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Bis(oxyphenylene)benzimidazoles: A novel class of anti-*Plasmodium falciparum* agents

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ABSTRACT

A small library of 26 2,2′-[alkane-α,ω-diylbis(oxyphenylene)]bis-1*H*-benzimidazoles has been prepared and evaluated against *Giardia intestinalis*, *Entamoeba histolytica*, *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani*, and *Plasmodium falciparum*. Among the tested compounds, eight derivatives (**17, 19, 20, 24, 27, 30, 32** and **35**) exhibited an anti-*Plasmodium falciparum* activity characterized by IC_{50} values in the range of 180–410 nM (0.11–0.21 μg/mL) and selectivity indexes (IC_{50} rat skeletal myoblasts L6 cells vs IC_{50} *P. falciparum* K1 strain) varying between 92 and more than 450. Two of the eight novel drug leads, namely compounds **19** and **32**, were also active against *G. intestinalis* and *L. donovani* with selectivity indexes of 122 and >164 respectively.

1. Introduction

The benzimidazole skeleton has attracted and still attracts much attention from medicinal chemists because of its structural resemblance to various moieties present in fundamental constituents of proteins and nucleic acids. Benzimidazoles are also well-known for their broad spectrum of anti-parasitic properties. For example, thiabendazole (**1**, Fig. 1) is used primarily to control mold, blight, and other fungi in fruits and vegetables.[1] Thiabendazole (**1**), ciclobendazole (**2**), mebendazole (**3**), flubendazole (**4**), fenbendazole (**5**), and albendazole (**6**) are prescribed to eliminate gastrointestinal parasites and worms in wild and domestic animals, livestock, as well as humans.[2–7] Albendazole (**6**) is also used for the treatment of lymphatic filariasis and giardiasis in some parts of the world.[8,9] During the last decade some bisbenzimidazoles have been screened for inhibition of hepatitis C virus serine proteases[10,11] (e.g., **7**), and anti-cancer drug leads[12–15] (e.g., **8**). Meanwhile, structurally related congeners of **8** (e.g., **9**) and other bisbenzimidazoles (e.g., **10**) have been evaluated as anti-microbial, anti-fungal, and anti-parasitic agents.[16–20] For example we[18] have established that 2,2′-[1,5-pentanediylbis(oxy-1,4-phenylene)]bis-1*H*-benzimidazole **15** could be considered as a promising hit against *L. donovani*. Later Navarrete and co-workers[19] demonstrated that the 5,5′-dimethoxy analog exhibited significant in vitro effect against *Trichomonas vaginalis*, *Giardia lamblia*, *Entamoeba histolytica*, and *Leishmania mexicana* also.

Because of our combined interest in the chemistry of benzimidazoles[21,22] and in the study of their structure–activity relationships[18,23–25] we now wish to report on the preparation of a library of bisbenzimidazoles whose structures are depicted in Figure 2 and on their ability to inhibit (or not) the growth of various strains of parasites.

2. Results and discussion

2.1. Chemistry

The targeted bisbenzimidazoles were obtained by a two-step sequence following experimental protocols already described.[18,21–27] All compounds have been fully characterized by their spectroscopic data (^{1}H NMR, ^{13}C NMR, IR, HRMS) and purity assessed by HPLC. In the ^{13}C NMR spectra some signals due to the benzimidazole moieties were sometimes broadened or even not observed. As reported in the literature[28,29] this is linked to the well-known prototropic tautomerism in the (benz)azoles series. From the HPLC analyses, the purity of the compounds ranged from 95.6% to 100%.

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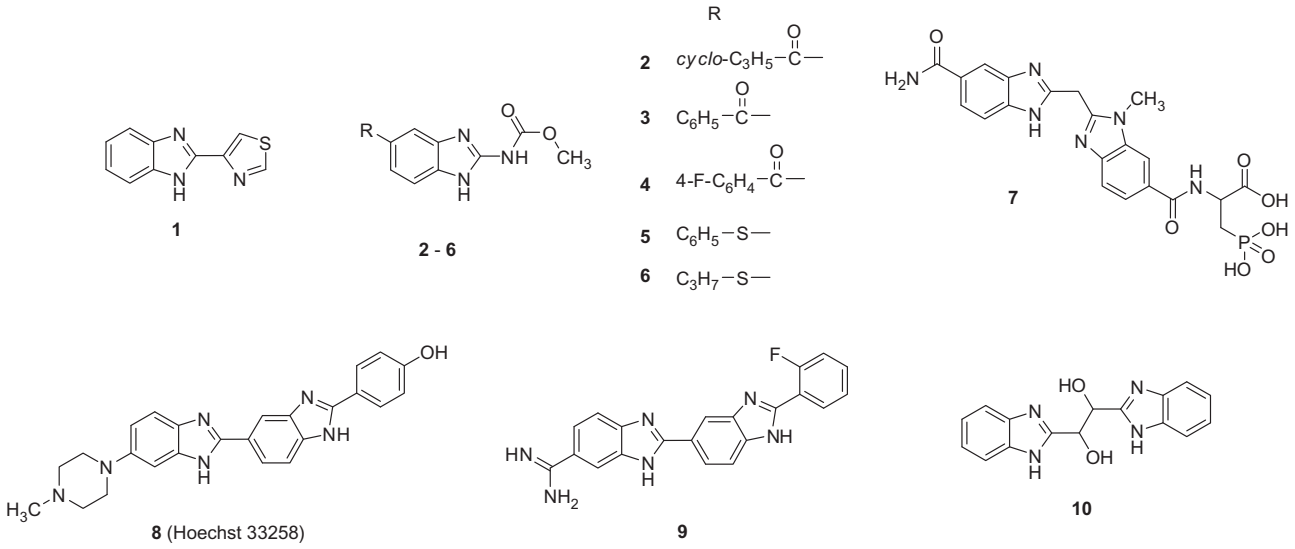

Figure 1. Some benzimidazole-containing derivatives of pharmacological interest.

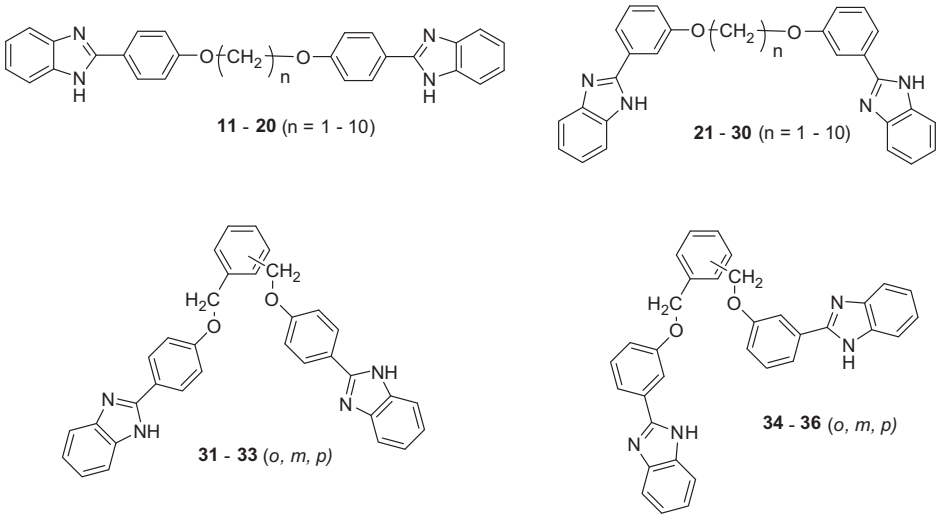

Figure 2. Structure of the bisbenzimidazoles studied in this work.

2.2. Biological assays

The so-obtained 26 bisbenzimidazoles were evaluated in vitro for their cytotoxicity towards the non-carcinoma L-6 rat skeletal myoblasts cell line and for their activity against various protozoan parasites infecting people in developing countries essentially (but not exclusively), namely *Giardia intestinalis*, *E. histolytica*, *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *L. donovani*, and *Plasmodium falciparum*.

The structures of the reference drugs are represented in Figure 3. Experimental data and some relevant calculated data (IC_{50} values, selectivity index, molecular weights and Log*P*) are compiled in Tables 1 and 2. The calculated partition coefficients of these compounds ranged from 6.83 to 9.28 indicating high lipid and low aqueous solubility. The high lipophilicity of the tested compounds could present some solubility problems and may enhance binding to plasma proteins in the assay mixture, thereby decreasing the real potency of the compounds. With this limitation in mind, the aim of this work is limited to identification of some potential active leads and not to determine why some compounds were inactive.

2.2.1. Cytotoxicity towards rat skeletal myoblasts, L-6 cells[30]

The L-6 cell line consists of rat muscle precursor cells. It is a commercially available cell line with low culture medium requirements. It can readily be handled and stops proliferation when reaching confluency.

Most compounds in this study were characterized by an IC_{50} value in the range of 1–12 μg/mL (3–25 μM). Derivatives **17**, **19**, **20**, **27**, **30**, **32**, and **35** emerged as the less toxic members of the library.

2.2.2. Anti-*Giardia* intestinalis activity[31]

Giardiasis is a form of contagious dysentery that is often related to poor sanitary conditions. It is caused by swallowing resting cysts of a protozoan parasite, *Giardia intestinalis*, which can be found in water, food, and soil. The illness is very common in developing countries but also affects all other parts of the world.

Metronidazole (a 2-nitroimidazole derivative, see Fig. 3), the first-line drug used in the treatment of giardiasis, was chosen as the reference drug for that study. It exhibited an IC_{50} value of 0.267 μg/mL (1.56 μM) against *Giardia intestinalis* G1. Four compounds (**13**, **15**, **17**, and **19**) were efficient against that protozoan at a dose lower than 1.50 μg/mL. Amazingly those four

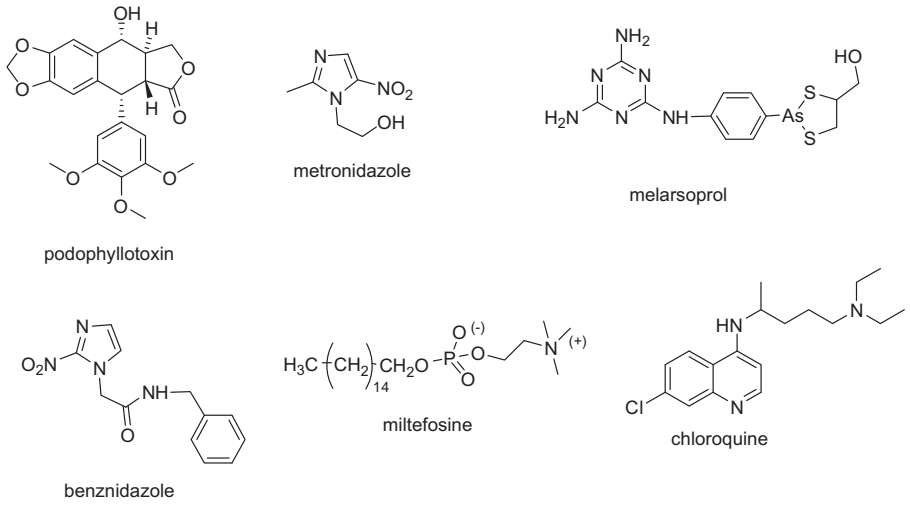

Figure 3. Reference drugs used in this study.

Table 1
IC_{50} values determined towards rat skeletal myoblasts L-6 cells, *Gardia intestinalis*, *Entemoeba hystolytica*, *T. brucei rhodesiense*, *T. cruzi*, and *L. donovani*

Compound[a]	IC_{50}[b] (μM)					
	L-6 cells	*G. intestinalis*	*E. hystolytica* Hk-9	*T. brucei rhodesiense*	*T. cruzi*	*L. donovani*
Podophyllotoxin	0.012					
Metronidazole		1.560	5.820			
Melarsoprol				0.010		
Benznidazol					1.134	
Miltefosine						0.445
11 (*n* = 1)	14.40			2.13	11.26	16.49
12 (*n* = 2)	25.38	124.08	190.82	154.13	133.15	> 202
13 (*n* = 3)	3.56	**0.74**	**3.37**	1.50	3.91	2.67
14 (*n* = 4)	7.84	17.85	**8.13**	2.76	5.75	3.25
15 (*n* = 5)	6.82	**0.82**	22.72	1.02	2.42	**0.80**
16 (*n* = 6)	9.61	3.58	>199	7.22	6.05	6.59
17 (*n* = 7)	**48.39**	**1.34**	21.74	1.08	14.75	1.53
18 (*n* = 8)	19.80	10.44	65.75	39.12	56.97	98.22
19 (*n* = 9)	**101.25**	**0.83**	30.72	0.81	> 165	5.93
20 (*n* = 10)	**45.98**	22.08	19.29	9.34	88.45	123.80
21 (*n* = 1)	18.94			14.20	11.63	6.19
22 (*n* = 2)	5.04	11.65	83.99	16.57	4.68	24.10
23 (*n* = 3)	9.82	56.07	78.26	8.01	8.92	3.91
24 (*n* = 4)	21.23	36.03	211	18.48	23.94	9.99
25 (*n* = 5)	3.56	18.46	27.84	1.33	**1.70**	1.19
26 (*n* = 6)	7.98	17.71	73.70	2.37	3.88	2.57
27 (*n* = 7)	**118.34**	25.76	56.73	1.37	1.82	2.69
28 (*n* = 8)	13.08	52.44	23.54	4.50	4.28	5.90
29 (*n* = 9)	21.92	45.53	46.21	2.37	4.98	6.59
30 (*n* = 10)	**72.20**	171.95	22.19	2.08	83.23	13.30
31 (*ortho*)	5.89			1.32	6.07	2.33
32 (*meta*)	**>172**			3.56	113.20	**1.05**
33 (*para*)	16.01			2.12	26.31	**0.80**
34 (*ortho*)	7.02			2.66	2.53	3.00
35 (*meta*)	**79.58**			3.60	5.99	4.63
36 (*para*)	10.39			24.01	10.89	8.44

Compounds with low cytotoxicity toward L-6 cells or significant activity against the protozoan parasites are shown in bold.

[a] The number of methylene groups or the relative positions of the substituents in the central ring are given in the brackets.

[b] The IC_{50} values are the average of duplicate determinations, the individual values vary less than a factor 2. Standard deviations or confidence intervals could not be calculated.

bisbenzimidazoles contain a central chain constituted by 3, 5, 7, and 9 methylene groups respectively and all of them are characterized by the presence of the benzimidazole moieties in the *para* position relative to the ether bonds linking the central chain and the phenylene rings. In that series of *para*-substituted congeners, cytotoxicity against L-6 cells roughly decreased when the length of the central alkanediyl chain increases, therefore the most interesting hit for further studies is derivative **19** for which a selectivity index (IC_{50} L-6 cells/IC_{50} parasite) of 123 could be calculated. Compound **19** also showed a high selectivity index of 344 against *P. falciparum*, and was relatively non-toxic to the L-6 cells. Therefore this compound is an interesting lead that deserves further attention.

It is interesting to note that Navarrete and co-workers[19] reported that compound **15** was less active against *G. lamblia* and more active against *E. histolytica* when compared to metronidazole, but the strains of the parasites and the protocols used were different.

Table 2
IC_{50} values determined towards *P. falciparum* and some calculated values (selectivity indexes, molecular weights, and log *P*)

Compound[a]	IC_{50}		SI[b]	MW	*c*Log *P*[c]
	μg/mL	μM			
	P. falciparum				
Chloroquine	0.059	0.184		319.87	5.01
11 (*n* = 1)	0.30	0.69	21	432.47	6.88
12 (*n* = 2)	0.90	2.01	13	446.50	6.89
13 (*n* = 3)	0.13	0.28	13	460.53	7.16
14 (*n* = 4)	0.21	0.44	18	474.55	7.43
15 (*n* = 5)	0.13	0.27	26	488.58	7.94
16 (*n* = 6)	0.12	0.24	40	502.61	8.37
17 (*n* = 7)	0.12	0.23	**208**	516.63	8.67
18 (*n* = 8)	0.16	0.30	67	530.66	8.90
19 (*n* = 9)	0.16	0.29	**344**	544.69	9.09
20 (*n* = 10)	0.10	0.18	**257**	558.71	9.24
21 (*n* = 1)	0.73	1.69	11	432.47	6.83
22 (*n* = 2)	1.15	2.58	2	446.50	6.84
23 (*n* = 3)	1.11	2.41	4	460.53	7.11
24 (*n* = 4)	0.11	0.23	**92**	474.55	7.38
25 (*n* = 5)	0.17	0.34	10	488.58	7.89
26 (*n* = 6)	0.14	0.28	29	502.61	8.33
27 (*n* = 7)	0.21	0.41	**291**	516.63	8.64
28 (*n* = 8)	0.24	0.45	29	530.66	8.88
29 (*n* = 9)	0.31	0.57	39	544.69	9.07
30 (*n* = 10)	0.20	0.36	**202**	558.71	9.28
31 (*ortho*)	0.18	0.34	17	522.60	8.34
32 (*meta*)	0.20	0.38	**>450**	522.60	8.36
33 (*para*)	0.12	0.23	70	522.60	8.38
34 (*ortho*)	0.18	0.34	20	522.60	8.33
35 (*meta*)	0.21	0.40	**198**	522.60	8.34
36 (*para*)	0.10	0.19	54	522.60	8.34

Compounds with high selectivity indexes are shown in bold.
[a] The number of methylene groups or the relative positions of the substituents in the central ring are given in the brackets.
[b] Selectivity index = IC_{50} L-6 cells/IC_{50} parasite.
[c] From www.molinspiration.com.

2.2.3. Anti-*Entamoeba* histolytica Hk-9 activity[31]

E. histolytica is another parasitic protozoal organism that is responsible for amebiasis that manifests under the forms essentially of amebic dysentery or amebic liver abscess. These diseases are prevalent in tropical countries with an estimated 40 million cases and 100,000 deaths annually. They come in second place, after malaria, in terms of mortality.

Metronidazole is prescribed also to cure amebiasis and was again the reference compound for that study with an IC_{50} value of 5.82 μM against *E. histolytica* HK-9. Only two compounds, namely **13** and **14**, were found to be active enough against the parasite to deserve attention. However they were characterized with poor selectivity indexes (IC_{50} L-6 cells/IC_{50} parasite) neighboring one unit and therefore they had to be classified as devoid of practical interest.

2.2.4. Anti-Trypanosoma activity[32–35]

T. brucei rhodesiense is responsible for sleeping sickness, which is an endemic disease affecting several dozens of thousands people across sub-Saharan Africa, most of them being undiagnosed and therefore untreated. *T. cruzi* is a different parasite found in Central and South America. It causes Chagas disease. Approximately 20,000 persons die from Chagas disease every year. Depending on the strain considered, two different drugs of reference were used: melarsoprol in the case of *T. brucei rhodesiense* and benznidazole (a monobenzimidazole derivative) in the case of *T. cruzi*.

Deceptively the lowest IC_{50} value recorded against *T. brucei rhodesiense* for a bisbenzimidazole considered in this study remained 100-fold higher than the value measured for melarsoprol. None of the tested compounds were more active than benznidazole or showed good selectivity against *T. cruzi*.

2.2.5. Anti-*Leishmania* donovani activity[34,36–39]

Leishmaniases are visceral, cutaneous, and mucocutaneous diseases caused by intracellular protozoan parasites of the genus *Leishmania*. The World Health Organization reports that several million people are infected each year with visceral leishmaniasis. Depending on the geographic area, either *L. donovani* or *Leishmania infantum* is the causative agent.

The activities of bisbenzimidazoles **11**–**36** were compared to that of miltefosine in this assay. An IC_{50} value of 0.45 μM was determined for the reference drug whereas the lowest IC_{50} values measured in the bisbenzimidazoles library were 0.80 μM for **15** and 0.80 μM for **33**. Unfortunately, those two derivatives were also toxic in the presence of the rat skeletal myoblasts and were therefore excluded from any further development. However compound **32** in which the central phenylene ring is substituted at the *meta* positions is a good lead based on its selectivity index of >164.

2.2.6. Anti-*Plasmodium* falciparum activity[36,40]

It is estimated that over one billion people (one sixth of the world population) suffers from one or more tropical protozoal infections. Among them malaria, caused by *P. falciparum*, is the most dangerous disease with the highest rates of complications and mortality. The World Health Organization reports that in 2008 there were 247 million cases of the sickness causing almost one million deaths. Fight against female *Anopheles* mosquitoes, the vector of *Plasmodium*, can be affected by indoor spraying with insecticides or distribution of insecticide treated nets to cover beds. Chemoprophylaxis and chemotherapy, including the artemisin-based combination therapies (ACT) constitute other ways to eliminate the parasite. However excessive use of those chemical weapons led to numerous mutations and resistance in *Plasmodium*, thus justifying a continuous search for novel anti-malarial agents. The WHO Special Program for Research and Training in Tropical Diseases defines an activity criterion to have an IC_{50} value lower than 0.20 μg/mL. Interestingly only six out of the 26 members of the library exhibited IC_{50} values higher than 0.30 μg/mL, six compounds exhibited values ranging from 0.20 to 0.24 μg/mL and fourteen derivatives were active at doses lower than 0.20 μg/mL.

It must be pointed out that the majority of the less active substances are those in which the linker is constituted by a short alkanediyl chain of one, two, or eventually three carbon atoms, namely **11**, **12**, **21**, **22**, **23** (derivative **29** is the sixth substance of the group of the poorly active substances). All 20 other compounds were characterized by IC_{50} values ranging from 0.10 to 0.24 μg/mL, which corresponds to molar concentrations comprised between 180 and 450 nM. This means that those compounds are as active, in vitro, as chloroquine, the reference drug, or at the worst 2.5-fold less active than chloroquine which has an IC_{50} value of 0.059 μg/mL (184 nM).

As far as cytotoxicity is concerned, inspection of the selectivity indexes (IC_{50} L-6 cells/IC_{50} parasite) reported in Table 2 indicated that eight bisbenzimidazoles could emerge as promising drug leads. Those are the bisbenzimidazoles **17**, **19**, **20**, **24**, **27**, **30**, **32**, **35**, for which selectivity indexes of 208, 344, 257, 92, 291, 202, >450, and 198 respectively have been calculated. The main drawback of those novel anti-*Plasmodium falciparum* agents is their low water solubility which could affect their bioavailability when administered by the oral route. Indeed calculated partition coefficients between *n*-octanol and water (*c*log *P*, see Table 2) are higher than 7 units and therefore do not obey the Lipinski's rules.[41] That drawback could however be circumvented by preparing protonated forms of the bisbenzimidazoles. Indeed, softwares used to calculate log *P* values predicted a decrease of several units when going from the neutral bisbenzimidazole species to the diprotonated ones. In our opinion, molecular weights, which are at the limit of

the violation of the Lipinski's rules, would not hamper further development of the hits identified in this work.

Chloroquine and some other anti-malarial drugs are known to exert their effect by inhibiting a detoxication process by which *Plasmodium*, in its food vacuole, converts heme, a fatal poison for the parasite, into hemozoin, the non-toxic malaria pigment.[42–44] Such a chemical transformation can readily be mimicked in a cell-free assay.[45–50] In particular, Egan[45] reported that, under acidic condition (pH 5.0) similar to that found in the lysosomal vacuole of *P. falciparum*, ferriprotoporphyrin IX chloride (hemin) evolved to yield a precipitate of β-hematin, the synthetic form of hemozoin. However, that reaction can be inhibited in the presence of some chemicals, which include quinine, chloroquine, and amodiaquin. Therefore observation of that inhibition constitutes a simple test enabling to identify potential anti-*Plasmodium falciparum* agents. In our hands, a randomized series of bisbenzimidazoles described in this work (**13**, **14**, **15**, **16**, **17**, **22**, **23**, **24**, and **27**) have been evaluated in that cell-free assay and have been shown to effectively inhibit precipitation of β-hematin from hemin in an aqueous acetate buffer. These results suggest that the tested derivatives could contribute to the accumulation of heme in the food vacuole of *Plasmodium* and in this way lead to the death of the parasite. Similar conclusions had already been made to explain the anti-*Plasmodium* activity of pentamidine[49] and structurally related bisbenzamidines,[50] but obviously other modes of action cannot be excluded.[51,52]

3. Conclusion

A small library of 26 bisbenzimidazoles have been designed, prepared, and evaluated for their in vitro effects toward rat skeletal myoblasts and several protozoan parasites responsible for diseases essentially infecting large number of people living in developing countries. Those parasites are *Giardia intestinalis*, *E. histolytica*, *T. brucei rhodesiense*, *T. cruzi*, *L. donovani*, and *P. falciparum*. From the collected data, it is clear that the library of benzimidazoles constitutes an interesting source for the identification of a novel drug lead for the fight against *P. falciparum*. Indeed more than two-third of the derivatives was significantly active against the parasite and almost one-third is characterized by selectivity indexes (IC_{50} L-6 cells/IC_{50} parasite) higher than 90 and even exceeding 450. In addition let us emphasize that the bisbenzimidazoles described in this work are readily accessible from cheap precursors and are characterized by simple chemical structures devoid of stereogenic centers. These promising in vitro results however need to be confirmed by in vivo screenings, which will be a challenge because the physicochemical properties of these derivatives must be improved prior to the in vivo studies. In particular their solubility in water has to be increased and appropriate formulations should be found in order to reach acceptable oral absorption. Based on the leads identified in this work, future studies are directed to the preparation of novel compounds bearing substituents that could modulate not only the physicochemical properties, but also increase their selectivity indexes.

4. Experimental

^{1}H NMR spectra were obtained using a Varian Inova instrument (500 MHz), ^{13}C NMR spectra using a Bruker Advance 500 (125 MHz), chemical shifts (δ) are given in ppm using TMS as internal reference and coupling constants (J) in Hz. IR spectra were recorded on a Perkin-Elmer Spectrum One spectrometer operating in the diffuse reflectance mode. High resolution mass spectra (HRMS) were recorded on a Waters Q-ToF 2 instrument. A Merck Hitachi LaChrom HPLC equipped with a Hibar® 125-4 Purospher® STAR RP-18e column has been used (solvents: methanol/water) to assess purity of the final compounds. Solvents and reagents were commercially available (Aldrich, Alfa Aesar, Acros Organics, Fisher Scientific) and were used without further purification.

4.1. Chemistry

4.1.1. Preparation of the bisbenzaldehydes

A mixture of a hydroxybenzaldehyde (6.72 g, 55 mmol), a dibromoalkane (25 mmol), and tetrabutylammonium sulfate (0.34 g; 1 mmol) in an aqueous 4 M solution of sodium hydroxide was heated under reflux for 7 h. After cooling the precipitate was filtered and successively washed with water, and ether. Yields (not optimized) ranged from 20% (for the precursor of **11**) to 95% for the precursors of **25**, **32**, **34**, and **35**). The bisbenzaldehydes were pure enough to be engaged in the second step.

The bisbenzaldehydes precursors of **11**–**20**,[53] **21**,[54] **22**,[55] **23**,[56] **24**,[57] **25**,[58] **26**,[58] **27**,[59] **28**–**30**,[58] **31**,[60] **32**,[61] **33**,[62] **34**,[63] **35**,[64] and **36**.[65] have been described in the literature.

4.1.2. Preparation of the bisbenzimidazoles

A mixture of pyridine (1.62 mL; 1.58 g; 20 mmol) and thionyl chloride (1.46 mL; 2.38 g; 20 mmol) in dichloromethane (50 mL) was slowly added to a solution of bisbenzaldehyde (10 mmol) in dichloromethane (25 mL) maintained at 0 °C. After addition, the reaction medium was allowed to warm up to room temperature for 10 min and a solution of 1,2-phenylenediamine (4.32 g; 40 mmol) in dichloromethane (25 mL) was slowly added. The mixture was stirred overnight. The solid was filtered and successively washed with *N,N*-dimethylformamide (or acetonitrile when the bisbenzimidazole was soluble in *N,N*-dimethylformamide), hot water, and hot ethanol. The so-obtained dihydrochloride salt (100 mg) was neutralized in a hot mixture of NaOH 0.4 M (4 mL) and ethanol (1 mL). Yields have not been optimized.

The bisbenzimidazoles **13**–**17** and **25** have been described in the literature.[18]

4.1.2.1. 2,2′-[Methylenebis(oxy-1,4-phenylene)]bis-1*H*-benzimidazole (11). Yield: 70%. Mp: >300 °C. ^{1}H NMR (DMSO-d_6): 8.1 (d, 4H, J = 8 Hz); 7.6 (d, 2 H, J = 7 Hz); 7.5 (d, 2 H, J = 7 Hz); 7.2 (m, 8 H); 6.0 (s, 2 H) ppm. ^{13}C NMR (DMSO-d_6): 157.5; 151.0; 144.8; 135.0; 128.8; 124.5; 122.2; 121.5; 118.6; 116.5; 111.1; 89.9 ppm. IR: 3300–2200; 3058; 1616; 1500; 1439; 1233 cm^{-1}. HRMS: found 455.1484, calcd 455.1470 for $C_{27}H_{20}N_4O_2$ + Na.

4.1.2.2. 2,2′-[1,2-Ethanediylbis(oxy-1,4-phenylene)]bis-1*H*-benzimidazole (12). Yield: 50%. Mp: >300 °C. ^{1}H NMR (DMSO-d_6): 8.1 (d, 4 H, J = 8 Hz); 7.6 (d, 2H, J = 7 Hz); 7.5 (d, 2 H, J = 7 Hz); 7.2 (m, 8 H); 4.5 (s, 4 H) ppm. ^{13}C NMR (DMSO-d_6): 159.7; 151.3; 143.8; 134.9; 128.1; 122.9; 122.1; 121.5; 118.5; 114.9; 111.0; 66.5 ppm. IR: 3300–2200; 3057; 1609; 1496; 1437; 1246 cm^{-1}. HRMS: found 447.1823, calc. 447.1821 for $C_{28}H_{23}N_4O_2$.

4.1.2.3. 2,2′-[1,8-Octanediylbis(oxy-1,4-phenylene)]bis-1*H*-benzimidazole (18). Yield: 35%. Mp: 292–294 °C (decomposition). ^{1}H NMR (DMSO-d_6): 8.1 (d, 4 H, J = 8 Hz); 7.6 (d, 2 H, J = 7 Hz); 7.5 (d, 2 H, J = 7 Hz); 7.2 (m, 8 H); 4.1 (t, 4 H, J = 6 Hz); 1.8 (m, 4 H, J = 6 Hz); 1.5 (br, 4 H); 1.4 (br, 4 H) ppm. ^{13}C NMR (DMSO-d_6): 160.1; 151.4; 143.8; 135.0; 128.0; 122.5; 122.0; 121.4; 118.4; 114.8; 111.0; 67.6; 28.7; 28.6; 25.4 ppm. IR: 3300-2200; 2938; 1614; 1551; 1503; 1445 1258 cm^{-1}. HRMS: found 531.2762, calcd 531.2760 for $C_{34}H_{35}N_4O_2$.

4.1.2.4. 2,2′-[1,9-Nonanediylbis(oxy-1,4-phenylene)]bis-1*H*-benzimidazole (19). Yield: 50%. Mp: 271–273 °C (decomposition). ^{1}H NMR (DMSO-d_6): 8.1 (d, 4 H, J = 8 Hz); 7.6 (d, 2 H, J = 7 Hz); 7.5 (d, 2 H, J = 7 Hz); 7.2 (m, 8 H); 4.1 (t, 4 H, J = 6 Hz); 1.8 (m, 4 H, J = 6 Hz); 1.5 (br, 4 H); 1.4 (br, 6 H) ppm. ^{13}C NMR

(DMSO-d_6): 160.0; 151.4; 143.8; 134.9; 128.0; 122.5; 122.0; 121.4; 118.4; 114.8; 111.0; 67.64; 28.9; 28.7; 28.6; 25.5 ppm. IR: 3300–2200; 2933; 1613; 1435; 1256 cm^{-1}. HRMS: found 567.2745, calcd 567.2736 for $C_{35}H_{36}N_4O_2$ + Na.

4.1.2.5. 2,2′-[1,10-Decanediylbis(oxy-1,4-phenylene)]bis-1*H*-benzimidazole (20). Yield: 50%. Mp: 246–248 °C (decomposition). ^{1}H NMR (DMSO-d_6): 8.1 (d, 4 H, J = 8 Hz); 7.6 (d, 2 H, J = 7 Hz); 7.5 (d, 2 H, J = 7 Hz); 7.2 (m, 8 H); 4.1 (t, 4 H, J = 7 Hz); 1.8 (m, 4 H, J = 7 Hz); 1.4 (br, 4 H); 1.3 (br, 4 H) ppm. ^{13}C NMR (DMSO-d_6): 159.3; 150.6; 142.5; 134.0; 127.2; 122.5; 121.7; 117.7; 114.0; 110.0; 66.9; 28.1; 28.0; 27.8; 24.7 ppm. IR: 3300–2200; 2924; 1614; 1498; 1436; 1252 cm^{-1}. HRMS: found 559.3069, calcd 559.3073 for $C_{36}H_{39}N_4O_2$.

4.1.2.6. 2,2′-[Methylenebis(oxy-1,3-phenylene)]bis-1*H*-benzimidazole (21). Yield: 35%. Mp: 289–291 °C (decomposition). ^{1}H NMR (DMSO-d_6): 8.0 (t, 2 H, J = 2 Hz); 7.9 (d, 2 H, J = 8 Hz); 7.7 (d, 2 H, J = 6 Hz); 7.5 (m, 4 H); 7.2 (m, 6 H); 6.1 (s, 2 H) ppm. ^{13}C NMR (DMSO-d_6): 156.8; 150.8; 144.8; 135.0; 131.7; 130.3; 122.6; 121.7; 120.5; 118.9; 117.7; 114.1; 111.3; 90.1 ppm. IR: 3300–2200; 2798; 1590; 1490; 1445; 1205 cm^{-1}. HRMS: found 455.1483, calcd 455.1484 for $C_{27}H_{20}N_4O_2$ + Na.

4.1.2.7. 2,2′-[1,2-Ethanediylbis(oxy-1,3-phenylene)]bis-1*H*-benzimidazole (22). Yield: 20%. Mp: >300 °C. ^{1}H NMR (DMSO-d_6): 8.0 (t, 2 H, J = 2 Hz); 7.9 (d, 2 H, J = 8 Hz); 7.7 (d, 2 H, J = 6 Hz); 7.5 (m, 4 H); 7.2 (m, 6 H); 4.5 (s, 4 H) ppm. ^{13}C NMR (DMSO-d_6): 158.7; 151.0; 143.7; 134.9; 131.5; 130.2; 122.6; 121.7; 119.1; 118.9; 116.4; 112.0; 111.3; 66.5 ppm. IR: 3200–2500; 3049; 1589; 1490; 1446; 1400; 1233 cm^{-1}. HRMS: found 447.1812, calcd 447.1821 for $C_{28}H_{23}N_4O_2$.

4.1.2.8. 2,2′-[1,3-Propanediylbis(oxy-1,3-phenylene)]bis-1*H*-benzimidazole (23). Yield: 20%. Mp: 285–286 °C (decomposition). ^{1}H NMR (DMSO-d_6): 8.0 (t, 2 H, J = 2 Hz); 7.9 (d, 2 H, J = 8 Hz); 7.7 (d, 2 H, J = 6 Hz); 7.5 (m, 4 H); 7.2 (m, 6 H); 4.3 (t, 4 H, J = 6 Hz); 2.3 (m, 2 H, J = 6 Hz) ppm. ^{13}C NMR (DMSO-d_6): 158.9; 151.0; 131.4; 130.2; 122.4; 118.8; 116.4; 111.9; 64.5; 28.7 ppm. IR: 3300-2200; 2960; 1590; 1489; 1455; 1235 cm^{-1}. HRMS: found 461.1989, calcd 461.1978 for $C_{29}H_{25}N_4O_2$.

4.1.2.9. 2,2′-[1,4-Butanediylbis(oxy-1,3-phenylene)]bis-1*H*-benzimidazole (24). Yield: 35%. Mp: 287–287 °C (decomposition). ^{1}H NMR (DMSO-d_6): 8.0 (t, 2 H, J = 2 Hz); 7.9 (d, 2 H, J = 8 Hz); 7.7 (d, 2 H, J = 6 Hz); 7.5 (m, 4 H); 7.2 (m, 6 H); 4.2 (t, 4 H, J = 6 Hz); 2.0 (t, 4 H, J = 6 Hz) ppm. ^{13}C NMR (DMSO-d_6): 159.0; 151.1; 131.3; 130.1; 122.2; 118.7; 116.3; 112.1; 67.4; 25.4 ppm. IR: 3300-2200; 2922; 1589; 1491; 1455; 1401; 1224 cm^{-1}. HRMS: found 475.2125, calc. 475.2134 for $C_{30}H_{27}N_4O_2$.

4.1.2.10. 2,2′-[1,6-Hexanediylbis(oxy-1,3-phenylene)]bis-1*H*-benzimidazole (26). Yield: 40%. Mp: 294–296 °C (decomposition). ^{1}H NMR (DMSO-d_6): 8.0 (t, 2 H, J = 2 Hz); 7.9 (d, 2 H, J = 8 Hz); 7.7 (d, 2 H, J = 6 Hz); 7.5 (m, 4 H); 7.2 (m, 6 H); 4.1 (t, 4 H, J = 6 Hz); 1.8 (m, 4 H); 1.6 (m, 4 H) ppm. ^{13}C NMR (DMSO-d_6): 157.5; 149.6; 142.1; 133.4; 129.6; 128.5; 121.0; 120.1; 117.3; 117.1; 114.8; 110.4; 109.8; 66.0; 27.1; 23.8 ppm. IR: 3300–2200; 2929; 1603; 1541; 1492; 1457; 1364; 1237 cm^{-1}. HRMS: found 525.2272, calcd 525.2266 for $C_{32}H_{30}N_4O_2$ + Na.

4.1.2.11. 2,2′-[1,7-Heptanediylbis(oxy-1,3-phenylene)]bis-1*H*-benzimidazole (27). Yield: 35%. Mp: 258–259 °C (decomposition). ^{1}H NMR (DMSO-d_6): 8.0 (t, 2 H, J = 2 Hz); 7.9 (d, 2 H, J = 8 Hz); 7.7 (d, 2 H, J = 6 Hz); 7.5 (m, 4 H); 7.2 (m, 6 H); 4.1 (t, 4 H, J = 6 Hz); 1.8 (m, 4 H, J = 6 Hz); 1.5 (br, 6 H) ppm. ^{13}C NMR (DMSO-d_6): 159.1; 151.1; 143.7; 134.9; 131.4; 130.1; 122.6; 121.7; 118.8; 118.6; 116.3; 112.0; 111.3; 67.6; 28.6; 28.5; 25.5 ppm. IR: 3300–2200; 2937; 1603; 1492; 1463; 1275; 1242 cm^{-1}. HRMS: found 539.2441, calcd 539.2423 for $C_{33}H_{32}N_4O_2$ + Na.

4.1.2.12. 2,2′-[1,8-Octanediylbis(oxy-1,3-phenylene)]bis-1*H*-benzimidazole (28). Yield: 30%. Mp: 270–271 °C (decomposition). ^{1}H NMR (DMSO-d_6): 8.0 (t, 2 H, J = 2 Hz); 7.9 (d, 2 H, J = 8 Hz); 7.7 (d, 2 H, J = 6 Hz); 7.5 (m, 4 H); 7.2 (m, 6 H); 4.1 (t, 4 H, J = 6 Hz); 1.8 (m, 4 H, J = 6 Hz); 1.5 (br, 4 H); 1.4 (br, 4 H) ppm. ^{13}C NMR (DMSO-d_6): 159.0; 151.1; 143.7; 134.9; 131.4; 130.1; 122.6; 120.7; 118.8; 118.6; 116.3; 111.9; 111.3; 67.6; 28.7; 28.6; 25.5 ppm. IR: 3300–2200; 2930; 1602; 1491; 1458; 1235 cm^{-1}. HRMS: found 531.2768, calcd 531.2760 for $C_{34}H_{35}N_4O_2$.

4.1.2.13. 2,2′-[1,9-Nonanediylbis(oxy-1,3-phenylene)]bis-1*H*-benzimidazole (29). Yield: 30%. Mp: 226–228 °C (decomposition). ^{1}H NMR (DMSO-d_6): 8.0 (t, 2 H, J = 2 Hz); 7.9 (d, 2 H, J = 8 Hz); 7.7 (d, 2 H, J = 6 Hz); 7.5 (m, 4 H); 7.2 (m, 6 H); 4.1 (t, 4 H, J = 6 Hz); 1.8 (m, 4 H, J = 6 Hz); 1.5 (br, 4 H); 1.4 (br, 6 H) ppm. ^{13}C NMR (DMSO-d_6): 159.0; 151.1; 131.3; 130.6; 122.2; 118.6; 116.3; 112.0; 67.6; 29.0; 28.7; 28.6; 25.5 ppm. IR: 3300–2200; 2920; 1604; 1539; 1492; 1456; 1236 cm^{-1}. HRMS: found 545.2928, calcd 545.2917 for $C_{35}H_{37}N_4O_2$.

4.1.2.14. 2,2′-[1,10-Decanediylbis(oxy-1,3-phenylene)]bis-1*H*-benzimidazole (30). Yield: 20%. Mp: 251–253 °C (decomposition). ^{1}H NMR (DMSO-d_6): 8.0 (t, 2 H, J = 2 Hz); 7.9 (d, 2 H, J = 8 Hz); 7.7 (d, 2 H, J = 6 Hz); 7.5 (m, 4 H); 7.2 (m, 6 H); 4.1 (t, 4 H, J = 6 Hz); 1.8 (m, 4 H, J = 6 Hz); 1.4 (br, 4 H); 1.3 (br, 8 H) ppm. ^{13}C NMR (DMSO-d_6): 159.0; 151.1; 131.3; 130.1; 122.2; 118.6; 116.3; 114.6; 111.9; 67.58; 28.9; 28.7; 28.6; 25.5 ppm. IR: 3300–2200; 2928; 1601; 1460; 1236 cm^{-1}. HRMS: found 559.3085, calcd 559.3073 for $C_{36}H_{39}N_4O_2$.

4.1.2.15. 2,2′-[1,2-Phenylenebis(methyleneoxy-1,4-phenylene)]bis-1*H*-benzimidazole (31). Yield: 60%. Mp: >300 °C. ^{1}H NMR (DMSO-d_6): 8.1 (d, 4 H, J = 8 Hz); 7.6–7.4 (m, 8 H); 7.2 (d, 4 H, J = 8 Hz); 7.2 (dd, 4 H, J = 6 Hz and 3 Hz); 5.4 (s, 4 H) ppm. ^{13}C NMR (DMSO-d_6): 159.6; 151.2; 135.0; 128.6; 128.2; 128.0; 123.0; 121.8; 67.2 ppm. IR: 3300–2200; 2881; 1611; 1499; 1435; 1246; 1176 cm^{-1}. HRMS: found 545.1945, calc. 545.1953 for $C_{34}H_{26}N_4O_2$ + Na.

4.1.2.16. 2,2′-[1,3-Phenylenebis(methyleneoxy-1,4-phenylene)]bis-1*H*-benzimidazole (32). Yield: 40%. Mp: 242–244 °C (decomposition). ^{1}H NMR (DMSO-d_6): 8.1 (d, 4 H, J = 8 Hz); 7.6 (br, 2 H); 7.5–7.4 (br, 8 H); 7.2–71 (m, 8 H); 5.2 (s, 4 H) ppm. ^{13}C NMR (DMSO-d_6): 159.7; 151.3; 137.1; 128.7; 128.0; 127.4; 127.0; 122.9; 121.8; 115.2; 69.3 ppm. IR: 3300–2200; 3053; 1613; 1435; 1251 cm^{-1}. HRMS: found 545.1939, calc. 545.1953 for $C_{34}H_{26}N_4O_2$ + Na.

4.1.2.17. 2,2′-[1,4-Phenylenebis(methyleneoxy-1,4-phenylene)]bis-1*H*-benzimidazole (33). Yield: 70%. Mp: 289–291 °C (decomposition). ^{1}H NMR (DMSO-d_6): 8.1 (d, 4 H, J = 8 Hz); 7.6 (br, 2 H); 7.5 (s, 4 H); 7.5 (br, 2 H); 7.3 (d, 4 H, J = 8 Hz); 7.2 (br, 4 H); 5.2 (s, 4H) ppm. ^{13}C NMR (DMSO-d_6): 159.7; 151.3; 143.9; 136.6; 135.0; 128.0; 127.8; 123.0; 122.1; 121.4; 118.5; 114.6; 111.0; 69.2 ppm. IR: 3300–2200; 3060; 1614; 1499; 1248 cm^{-1}. HRMS: found 545.1960, calcd 545.1953 for $C_{34}H_{26}N_4O_2$ + Na.

4.1.2.18. 2,2′-[1,2-Phenylenebis(methyleneoxy-1,3-phenylene)]bis-1*H*-benzimidazole (34). Yield: 30%. Mp: >300 °C. ^{1}H NMR (DMSO-d_6): 7.8 (s, 2 H); 7.8 (d, 2 H, J = 8 Hz); 7.7–7.4

(m, 10 H); 7.2 (m, 6 H) 5.4 (s, 4 H) ppm. ^{13}C NMR (DMSO-d_6): 158.7; 151.0; 143.7; 135.1; 134.9; 131.5; 130.1; 128.7; 128.2; 122.6; 121.7; 119.1; 118.9; 116.5; 112.6; 111.3; 67.3 ppm. IR: 3300–2200; 2880; 1589; 1533; 1455; 1226 cm^{-1}. HRMS: found 545.1955, calcd 545.1953 for $C_{34}H_{26}N_4O_2$ + Na.

4.1.2.19. 2,2′-[1,3-Phenylenebis(methyleneoxy-1,3-phenylene)]bis-1*H*-benzimidazole (35). Yield: 20%. Mp: 253–255 °C (decomposition). ^{1}H NMR (DMSO-d_6): 7.9 (s, 2 H); 7.8 (d, 2 H, J = 8 Hz); 7.7 (s, 1 H); 7.6-7.4 (m, 9 H); 7.2 (br, 4 H); 7.1 (dd, 2 H, J = 8 Hz and 2 Hz); 5.3 (s, 4 H) ppm. ^{13}C NMR (DMSO-d_6): 158.7; 151.0; 143.7; 137.2; 134.9; 131.5; 130.2; 128.7; 127.3; 126.9; 122.6; 121.7; 119.0; 118.9; 116.5; 112.5; 111.3; 69.3 ppm. IR: 3300–2200; 2874; 1590; 1540; 1455; 1229 cm^{-1}. HRMS: found 545.1967, calcd 545.1953 for $C_{34}H_{26}N_4O_2$ + Na.

4.1.2.20. 2,2′-[1,4-Phenylenebis(methyleneoxy-1,3-phenylene)]bis-1*H*-benzimidazole (36). Yield: 20%. Mp: >300 °C. ^{1}H NMR (DMSO-d_6): 7.9 (s, 2 H); 7.8 (d, 2 H, J = 8 Hz); 7.6 (br, 4 H); 7.6 (s, 4 H); 7.5 (t, 2 H, J = 8 Hz); 7.2 (dd, 4 H, J = 8 Hz and 3 Hz); 7.2 (dd, 2 H, J = 8 Hz and 3 Hz); 5.2 (s, 4 H) ppm. ^{13}C NMR (DMSO-d_6): 158.7; 151.0; 136.6; 131.5; 130.1; 127.8; 122.2; 119.1; 116.6; 112.6; 69.2 ppm. IR: 3300–2200; 2870; 1601; 1491; 1455; 1219 cm^{-1}. HRMS: found 545.1960, calc. 545.1953 for $C_{34}H_{26}N_4O_2$ + Na.

4.2. Biological assays

4.2.1. Cytotoxicity towards rat skeletal myoblasts, L-6 cells[66,67]

Assays were performed in 96-well microtiter plates, each well containing 100 μL of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% fetal bovine serum, and 4×10^4 L6 cells. Serial drug dilutions of seven threefold dilution steps covering a range from 90 to 0.123 μg/mL were prepared. After 72 h of incubation, the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Ten microliters of the viability marker resazurin (12.5 mg resazurin dissolved in 100 mL phosphate-buffered saline) were then added to each well and the plates incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Corporation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the microplate reader software Softmax Pro. Podophyllotoxin was the reference drug used.

4.2.2. Anti-*Giardia* intestinalis activity[68]

One hundred microliters of a slightly modified TPS-1 culture medium were added to wells of a 96-well microtiter plate. Serial drug dilutions covering a range from 100 to 0.137 μg/mL were prepared. Then a suspension (100 μL) of the *Giardia intestinalis* G1 strain was added to each well leading to an initial *Giardia* density of 5×10^5 microorganisms per mL. After 70 h of incubation at 37 °C in a nitrogen atmosphere, the plate was inspected under an inverted microscope to assure growth of the controls and sterile conditions. The medium was removed from all wells and replace by 100 μL of the viability marker resazurin (12.5 mg resazurin dissolved in 100 mL phosphate-buffered saline–PBS—further diluted 10× in PBS to afford a final concentration of 12.5 mg/L). Then the plate was sealed again and incubated for a further 2 h at 37 °C. The plate was finally read with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the software Softmax Pro (Molecular Devices Corporation, Sunnyvale, CA, USA). Decrease of fluorescence (inhibition) was expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. IC_{50} values were calculated from the sigmoidal inhibition curves. Metronidazole was used as reference drug.

4.2.3. Anti-*Entamoeba* histolytica activity[69]

Entamoeba histolytica Hk-9 trophozoites were cultivated in a TYI-S-33 culture medium supplemented with 10% heat-inactivated bovine serum and enriched with 3% vitamin-mix NCTC. A procedure similar to that described for the determination of the anti-*Giardia intestinalis* activity was developed. Metronidazole was also used as reference drug.

4.2.4. Anti-Trypanosoma activity

4.2.4.1. Anti-*Trypanosoma* brucei rhodesiense activity[70]. Minimum Essential Medium–MEM–(50 μL) supplemented with 25 mM HEPES, 1 g/L additional glucose, 1% MEM non-essential amino acids (100×), 0.2 mM 2-mercaptoethanol, 1 mM Na-pyruvate, and 15% heat inactivated horse serum were added to each well of a 96-well microtiter plate. Serial drug dilutions of seven threefold dilution steps covering a range from 90 to 0.123 μg/mL were prepared. Then 2×10^3 bloodstream forms of *T. brucei rhodesiense* STIB 900 in 50 μL were added to each well and the plate was incubated at 37 °C under a 5% CO_2 atmosphere for 70 h. Ten microliters resazurin solution (12.5 mg resazurin dissolved in 100 mL phosphate-buffered saline) were then added to each well and incubation continued for a further 2–4 h. Then the plate was read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Corporation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the microplate reader software Softmax Pro (Molecular Devices Corporation, Sunnyvale, CA, USA). Melarsoprol was used as reference drug.

4.2.4.2. Anti-*Trypanosoma* cruzi activity[71]. Rat skeletal myoblasts (L6 cells) were seeded in 96-well microtiter plates at 2000 cells/well in 100 μL RPMI 1640 medium with 10% FBS and 2 mM L-glutamine. After 24 h the medium was replaced by 100 μL per well containing 5000 trypomastigote forms of *T. cruzi* Tulahuen strain C2C4 containing the β-galactosidase (Lac Z) gene. After 48 h, the medium was removed from the wells and replaced by 100 μL fresh medium with or without a serial drug dilution of seven threefold dilution steps covering a range from 90 to 0.123 μg/mL. After 96 h of incubation, the plates were inspected under an inverted microscope to assure growth of the controls and sterility. Then, the substrate CPRG/Nonidet (50 μL) was added to all wells. A color reaction developed within 2–6 h and could be read photometrically at 540 nm. Data were transferred into the graphic programme Softmax Pro, which calculated IC_{50} values. Benznidazole was used as reference drug.

4.2.5. Anti-*Leishmania* donovani activity[72]

Amastigotes of *L. donovani* strain MHOM-ET-67/L82 were grown in axenic culture at 37 °C in SM medium at pH 5.4 supplemented with l0% heat-inactivated fetal bovine serum under an atmosphere of 5% CO_2 in air. One hundred microliters of culture medium with 10^5 amastigotes from axenic culture with or without a serial drug dilution were seeded in 96-well microtitre plates. Serial drug dilutions covering a range from 90 to 0.123 μg/mL were prepared. After 72 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. 10 μL of resazurin (12.5 mg resazurin dissolved in 100 mL phosphate-buffered saline) were then added to each well and the plates incubated for another 2 h. Then, the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Corporation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data

were analyzed using the software Softmax Pro (Molecular Devices Corporation, Sunnyvale, CA, USA). Decrease of fluorescence (inhibition) was expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves the IC_{50} values were calculated. Miltefosine was used as reference drug.

4.2.6. Anti-*Plasmodium* falciparum activity[73]

In vitro activity against erythrocytic stages of *P. falciparum* was determined by a modified [^{3}H]-hypoxanthine incorporation assay, using the chloroquine- and pyrimethamine-resistant K1 strain. Parasite cultures incubated in RPMI 1640 medium with 5% Albumax (without hypoxanthine) were exposed to serial drug dilutions in microtiter plates. After 48 h of incubation at 37 °C in a reduced oxygen atmosphere, 0.5 μCi ^{3}H-hypoxanthine was added to each well. Cultures were incubated for a further 24 h before they were harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a Betaplate™ liquid scintillation counter (Wallac, Zurich, Switzerland). The results were recorded as counts per minute (CPM) per well at each drug concentration and expressed as percentage of the untreated controls. IC_{50} values were calculated from graphically plotted dose–response curves. Chloroquine was used as reference drug.

Inhibition of formation of β-hematin in a cell-free assay was observed by using the following procedure. A stock solution (10 mL) of the studied compound (solution A) was prepared by dissolving 2 μmol of that compound in a mixture of DMSO (1 mL) and HEPES buffer 0.2 M (pH 7.0). A stock solution (10 mL) of hemin (solution B) was prepared by dissolving 2 μmol (1.3 mg) of hemin in a mixture of aqueous sodium hydroxide 0.1 M (5 mL) and HEPES buffer 0.2 M. The test solution of the studied compound was obtained by mixing solution A (1 mL), solution B (1 mL), acetate buffer pH 5 (1 mL) and HEPES buffer 0.2 M (2 mL). In the absence of benzimidazole, β-hematin precipitated within a few minutes.

References and notes

1. Chand-Goyal, T.; Spotts, R. A. *Postharvest Biol. Technol.* **1996**, *7*, 51–64.
2. Guggenmoos, R.; Akhtaruzzaman, K. M.; Rosenkaimer, F.; Gaus, W.; Bienzle, U.; Dietrich, M. *Tropenmed Parasitol.* **1978**, *29*, 423–426.
3. Fourestié, V.; Bougnoux, M. E.; Ancelle, T.; Liance, M.; Roudot-Thoraval, F.; Naga, H.; Pairon-Pennachioni, M.; Rauss, A.; Lejonc, J. L. *Parasitol. Res.* **1988**, *75*, 36–41.
4. Towsend, L. B.; Wise, D. S. *Parasitol. Today* **1990**, *6*, 107–112.
5. Cook, G. C. *Parasitol. Today* **1990**, *6*, 133–136.
6. Frayha, G. J.; Smyth, J. D.; Gobert, J. G.; Savel, J. *Gen. Pharmacol.* **1997**, *28*, 273–299.
7. Reuter, S.; Jensen, B.; Buttenschoen, K.; Kratzer, W.; Kern, P. *J. Antimicrob. Chemother.* **2000**, *46*, 451–456.
8. Addiss, D.; Critchley, J.; Garner, P.; Gelband, H.; Gamble, C. *Cochrane Database Syst. Rev.* **2004**, CD003753.
9. Karabay, O.; Tamer, A.; Gunduz, H.; Kayas, D.; Arinc, H.; Celebi, H. *World J. Gastroenterol.* **2004**, *10*, 1215–1217.
10. Yeung, K.-S.; Meanwell, N. A.; Qiu, Z.; Hernandez, D.; Zhang, S.; McPhee, F.; Weinheimer, S.; Clark, J. M.; Janc, J. W. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2355–2359.
11. Sperandio, D.; Gangloff, A. R.; Litvak, J.; Goldsmith, R.; Hataye, J. M.; Wang, V. R.; Shelton, E. J.; Elrod, K.; Janc, J. W.; Clark, J. M.; Rice, K.; Weinheimer, S.; Yeung, K.-S.; Meanwell, N. A.; Hernandez, D.; Staab, A. J.; Venagles, B. L.; Spencer, J. R. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3129–3133.
12. Singh, A. K.; Lown, J. W. *Anticancer Drug Des.* **2000**, *15*, 265–275.
13. Mann, J.; Barron, A.; Opoku-Boahen, Y.; Johansson, E.; Perkinson, G.; Kelland, L. R.; Neidle, S. *J. Med. Chem.* **2001**, *44*, 138–144.
14. Huang, G.; Li, G. R.; Wu, Z. G.; Song, Z. B.; Zhou, Y. Y.; Shuai, L.; Weng, X. C.; Zhou, X.; Yang, G. F. *Chem. Commun.* **2009**, 902–904.
15. Alanyali, F. S.; Arici, M.; Artagan, O.; Isikdag, I.; Ozkay, Y. *Z. Naturforsch., C: Biosci.* **2010**, *65*, 10–14.
16. Agh-Atagay, N. M.; Dulger, B.; Gucin, F. *Eur. J. Med. Chem.* **2003**, *38*, 875–881.
17. Walker, J.; Saravia, N. G. *J. Parasitol.* **2004**, *90*, 1155–1162.
18. Mayence, A.; Pietka, A.; Collins, M. S.; Cushion, M. T.; Tekwani, B. L.; Huang, T. L.; Vanden Eynde, J. J. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2658–2661.
19. Torres-Gomez, H.; Hernandez-Nunez, E.; Leon-Rivera, I.; Guerrero-Alvarez, J.; Cedillo-Rivera, R.; Moo-Puc, R.; Argotte-Ramos, R.; Rodriguez-Gutierrez, M. D. C.; Chan-Bacab, M. J.; Navarrete-Vazquez, G. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3147–3151.
20. Alp, M.; Goker, H.; Brun, R.; Yildiz, S. *Eur. J. Med. Chem.* **2009**, *44*, 2002–2008.
21. Van Gysel, A.; Maquestiau, A.; Vanden Eynde, J.J.; Mayence A.; Vanovervelt, J.C. EP19920870051; *Chem. Abstr.* **1993**, *119*, 95521.
22. Vanden Eynde, J. J.; Mayence, A.; Maquestiau, A.; Anders, E. *Bull. Soc. Chim. Belg.* **1993**, *102*, 357–364.
23. Mayence, A.; Vanden Eynde, J. J.; LeCour, L., Jr.; Walker, L. A.; Tekwani, B. L.; Huang, T. L. *Eur. J. Med. Chem.* **2004**, *39*, 547–553.
24. Cushion, M. T.; Walzer, P. D.; Collins, M. S.; Rebholz, S.; Vanden Eynde, J. J.; Mayence, A.; Huang, T. L. *Antimicrob. Agents Chemother.* **2004**, *48*, 4209–4216.
25. Huang, T. L.; Vanden Eynde, J. J.; Mayence, A.; Donkor, I. O.; Khan, S. I.; Tekwani, B. L. *J. Pharm. Pharmacol.* **2006**, *58*, 1033–1042.
26. Sarju, J.; Danks, T. N.; Wagner, G. *Tetrahedron Lett.* **2004**, *45*, 7675–7677.
27. Ridley, H. F.; Spickett, R. G.; Timmis, G. M. *J. Heterocycl. Chem.* **1965**, *2*, 453–456.
28. Fedorv, L. A.; Savarino, P.; Viskardi, G.; Rebrov, A. I.; Barni, E. *Acad. Sci. USSR, Chem. Ser.* **1992**, 223–2230.
29. Garcia, M. A.; Claramunt, R. M.; Solcan, T.; Milata, V.; Alkorta, I.; Elguero, J. *Magn. Reson. Chem.* **2009**, *47*, 100–104.
30. Kubish, R.; Stütz, E.; Ehret, R.; Drechsler, S.; Fleischer, M. *Procedia Chem.* **2009**, *1*, 730–733.
31. Petri, W. A., Jr. *Trends Parasitol.* **2003**, *19*, 523–526.
32. Delespaux, V.; de Koning, H. P. *Drug Resist. Updat.* **2007**, *10*, 30–50.
33. Sanchez-Sancho, F.; Campillo, N. E.; Paez, J. A. *Curr. Med. Chem.* **2010**, *17*, 423–452.
34. Nussbaum, K.; Honek, J.; Cadmus, C. M. C. V.; Efferth, T. *Curr. Med. Chem.* **2010**, *17*, 1594–1617.
35. Burri, C. *Parasitology* **2010**, *137*, 1987–1994.
36. Pathak, R.; Batra, S. *Anti-infect. Agents Med. Chem.* **2009**, *8*, 226–267.
37. Mondal, S.; Bhattacharya, P.; Ali, N. *Expert Rev. Anti-infective Ther.* **2010**, *8*, 919–944.
38. Ameen, M. *Clin. Exp. Dermatol.* **2010**, *35*, 699–705.
39. Ameen, M. *Expert Opin. Pharmacother.* **2010**, *11*, 557–569.
40. Baird, J. K. *N. Engl. J. Med.* **2005**, *352*, 1565–1577.
41. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug Delivery Rev.* **1997**, *23*, 3–25.
42. Egan, T. *Mini-Rev. Med. Chem.* **2001**, 113.
43. Ziegler, J.; Linck, R.; Wright, D. W. *Curr. Med. Chem.* **2001**, *8*, 171.
44. Kumar, A.; Katiyar, S. B.; Agarwal, A.; Chauhan, P. M. *Curr. Med. Chem.* **2003**, *10*, 1137.
45. Slater, A. F. G.; Swiggard, W. J.; Orton, B. R.; Flitter, W. D.; Goldberg, D. E.; Cerami, A.; Henderson, G. B. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 325.
46. Slater, A. F. G.; Cerami, A. *Nature* **1992**, *355*, 167.
47. Egan, T. J.; Ross, D. C.; Adams, P. A. *FEBS Lett.* **1994**, *352*, 54.
48. Wright, C. W.; Addae-Kyereme, J.; Breen, A. G.; Brown, J. E.; Cox, M. F.; Croft, S. L.; Gokcek, Y.; Kendrick, H.; Phillips, R. M.; Pollet, P. L. *J. Med. Chem.* **2001**, *44*, 3187.
49. Stead, A. M. W.; Bray, P. G.; Edwards, G.; de Koning, H. P.; Elford, B. C.; Stocks, P. A.; Ward, S. A. *Mol. Pharmacol* **2001**, *59*, 1298.
50. Mayence, A.; Vanden Eynde, J. J.; Huang, T. L. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1625–1628.
51. Katyar, S. K.; Gordon, V. R.; McLaughlin, G. L.; Edlind, T. D. *Antimicrob. Agents Chemother.* **1994**, *38*, 2086–2090.
52. Purfield, A. E.; Tidwell, R. R.; Meshnick, S. R. *Malaria J.* **2009**, *8*, 104–112.
53. Donahoe, H. B.; Benjamin, L. E.; Fennoy, L. V.; Greiff, D. *J. Org. Chem.* **1961**, *26*, 474–476.
54. Salunkhe, M. M.; Salunkhe, D. G.; Kanade, A. S.; Mane, R. B.; Wadgaonkar, P. P. *Synth. Commun.* **1990**, *20*, 1143–1147.
55. Matsumura, S.; Enomoto, H.; Aoyagi, Y.; Yoshikuni, Y.; Yagi, M.; Kura, K.; Shirahase, I. Jp Patent 1980–9173; *Chem. Abstr.* **1982**, *97*, 163421.
56. Kuvshinova, E. M.; Golubchikov, O. A.; Berezin, B. D. *Zh. Obshch. Khim.* **1991**, *61*, 1799–1804.
57. Golubchikov, O. A.; Korovina, S. G.; Kuvshinova, E. M.; Semeikin, A. S.; Shul'ga, A. M.; Perfilev, V. A.; Syrbu, S. A.; Berezin, B. D. *Zh. Org. Khim.* **1988**, *24*, 2378–2383.
58. Jaunin, R.; Stoll, A. *Helv. Chim. Acta* **1960**, *43*, 1864–1867.
59. Wagner, R. W.; Johnson, T. E.; Lindsey, J. S. *Tetrahedron* **1997**, *53*, 6755–6790.
60. Wright, M. E.; Mullick, S. *Polymer Preprints* **1992**, *33*, 1119–1120.
61. Rajakumar, P.; Srisailas, M. *Tetrahedron Lett.* **2003**, *44*, 2885–2887.
62. Suematsu, K.; Nakamura, K.; Takeda, J. *Colloid Polym Sci.* **1983**, *261*, 493–501 (1983).
63. Ben, I.; Castedo, L.; Saa, J. M.; Seijas, J. A.; Suau, R.; Tojo, G. *J. Org. Chem.* **1985**, *50*, 2236–2240.
64. Pigge, F. C.; Ghasedi, F.; Rath, N. P. *J. Org. Chem.* **2002**, *67*, 4547–4552.
65. Wagner, R. W.; Brown, P. A.; Johnson, T. E.; Lindsey, J. S. *J. Chem. Soc., Chem. Commun.* **1991**, 1463–1466.
66. Page, C.; Page, M.; Noel, C. *Int. J. Oncol.* **1993**, *3*, 473–476.
67. Ahmed, S. A.; Gogal, R. M.; Walsh, J. E. *J. Immunol. Methods* **1994**, *170*, 211–224.
68. Benere, E.; da Luz, R. A. I.; Vermeersch, M.; Cos, P.; Maes, L. *J. Microbiol. Methods* **2007**, *71*, 101–106.
69. Diamond, L. S. *J. Parasitol.* **1968**, *54*, 1047–1056.
70. Räz, B.; Iten, M.; Grether-Bühler, Y.; Kaminsky, R.; Brun, R. *Acta Trop.* **1997**, *68*, 139–147.
71. Buckner, F. S.; Verlinde, C. L.; La Flamme, A. C.; van Hoorhis, W. C. *Antimicrob. Agents Chemother.* **1996**, *40*, 2592–2597.
72. Mikus, J.; Steverding, C. L. *Parasitol. Int.* **2000**, *48*, 265–269.
73. Matile, H.; Pink, J. R. L. *Plasmodium falciparum* malaria parasite cultures and their use in immunology. In *Immunological Methods*; Lefkovits, I., Pernis, B., Eds.; Academic Press: San Diego, CA, USA, 1990; pp 221–234.