Y strain (DTU II) (Zingales *et al*. 2009) (5×10^6 per mL) were incubated for 2 and 24 h at 37 °C in RPMI in the presence or not of serial dilution of the compounds (up to 32 μM). After compound incubation, the death parasite rates were determined by light microscopy through the direct quantification of the number of live parasites using a Neubauer chamber, and the EC_{50} concentration (the compound concentration that reduces in 50% the number of parasites) was calculated (Timm *et al*. 2014). For the assay on intracellular forms, culture-derived trypomastigotes of *T. cruzi* (Tulahuen strain expressing β-galactosidase; DTU VI) (Zingales *et al*. 2009) used to infect L929 infected-cells cultures using a ratio of 10:1 (parasite: host cell). After 2 h, the cultures rinsed and further incubated for 48 h for establishment of the infection. Then, the compounds were added (initially using a fixed concentration of 10 μM followed by other set of assays using increasing non-toxic concentrations to the mammalian host cell for determination of EC_{50} values) and the cultures maintained at 37 °C for 96 h. After addition of 50 μL of the substrate [(CPRG – chlorophenol red glycoside) 500 mM] in 0·5% Nonidet P40 and incubation at 37 °C for 18 h, the absorbance at 570 nm was measured, and the results expressed as per cent of parasite growth inhibition (Romanha *et al*. 2010).

In all assays, at least three experiments ($n \leq 3$) were done using ≤2 replicates.

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Computational assessment of the drug-like properties of the tested compounds

ADMET properties of the studied amidines evaluated using the pkCSM approach, which uses graph-based signatures to develop predictive ADMET (Pires *et al*. 2015).

Statistical analysis

Statistical analysis was performed using Student's *t*-test with significance set at $p \leq 0{\cdot}05$.

Ethics

All procedures were conducted in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14).

RESULTS

Initially, the biological assays were carried out to evaluate the activity of these molecules upon BT forms (Y strain – DTU II), and their respective toxicity towards cardiac cells. Our findings demonstrate that after a short period of incubation (2 h), six out of the nine drugs demonstrate trypanosomicidal activity against BT, exhibiting EC_{50} values lower than 20 μM, while Bz was inactive (Table 1). DB2229 and DB2294 displayed an EC_{90} value <10 μM after only 2 h of compound treatment (Table 1) presenting a fast activity towards these forms. After 24 h, seven molecules (DB2228, DB2229, DB2232, DB2235, DB2255, DB2292 and DB2294) were more potent ($EC_{50} \leq 8{\cdot}3$ μM) than Bz (9·6 μM), being DB2292 about 3-fold more active than the reference drug (Table 1). The toxicity profile assessed using cardiac cell cultures to exclude compounds and concentrations that presented cardiotoxic characteristics evaluated by morphological, contractility and density analysis besides through cellular viability approach using a colorimetric methodology (PrestoBlue). Only DB2235 and DB2251 presented detectable toxicity up to the studied concentrations ($CC_{50} = 49 \pm 21$ and 62 ± 23 μM, respectively) (Table 1).

Next, further assays analysed the activity on intracellular forms of *T. cruzi*, using the Tulahuen strain transfected with β-galactosidase, as previous reported (Romanha *et al*. 2010). The trypanocidal action after 96 h of incubation using a fixed concentration of 10 μM showed that only the AIA DB2255 displayed a high inhibition of the parasite growth (88%), reaching similar activity to that of Bz (Table 2). Therefore, DB2255 was the only molecule selected for the next screening step, consisting of infection of L929 cells followed by incubation with non-toxic concentrations (up to 32 μM). DB2255 and Bz presented similar potency (EC_{50} values of $3{\cdot}6 \pm 0{\cdot}39$ and 3 ± 1 μM, respectively), but the reference drug exhibited higher selectivity (data not shown).

Mathematic parameters of drug likeness including, absorption, distribution, metabolism, excretion and toxicity properties were calculated using the pkCSM approach that uses graph-based signatures to develop predictive of ADMET (Pires *et al*. 2015). I*n silico* ADMET analysis demonstrated probability of human intestinal adsorption (>90%), while mutagenicity and inhibition of hERG1 were not predicted, besides giving acceptable predicted volumes of distribution (Tables 3 and 4).

DISCUSSION

In the last 40 years the only available treatment for CD has been two nitrohetocyclic agents, Bz and

Table 1. *In vitro* activity of the amidines and benznidazole on bloodstream trypomastigotes of the Y strain and on cardiac cells : EC_{50} and EC_{90} values after 2 and 24 h, CC_{50} values of CC after 24 h of incubation at 37 °C, respectively, and the corresponding selectivity index (SI)

	EC_{50} (mean ± S.D.) μM		EC_{90} (mean ± S.D.) μM		CC_{50} (mean ± S.D.) μM	
Compound	2 h	24 h	2 h	24 h	24 h	SI 24 h[a]
Bz	>32	9·6 ± 1·4	>32	30·6 ± 0·64	>1000	>104
DB2228	9·3 ± 0·75	8·3 ± 3·2	>32	25·9 ± 4·2	>96	>12
DB2229	6·3 ± 3·7	2·7 ± 0·3	9·8 ± 1·3	8·1 ± 1·37	>96	>36
DB2292	7·2 ± 1	3·1 ± 0·9[b]	24·4 ± 1·5	10·8 ± 2·9	>96	>31
DB2294	5·2 ± 2·9	3·9 ± 1·3[b]	9·6 ± 1·7	9 ± 0·5	>96	>25
DB2232	19·6 ± 0·3	7·4 ± 0·6	>32	30·6 ± 1·1	>96	>13
DB2235	>32	5·27 ± 3·7	>32	10·3 ± 0·35	49 ± 21	9
DB2251	>32	10·1 ± 2·5	>32	28·9 ± 1·8	62 ± 23	6
DB2253	>32	19 ± 8	>32	>32	>96	>5
DB2255	5·5 ± 2·25	3·6 ± 2·4[b]	>32	6·46 ± 3·2	>96	>27

[a] Based on EC_{50} 24 h.
[b] Student's *t*-test statistical analysis of studied compound and Bz: ($P < 0{\cdot}05$).

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Table 2. Activity of the amidines and benznidazole on L929 cell lines infected with *Trypanosoma cruzi* (Tulahuen strain transfected with β-galactosidase) after 96 h of incubation with 10 μM of each compound

Compounds	% of parasite growth inhibition	CC_{50}
Bz	83 ± 5	>100
DB2228	46 ± 18	>96
DB2229	50 ± 10	>96
DB2292	39 ± 22	>96
DB2294	7 ± 5	>96
DB2232	22 ± 10	>96
DB2235	58 ± 14	>96
DB2251	70 ± 17	>96
DB2253	70 ± 17	>96
DB2255	88 ± 11	>96

Nif, despite their severe side effects and low efficiency during the later chronic phase (Wilkinson and Kelly, 2009; Don and Ioset, 2013). The limitations of these therapies highlight the urgent need to find more effective and safer new compounds. Many compounds have been developed and screened with different experimental models of neglected diseases including CD (Bilbe, 2015). The azole anti-fungal inhibitors posaconazole (Pos) and ravuconazole (Rav) that act on the sterol 14α-demethylase (CYP51) enzyme although were very potent *in vitro* and *in vivo* (using dog and mouse models) (Urbina *et al*. 1998; Diniz *et al*. 2010; Keenan and Chaplin, 2015) unfortunately failed during clinical trials performed by the Drugs for Neglected Diseases initiative (Molina *et al*. 2014). In addition, another recent clinical trial called 'Benznidazole Evaluation for Interrupting Trypanosomiasis' (BENEFIT) designed to evaluate the efficacy and safety of Bz compared with placebo, did not demonstrate protection by this drug against clinical outcomes among patients with chronic CD. Often, in drug development for CD, as well as for other pathologies, there is a lack of direct translation between pre-clinical *in vitro* and *in vivo* results and clinical outcomes. Experimental chemotherapy for CD presents serious challenges in part due to experimental difficulties related to reliable demonstration of a sterile cure, particularly during the chronic stage of infection when parasite burden is low and tissue distribution is not fully understood (Chatelain and Konar, 2015; Francisco *et al*. 2015).

Our group has studied the *in vitro* and *in vivo* activity of AA and analogues and the bulk of the data revealed very promising action of these cations against intracellular pathogens, including *T. cruzi* (Soeiro *et al*. 2013). Presently, nine aromatic compounds were evaluated by phenotypic and *in silico* studies. The mono-amidines (DB2228, DB2229, DB2292 and DB2294) with tethered aryl rings chosen due to previous observation that this class display potent effect against this parasite (Simões-Silva *et al*. 2016). Three of the four diamidines (DB2235, DB2251 and DB2253) are analogues of furamidine and one (DB2232) is an extended bis-amidino benzimidazole, which represents another class of highly active diamidines. Lastly, one novel bis-AIA (DB2255) results from a simple modification of the structure of the highly active anti-*T. cruzi* compound DB766 (Batista *et al*. 2010). In DB2255, the central furan ring of DB766 replaced with a non-aromatic 5-membered imidazolidin-2-one ring.

Results of calculations using the pkCSM approach for estimation of ADMET and other drug-like properties are important to consider at an early stage in the drug discovery process (Pires *et al*. 2015). The *in silico* estimation of ADMET properties showed that only DB2229, DB2235, DB2251 and DB2253 are likely to permeate Caco2 cells, with values near of the adopted threshold of 0·9. In addition, DB2228, DB2229, DB2251, DB2253, DB2255, DB2292 and DB2294 are predicted to show good adsorption (above 90%) by human intestines and reasonable predicted volume of distribution. Regarding the toxicity predictors, none expected to be mutagenic nor inhibitors of hERGI, whereas all compounds are expected to inhibit hERGII and have hepatotoxic profile as has also the reference drug, Bz.

Regarding the biological phenotypic assays, seven of out nine amidines presently screened against bloodstream forms resulted in parasite death rates similar to Bz including mono-amidines DB2228, DB2229, DB2292, DB2294, diamidines DB2232, DB2235 and AIA DB2255. Another important characteristic of some (DB2228, DB2229, DB2235, DB2292 and DB2294) was the ability to fast kill the parasite exhibiting anti-trypomastigote activity after 2 h of exposure while Bz was completely inactive at this time of drug exposure. When these aromatic compounds were tested against the intracellular amastigotes the bis-AIA DB2255 that was one of the best molecules against BT forms, also presented anti-parasitic effect in the same range as Bz, even using a different parasite strain and DTUs (Y and Tulahuen for BT and intracellular forms, corresponding to DTU II and VI, respectively). These data corroborate our previous findings that demonstrated the promising trypanocidal phenotypic effect of bis-AIAs (De Araujo *et al*. 2014; Timm *et al*. 2014). Data using trypomastigotes collected from infected cell lines reported EC_{50} values of 2·8 and 15·2 μM for pentamidine exposure using Y and Dm28c strains, respectively (Díaz *et al*. 2014). However, DB2255 was less potent than other studied AIAs such as DB766 which gives EC_{50} values at <0·1 μM (Batista *et al*. 2010). This result demonstrates that to achieve high anti-*T. cruzi* activity using the DB766 scaffold a central five membered

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Table 3. *In silico* ADME

	DB2228	DB2229	DB2232	DB2235	DB2251	DB2253	DB2255	DB2292	DB2294	Bz
Absorption										
$CaCo_2$ permeability (log cm s^{-1})	0·316	0·858	0·086	0·824	1·124	0·998	0·026	0·163	0·199	0·479
Intestinal absorption (human, %)	97·185	100	86·259	82·685	94·223	93·881	90·08	96·671	93·916	68·885
Skin permeability (logKp)	−2·741	−3·011	−2·947	−3·713	−3·51	−3·629	−2·838	−2·818	−2·759	−2·893
Distribution										
VDss (human) (log VDss (L kg^{-1}))	0·645	0·709	0·318	0·482	0·93	0·705	0·449	0·573	0·521	−0·104
Fraction unbound (human)	0·307	0·2	0·278	0·35	0·278	0·316	0·187	0·213	0·221	0·503
BBB permeability	−1·185	−0·85	−1·086	−0·157	0·13	0·007	−1·265	−1·249	−1·465	−0·619
CNS permeability	−1·913	−2·298	−2·944	−2·439	−2·095	−2·151	−2·526	−2·766	−3·072	−2·995
Metabolism										
CYP2D6 substrate	No	No	No	No	No	No	No	No	No	No
CYP3A4 substrate	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
CYP1A2 inhibitor	No	No	No	No	No	No	No	No	No	No
CYP2C19 inhibitor	No	No	No	No	No	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	No	No	No	No	No	No	No
CYP2D6 inhibitor	No	No	No	No	No	No	No	No	No	No
CYP3A4 inhibitor	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	No
Excretion										
Total clearance log(ml min^{-1} kg^{-1})	1·117	1·322	1·74	1·177	0·598	0·628	0·605	1·459	1·649	0·625

Table 4. *In silico* toxicity

	DB2228	DB2229	DB2232	DB2235	DB2251	DB2253	DB2255	DB2292	DB2294	Bz
AMES toxicity	No	No	No	No	No	No	No	No	No	Yes
Max. tolerated dose (human) (log mg kg^{-1} day^{-1})	0·346	−0·896	−0·719	−0·109	−0·494	−0·233	−0·624	−0·838	−0·741	0·984
hERG I inhibitor	No	No	No	No	No	No	No	No	No	No
hERG II inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Oral rat acute toxicity (lD50) (mol kg^{-1})	2·776	3·007	2·577	2·451	2·887	2·785	2·841	2·758	2·53	2·454
Oral rat chronic toxicity (LOAEL) log(mg kg^{-1}_bw day^{-1})	2·577	0·511	0·981	1·483	1·085	1·213	0·407	0·471	0·436	1·649
Hepatotoxicity	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Skin sensitisation	No	No	No	No	No	No	No	No	No	No
T. Pyriformis toxicity pIGC50 (log ug L^{-1})	0·285	0·3	0·308	0·946	0·475	0·807	0·329	0·29	0·287	1·227
Minnow toxicity log LC50 (mM)	0·276	0·125	0·822	1·156	0·726	0·925	−0·069	−0·025	−0·19	1·649

hetero aromatic ring is required. In addition, is important to take into consideration that a hit compound for CD must be active against both parasite stages and upon the different DTUs in order to be given in the distinct endemic areas of this neglected disease (Chatelain, 2015).

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CONFLICT OF INTEREST

None.

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