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Conjugation to 4-aminoquinoline improves the anti-trypanosomal activity of Deferiprone-type iron chelators

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ABSTRACT

Iron is an essential growth component in all living organisms and plays a central role in numerous biochemical processes due to its redox potential and high affinity for oxygen. The use of iron chelators has been suggested as a novel therapeutic approach towards parasitic infections, such as malaria, sleeping sickness and leishmaniasis. Known iron chelating agents such as Deferoxamine and the 3-hydroxypyridin-4-one (HPO) Deferiprone possess anti-parasitic activity but suffer from mammalian toxicity, relatively modest potency, and/or poor oral availability. In this study, we have developed novel derivatives of Deferiprone with increased anti-parasitic activity and reduced cytotoxicity against human cell lines. Of particular interest are several new derivatives in which the HPO scaffold has been conjugated, via a linker, to the 4-aminoquinoline ring system present in the known anti-malaria drug Chloroquine. We report the inhibitory activity of these novel analogues against four parasitic protozoa, *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania infantum* and *Plasmodium falciparum*, and, for direct comparison, against human cells lines. We also present data, which support the hypothesis that iron starvation is the major cause of growth inhibition of these new Deferiprone–Chloroquine conjugates in *T. brucei*.

1. Introduction

Neglected tropical diseases such as malaria, sleeping sickness and leishmaniasis continue to pose a very considerable health threat to significant parts of the population, particularly in the developing world.[1] The causative agents of these diseases are different parasitic protozoa, namely *Plasmodium falciparum* (malaria), *Trypanosoma brucei* (human African sleeping sickness), *Trypanosoma cruzi* (Chagas disease) and *Leishmania* sp. (leishmaniasis). In 2008, the WHO recorded nearly 1 million deaths caused by malaria, mostly among African children.[2] Cases of African sleeping sickness, also known as Human African Trypanosomiasis (HAT), have been reported in 36 sub-Saharan countries, and despite significant progress in the control and treatment of HAT over the past decade, the estimated incidence of HAT still reaches 10,000 cases per annum.[3] Chagas disease is a major cause of morbidity and mortality in many regions of South America, with an estimated prevalence of 16–18 million people infected and approximately 45,000 deaths per year due to cardiac disease.[4] Cases of Visceral Leishmaniasis (VL) have been reported in 98 countries, with an estimated prevalence of 12 million cases worldwide and an incidence of 500,000 new cases per year.[5] If untreated, all of these diseases, especially HAT and VL, can be fatal.

Current treatment options for these parasitic infections focus mainly on chemotherapy. However, existing therapies often rely on outdated and toxic drugs that were identified decades ago, such as the arsenical melarsoprol for HAT[6] and pentavalent antimonials for VL.[7] In the case of Chagas disease, benznidazole and nifurtimox are the only available drugs for the treatment of acute infections, but are ineffective and too toxic against the chronic stage of the disease.[8] In addition, the clinical use of both drugs can be compromised by significant side effects such as peripheral polyneuropathy, depression of bone marrow, and allergic dermopathy.[9] These limitations of the few existing treatments for parasitic infections, together with growing resistance development, have created an urgent medical need for the development of new anti-parasitic agents.[1] The identification of such agents, ideally with oral availability and broad-spectrum activity against several of these parasites, is therefore a highly topical area of research.[10–13]

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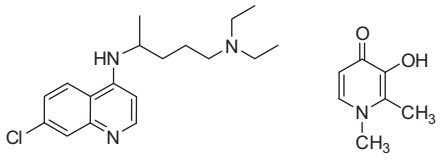
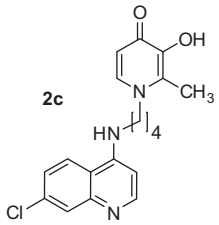
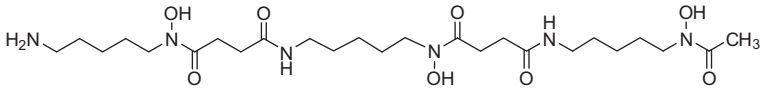
Table 1
Activity of reference compounds Chloroquine, Desferoxamine (DFO) and Deferiprone, and of Deferiprone–CQ hybrid **2c**, against different parasites and mammalian cell lines



Chloroquine

Deferiprone (**1a**)





Desferoxamine

	GI_{50} (μM)			
	CQ	DFO	Deferiprone (**1a**)	Hybrid (**2c**)
P. falciparum	0.0025[a]	28	67[b]	2.4
T. brucei	15.6	2.9[c]	>100	3.8
T. cruzi	22.2	n.a.[d]	>100	6.5
L. infantum	3.7	150[e]	75[e], >100[f]	27.0
HL60	128	18.4[c]	>100	50.3
K562	n.a.[d]	33.1	n.a.[d]	38.2

[a] From Ref. 13.
[b] From Ref. 25.
[c] From Ref. 18.
[d] Data not available.
[e] From Ref. 36.
[f] This study.

Iron has a central role in numerous biochemical processes due to its redox potential and high affinity for oxygen.[14] In protozoa, as well as fungi and bacteria, iron is an essential growth factor,[15] and iron chelation has been suggested as a potential new strategy to combat parasitic infections.[16] It has previously been shown that the iron chelator desferoxamine (DFO, Table 1) compromises the activity of Fe(III)-containing enzymes in parasitic protozoa such as ribonucleotide reductase, which is directly involved in DNA synthesis, by chelating cellular iron.[17–20] Consequently, iron chelators interfere with the growth of protozoan parasites, and DFO has been reported to successfully clear human hosts from *P. falciparum* in over 90% of those treated,[21] and to also possess cytotoxicity against *T. brucei*.[18,19] While these results illustrate the potential of DFO and related iron chelators for anti-parasitic applications, the clinical use of DFO itself is compromised by its poor oral availability.[21,22]

In contrast to DFO, iron chelators of the 3-hydroxypyridin-4-one (HPO) class such as the marketed drug Deferiprone (**1a**, Table 1) are orally active, which is partly due to their relatively low-molecular weight compared to DFO. Despite a molecular weight of <140, **1a** and related HPOs are effective iron chelators with a high selectivity for Fe(III), due to the formation of 3:1 complexes with the metal (Fig. 1). Deferiprone itself, which is used clinically for the treatment of iron overload,[23] has also been assessed for its anti-malarial potential. Although the drug has moderate in vitro activity against *P. falciparum* (Table 1), its in vivo anti-malarial activity, at dosages that are effective for treating iron overload, is poor.[24] Structural modifications in position N1, including the introduction of acidic functionalities, did not significantly improve the in vitro activity of **1a**.[21,25] In addition, **1a** as well as these N1-modified derivatives had only moderate selectivity for *P. falciparum* and also showed significant cytotoxicity against mammalian cells.[25,26]

In order to improve the anti-parasitic activity of **1a**, we have designed novel derivatives of **1a**, in which the HPO scaffold has been

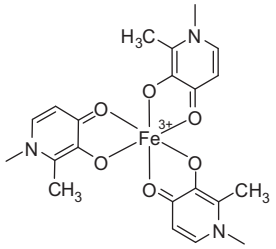
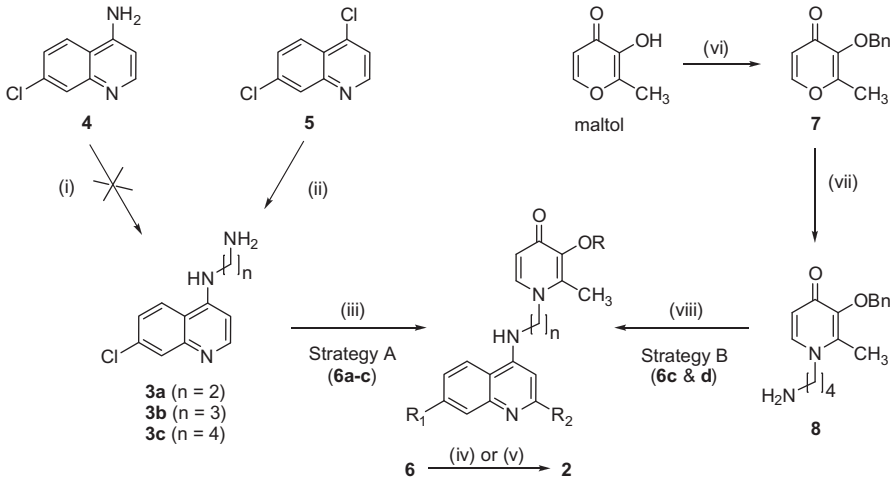


Figure 1. Deferiprone (**1a**) in complex with Fe(III).

conjugated, via a linker at N1, to the 4-aminoquinoline ring system present in the known anti-malarial drug Chloroquine. Previously, such a conjugation strategy with Chloroquine has been used successfully with other bioactive moieties.[27–31] Herein we present the results from the application of this approach to iron chelators of the HPO class. We have synthesized a series of new Deferiprone–Chloroquine hybrids and investigated their activity against *P. falciparum* and, in two cases, also against three other protozoal parasites (*T. brucei, T. cruzi* and *Leishmania infantum*). Although conjugation to Chloroquine resulted in improved anti-malarial activity relative to the parent **1a**, the new hybrids were less active than Chloroquine alone. Intriguingly, however, one of the new Deferiprone–Chloroquine hybrids showed increased activity against both trypanosome species (*T. brucei* and *T. cruzi*) compared to both **1a** and Chloroquine. Previously, **1a** had never been evaluated against *Trypanosoma*, and these are the first examples for the anti-trypanosomal activity of this class of iron chelator. Additional mode-of-action experiments with *T. brucei* strongly indicate that iron deprivation contributes significantly to the anti-trypanosomal activity of the new Deferiprone–Chloroquine conjugates. Taken together, our results suggest that the conjugation of HPO iron



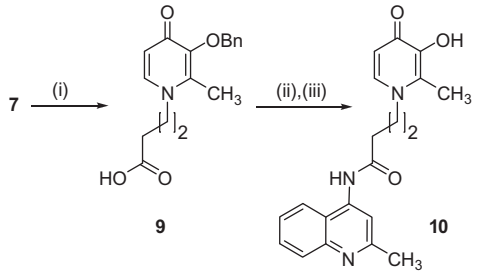
protected species (R = Bn)	deprotected species (R = H)	deprotection conditions	R_1	R_2	n
6a	**2a**	(iv)	Cl	H	2
6b	**2b**	(iv)	Cl	H	3
6c	**2c**	(iv)	Cl	H	4
6c	**2d**	(v)	H	H	4
6d	**2e**	(iv)	H	CH_3	4

Scheme 1. Synthesis of HPOs **2a**–**e** conjugated to Chloroquine and Chloroquine derivatives. Reagents and conditions: (i) 2-(4-bromobutyl)isoindoline-1,3-dione, $NaHCO_3$, DMF or DMSO or MeCN or EtOH; (ii) **3a**: ethylenediamine (9 equiv), reflux, 12 h; **3b**: 1,3-diaminopropane (7 equiv), reflux, 12 h; **3c**: 1,4-diaminobutane (5 equiv), 160–180 °C, 18 h; (iii) **7**, 2 N NaOH, pH 13, aqueous EtOH (25%), reflux, 12 h; (iv) 6 N HCl, reflux, 2 h; (v) 5% Pd/C, H_2, ethanol, aq HCl; (vi) NaOH (1.1 equiv), BnBr (1.1 equiv), aq MeOH (50%), reflux, 6 h; (vii) 1,4-diaminobutane (1 equiv), 10 N NaOH, pH 13, reflux, 18 h; (viii) **6c**: 4,7-dichloroquinoline (1 equiv), or **6d**: 4-chloroquinaldine (1 equiv), DMSO, 100–110 °C, 12 h.

chelators to a second bioactive moiety is a promising strategy against *Trypanosoma* parasites.

2. Chemistry

The new Deferiprone derivatives **2a**–**e**, which are conjugated via an alkyl linker to various 4-aminoquinolines, were prepared in analogy to our previously reported syntheses for simple N1-substituted HPOs (Scheme 1).[32] The central step in this approach is the installation of the N1-substituent via the double Michael addition of a suitable primary amine to the benzyl-protected maltol derivative **7**. In order to prepare the HPO derivatives **2a**–**e**, we employed two different variations of this general strategy (Scheme 1). For the preparation of HPOs **2a–c**, we followed strategy A, which is characterized by the installation of the linker at the Chloroquine fragment prior to the double Michael addition. For this strategy, the primary amines **3a**–**c** were required as intermediates. In initial trials, all attempts to alkylate aminoquinoline **4** by reaction with *N*-(4-bromobutyl)phthalimide were unsuccessful, probably due to the limited nucleophilicity of the aromatic amino group. In order to overcome this reactivity problem, we switched to a S_nAr strategy, as also described recently by Pérez et al.[28] Thus, 4,7-dichloroquinoline **5** was reacted in neat solution of different diaminoalkanes to provide the desired amines **3a–c** in moderate yields (48–66%). Conveniently, **3a–c** were afforded as white crystals after re-crystallization from ethanol/ether. In the next step, the primary amines **3a–c** were reacted with benzyl-protected maltol **7** in aqueous ethanol and in the presence of catalytic amounts of sodium hydroxide to give the double Michael addition products **6a–c**. Interestingly, we found that the base-catalyzed Michael addition reaction is pH sensitive. The best yields were achieved at pH 13. Finally, removal of the benzyl protecting group under acidic conditions gave the target HPOs **2a–c**.



Scheme 2. Synthesis of HPO **10** containing an amide group in the linker. Reagents and conditions: (i) GABA (1.2 equiv), aq EtOH (50%), 10 N NaOH, pH 13, reflux, 18 h; (ii) 4-aminoquinaldine (1 equiv), DCC (1.2 equiv), DMF, 70 °C, 12 h; (iii) Pd/C, H_2, ethanol, pH 1 (HCl).

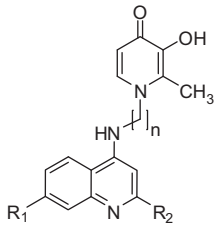
HPOs **2d–e** were obtained via synthetic strategy B, in which the N-substituted HPO core was prepared by a double Michael addition of 1,4-diaminobutane to **7** (Scheme 1). The resulting primary amine **8** was then used in an S_N reaction with, respectively, 4,7-dichloroquinoline or 4-chloroquinaldine, to provide the benzyl-protected HPOs **6c** and **6d**. Removal of the benzyl protecting group in **6c** under acidic conditions provided an alternative synthetic entry to HPO **2c**, while deprotection of **6c** by catalytic dehydrogenation led to concomitant dehalogenation in position 7, thus affording HPO **2d**. Analogously, deprotection of **6d** by catalytic hydrogenation gave target HPO **2e**.

Finally, we also prepared derivative **10** in which the linker contains an amide functionality (Scheme 2). For the preparation of **10**, **7** was reacted with γ-aminobutyric acid (GABA) to afford the free carboxylic acid **9**. Standard coupling conditions with DCC, DMAP and 4-aminoquinaldine gave the desired amide and, after removal of the benzyl protecting group via catalytic hydrogenation, the target HPO **10**.

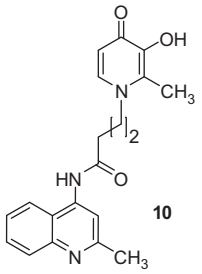
3. Biological results

We have previously shown that Deferiprone (**1a**) has modest activity against *P. falciparum* (Table 1).[25] Conjugation of the HPO

Table 2
Anti-plasmodium activity of HPOs **2a–e** and **10**



2a-e

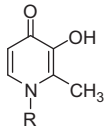


10

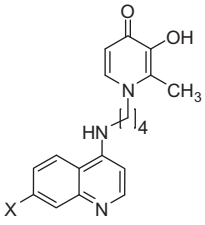
Compd	*n*	R^1	R^2	GI_{50} (μM)		SI[b]
				P. falciparum[a]	K562	
2a	2	Cl	H	7.0	182.2	26
2c	4	Cl	H	2.4	38.2	16
2d	4	H	H	1.6	27.1	17
2e	4	H	CH_3	9.0	204.2	23
10	n/a	n/a	n/a	6.0	127.7	21

[a] Strain ITG2G1.
[b] Selectivity index.

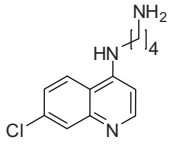
Table 3
Anti-trypanosomal and anti-leishmanial activity of HPOs **1a–d**, **2c**, **2d** and **3c**



1a-d



2c (X = Cl)
2d (X = H)



3c (X = Cl)

Compd	R	GI_{50} (μM)			
		T. brucei	*T. cruzi*	*L. infantum*[a]	HL60
1a	CH_3	>100	>100	>100	>100
1b	C_8H_{17}	3.6 ± 0.2	3.0 ± 0.3	3.2 ± 0.4	11.2 ± 0.4
1c	$C_{10}H_{21}$	1.4 ± 0.05	0.8 ± 0.1	0.7 ± 0.03	6.3 ± 0.2
1d	$C_2H_4NH_2$	>100	>100	>100	111 ± 2
2c	n.a.	3.8 ± 1.0	6.5 ± 0.1	27.1 ± 7.0	50.3 ± 1.1
2d	n.a.	7.6 ± 0.4	>100	>100	>100
3c	n.a.	15.8 ± 1.3	46.3 ± 13.4	42.4 ± 4.8	>100

[a] Promastigote form.

scaffold in **1a** via a linker to different 4-aminoquinolines, the pharmacophore of the known anti-malarial Chloroquine (CQ) significantly improved anti-plasmodial activity (Table 2). All of the new HPO-conjugates **2a–e** and **10** showed enhanced anti-plasmodial activities compared to the parent **1a**, as well as a favorable cytotoxicity profile, with 16- to 26-fold selectivity for *P. falciparum* over a mammalian cell line. However, **2a–e** and **10** were less active than CQ alone. While low micromolar activity against *P. falciparum* was observed with all analogues in this series, irrespective of the nature or length of the linker, the best results were achieved with analogues **2c** and **2d**, both in terms of anti-plasmodium activity and parasite selectivity. We therefore selected these two conjugates for further studies with other parasites.

While Deferiprone (**1a**) and some N1-substituted derivatives have previously been tested against *P. falciparum* and *Plasmodium berghei*,[21,25,26] the activity of this class of iron chelators against *Trypanosoma* has never been evaluated. To assess the anti-trypanosomal activity of **1a** and its derivatives **2c** and **2d**, we used established viability assays for these parasites.[33–35] While the parent compound **1a** (Deferiprone) had moderate activity against *Plasmodium*, it was inactive against *T. brucei* and *T. cruzi* at concentrations up to 100 μM (Table 1). The Deferiprone conjugates **2c** and **2d**, on the other hand, showed activity against *T. brucei* at low micromolar concentrations, similar to their activity against *P. falciparum* (Table 3). Compound **2c**, but not **2d**, was also active against *T. cruzi*. This anti-trypanosomal activity cannot be attributed exclusively to the presence of the Chloroquine fragment, as both CQ itself and the Chloroquine-based synthetic precursor **3c**, which corresponds to the Chloroquine-conjugate **2c**, but without the HPO fragment, were four- to sixfold less active against both trypanosome species than **2c**.

Chloroquine-conjugate **2c**, but not its close structural analogue **2d**, also displayed activity against *L. infantum* promastigotes in our anti-leishmanial assay (Table 3).[34] Against this parasite, **2c** was at least four times more active than its parent Deferiprone **1a**, which did not show any activity in our own experiments at concentrations up to 100 μM (Table 1), even though moderate activity against *L. infantum* has previously been reported for **1a**.[36] *Leishmania* promastigotes were approximately fourfold more resistant to **2c** than *T. cruzi* trypomastigotes. It has previously been observed that *Leishmania* parasites are less susceptible to drugs than *T. cruzi*.[35] The different bioactivities of the new HPO-conjugate **2c** against these two parasites are therefore not entirely unexpected. Compound **2c** was also tested against the intracellular amastigote forms of *L. infantum*, but showed no activity at the highest concentration that was tested (60 μM). This may be due to the different metabolism of amastigotes, which live in the unusual intracellular milieu of macrophages, and/or the limited uptake of **2c** by macrophages. In this context it is interesting to note that the substitution of Cl (**2c**) with H (**2d**) abolished the toxicity to mammalian cells, but also reduced anti-leishmanial activity.

Taken together, these results suggest that conjugation of **1a** to a Chloroquine-like fragment leads to synergistic, anti-parasitic effects, which are particularly pronounced against trypanosomes, but more modest against *Leishmania*. To understand the role of the Chloroquine fragment for the anti-parasitic activity of the new HPO–Chloroquine conjugates, we also investigated the activity of selected Deferiprone derivatives with simple alkyl substituents in position N1 against *T. brucei*, *T. cruzi* and *L. infantum*. Interestingly, the octyl- and decyl-substituted derivatives **1b** and **1c** showed potent activity in the low micromolar range against all three parasites (Table 3). However, both iron chelators were also cytotoxic towards HL-60 cells at only slightly higher concentrations, resulting in a relatively modest selectivity index (Table 4). Previously, partition coefficients (log*P* values) close to 1 have been

Table 4
Selectivity indices and log*P* values of HPOs **1a–c**, **2c** and **2d**

Code	R	X	SI[a]			Log*P*[b]
			T. brucei	*T. cruzi*	*L. infantum*	
1a	CH_3	n/a[c]	n.d.[d]	n.d.[d]	n.d.[d]	0.40
1b	C_8H_{17}	n/a[c]	3	4	4	3.56
1c	$C_{10}H_{21}$	n/a[c]	5	8	9	4.45
2c	n/a[c]	Cl	13	8	2	1.40
2d	n/a[c]	H	>13	n.d.[d]	n.d.[d]	1.25
CQ	n/a[c]	n/a[c]	8	6	35	n.d.[d]

[a] Selectivity index versus HL60 cells.
[b] Calculated *c*log*P* values for **1a–1c**, experimentally determined log*P* values for **2c** and **2d**, see Supplementary data for details.
[c] Not applicable.
[d] Not determined.

suggested as favorable for both inhibition of iron release and membrane permeability,[26] while higher log*P* values have been associated with increased toxicity of Deferiprone derivatives.[26] This correlation between lipophilicity and toxicity appears to hold for the new derivatives **1b** and **1c**, which both have *c*log*P* values greater than 3.5 (Table 4). Experimentally determined log*P* values for Chloroquine-conjugates **2c** and **2d**, on the other hand, were significantly lower, and both **2c** and **2d** had a more favorable selectivity profile than either **1b**, **1c** or Chloroquine alone, at least against *T. brucei* (Table 4). A possible explanation for these results is that Deferiprone analogues with a simple alkyl substituent in position N1 (e.g., **1b**, **1c**) may act non-specifically on iron depletion within the cellular compartment of both parasite and mammalian cells, resulting in their general cytotoxicity. In contrast, Chloroquine-conjugates **2c** and **2d** may have a certain preference for parasite cells, at least in the case of *T. brucei*. An alternative explanation for the non-specific cytotoxicity of **1b** and **1c** may be that molecules with a long *N*-alkyl substituent attached to a hydrophilic fragment can induce membrane disruption.[37]

To further investigate the anti-parasitic activity of the hybrid molecules, we next carried out mode-of-action studies. First, we determined important physicochemical parameters for the new HPO–Chloroquine conjugates, namely their pK_a values and absolute stability constant for Fe(III), pFe^{3+}. The HPO–Chloroquine conjugates are characterized by three possible protonation states pK_{a1} (protonation of the 4-oxo group), pK_{a2} (protonation of the quinoline ring nitrogen) and pK_{a3} (dissociation of the 3-OH group; Supplementary data). For compounds **2a**–**2e**, pK_{a2} values range from 7.5 to 9.6, indicating that a significant proportion of each compound is protonated at pH 7.4 (Supplementary data). Consequently, the neutral fraction of each HPO–Chloroquine conjugate is relatively small, and only a minor percentage would be expected to readily cross biological membranes through passive diffusion. **2e** with the highest pK_{a2} of all compounds has the lowest distribution coefficient $D_{7.4}$, whereas **2c** with an pK_{a2} of 8.2 has the highest $D_{7.4}$ (Supplementary data). This indicates that in this series, a pK_{a2} of ~8 leads to the best distribution profile, in keeping with the superior bioactivity of **2c** and **2d** against *P. falciparum*.

To assess the efficacy of HPO–Chloroquine conjugates as iron chelators we next determined pFe^{3+} over a pH range of 2–11. For these experiments, **2e** was selected as the best suited HPO–Chloroquine conjugate due to its relatively good solubility in aqueous media. Using a spectrophotometric titration method, we determined the pFe^{3+} value of **2e** as 21.1 at pH 7.45. Interestingly, this value falls between the pFe^{3+} values of **1a** (19.3) and DFO (26.6). This result therefore suggests that conjugation to CQ does not interfere with the iron chelation capacity of HPOs, and that the new HPO–Chloroquine conjugates remain potent iron chelators.

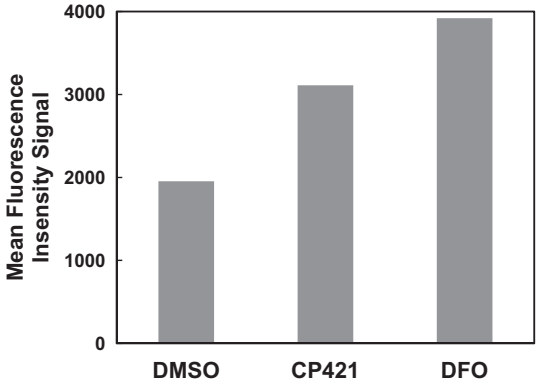


Figure 2. Accumulation of fluorescein-labeled transferrin in bloodstream forms of *T. brucei* upon incubation with **2d** (CP421) and DFO. Conditions: Trypanosomes (5×10^5/mL) were incubated with 50 μM **2d** (CP421), 25 μM DFO or DMSO (control) in Baltz medium supplemented with 16.7% heat-inactivated FBS. After 24 h incubation, trypanosomes were harvested and washed three times with Baltz medium supplemented with 2% BSA. Then, the cells were incubated with 50 μg/mL fluorescein-labeled transferrin in Baltz medium/2% BSA for 2 h. After washing twice with PBS/1% glucose, cells were fixed with 2% formaldehyde/0.05% glutaraldehyde, and analyzed by flow cytometry using a DB Accuri C6 flow cytometer.

Finally, we investigated the relevance of iron chelation for the anti-trypanosomal activity of the HPO–Chloroquine conjugates in live trypanosomes. For these mode-of-action experiments we focused on *T. brucei*, as relatively straightforward assays exist to investigate the effect of iron depletion with bloodstream forms of this parasite. It has previously been shown that under conditions of iron deprivation, *T. brucei* upregulates the expression of their transferrin receptor (*Tb*TfR).[38] *Tb*TfR expression therefore is an indicator of the parasite's iron status, and we studied the upregulation of *Tb*TfR by the parasite in the presence of **2d**. As a measure of *Tb*TfR upregulation, we quantified the uptake of fluorescently labeled transferrin by *T. brucei* using FACS analysis. Under these conditions, the parasite showed an increased uptake of transferrin by a factor of 1.6 in the presence of **2d**, compared to control cells incubated with DMSO (Fig. 2). Transferrin uptake was also increased, by a factor of 2.0, by the known iron chelator DFO. These results suggest that the capacity of **2d** to act as an iron chelator under cellular conditions is responsible, at least in part, for its anti-trypanosomal activity. This interpretation is further confirmed by the observation that pre-incubation of **2d** with $FeCl_3$, and thus saturation of the iron chelator with Fe(III), results in a complete loss of activity against *T. brucei* in our cytotoxicity assay. It should be noted that the iron-saturated compound **2d** is not charged but neutral (Fig. 1) and therefore able to penetrate the parasite plasma membrane and diffuse into the cytosol. However, the excess concentration of free Fe(III) ions in solution most likely scavenges all of the iron chelator, leaving the parasite with enough iron (e.g., from transferrin) to maintain viability.

4. Conclusion

We describe herein the anti-parasitic activity of several derivatives of the known iron chelator Deferiprone (**1a**). Derivatives of HPO **1a** with simple alkyl substituents in position N1 are generally cytotoxic both against parasites and mammalian cells. In contrast, a new class of N1-substituted HPO derivatives conjugated to the known anti-malarial Chloroquine showed preferential activity against parasitic cells, in particular in the case of *P. falciparum* and *T. brucei*. The new HPO derivatives retain their potent iron chelating capacity, which very likely forms the basis for their anti-parasitic activity. Importantly, the activity of the most potent conjugate **2c** against *T. brucei* and *T. cruzi* is clearly improved relative to both parent compounds. Thus, the anti-trypanosomal activity of **2c** was increased >25-fold against *T. brucei* and >15-fold against *T. cruzi* compared to Deferiprone, and about fourfold against both species compared to Chloroquine.

These results represent a promising starting point to revisit the application of iron chelators, in particular of the HPO class, as anti-parasitic agents. Our findings suggest that, at least against trypanosomes, our conjugation strategy leads to synergistic effects between the HPO and Chloroquine fragments, while retaining reasonable selectivity indices. The good anti-trypanosomal activity is particularly remarkable, as neither Deferiprone, nor Chloroquine alone, are potent anti-trypanosomal agents. Results from pK_a studies indicate that the neutral, membrane-permeant fraction of even the best HPO–Chloroquine conjugates in this series is relatively small. Modification of their acid/based properties may therefore represent a promising strategy for further optimisation of the anti-trypanosomal activity of this class of iron chelators.

5. Experimental section

5.1. General

All chemicals were purchased from commercial sources and were used as received, unless otherwise noted. Compounds **1a–d** were prepared as previously reported.[32] Dry solvents were obtained commercially or dried under standard procedures. Reactions were carried out under normal atmosphere, unless stated otherwise. Reaction products were purified by flash chromatography and characterized by ^{1}H NMR, ^{13}C NMR, ESI-MS, HRMS-MS, IR and TLC. Flash chromatography columns were packed wet and the separation was carried out at 1 bar. NMR spectra were recorded on a Varian VXR 400S spectrometer at 400 MHz or on a PerkinElmer R32 at 90 MHz. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. Signals are referenced to the residual protons of the respective deuterated solvent. High- and low-resolution mass spectra (LR-MS and HR-MS) were obtained on a LTQ Orbitrap XL mass spectrometer with electrospray ionization at the EPSRC National Mass Spectrometry Service Centre, Swansea. IR spectra were recorded on a PerkinElmer 298. Analytical HPLC was carried out on a PerkinElmer Series 200 machine equipped with a Supelcosil™ LC-18-T column (5 μm, 25 cm × 4.6 mm), a column oven (set to 35 °C), and a diode array detector. Compound purity was determined under the following conditions: 0–100% MeOH over 60 min (flow rate: 1 mL/min); detection wavelengths: 280 and 354 nm. Thin layer chromatography (TLC) was performed on pre-coated aluminum plates (Silica Gel 60 F_{254}, Merck). Compounds were visualized by exposure to UV light (254 nm wavelength) or by staining with ninhydrin, molybdic acid or $KMnO_4$ solution.

5.2. Synthesis

5.2.1. 3,4-Dihydroxy-2-methyl-1-{4-[(7-chloro-4-quinolyl)amino]ethyl}pyridinium chloride (2a)

To a solution of **7** (1.5 g, 0.0068 mol) in aq ethanol (83% v/v, 36 mL) was added **3a** (2.2 g, 0.010 mol). The pH was adjusted to 13 with aq NaOH (10 N) and the reaction was heated to reflux for 12 h. The solvents were removed and the residue was dissolved in water. The pH was adjusted to 1 with aq HCl, and the aqueous solution was washed with ether. The pH was adjusted to 7, and the aqueous solution was extracted with $CHCl_3$. The combined organic extracts were dried over $NaSO_4$ and the solvent was removed in vacuo. To remove the protecting group, the residue was taken up in aqueous HCl (6 N, 30 mL) and heated to reflux for 2 h. The solvents were removed and the resulting solid was recrystallized from methanol to give 1.1 g of the title compound as a white solid (39% yield). Mp: 296–303 °C. ^{1}H NMR (90 MHz, DMSO-d_6, ppm) δ: 2.42 (s, 3H, CH_3), 3.88 (m, 2H, CH_2NH_2), 4.57 (m, 2H, $NHCH_2$), 6.70–8.40 (m, 7H, 7 × ArCH). IR (KBr, cm^{-1}): 2400–3300 (ν_{OH}, ν_{NH}). LR-MS: m/z 330 (100%, $[M+H]^+$), 154 (63%).

5.2.2. 3,4-Dihydroxy-2-methyl-1-{4-[(7-chloro-4-quinolyl)amino]propyl}pyridinium chloride (2b)

To a solution of **7** (1.5 g, 0.006 mol) in aq ethanol (83% v/v, 60 mL) was added **3b** (2.07 g, 0.010 mol) followed by aq NaOH (2 N) until pH 12.5 was reached. The reaction mixture was heated to reflux overnight. The solvents were removed and the residue was purified by column chromatography ($CHCl_3$:MeOH 9:1 + 0.9% NH_3) to afford 4.3 g of **6b** (62% yield). To remove the protecting group, this material was dissolved in aqueous HCl (12 N, 30 mL) and heated to reflux for 2 h. Recrystallisation from methanol/ether gave the title compound as a white solid. Mp: 253.3–254.8 °C. ^{1}H NMR (90 MHz, DMSO-d_6, ppm) δ: 1.90–2.40 (m, 2H, CH_2), 2.40 (s, 3H, CH_3), 3.30–3.60 (m, 2H, CH_2NH), 4.23–4.50 (m, 2H, NCH_2), 6.60–8.78 (m, 7H, 7 × ArCH). IR (KBr, cm^{-1}): 2400–3300 (ν_{OH}, ν_{NH} LR-MS: m/z 344 (100%, $[M+H]^+$), 219 (42%), 191 (31%).

5.2.3. 3,4-Dihydroxy-2-methyl-1-{4-[(7-chloro-4-quinolyl)amino]butyl}pyridiniumchloride (2c)

Strategy A: To a solution of **7** (1.3 g, 0.006 mol) in aq ethanol (83% v/v, 30 mL) was added **3c** (1 g, 0.004 mol) followed by aq NaOH (2 N) until pH 13 was reached. The mixture was refluxed over night, concentrated and extracted with $CHCl_3$ (3 × 50 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated. Purification by flash chromatography ($CHCl_3$/MeOH 8:2 + 0.25–0.3% aq NH_3) afforded 0.9 g of **6c** as a yellow-orange solid. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 1.61–1.74 (m, 4 H, β, γ-CH_2), 2.16 (s, 3H, CH_3) 3.38–3.44 (m, 2H, δ-CH_2), 3.94 (t, 2H, $^{3}J_{H,H}$ = 6.8 Hz, α-CH_2), 5.00 (s, 2H, CH_2), 6.16 (d, 1H, $^{3}J_{H,H}$ = 7.4 Hz, 3-HPO-CH), 6.65 (d, 1H, $^{3}J_{H,H}$ = 6.3 Hz, 3-Ar-CH), 7.29–7.39 (m, 5H, 5 × Ar-CH), 7.56 (dd, 1H, $^{3,4}J_{H,H}$ = 2.2, 9.0 Hz, 6-Ar-CH), 7.68 (d, 1H, $^{3}J_{H,H}$ = 7.4 Hz, 2-HPO-CH), 7.91–7.97 (m, 1H, 8-Ar-CH), 8.45 (d, 1H, $^{3}J_{H,H}$ = 6.3 Hz, 2-Ar-CH), 8.58 (d, 1H, $^{3}J_{H,H}$ = 9.0 Hz, 5-Ar-CH), 8.69–8.74 (m, 1H, NH). ^{13}C NMR (400 MHz, DMSO-d_6, ppm) δ: 11.9 (CH_3), 24.4, 27.7 (β-, γ-CH_2), 42.2 (δ-CH_2), 52.4 (α-CH_2), 71.8 (CH_2), 98.6 (3-Ar-CH), 116.0 (3-HPO-CH), 116.5 (Ar-C), 123.1 (8-Ar-CH), 125.3 (5-Ar-CH), 125.4 (6-Ar-CH), 127.8 (Ar-CH), 128.2 (Ar-CH), 128.4 (Ar-CH), 135.7 (Ar-C), 137.7 (Ar-C), 139.6 (2-HPO-CH), 140.8 (Ar-C), 143.7 (Ar-C), 145.3 (Ar-C), 147.1 (2-Ar-CH), 152.8 (Ar-C), 171.8 (C=O). LR-MS: m/z 447.1 (100%); HR-MS: 447.0729 $[M]^-$, calculated for $C_{26}H_{26}ClN_3O_2$: 447.1714. This material was dissolved in aq HCl (6 N, 30 mL) and the solution was heated to reflux for 2 h to remove the protecting group. The reaction mixture was concentrated in vacuo, and the resulting precipitate was recrystallized from ethanol/acetone, to give the title compound **2c** as a white solid. *Strategy B*: A mixture of **8** (2.7 g, 0.0094 mol) and **5** (1.86 g, 0.0094 mol) in DMSO (30 mL) was heated to 100–110 °C overnight. The solvent was removed in vacuo and the residue was purified as described in Strategy A, to give 2.1 g of **6c** as a white solid in 50% yield. Deprotection of **6c** and subsequent purification was carried out as described in Strategy A, to give the title compound **2c** as a white solid. Mp: 130.1–131.9 °C. HPLC: 20.6 min (99%). ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 1.68–1.79 (m, 2H, γ-CH_2), 1.83–1.92 (m, 2H, β-CH_2), 2.53 (s, 3H, CH_3), 3.54–3.61 (m, 2H, δ-CH_2), 4.38 (t, 2H, $^{3}J_{H,H}$ = 7.4 Hz, α-CH_2), 6.89 (d, 1H, $^{3}J_{H,H}$ = 7.2 Hz, 5-HPO-CH), 7.18 (d, 1H, $^{3}J_{H,H}$ = 7.0 Hz, 3-Ar-CH), 7.77 (dd, 1H, $^{3,4}J_{H,H}$ = 2.1, 9.1 Hz, 6-Ar-CH), 8.07 (d, 1H, $^{4}J_{H,H}$ = 2.1 Hz, 8-Ar-CH), 8.26 (d, 1H, $^{3}J_{H,H}$ = 7.0 Hz, 2-Ar-CH), 8.55 (d, 1H, $^{3}J_{H,H}$ = 7.2 Hz, 6-HPO-CH), 8.77 (d, 1H, $^{3}J_{H,H}$ = 9.1 Hz, 5-Ar-CH), 9.56 (t(br), 1H, $^{3}J_{H,H}$ = 5.4 Hz), 14.27 (s(br), 1H, OH). ^{13}C NMR (400 MHz, DMSO-d_6, ppm) δ: 12.5 (CH_3), 24.2 (CH_2), 26.9 (CH_2), 42.5 (CH_2), 55.3 (CH_2), 98.6 (2′-HPO-CH), 110.7 (3-Ar-CH), 115.5 (Ar-C), 119.1 (8-Ar-CH), 125.9 (5-Ar-CH), 126.9 (6-Ar-CH), 138.0 (Ar-C), 138.5 (2-Ar-CH), 142.9 (Ar-C), 143.2 (Ar-C), 155.4 (Ar-C), 166.3 (C=O). IR (KBr, cm^{-1}) 2500–3300 (ν_{OH}, ν_{NH}). LR-MS: m/z 358 (100%, $[M+H]^+$), 233 (75%), 154 (38%).

5.2.4. 3,4-Dihydroxy-2-methyl-1-{4-[(4-quinolyl)amino]butyl}pyridinium chloride (2d)

The title compound was prepared from **8** and **5** as described in the synthesis for **9c** (Strategy B), but with a different deprotection procedure. The primary reaction product **6c** was dissolved in ethanol, the pH was adjusted to 1 with aq HCl, and hydrogenation was carried out in the presence of 5% Pd/C to give the title compound **2d** as a white solid. Mp: 216.5–221.0 °C. ^{1}H NMR (400 MHz, D_2O, ppm) δ: 1.80–1.89 (m, 2H, CH_2), 1.98–2.06 (m, 2H, CH_2), 2.50 (s, 3H, CH_3), 3.58 (t, 2H, $^{3}J_{H,H}$ = 6.6 Hz, CH_2), 4.38 (t, 2H, $^{3}J_{H,H}$ = 6.9 Hz, CH_2), 6.65 (d, 1H, $^{3}J_{H,H}$ = 7.0 Hz, 3-HPO-Ar-CH), 6.93 (d, 1H,

$^3J_{H,H}$ = 6.9 Hz, 3-Ar-CH), 7.65 (at, 1H, $^3J_{H,H}$ = 7.6, 8.4 Hz, 7-Ar-CH), 7.75 (d, 1H, $^3J_{H,H}$ = 8.4 Hz, 5-Ar-CH), 7.90 (at, 1H, $^3J_{H,H}$ = 7.6, 8.4 Hz, 6-Ar-CH), 7.99 (d, 1H, $^3J_{H,H}$ = 6.9 Hz, 2-Ar-CH), 8.03 (d, 1H, $^3J_{H,H}$ = 8.4 Hz, 8-Ar-CH), 8.21 (d, 1H, $^3J_{H,H}$ = 7.0 Hz, 2-HPO-Ar-CH). ^{13}C NMR (400 MHz, D_2O, ppm) δ: 15.1 (CH_3), 26.1 (CH_2), 29.3 (CH_2), 45.1 (CH_2), 59.2 (CH_2), 100.8(3-HPO-Ar-CH), 113.6 (3-Ar-CH), 119.4 (Ar-C), 122.8 (5-Ar-CH), 124.7 (8-Ar-CH), 130.0 (7-Ar-CH), 136.6 (6-Ar-CH), 140.1 (Ar-C), 141.5 (2-Ar-CH), 144.2 (2-HPO-Ar-CH), 145.7 (Ar-C), 158.5 (Ar-C), 161.6 (C=O). IR (KBr, cm^{-1}): 2500–3500 (ν_{OH}, ν_{NH}). LR-MS: m/z 324 (45%, $[M+H]^+$), 233 (100%).

5.2.5. 3,4-Dihydroxy-2-methyl-1-{4-[2-methyl-4-quinolyl)amino]butyl}pyridinium chloride (2e)

A solution of **8** (2.5 g, 0.0087 mol) and 4-chloroquinaldine (1.5 g, 0.0087 mol) in DMSO was maintained at 110 °C overnight. The solvent was removed in vacuo and the residue was purified by column chromatography ($CHCl_3$/MeOH 8:2 + 0.5% NH_3) to afford 2.0 g of **6d** (54% yield). To remove the protecting group, this material was dissolved in aqueous HCl (6 N, 50 mL) and heated to reflux for 2 h. The solvent was removed in vacuo. Recrystallization of the residue from methanol/ether afforded the title compound **2e** as a white solid. Mp: 180 °C. ^{1}H NMR (90 MHz, DMSO-d_6, ppm) δ: 1.70 (m, 4H, 2 × CH_2), 2.40 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 3.30–3.55 (m, 2, CH_2NH), 4.10–4.45 (m, 2H, CH_2N), 6.50–8.90 (m, 7H, 7 × ArCH). IR (KBr, cm^{-1}) 2750–3500 (ν_{OH}, ν_{NH}). LR-MS: m/z 338 (100%, $[M+H]^+$), 213 (42%).

5.2.6. 7-Chloro-4-(2′-aminoethyl)quinoline (3a)

A mixture of **5** (5 g, 0.025 mol) and ethylene diamine (13.485 g, 0.224 mol) was heated to reflux for 12 h. The crude product was purified by column chromatography ($CHCl_3$:MeOH 9:1 + 4% NH_3). The solvents were removed in vacuo and the brown solid was washed with $CHCl_3$, to give 3.60 g of **3a** as a white solid (66% yield). Mp: 228.5–230.1 °C. ^{1}H NMR (90 MHz, DMSO-d_6, ppm) δ: 2.80–2.97 (m, 2H, CH_2-NH_2), 3.25–3.47 (m, 2H, $NHCH_2$), 6.15–6.80 (m, 3H, NH, NH_2), 7.01–8.24 (m, 5H, 5 × ArCH).

5.2.7. 7-Chloro-4-(3′-aminopropyl)quinolone (3b)

A mixture of **5** (5.0 g, 0.025 mol) and 1,3-diaminopropane (15 mL, 0.176 mol) was heated to reflux for 12 h. The reaction mixture was concentrated and the crude product was washed with $CHCl_3$. The residue was purified by column chromatography ($CHCl_3$:MeOH 9:1 + 1% NH_3). Subsequent recrystallization from MeOH/Et_2O afforded 4.4 g of **3b** as a pale-yellow solid (74% yield). Mp: 104.5–105.5 °C. ^{1}H NMR (90 MHz, DMSO-d_6, ppm) δ: 1.50–1.82 (m, 2H, CH_2), 2.41–2.65 (m, 2H, CH_2NH_2), 3.03–3.30 (m, 2H, $NHCH_2$), 6.20–8.30 (m, 5H, 5 × ArCH).

5.2.8. 4-(4′-Aminobutylamino)-7-chloro-quinoline (3c)

A mixture of **5** (5 g, 0.025 mol) and 1,4-diaminobutane (11.02 g, 0.125 mol) was heated to reflux for 18 h at 160–180 °C. To the suspension was added chloroform and the mixture was concentrated in vacuo. Trituration of the resulting solid with DMF followed by filtration and washing with chloroform yielded a white solid. Recrystallization from MeOH/Et_2O gave 3.85 g of **3c** as white crystals (62% yield). Mp: 242.2–244.8 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 1.49–1.57 (m, 2H, β-CH_2), 1.61–1.70 (m, 2H, γ-CH_2), 3.11–3.16 (m, 2H, δ-CH_2), 3.24–3.30 (m, 2H, α-CH_2), 6.48 (d, 1H, $^3J_{H,H}$ = 5.5 Hz, 3-Ar-CH), 7.37 (t(br), 1H, $^3J_{H,H}$ = 5.1 Hz, NH), 7.77 (d, 1H, $^4J_{H,H}$ = 2.2 Hz, 8-Ar-CH), 7.97–8.05 (m, 2H, NH2), 8.28 (d, 1H, $^3J_{H,H}$ = 9.0 Hz, 5-Ar-CH), 8.39 (d, 1H, $^3J_{H,H}$ = 5.5 Hz, 2-Ar-CH). ^{13}C NMR (400 MHz, DMSO-d_6, ppm) δ: 25.2 (γ-CH_2), 26.7 (β-CH_2), 36.8 (δ-CH_2), 42.0 (α-CH_2), 98.7 (3-Ar-CH), 117.4 (Ar-C), 124.1 (6-Ar-CH), 124.2 (5-Ar-CH), 127.3 (8-Ar-CH), 133.5 (Ar-C), 150.2 (Ar-C), 151.7 (2-Ar-CH), 161.0 (Ar-C).

5.2.9. 2-Methyl-3-benzyloxy-4-(4*H*)-pyranone (7)

To a solution of maltol (50 g, 0.397 mol) in methanol (450 mL) was added aq sodium hydroxide (8.7 M, 50 mL of) and benzylbromide (74.66 g, 0.437 mol). The reaction mixture was heated to reflux for 6 h. After removal of solvent in vacuo, the oily residue was taken up in DCM (190 mL), washed with aq sodium hydroxide (5%, 2 × 190 mL) and water (2 × 190 mL). The organic fraction was dried over anhydrous sodium sulfate, filtered and concentrated to yield as an orange oil which solidified upon cooling. Recrystallization from Et_2O gave 69.9 g of **7** as colorless needles (81% yield). Mp: 33–34 °C. ^{1}H NMR (90 MHz, DMSO-d_6, ppm) δ: 1.99 (s, 3H, CH_3), 5.00 (s, 2H, CH_2), 6.14–6.24 (m, 1H, ArCH), 7.20 (s, 5H, 5 × ArCH), 7.39–7.49 (m, 1H, ArCH).

5.2.10. 1-(4′-Aminobutyl)-2-methyl-3-benzyloxy-4-(1*H*)-pyridone (8)

To a solution of **7** (4.1 g, 0.046 mol) in aq ethanol (50%, 160 mL) was added 1,4-diaminobutane (10 g, 0.046 mol). The pH of the reaction mixture was adjusted to pH 13 with aq sodium hydroxide (10 N). The reaction was heated to reflux for 18 h. The solvents were removed in vacuo and the residue was taken up in $CHCl_3$. The organic was washed with water, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by column chromatography ($CHCl_3$:MeOH 8:2 + 0.5–2% NH_3) to afford 6.34 g of **8** as an orange oil (48% yield). ^{1}H NMR (90 MHz, DMSO-d_6, ppm) δ: 0.7–1.6 (m, 4H, 2 × CH_2), 1.90 (s, 3H, CH_3), 2.13–2.34 (m, 2H, CH_2NH_2), 2.68 (s, 2H, NH_2), 3.41–3.66 (m, 2H, NCH_2), 4.70 (s, 2H, CH_2Bn), 5.73–5.88 (m, 1H, CHC=O), 7.02 (s, 5H, 5 × ArCH), 7.19–7.31 (m, 1H, CHC*H*N).

5.2.11. 3,4-Dihydroxy-2-methyl-1-{4-[(2-methyl-4-quinolyl)amino]4-oxo-butyl}pyridinium chloride (10)

To a solution of **7** (5 g, 0.023 mol) in ethanol (60 mL) was added a solution of GABA (2.75 g, 0.276 mol) in water (60 mL). Aq NaOH (10 N) was added to the reaction until pH 13 was attained. The reaction mixture was heated to reflux for 18 h. The solvent was reduced to 200 mL and the pH was adjusted 4 with aq HCl. The aqueous solution was extracted with DCM (3 × 50 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated to afford 4.9 g of **9** as a white solid. This material was used in the next step without further purification. A mixture of **9** (6 g, 0.020 mol), 4-aminoquinaldine (3.15 g, 0.020 mol) and DCC (3.55 g, 0.022 mol) in DMF (30 mL) was heated with stirring at 70 °C overnight. The solvent was removed in vacuo and the residue was purified by column chromatography ($CHCl_3$:MeOH 9:1 + 0.5% NH_3) to afford an orange oil which solidified upon cooling. Recrystallization from MeOH/Et_2O gave 5.4 g of benzylated **10** as white crystals (61% yield). To remove the protecting group, this material was dissolved in ethanol and hydrogenated in the presence of 5% Pd/C to give **10** as a white solid. Mp: 275 °C. ^{1}H NMR (90 MHz, DMSO-d_6, ppm) δ: 1.69–1.90 (m, 2H, CH_2), 1.98 (s, 3H, CH_3), 2.48 (m, 2H, CH_2), 2.51 (s, 3H, CH_3), 3.45–3.75 (m, 2H, CH_2N), 4.80 (s, 2H, CH_2Bn), 5.90–6.01 (d, 1H, CHC=O), 6.88–7.80 (m, 5H, 5 × ArCH), 7.00 (s, 5H, 5 × ArCH), 8.16–8.20 (m, 1H, CH-N). IR (KBr, cm^{-1}): 2500–3500 (ν_{OH}, ν_{NH}), 1500 (ν_{NH}-C=O). LR-MS: m/z 352 (100%), 227 (51%), 154 (40%).

5.3. Ligand pK_a values and stability constant of iron(III) complexes

Iron chloride (17.906 mM in 1% HCl, atomic absorption standard, Aldrich) was utilized in this study. Analytical grade reagent HCl (37%) and KOH (10 M) ampoules (Fisher), HPLC grade water and methanol (Fisher) were used in the preparation of all solutions. The automatic titration system used in this study comprises of an autoburette (Metrohm Dosimat 765, 1 mL syringe) and Mettler

Toledo MP230 pH meter with Metrohm AgCl electrode (6.0133.100) and a reference electrode (6.0733.100). 0.1 M KCl electrolyte solution was used to maintain the ionic strength. The temperature of the test solutions was maintained at 25 ± 0.1 °C by a Techne TE-8J temperature controller. The solution under investigation was stirred vigorously. A Gilson mini-plus3 pump with speed capability (20 mL/min) was used to circulate the test solution into a Hellem quartz flow cuvette.[39] For stability constant determinations, a 50 mm path length cuvette was used and for pK_a determinations a cuvette path length of 10 mm was used. Automatic titration and spectral scans adopted the following protocol: the pH value of the solution was increased or decreased by 0.1 pH unit by the addition of KOH or HCl from the autoburette over the pH range of 2–12. When pH readings varied by <0.001 pH unit over a 3 s period, the solution was allowed to reach equilibrium (for stability constant determinations a period of 5 min was adopted, for pK_a determinations a period of 1 min was adopted). The spectrum of the solution was then recorded. The cycle was then repeated automatically, until the defined end point pH value was achieved. Following electrode calibration, samples with an appropriate concentration to give measurable absorbance were titrated in pH range 2–12 for pK_a determination. For affinity studies, the iron:ligand ratio was kept at 5:1 ($[iron]_{total}$ 1 mM). Insoluble iron(III) complexes were measured in 1:1 methanol/water mixtures, the results were converted to those corresponding to aqueous conditions. The titration data were analyzed by pHab.[39,40] Affinity is expressed as the pM value. pM is defined as the negative logarithm of the metal ion concentration under the following conditions: $[Metal\ Ion]_{total} = 10^{-6}$ M, $[Ligand]_{total} = 10^{-5}$ M at pH 7.4.

5.4. Biological assays

The activity of test compounds against *P. falciparum* (strain ITG2G1) was determined as previously described.[41] Other bioassays were carried out as follows:

5.4.1. Cytotoxicity assay

Human myeloid leukemia HL-60 cells were propagated in RPMI 1640 medium supplemented with 2 mM L-glutamine and 16.7% FBS. HL-60 cells were incubated at 37 °C and 5% CO_2 and seeded in 24-well plates at an initial density of 10^5 cells/mL. Cell viability in the presence or absence of inhibitors was evaluated using Alamar Blue, as described for the *T. brucei* activity assay.

5.4.2. Anti-trypanosomal activity assay: *T. brucei*

Bloodstream form *T. brucei* TC211 was cultured in Baltz Medium supplemented with 16.7% heat-inactivated fetal bovine serum (FBS). Cells were seeded in 24-well plates containing medium (1 mL) and various concentrations of test compounds dissolved in DMSO. Controls were performed in the presence of DMSO only. The final DMSO concentration was 0.5% and had no effect on cell growth. To maintain the cells in a logarithmic growth phase during the course of the experiment, *T. brucei* was seeded at an initial density of 10^4 cells/mL. After 24 h, Alamar Blue was added. After an incubation period of 48 h, the absorbance at 570 nm was read, using 630 nm as the reference wavelength. Melarsoprol was used as a positive control (GI_{50} 3.8 nM, $CI_{95\%}$ 3.46–4.14). Each assay was performed in duplicate or triplicate. GI_{50} values were calculated using the function 'log(inhibitor) versus normalized response—Variable slope' in GraphPad Prism 5.0a for Mac OS X.

5.4.3. Anti-trypanosomal activity assay: *T. cruzi*

Trypomastigotes of *T. cruzi* were maintained in LLC-MK2 cells (ATCC CCL 7). Trypomastigotes were counted in a Neubauer hemocytometer and seeded at 1×10^6 cells per well in 96-well microplates. Compounds were incubated to the highest concentration of 100 μM for 24 h at 37 °C in a 5% CO_2-humidified incubator. Benznidazole was used as a positive control (GI_{50} 440.7 μM, $CI_{95\%}$ 406.2–478.4). The trypomastigote viability was based on the cellular conversion of the soluble tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyl-tetrazolium bromide) into the insoluble formazan by mitochondrial enzymes.[34,35]

5.4.4. Anti-leishmanial activity assay

The anti-leishmanial activity of compounds was determined against *L. infantum* (MHOM/BR/1972/LD) promastigotes. Parasites were maintained in M-199 medium supplemented with 10% calf serum and 0.25% hemin at 24 °C. Compounds were incubated to the highest concentration of 100 μM for 48 h at 24 °C and the viability of parasites was colorimetrically determined by the mitochondrial oxidation of MTT. Amphotericin B was used as a positive control (GI_{50} 0.17 μM, $CI_{95\%}$ 0.14–0.21). Compounds were also tested against intracellular amastigotes of *L. infantum*, using peritoneal macrophages as host cells. Compounds were incubated for 120 h at 37 °C in a 5% CO_2-humidified incubator, using meglumine antimonate (Glucantime) as a standard drug. The infection rate was determined by light microscopy by the number of infected macrophages out of 500 cells in duplicate.[42]

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Supplementary data

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