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Synthesis and Antiprotozoal Activity of *N*-Alkoxy Analogues of the Trypanocidal Lead Compound 4,4'-Bis(imidazolinylamino)diphenylamine with Improved Human Blood–Brain Barrier Permeability

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Abstract. To improve the blood–brain barrier permeability of the trypanocidal lead compound 4,4'-bis(imidazolinylamino)diphenylamine (**1**), five *N*-alkoxy analogues were synthesized from bis(4-isothiocyanatophenyl)amine and *N*-alkoxy-*N*-(2-aminoethyl)-2-nitrobenzenesulfonamides following successive chemical reactions in just one reactor ("one-pot procedure"). This involved: a) formation of a thiourea intermediate, b) removal of the amine protecting groups, and c) intramolecular cyclization. The blood–brain barrier permeability of the compounds determined in vitro by transport assays through the hCMEC/D3 human cell line, a well-known and characterized human cellular blood–brain barrier model, showed that the *N*-hydroxy analogue **16** had enhanced blood–brain barrier permeability compared with the un-substituted lead compound. Moreover, this compound displayed low micromolar IC_{50} against *Trypanosoma brucei rhodesiense* and *Plasmodium falciparum*, and moderate activity by intraperitoneal administration in the STIB900 murine model of acute sleeping sickness.

Keywords: prodrug, *Trypanosoma*, *Plasmodium*, chemotherapy, hCMEC/D3 cell line, blood–brain barrier, imidazoline, *N*-alkoxy-imidazoline.

Introduction

Human African trypanosomiasis (HAT[‡] or sleeping sickness), a neglected tropical disease caused by subspecies of the protozoan parasite *Trypanosoma brucei*, is endemic in several regions of sub-Saharian Africa. It also represents a serious economic and social burden to some of Africa's poorest countries.[1] According to the last epidemiologic update by the World Health Organization (WHO),[2] 50000 to 70000 people were infected in 2006. However, this may remain only a short-term view as no long-term systematic screening of the population at risk is currently ongoing.[3]

Chemotherapy of HAT relies on four old drugs (suramin, pentamidine, melarsoprol and eflornithine) which have unacceptable toxicity and require parenteral administration. This, greatly complicates the treatment of patients in disease endemic countries with no basic health care systems.[4, 5] The treatment options available for the late-stage disease involving central nervous system (CNS) infection are particularly deficient as they mostly rely on a highly toxic arsenical derivative, melarsoprol, which provokes deadly post-treatment reactive encephalopathy in up to 10% of the cases.[4] Even though the recent inclusion of the combination therapy Nifurtimox-eflornithine as simplified stage 2 treatment for *T. b. gambiense* sleeping sickness improves the therapeutic arsenal against late-stage HAT, the discovery of new orally active drugs that are able to cure the acute and CNS stages of HAT remains a priority in tropical medicine.[1]

In previous reports, we have shown that 4,4'-bis(imidazolinylamino)diphenylamine dihydrochloride (**1**, Chart 1) displayed excellent antitrypanosomal[6] and antiplasmodial[7]

[‡] Abbreviations: BBB: blood-brain barrier; CNS: central nervous system; DMF: *N,N*-dimethylformamide; DIPEA: *N,N*-diisopropylethylamine; EtOAc: ethyl acetate; Et_2O: diethyl ether; HAT: human African trypanosomiasis; LY: lucifer yellow; Ns (nosyl): 2-nitrobenzenesulfonamide.

activity in vitro. The crystal structure of **1** bound to its preferred DNA binding site 5'-AATT has suggested a basis for understanding the antitrypanosomal action of this compound.[8] Further studies in vivo have shown that this trypanocidal lead compound was curative by intraperitoneal administration in murine models of acute *T. b. brucei* and *T. b. rhodesiense* infections, but not in the late-stage disease involving CNS infection.[9] A reason for the lack of effectiveness against the CNS-stage is a poor brain penetration that is most likely due to the positively charged imidazolinylamino groups (pK_a = 9.9)[10] at physiological pH, which limit its passage through the blood-brain barrier (BBB). The same drawback has been observed with diamidine drugs such as pentamidine or diminazene which have been used for decades as a first line treatment for early stage gambiense sleeping sickness and cattle trypanosomiasis (Nagana), respectively. Previous reports in the literature have shown that derivatization of the amidine group as amidoxime prodrug was useful to overcome this problem and improve the oral bioavailibility and CNS delivery of diamidines. These amidoxime prodrugs are metabolized to the amidine active compounds through sequential oxidative *O*-demethylation by cytochrome P450 isoforms 1A1, 1A2 and 1B1 and cytochrome *b*5 catalyzed reductive *N*-dehydroxylation reactions.[11-21]

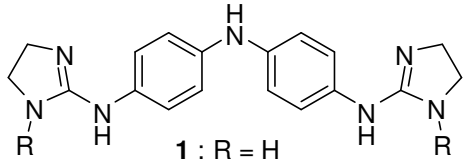
More recently, similar prodrug strategies (e.g., *N*-substituted bis-C-alkyloxadiazolones,[22] *N,N'*-dihydroxyamidines,[23] O-carboxymethylamidoximes,[24] diacetyldiamidoximeester[25]) for the oral delivery of amidines and guanidines were also reported.

In line with these findings, we have developed a similar approach (i.e., synthesis of *N*-alkoxy analogues) to reduce the pK_a of the imidazolinylamino moiety in order to improve the pharmacokinetics of compound **1.** We also sought to establish the influence of replacing the imidazolylamino moiety with a closely related heterocycle (i.e., 4,5-

dihydrothiazolinylamine) that has lower pK_a value so as to avoid problems of being ionized at physiological pH (i.e., the pK_a of thiazolines is ca. 3 units lower than the pK_a of imidazolines; e.g., 2-aminoimidazole: pK_a = 8.46;[26] 2-aminothiazole, pK_a = 5.39).[27] Hence, five different *N*-alkoxy substituted analogues of **1** (Chart 1) and the 4,5-dihydrothiazolinylamino derivative **17** (Scheme 2) were synthesized. The antitrypanosomal activity of the target compounds was checked in vitro against the STIB900 *Trypanosoma brucei rhodesiense* strain. As additional data, we also checked the antiprotozoal activity of the new compounds against 3 related parasites (*Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum*).

Further, the activity of the new compounds was assayed in vivo in the murine model of acute *T. brucei* infection. Additionally, the BBB permeability of the compounds was determined in vitro by permeability assays through the hCMEC/D3 human endothelial cell monolayer.[28, 29] This cell line holds most of the specific properties of in vivo BBB, including cell-cell junction expression of tight junction proteins (claudin-5, JAM-A, Zo-1) and the polarized expression of a variety of functional transporters.[30, 31] By comparing the permeability coefficients of the new derivatives and the lead compound **1,** some conclusions regarding the influence of *N*-alkoxy substitution on the human BBB permeability can be drawn.

Chart 1. Lead compound (**1**) and new *N*-alkoxy analogues.

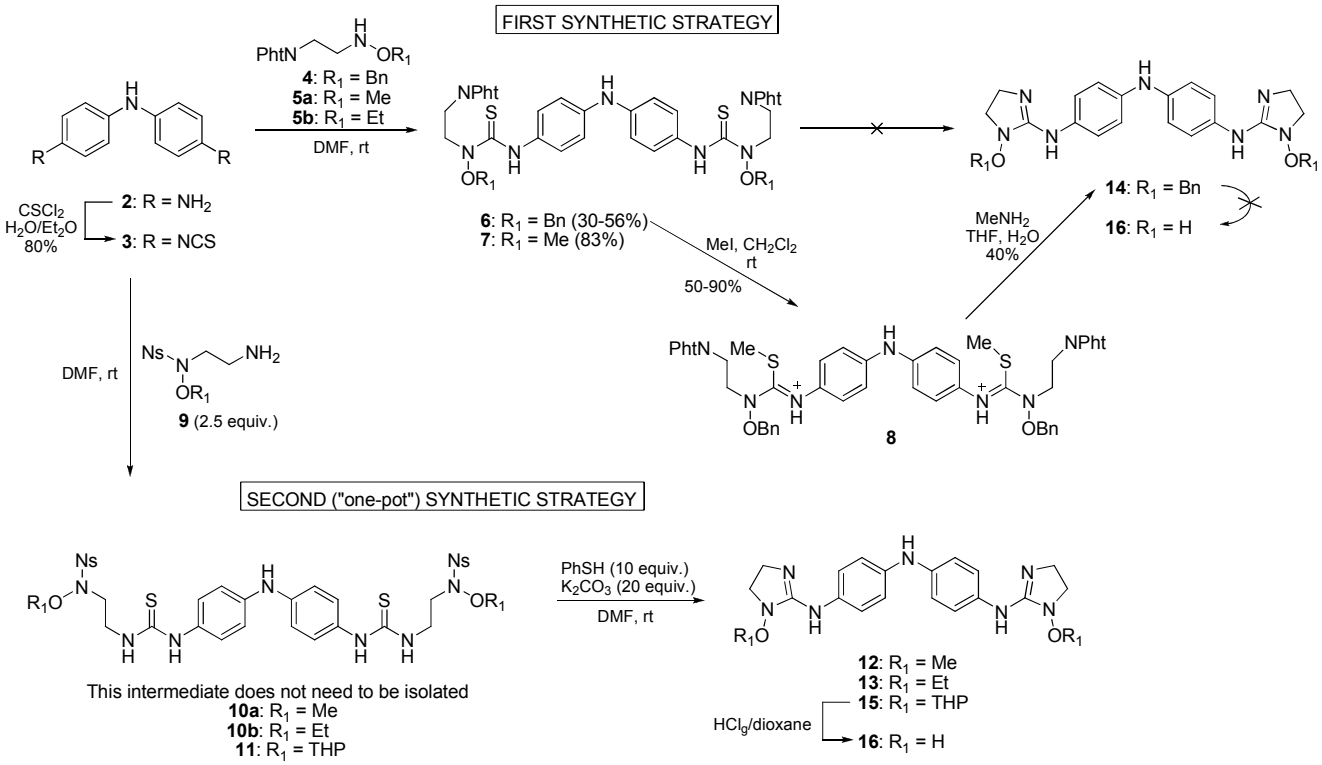


1 : R = H
12: R = OMe
13: R = OEt
14: R = OBn
15: R = OTHP
16: R = OH

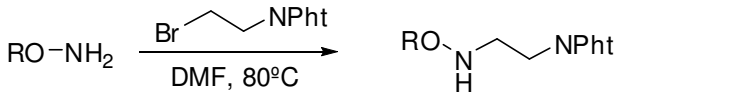
Results

Chemistry. We attempted two different synthetic strategies in order to obtain the *N*-alkoxy-substituted derivatives of **1**. Both approaches relied on the reaction of two equivalents of a functionalized ethylenediamine precursor (**4**–**5**, or **9**) with the isothiocyanate **3**, previously prepared in 80% yield from 4,4'-diaminodiphenylamine (Scheme 1).[32] The thiourea intermediates obtained (**6**–**7**, and **10**–**11**, respectively) were subsequently subjected to intramolecular cyclization after adequate removal of the amine protecting groups.

Scheme 1. Synthetic routes for the preparation of 1-alkoxy-2-arylaminoimidazolines



Regarding the first synthetic strategy, the *N*-alkoxyethylenediamine precursors **4, 5a,** and **5b** were prepared in low yield following the protocol previously described for compound **4** (Equation 1).[33, 34]



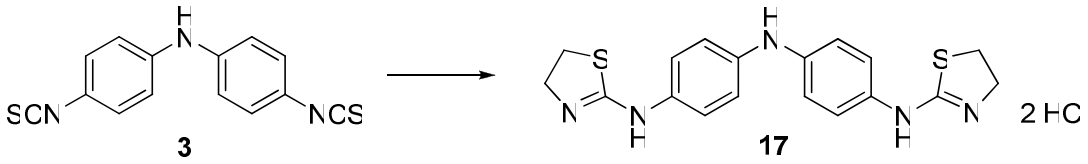
4: R = Bn (30-70%, 14 mmol scale)
5a: R = Me (20%, 4 mmol scale)
5b: R = Et (25%, 1.5 mmol scale)

The reaction of **3** with 2.5 equivalents of **4**, or **5a**, gave **6** and **7**, respectively (Scheme 1). Attempts to optimize the conditions for the deprotection of the amino groups with hydrazine/EtOH and the subsequent in situ cyclization to the target compound **14** were disappointing, leading to several by-products. Hence, the thiourea **6** was activated via formation of the methylisothiouronium salt **8,** which was obtained almost quantitatively (excess CH_3I/ CH_2Cl_2/ rt / 24h) with a purity > 80%. Partial deprotection of one benzyl group accounted for the lower purity of the product. Longer reaction times slightly increased the yield of the deprotection product. Attempts of purification by chromatography were unsuccessful so the compound was used in the cyclization step without further purification. The deprotection of the amino groups ($MeNH_2$/ THF-H_2O) and the consequent *in situ* spontaneous cyclization afforded **14**, which was isolated in 40% yield after silica chromatography. Our attempts to scale up the synthesis of **5a** and **5b** were unsuccessful so we explored a different strategy to obtain the corresponding methoxy **12** and ethoxy analogues **13**. As an alternative, we considered the deprotection of the benzyl protecting groups of **14** and the subsequent alkylation of **16** to the corresponding alkoxy derivatives. However, all of the attempts to remove the benzyl groups, either by catalytic hydrogenolysis or by other classical methods,[35] gave poor yields of the desired product. Hence, an alternative one-pot procedure strategy was

developed to prepare the 1-methoxy- (**12**), 1-ethoxy- (**13**), 1-benzyloxy- (**14**) and 1-tetrahydropyranyloxy- (**15**) derivatives (Scheme 1).[36] Treatment of **15** with HCl_g saturated dioxane solution afforded the 1-hydroxy analogue **16** as its dihydrochloride salt. The intermediates **10a** and **10b** were isolated in separate experiments and fully characterized.

The 4,5-dihydrothiazolinylamino derivative **17** was prepared by reaction of **3** with 2-bromoethanamine hydrobromide in the presence of triethylamine (Scheme 2).

Scheme 2.[a]



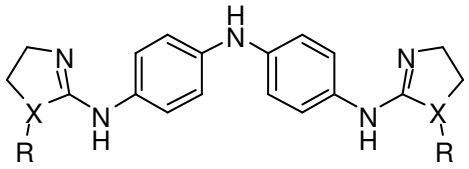
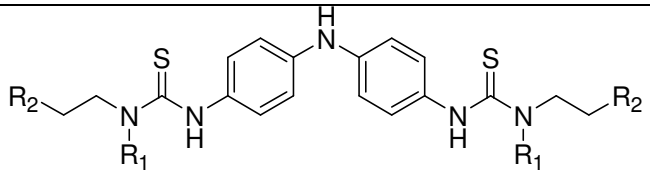
[a] Reagents and conditions. (i) 2-bromoethanamine hydrobromide, Et_3N, CH_2Cl_2, rt; (ii) HCl_g/dioxane, MeOH.

In Vitro Antiprotozoal Activity. The target compounds (**12**–**16**, and **17**) and the synthetic intermediates (**6**, **7**, **10**, and **11**) were screened in vitro against *T. b. rhodesiense* and 3 other related parasites: *T. cruzi*, *L. donovani* and *P. falciparum* (Table 1). The *N*-substituted compounds **12**–**16** showed submicromolar IC_{50} values against *T. b. rhodesiense* and *P. falciparum*. Nevertheless, these values represented a 5-fold (**14, 16**), 10-fold (**12**, **13**) and 20-fold (**15**) loss of activity against *T. brucei*, respectively, compared with the lead compound **1**. The antiplasmodial activity was also decreased, in comparison with **1**, by 20-fold (**14**), 50-fold (**12**, **13**), 100-fold (**15**), and 7-fold for

compound **16**. The latter notably conserved nanomolar activity (IC_{50} = 55 nM) and high selectivity (SI = 1673) against this parasite. Previous reports have underlined the importance of the positively charged imidazolylamino moieties of **1** for antitrypanosomal activity and for binding to DNA minor groove. [7-9] Hence, the 5- to 20-fold higher IC_{50} observed in vitro for the uncharged 1-alkoxy-substituted derivatives **12**–**16** was not unexpected. The lower activity of the 4,5-dihydrothiazolinylamino analogue **17** with respect to the imidazolylamino compound **1** (i.e., 160-fold against *T. b. rhodesiense* and 270-fold against *P. falciparum*) confirmed these findings. The higher pK_a of the imidazoline structure (pK_a = 9.9 for **1**[10]) ensures a higher proportion of the ionized form of the molecule at physiological pH, which proved important for high activity in this series.

None of the new compounds showed significant activity against axenically grown amastigotes of *L. donovani.* On the contrary, one compound, **14,** presented an IC_{50} value 3-times higher than the reference drug benznidazole (1.64 μM) on intracellular *T. cruzi* amastigotes. The synthetic intermediates (urea derivatives) **6**, **7**, **10a**, and **11b** were poorly active on the four parasites with IC_{50} in the high micromolar range (Table 1).

Table 1. In Vitro Antiprotozoal Activity of *N*-substituted Analogues **12-17** and synthetic intermediates **6**, **7**, and **10**.

			IC_{50} (μM)				
			T. b. r.[a] (SI)[b]	*T. c.* (SI)[c]	*L. d.* (SI)[d]	*P. f.* (SI)[e]	Cytotox. L6-cells[f]
Cmpd	X	R					
1[g]	N	H	0.069 (3072)	na[h]	na	0.009 (24091)	212
12	N	OMe	0.83 (65.6)	136	39	0.45 (120)	54
13	N	OEt	0.73 (55)	87	62	0.59 (68)	40
14	N	OBn	0.32 (34.7)	6.6	104	0.20 (56)	11
15	N	OTHP	1.18 (17)	24	41	0.75 (26)	20
16	N	OH	0.37 (236)	> 204	58	0.055 (1673)	92
17	S	nil	11.1 (4.7)	30	120	2.43 (21)	52
Cmpd	R_1	R_2					
6	OBn	-NPht	86.7	>100	36	1.36	>102
7	OMe	-NPht	73.8	>124	69	2.64	>124
10a	H	-N(Ns)OMe	61.3	>108	68	1.81	>107
10b	H	-N(Ns)OEt	14.9	>104	14	1.94 (35)	67

[a] *T. brucei rhodesiense* STIB900 strain. Control: melarsoprol, IC_{50} = 12.6 nM; [b] Selectivity index = [IC_{50} (L6-cells) / IC_{50} (parasite)]; [c] Amastigotes of *T. cruzi* Tulahuen C4 strain. Control: benznidazole, IC_{50} = 1.64 μM; [d] *L. donovani* strain MHOM/ET/67/L82 axenically grown amastigotes. Control: miltefosine, IC_{50} = 0.275 μM; [e] *P. falciparum* K1 erythrocytic stages. Control: chloroquine, IC_{50} = 0.169 μM; [f] Rat skeletal myoblast L-6 cells; [g] Data taken from ref. [6, 7]. [h] Not available. See reference [37] for detailed experimental procedures.

In Vivo Antitrypanosomal Activity. In the *T. b. rhodesiense* STIB900 mouse model of acute infection, which mimics the first stage of the disease, the lead compound **1** was curative when administered intraperitoneally at 4×20 mg/kg.[9] However, it was hardly effective by oral route in this model (i.e., 10% increase in mean relapse days compared to control, no cures), and totally inactive in the GVR35 mouse model that mimics the late stage of the disease involving CNS infection.[9] The newly synthesized *N*-alkoxy analogues of **1** were designed as possible prodrugs with improved oral bioavailability and enhanced CNS uptake. In order to check their in vivo activity, compounds **12**–**16** were administered by intraperitoneal (4×20 mg/kg) and oral route (4×50 mg/kg) to *T. brucei* infected mice (Table 2). Compounds **12**–**15** did not show any activity in this model of first stage HAT (i.e., same mean time of relapse as the untreated mice), possibly due to a poor bioactivation in vivo. On the contrary, compound **16** was moderately active by ip administration increasing the mean time of relapse 3.6-fold compared to control.

Table 2. In Vivo Antitrypanosomal Activity of *N*-substituted Analogs of **1** in the *T. b. rhodesiense* (STIB900) mouse model.

Compound	R	Dosage route[b]	Dosage (mg/kg)	Cured[c]/Infected	Mean relapse days
Control		-	-	0/4	7[d]
1	H	ip	4 × 20	4/4[e]	> 60
		po	4 × 50	0/4	7.75
12, 13, 14, 15	OMe, OEt, OBn, OTHP	ip	4 × 20	0/4	7
		po	4 × 50	0/4	7
16	OH	ip	4 × 20	0/4	25.3
		po	4 × 50	0/4	7

[a] See experimental section for details of STIB 900 (*T. b. rhodesiense*) model. [b] ip = intraperitoneal, po = per os. [c] Number of mice that survive and are parasite free for 60 days. [d] Control mice were always positive and were euthanized on day 7. [e] Data taken from ref. [9].

In Vitro Determination of Human Blood-Brain Barrier Permeability. We have mentioned before that compound **1** did not show in vivo activity in the murine model of CNS-stage sleeping sickness.[9] Because crossing the BBB is a requirement for drugs targeting the late-stage disease, we decided to assess the in vitro BBB permeability of the lead compound **1** and the new *N*-alkoxy analogues **12**, **13**, **14** and **16**. The immortalized human brain endothelial cell line hCMEC/D3[29] was used for the drug transport studies. This cell line has proved useful as a human BBB model,[28] so the results obtained in this study are particularly relevant from a therapeutic point of view.

The clearance principle (i.e., the slope of the volume cleared versus time) was used to calculate the permeability coefficients as usually performed (see experimental part for details).[38-40] The fluorescent dye lucifer yellow (LY) was used as a marker of passive diffusion through cell-cell junctions; a low permeability to LY reflects the integrity of the endothelial monolayer and the relevance of cell-cell junction complexes (see experimental part). First, we validated the good quality of the BBB model in the presence of the compounds by finding the concentration of the compounds which could be applied to the endothelial cells without affecting LY permeability. Hence, permeability assays of LY in the presence of the new compounds were performed. Compounds **1**, **12**, **13**, and **16** did not affect the endothelial monolayer integrity (i.e., a low permeability to LY was conserved) at concentrations of 100 μM whereas **14** required a concentration of 10 μM (Figure 1). Compound **14**, at this concentration,

made the analysis of the results unreliable (i.e., beyond the sensitivity limits of our HPLC detection method). This suggests a very low permeability through the in vitro BBB for this compound.

The measured permeability values for the lead compound (**1),** the O-methoxy (**12)**, and O-ethoxy (**13)** derivatives were not significantly different from the one measured for the poorly permeable compound LY. In contrast, the 1-hydroxy analogue (**16**) had significant higher permeability than LY and also than compound **1** (nearly 3-fold). These results show that N-substitution of the imidazolylamino group with OH enhances the in vitro BBB permeability of this molecule whereas O-alkyl substituents do not improve the in vitro BBB permeability.

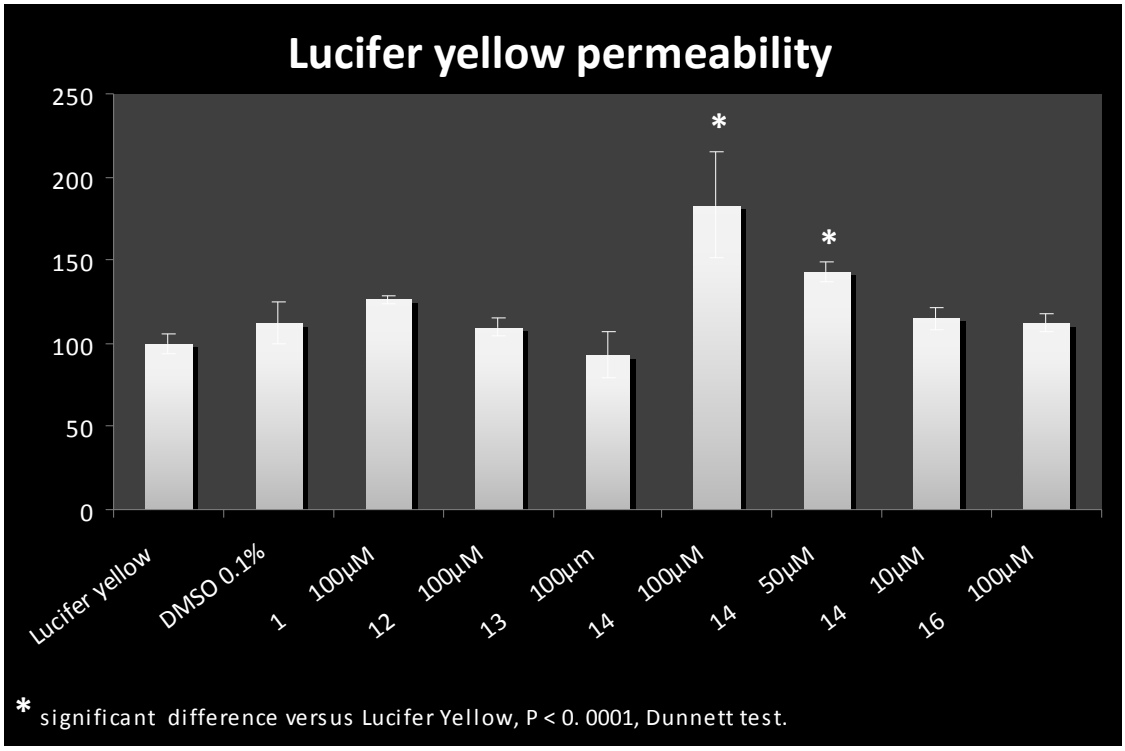


Figure 1. Endothelial monolayer integrity measured as a function of LY permeability in the presence of compounds **1**, **12**, **13**, **14**, and **16** at different concentrations. Increase in LY permeability in presence of the tested compound is an indication of loss of endothelial monolayer integrity. See experimental part for details. Data represent means of 3 independent culture inserts per condition.

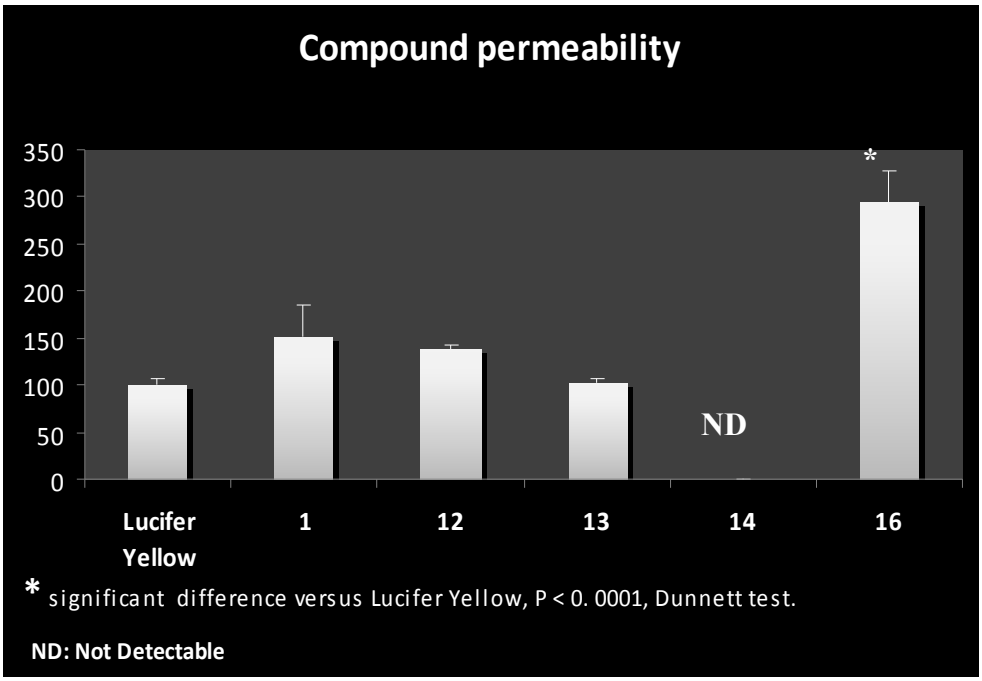


Figure 2. Permeability values of compound **1** and its *N*-OMe (**12**), *N*-OEt (**13**), and *N*-OH (**16**) analogues compared to LY. Permeability values are represented as a percentage of the permeability value of LY (normalized to 100%). Data represent means of 3 independent culture inserts per condition.

Discussion

Dicationic drugs are very effective antimicrobial agents that have been used for the treatment of African trypanosomiasis and leishmaniasis for decades. Despite their excellent antiparasitic activity this kind of drugs presents a major drawback, namely a poor absorption through biological barriers such as the gastrointestinal tract or the BBB (e.g., the diamidine drugs pentamidine, berenil or furamidine are not orally active and do not cure late-stage HAT). Lowering the pK_a of the amidine moiety by attaching OR groups (R = H, alkyl) to the nitrogen atoms to generate orally active prodrugs has been a successful strategy in a number of cases.[11, 13, 21, 41] Our objective was the study of *N*-

alkoxy derivatives of a dicationic bisaminoimidazolinyl trypanocidal lead compound, **1**, showing the same drawback as the above mentioned diamidines.

As expected for a prodrug requiring in vivo metabolic activation, the *N*-alkoxy derivatives displayed reduced activity (in the submicromolar range) in the parasite susceptibility assay in vitro. Furthermore, one of the new *N*-alkoxy derivatives, **16**, was active in vivo in the STIB900 mouse model after intraperitoneal administration. Even though this moderate effect could be attributed to the fair intrinsic activity observed in vitro for this compound (IC_{50} = 0.37 μM), it is likely due to a partial bioactivation of **16** to the lead compound **1**. In fact, the 2-aminoimidazolinyl moiety can be viewed as a cyclic guanidine[42] and the in vivo reduction of *N*-hydroxylated guanidines and amidines by microsomes and mitochondria of different organs from different species (e.g., kidney, liver, brain) has been established before.[12, 43, 44] This result is important because it is compound **16** that showed the highest in vitro BBB permeability in our assays. The in vivo activity of **16** in the mouse model of CNS-stage infection was not yet tested because of its limited efficacy in the acute STIB900 model. However, the synthesis of this class of prodrugs with more potent lead candidates is warranted.

On the one hand, the lack of in vivo activity of the alkoxy derivatives **12**, **13** and **14,** despite of similar inherent activities in vitro (IC_{50} values of 0.83, 0.73, and 0.32 μM, respectively), may possibly be attributed to a poor bioactivation. As far as we know, the bioactivation of *N*-alkoxy prodrugs has been described for amidines but not for guanidines.On the other hand, this might be explained by a high fraction of protein binding or other unfavourable pharmacokinetics.

The P_e values measured in vitro with the human brain endothelial cell line hCMEC/D3 for the lead compound **1,** and the O-alkyl substituted derivatives **12** and **13,** were

comparable to the P_e values of the control molecule LY that presents a very low brain uptake (see experimental section for more details). Thus, compound **1** scarcely penetrates the BBB which possibly explains why this molecule is inactive in the murine model of late-stage HAT.[9] The derivatization of the imidazoline nitrogen with OMe or OEt did not improve the BBB permeability whereas a free OH group increased the permeability by almost 3-fold compared with the lead compound **1**. In comparison, the benzodiazepine drug diazepam that is used as anxiolytic and has large BBB uptake[45] shows 10-fold higher permeability than LY using the hCMEC/D3 cell line (data not shown). This means that compound **16** shows an intermediate in vitro permeability to the brain.

This result was somewhat unexpected as increasing the number of hydrogen bond donors and the polar surface area is generally associated with a reduced BBB permeability.[46] To try rationalize these results, we calculated some physicochemical parameters that are generally accepted as important for a compound to be readily BBB permeable (Table 3).[47] The molecular hydrophobicity of a compound, measured as the octanol/water partition coefficient (logP), is an important physicochemical property associated with biological membrane permeation. For ionizable molecules to be BBB permeable, values of octanol/water distribution coefficients, log$D_{7.4}$, in the range between 1 and 3 are recommended.[48] As shown in Table 3, two compounds only, **12** and **16**, have log$D_{7.4}$ values within this range. Thus, adequate hydrophobicity and low pK_a values may explain the enhanced in vitro BBB permeability observed for **16.** However, these data do not explain the low permeability of **12** measured in vitro. Taken togrether, these findings show that subtle modification of the nitrogen substituent (e.g., OH ↔ OMe) greatly affects the BBB permeability of the bis-2-imidazolinylamino compounds.

Table 3. Calculated physicochemical parameters of the compounds: molecular weight (MW), polar surface area (PSA), octanol/water distribution coefficient at pH 7.4 (log $D_{7.4}$), H-bond donors (HBD), and pK_a.

Cmpd	MW[a]	PSA[b]	log $D_{7.4}$[b]	pK_a (N3; N3') [b,c]	HBD[d]
1[e]	408.33	88.09	0.1	8.02; 8.63	5
12	395.46	85.75	2.51	5.73; 6.34	3
13	423.51	85.75	3.22	5.81; 6.41	3
14	547.65	85.75	5.95	5.77; 6.37	3
15	535.64	104.21	4.31	5.85; 6.45	3
16	367.40	107.75	1.76	5.64; 6.24	5
17	369.51	60.81	3.92	6.76; 7.37	3

[a] Molecular weight (g/mol). [b] The polar surface area (Å^2), Log$D_{7.4}$ and pK_a were calculated using ChemAxon software MarvinSketch v.5.3.8. [c] Calculated pK_a values of the N3-nitrogen of the imidazoline rings. [d] Number of H-bond donors. [e] Dihydrochloride salt.

Conclusions

Several *N*-alkoxy analogues of compound **1** were designed to reduce the basicity of the aminomidazolinyl groups and potentially improve the oral and CNS absorption of that trypanocidal lead compound. Four of these derivatives showed submicromolar IC_{50} values in vitro against *T. brucei* and *P. falciparum*, but only one compound, the *N*-hydroxy-2-imidazolylamino analogue **16**, displayed moderate activity in vivo in the *T. brucei* STIB900 murine model at a dosage of 20mg/kg ip. This modest activity is possibly due to an incomplete bioactivation of the prodrug in vivo that does not permit

to reach the curative dose of the active compound. Even though other pharmacokinetic aspects such as plasma protein binding cannot be ruled out at this time, the in vivo activity displayed by **16** means that a substantial fraction of the active compound is unbound and free to reach its target in vivo. Further work to fully understand the pharmacokinetics of these compounds will be needed but this shall be the subject of a different study.

More importantly, this compound also had the highest BBB permeability of the series (i.e., almost 3-fold increase with respect to lucifer yellow) in the in vitro transport assay involving the human brain endothelial cell line hCMEC/D3. These results suggest that *N*-hydroxy-2-imidazolylamino analogues may possibly be used as prodrugs of the imidazolylamino group with enhanced BBB penetration. Efforts are ongoing towards the discovery of more potent drugs able to cross the BBB for the treatment of late-stage HAT.

Experimental section

Chemistry. All dry solvents were purchased from Aldrich (Sigma-Aldrich Quimica SA, Madrid, Spain) in Sure/Seal bottles. All reactions requiring anhydrous conditions or an inert atmosphere were performed under a positive pressure of N_2. All reactions were monitored by Thin Layer Chromatography (TLC) using silica gel 60 F_{254} plates (Merck-España, Madrid, Spain) or HPLC–MS. Chromatography was performed with Isolute SI prepacked columns (Biotage). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Advance 300 or Varian Inova 400 spectrometer. Chemical shifts of the ^{1}H NMR spectra were internally referenced to the residual proton resonance of the deuterated solvents: $CDCl_3$ (7.26 ppm), D_2O (δ 4.6 ppm), CD_3OD (3.49 ppm) and DMSO-d_6 (δ 2.49 ppm). *J*

values are given in Hz. Melting points were determined in open capillary tubes with a SMP3–Stuart Scientific apparatus and are uncorrected. All compounds are >95% pure by HPLC or combustion analysis otherwise noted. Elemental analysis was performed on a Heraeus CHN–O Rapid analyser. Analytical results were within ± 0.4% of the theoretical values unless otherwise noted. Analytical HPLC–MS was run with an Xbridge C18–3.5 μm (2.1×100mm) column on a Waters 2695 separation module coupled with a Waters Micromass ZQ spectrometer using electrospray ionisation (ES^+). The following HPLC conditions were used: column temperature = 30 ºC, gradient time = 15 min, H_2O/CH_3CN (5:95 → 95:5) (HCO_2H 0.1%), flow rate = 0.25 mL/min, UV detection: diode array (λ = 190−400 nm).

1. First synthetic route for the synthesis of 1-alkoxy-2-arylaminoimidazolines 12 and 14.

Bis(4-isothiocyanatophenyl)amine (3). The free diamine was obtained from technical grade 4,4'-diaminodiphenylamine sulphate as described earlier.[49] Thiophosgene (1.67 mL, 21.9 mmol) [CAUTION: wear adequate protection and work in a well ventilated fumehood] was added with a syringe to a mechanically stirred mixture of 4,4'-diaminodiphenylamine (1.985 g, 10 mmol) in 80 mL of Et_2O/H_2O (3/1 v/v). The reaction was almost instantaneous. The mixture was stirred vigorously overnight and the precipitate was collected by filtration to give the isothiocyanate **3** as a greenish solid (1.58 g, 56%). Another crop was recovered from the filtrate (0.85 g, 30%); mp 182–183 ºC; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.15 (d, *J* = 8.8, 4H), 7.00 (d, *J* = 8.8, 4H), 5.86 (br, *NH,* 1H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 139.2 (*C*), 132.5 (N*C*S), 125.3 (*CH*), 122.6 (*C*), 117.6 (*CH*); Anal. ($C_{14}H_9N_3S_2$) C, H, N.

2-(2-(Benzyloxyamino)ethyl)isoindoline-1,3-dione (4).[33, 34] A mixture of O-benzylhydroxylamine hydrochloride (12.71 mmol, 2.03 g, 1 equiv.), sodium hydroxide 5% solution (25 mL) and diethyl ether (Et_2O) (75 mL) was stirred for 15 min. The organic layer was extracted, dried over $MgSO_4$ and evaporated under vacuum to give the free base of O-benzylhydroxylamine ($BnONH_2$) as colorless oil (1.59 g, 100%). A mixture of $BnONH_2$ (1.59 g, 12.68 mmol, 2 equiv.) and 2-bromoethylphthalimide (1.61 g, 6.34 mmol, 1 equiv.) was heated at 80 ºC for 4 days. Ethyl acetate (EtOAc) was added to the cold reaction and the precipitate was filtered off. The filtrate was concentrated under vacuum to give a yellow oil. Recrystallization from MeOH yielded white crystals (433 mg, 23%): R_f = 0.38 (hexane/EtOAc 2:1); mp (MeOH) 83–85 ºC (with previous softening) [lit.[33] 92–94 ºC]. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.84–7.68 (m, 4H, ArH_{Pht}), 7.36–7.25 (m, 5H, Ar*H*), 5.63 (s, 1H, NH), 4.69 (s, 2H, CH_2O), 3.89 (t, J = 5.7, 2H, NCH_2), 3.20 (t, J = 6.0, 2H, CH_2NH). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 168.9 (*CO*), 138.1 (*C*-1, Ar), 134.3 (*CH*, Pht), 132.6 (*C*, Pht), 129.4 (*CH*-4, Ar), 129.3 (*CH*-3, Ar), 128.2 (*CH*-2, Ar), 123.5 (*CH*CO, Pht), 76.3 (OCH_2), 50.4 (CH_2NH), 36.6 (NCH_2). LRMS (ES^+) *m/z* = 297.0 [(M+H), 100%]; Anal ($C_{17}H_{16}N_2O_3$) C, H, N.

2-(2-(Methoxyamino)ethyl)isoindoline-1,3-dione (5a). *N,N*-Diisopropylethylamine (DIPEA) (3.7 mmol, 0.6 mL, 2 equiv.) was added to O-methylhydroxylamine hydrochloride (3.7 mmol, 307 mg, 2 equiv.) in acetonitrile under N_2 atmosphere. The mixture was stirred for few minutes and 2-bromoethylphthalimide (1.8 mmol, 467 mg, 1 equiv.) was added. The reaction was heated at 80 ºC for 43 h. The cold reaction mixture was diluted with EtOAc and the precipitate was filtered off. The filtrate was concentrated under vacuum to give a yellow oil. Purification by flash chromatography with hexane/EtOAc (9:1) yielded the product as a white solid (80 mg, 20%): R_f = 0.26 (hexane/EtOAc 2:1); mp 78–80 ºC. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.85–7.68 (m, 4H,

ArH_{Pht}), 5.63 (t, 1H, N*H*), 3.88 (t, *J* = 5.7, 2H, NCH_2), 3.51 (s, 3H, OCH_3), 3.19 (q, *J* = 5.7, 2H, CH_2NH). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 168.8 (*CO*), 134.4 (*CH*, Pht), 132.5 (*C*, Pht), 123.7 (*CH*, Pht), 62.4 (OCH_3), 49.9 (CH_2NH), 35.9 (NCH_2). LRMS (ES^+) *m/z* = 221.0 [(M+H), 100%]. Anal ($C_{11}H_{16}N_2O_5 \cdot 2H_2O$) calcd: C, 51.56; H, 6.29; N, 10.93. Found: C, 51.74; H, 4.90; N, 11.39.

1,1´-(4,4´-Azanediylbis(4,1-phenylene))bis(3-(benzyloxy)-3-(2-(1,3-dioxoisoindolin-2-yl)ethyl)thiourea) (6). Compound **4** (860 mg, 2.9 mmol, 2.5 equiv.) was added to the isothiocyanate **3** (329 mg, 1.7 mmol, 1 equiv.) in dry *N,N*-dimethylformamide (DMF) (5 mL). The reaction mixture was stirred 4 days at room temperature. The solvent was evaporated and the resulting yellow oil was purified by flash chromatography (10g SI cartridge) with Hexane/EtOAc: 4/1→1/1. Further rerystallization from EtOAc/hexane yielded **6** as a yellow solid (427 mg, 42%): R_f = 0.26 (EtOAc/hexane 1:1); mp 118–120 ºC (EtOAc/hexane). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.32 (s, 2H, Ph*NH*CS), 7.79 (dd, *J* = 3.0 and 5.4, 2H, ArH_{Pht}), 7.65 (dd, *J* = 3.0 and 5.4, 2H, ArH_{Pht}), 7.35–7.31 (m, 10H, ArH_{Bn}), 7.01 (d, *J* = 8.7, 4H, Ar*H*), 6.90 (d, *J* = 8.7, 4H, Ar*H*), 5.85 (s, 1H, Ph*NH*Ph), 4.87 (s, 4H, OCH_2Ph), 4.52 (t, *J* = 4.6, 4H), 4.09 (t, *J* = 4.6, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 181.2 (*CS*), 168.2 (*CO*), 141.2 (NH*C*), 133.9 (*C*CH_2O+*CH*$_{Pht}$), 132.1 (C_{Pht}), 130.8 (*C*NHCS), 129.3 (CH_m, Bn), 129.2 (CH_p, Bn), 128.8 (CH_o, Bn), 126.8 (*CH*, aniline), 123.1 (C*CH*CH, Pht), 117.4 (*CH*, aniline), 60.3 (OCH_2), 48.8 (CH_2N), 34.8 (CH_2N); LRMS (ES^+) *m/z* 877.0 (M+H). Anal ($C_{48}H_{41}N_7O_6S_2$) C, H, N.

1,1'-(4,4'-Azanediylbis(4,1-phenylene))bis(3-(2-(1,3-dioxoisoindolin-2-yl)ethyl)-3-methoxythiourea) (7). Compound **7** was obtained as an orange solid (55 mg, 83%) by the same procedure used for **6**: R_f = 0.74 (CH_2Cl_2/MeOH 95:5); mp 194–196 ºC

(decomp.). ^{1}H NMR (300 MHz, DMSO–d_6) δ 9.82 (s, 2H, *NH*CS), 8.10 (s, 1H, Ph*NH*Ph), 7.86–7.78 (m, 8H, *Pht)*, 7.05 (d, *J* = 8.7, 4H, *Ph*), 6.92 (d, *J* = 8.7, 4H, *Ph*), 4.38 (t, *J* = 4.7, 4H, CH_2CH_2), 3.90 (t, *J* = 4.7, 4H, CH_2CH_2), 3.68 (s, 6H, OCH_3). ^{13}C NMR (75 MHz, DMSO–d_6) δ 179.5 (*CS*), 168.1 (*CO*), 141.5 (NH*C*), 134.6 (CH_{Pht}), 132.3 (C_{Pht}), 131.6 (*C*NHCS), 128.0 (*CH*, aniline), 123.3 (CH_{Pht}), 116.3 (*CH*, aniline), 61.6 (OCH_3), 47.7 (CH_2N), 35.0 (CH_2N). LRMS (ES^+) *m/z* 724.10 [(M+H), 100%]. Anal ($C_{36}H_{33}N_7O_6S_2$) C, H, N.

(*N,N′Z,N,N′Z*)-Dimethyl-*N,N′*-4,4'-azanediylbis(4,1-phenylene)bis(*N*-benzyloxy-*N*-(2-(1,3-dioxoisoindolin-2-yl)ethyl)carbamimidothioate) (8). An excess of methyl iodide (2 mL) was added to a stirred solution of **6** (408 mg, 0.5 mmol) in CH_2Cl_2 (9 mL). The mixture was stirred at room temperature for 6 days. Vacuum evaporation of the solvents gave the product as a yellow solid (470 mg, quantitative). The product was pure enough to be used in the next reaction: R_f = 0.72 (EtOAc/Hexane 1:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.85–7.73 (m, 8H, *Pht*), 7.39–7.30 (m, 10H, CH_2Ph), 7.07 (m, 8H, *Ph*), 5.08 (s, 4H, OCH_2Ph), 4.31 (t, 4H, CH_2N), 4.04 (t, 4H, CH_2N), 2.12 (s, 6H, SCH_3); LRMS (ES^+) *m/z* 904.5 (M+H). HPLC = 80% pure.

N^1-(1-(Benzyloxy)-4,5-dihydro-1*H*-imidazol-2-yl)-N^4-(4-(1-(benzyloxy)-4,5-dihydro-1*H*-imidazol-2-ylamino)phenyl)benzene-1,4-diamine (14). An excess of methylamine 2.0 M solution in THF (15 mL) was added to a solution of **8** (1.37 g, 1.5 mmol) in H_2O (10 mL). The reaction mixture was stirred for 24 h and evaporated to dryness. The oily residue was washed with H_2O, filtered off and purified by flash chromatography with CH_2Cl_2/MeOH (97:3) to yield the title compound as a brown solid (250 mg, 30%). Dihydroiodide salt of **14**: mp 152–154 ºC (with previous softening). ^{1}H NMR (300 MHz, CD_3OD) δ 7.56–7.45 (m, 10H, CH_2Ph), 7.23 (m, 8H,

Ph), 5.49 (s, 1H, *NH*), 5.10 (s, 4H, OCH_2), 3.54 (s, 8H, CH_2CH_2). ^{13}C NMR (75 MHz, CD_3OD) δ 162.3 (*C*=N), 144.6 (*C*; 4,4'-Ph), 136.6 (*C*, Bn), 130.9 (CH_m, Bn), 130.2 (CH_p, Bn), 129.8 (CH_o, Bn), 128.2 (*C*; 1,1'-Ph), 127.5 (CH_{Ph}), 119.2 (CH_{Ph}), 79.7 (OCH_2), 52.7 (BnONCH_2), 42.1 (CH_2N). LRMS (ES^+) *m/z* 548.39 (M+H). Anal ($C_{32}H_{35}I_2N_7O_2 \cdot 2\ H_2O$) calcd: C, 45.78; H, 4.68; N, 11.68. Found: C, 45.65; H, 2.87; N, 12.09%. HPLC > 92% pure.

2. Second synthetic route for the synthesis of 1-alkoxy-2-arylaminoimidazolines 12–16. The 1-methoxy- (**12**), 1-ethoxy- (**13**), 1-benzyloxy- (**14**) and 1-tetrahydropyranyloxy- **(15)** derivatives were synthesised following the one-pot procedure previously reported by us. The products showed satisfactory spectroscopic and analytical data as reported previously.[36] In separate experiments, the thiourea intermediates (**10a** and **10b**) were also isolated and fully characterized. The spectroscopic data are given below.

***N,N*'-(2,2'-(4,4'-Azanediylbis(4,1-phenylene)bis(azanediyl))bis(thioxomethylene)bis (azanediyl)bis(ethane-2,1-diyl))bis(*N*-methoxy-2-nitrobenzenesulfonamide) (10a).** A solution of isothiocyanate **3** (0.30 mmol, 85 mg, 1 equiv.) in dry DMF (1 mL) was added to a solution of *N*-(2-aminoethyl)-*N*-methoxy-2-nitrobenzenesulfonamide (**9**, R_1 = Me)[36] (0.60 mmol, 277 mg, 2 equiv.) in dry DMF (5 mL). The resulting mixture was stirred at room temperature for 2 days. Water was added to give a precipitate that was filtered off. Purification by flash chromatography with hexane/ethyl acetate (1:1) yielded the product as a yellowish solid (67 mg, 27%): R_f = 0.65 (EtOAc/hexane 8:2); mp (CH_2Cl_2) 120–122 ºC (with previous softening). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.01 (d, *J* = 7.8, 2H, ArH_{Ns}), 7.85–7.67 (m, 4H, ArH_{Ns}), 7.75 (s, 2H, *NH*CS), 7.58 (d, *J* = 7.8, 2H, ArH_{Ns}), 7.13 (m, 8H, Ar*H*), 6.27 (t, *J* = 4.8, 2H, NHCH_2), 6.07 (s, 1H, *NH*), 3.90

(m, 4H), 3.78 (s, 6H, O*CH*$_3$), 3.35 (t, *J* = 4.4, 4H). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 181.7 (*C*S), 150.0 (*C*NO_2), 142.7 [Ar*C*-NH), 135.7 (*CH*-5, Ns), 132.8 (*CH*-3, Ns), 131.7 (*CH*-6, Ns), 128.9 (*C*SO_2, Ns), 127.9 (*CH*-3, Ar), 126.2 (*C*-4, Ar), 124.2 (*CH*-4, Ns), 119.5 (*CH*-2, Ar), 66.1 (O*CH*$_3$), 52.4, 42.9. LRMS (ES^+) *m/z* = 833.7 [(M+H), 100%]. Anal ($C_{32}H_{35}N_9O_{10}S_4 \cdot 3\ H_2O$) calcd: C, 43.28; H, 4.65; N, 14.20; S, 14.44. Found: C, 43.86; H, 4.78; N, 14.05; S, 13.01. HPLC > 90% pure.

***N,N'*-(2,2'-(4,4'-Azanediylbis(4,1-phenylene)bis(azanediyl))bis(thioxomethylene) bis (azanediyl)bis(ethane-2,1-diyl))bis(*N*-ethoxy-2-nitrobenzenesulfonamide) (10b).** A solution of isothiocyanate **3** (1.7 mmol, 329 mg, 1 equiv.) in dry DMF (1 mL) was added to a solution of *N*-(2-aminoethyl)-*N*-ethoxy-2-nitrobenzenesulfonamide[36] (**9**, R_1 = Et) (2.9 mmol, 860 mg, 2.5 equiv.) in dry DMF (5 mL). The reaction mixture was stirred for 4 days at room temperature. The solvent was evaporated to give a yellow oil that was purified by flash chromatography with hexane/EtOAc (1:1). Crystallization from EtOAc/Hexane yielded the product as yellow oil (427 mg, 42%): R_f = 0.83 (EtOAc/hexane 1:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.03 (dd, *J* = 7.7 and 1.35, 2H, ArH_{Ns}), 7.83–7.71 (m, 4H, ArH_{Ns}), 7.59 (dd, *J* = 7.78 and 1.0, 2H, ArH_{Ns}), 7.58 (s, 2H, *NH*CS), 7.14 (m, 8H, Ar*H*), 6.24 (t, 2H, *NH*CH_2), 5.94 (s, 1H, *NH*), 4.06 (q, *J* = 7.0, 4H, O*CH*$_2$), 3.92 (m, 4H, NH*CH*$_2$), 3.39 (t, *J* = 4.8, 4H, N*CH*$_2$), 1.19 (t, *J* = 7.1, 6H, *CH*$_3$). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 181.2 (*C*S), 149.5 (*C*NO_2), 142.2 (Ar*C*-NH), 135.2 (*CH*-5, Ns), 132.4 (*CH*-3, Ns), 131.2 (*CH*-6, Ns), 128.4 (*C*SO_2, Ns), 127.4 (*CH*-3, Ar), 125.9 (*C*-4, Ar), 123.8 (*CH*-4, Ns), 119.1 (*CH*-2, Ar), 73.9 (O*CH*$_2$), 52.1 (*CH*$_2$), 42.5 (*CH*$_2$), 13.5 (*CH*$_3$). LRMS (ES^+) *m/z* = 862.47 (M+H). HPLC > 90% pure.

4,4'-Bis(1-hydroxyimidazolinylamino)diphenylamine (16). The THP-protected product **15**[36] (80 mg, 0.15 mmol) was dissolved in MeOH (2 mL) and treated with HCl_g saturated dioxane solution (5 mL). The reaction was stirred at room temperature for 5 h

and the solvent was removed under vacuum. The crude product was dissolved in MeOH and Et_2O was added. The flask was allowed to stand in the fridge overnight whereupon a precipitate settled at the bottom of the flask. The supernatant was discarded and Et_2O was added. The precipitate was triturated with spatula, collected and dried under vacuum to give the dihydrochloride salt of **16** as brown solid (56 mg, 85%); mp > 91.4 ºC (decomp.). ^{1}H NMR (DMSO-*d*6) δ 10.94 (brs, 2H, *OH*), 10.62 (s, 2H, *NH*), 8.87 (s, 2H, *NH*), 7.21 (d, *J* = 9.0, 4H, Ar*H-3*), 7.16 (d, *J* = 9.0, 4H, Ar*H-2*), 3.82–3.46 (m, 8H). ^{13}C NMR (DMSO-*d*6) δ 160.8 (*C*=N), 142.7 (*C*), 126.9 (*C*), 126.4 (*C*), 117.6 (*CH*), 53.3 (*CH*$_2$), 40.6 (*CH*$_2$). LRMS (ES$^+$) *m*/*z* 368.15 [(M+H)]. HPLC ≥ 95% pure.

4,4'-Bis(thiazolinylamino)diphenylamine (17). Triethylamine (3.18 mmol, 0.44 mL, 3 equiv.) was added dropwise to a solution of isothiocyanate **3** (1.06 mmol, 300 mg, 1 equiv.) and 2-bromoethanamine hydrobromide (2.65 mmol, 543 mg, 2.5 equiv.) in CH_2Cl_2 (20 mL). The reaction mixture was stirred at room temperature overnight. The precipitate was collected by filtration, rinsed with dichloromethane and water, successively. Recrystallization from boiling methanol/acetonitrile yielded a green solid (92 mg, 15%): R_f = 0.22 (CH_2Cl_2/MeOH 2/1); mp 82–84 ºC (with previous softening). A methanolic solution of **17** (2 mL) was stirred with HCl_g saturated dioxane solution (3 mL). The solvents were removed under vacuum affording the dihydrochloride salt of **17**: ^{1}H NMR (DMSO-*d*6, 300 MHz) δ 8.64 (brs, 2H, *NH*), 7.67 (s, 1H, *NH*), 7.25 (d, *J* = 7.9, 4H, Ar*H-2*), 6.88 (d, *J* = 8.7, 4H, Ar*H-3*), 3.85 (t, *J* = 7.1, 4H, N*CH*$_2$), 3.24 (t, *J* = 7.1, 4H, S*CH*$_2$). ^{13}C NMR (DMSO-*d*6, 300 MHz) δ 157.2 (*C*=N), 138.9 (Ar, *C*-2), 137.1 (Ar, *C*-4), 120.2 (Ar, 2-*CH*), 117.3 (Ar, 3-*CH*), 57.33 (*CH*$_2$N), 32.9 (*CH*$_2$S). LRMS (ES$^+$) *m/z* = 369.9 [(M+H), 100%]. Anal ($C_{18}H_{19}N_5S_2$·2HCl·2H_2O) C, H, N. HPLC > 95% pure.

Biology.

In vitro BBB permeability studies. Cell line culture. Immortalized human brain endothelial cells (hCMEC/D3 cell line) were seeded at the density of 50,000 cells per cm^2 in the upper compartment of six-well tissue culture plate inserts (Millicell CM inserts, 0.4 μm porosity, Millipore, Bellerica, MA, USA), pre-coated with rat tail collagen type I (Cultrex rat Collagen, R&D systems, Trevigen). They were then maintained for 10-12 days in EBM-2 basal medium (Lonza, Basel, Switzerland) containing 5% fetal bovine serum Gold (PAA, The Cell Culture Company, Pasching, Austria), 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), 1.4 μM hydrocortisone (Sigma, St. Louis, MO, USA), 10 mM HEPES (PAA Laboratories, Pasching, Austria), 5 $\mu g\ mL^{-1}$ ascorbic acid (Sigma, St Louis, MO, USA) and 1 $ng\ mL^{-1}$ bFGF (Sigma, St Louis, MO, USA), at 37 °C in 5% CO_2 atmosphere. The medium was changed every 3–4 days. Twenty four hours prior to permeability assays, fresh medium was supplemented with Simvastatin 1 nM (Calbiochem, La Jolla, CA, USA). In summary, endothelial cells were cultured in Boyden chamber-like system allowing them to separate 2 compartments, the upper or luminal compartment and the lower or abluminal one, which represent the blood and the cerebral one, respectively.

Permeability assays. Prior compound permeability study, it is essential to find a working concentration of the compounds that will not disturb the cellular complex between endothelial cells. For this purpose, endothelial monolayer quality was checked by following the passage of the small hydrophilic fluorescent molecule lucifer yellow (LY, Sigma, St Louis, MO, USA) when a fixed concentration of the studied compound (i.e., **12**, **13**, **14**, or **16**) was put on the cells in the luminal compartment. Note that LY is widely used and accepted as a marker of tight junction integrity.[40] If LY permeability

was affected by the presence of the compound, the concentration of the compound was decreased until a non-affecting dose was reached.

Compound **1** was directly dissolved in transport buffer whereas the *N*-alkoxy derivatives (**12**, **13**, **14**, and **16**) were dissolved in DMSO (Sigma) and then diluted with transport buffer to reach a concentration of 100 μM. The maximum quantity of DMSO in the stock solutions was 0.1%. The endothelial monolayer integrity in presence of 0.1% DMSO was checked, showing no significant difference compared with the buffer alone (See Figure 1).

So, briefly after 10-12 days of culture, coated culture inserts with and without endothelial cell were transferred to 6-well plates containing 2 mL of transport buffer (Hanks buffer saline solution with $CaCl_2$ and $MgCl_2$, 10 mM HEPES and 1 mM sodium pyruvate) in the abluminal chamber. At time 0, transport buffer, containing tested compound with or without LY (50 μM dissolved in cell culture tested water), was placed in each luminal chamber. Transport incubations occured at 37 °C, 95% humidity and 5% CO_2. At different times: 10, 25 and 45 min, each culture insert (with and without cell) was transferred to a new lower compartment containing fresh transport buffer. The amount of each compound in the lower compartments at different time points, in the upper one at the end of the experiment, and in the working solution was quantified either by fluorimetry (for LY) or by HPLC–MS (for compounds **12** ,**13**, **14**, and **16**).

Endothelial permeability was calculated from the clearance rate and the surface area, as previously described.[38, 39] In this way, a concentration-independent permeability value was obtained. The increment in cleared volume between successive sampling events is calculated by dividing the amount of solute during the interval time by the donor chamber concentration, which is the luminal one in this study, as described below:

The total cleared volume at each time point is calculated by summing the incremental cleared volumes up to the given time point:

Clearance (mL) = X/C_d

Where X is the amount of drug in the receptor chamber (the abluminal one) and C_d is the donor chamber concentration at each time-point. The average volume cleared is plotted versus time, and the slope estimated by linear regression analysis, which allows calculating total endothelial monolayer permeability noted PS_e:

$1 / PS_e = 1 / PS_t - 1 / PS_f$ and $P_e = PS_e / A$,

where PS_e is the permeablitiy–surface area product value for the endothelial cell monolayer, A is the surface area of the filter (in cm^2), PS_t and PS_f are the slopes of the clearance curves for the cell monolayer on coated culture insert filter and for the coated culture insert filter alone (meaning without cell monolayer), respectively. The PS_e values are divided by the surface area (A, in cm^2) of the culture insert to generate the endothelial permeability coefficient (P_e, in cm per min). These P_e values allow classifying the molecules into 3 categories: low-, intermediate- or high-brain uptake. Compound permeability values were expressed as an increase or decrease compared with a model of low brain uptake, LY permeability ($P_e = 3.04 \times 10^{-3}$ cm/min), which is given the value of 100%. LY was chosen as paracellular diffusion marker because of its properties: it is a small (457.3 Da) and hydrophilic molecule that has no transporter on endothelial cells (data not shown).

HPLC analysis of the results. 1 mL samples for each time point were taken directly from the basal compartment and transferred to 1.5 mL Waters HPLC vials that were stored in the freezer. The samples were defrost and shaken with an orbital shaker for 20 min just before running the HPLC analysis. Analytical HPLC–MS was run with a

Waters Sunfire C18–3.5 μm (4.6×50mm) column on a Waters 2695 separation module coupled with a Waters Micromass ZQ spectrometer using electrospray ionization (ES^+). The following HPLC conditions were used: column temperature = 30 ºC, flow rate = 1 mL/min, gradient time = 5 min, the solvent mixture was H_2O:CH_3CN (HCO_2H 0.1%%) with the following proportions: compound **1** (100:0 → 90:10), compound **12** (98:2 → 50:50), compounds **13** and **14** (95:5 → 50:50), and compound **16** (98:2 → 70:30). The compounds were detected by UV at the following wavelengths: λ = 296 nm (compound **1**), 300 nm (compounds **12** and **13**), 303 nm (compound **14**), and 298 nm (compound **16**). Each sample was injected 3 times and the mean value of the compound UV peak area was used to determine the concentration of the sample according to the calibration curves. Calibration curves were obtained for each compound using 6 different concentrations (1, 10, 30, 75, 150, 375 μM). Each concentration was tested in triplicate and the mean value of the compound UV peak area *vs*. concentration was plotted. The equation for the calibration curve was obtained by linear regression analysis using the Microsoft Excel program.

In vitro antiprotozoal activity. IC_{50} values against bloodstream forms of *T. b. rhodesiense* strain STIB900, amastigotes of *L. donovani* strain MHOM/ET/67/L82, and cytotoxicity on rat myoblasts L-6 cells were determined using the Alamar blue assay.[50, 51] IC_{50} values against erythrocytic stages of *P. falciparum* was determined by a [^{3}H]hypoxanthine incorporation assay using the chloroquine- and pyrimethamine-resistant strain K1.[52] IC_{50} values against trypomastigote forms of *T. cruzi* Tulahuen strain C2C4 containing the β-D-galactosidase (LacZ) gene were determined using a colorimetric assay with the substrate chlorophenyl red β-D-galactopyranoside (CPRG)-Nonidet.[53] Detailed experimental protocols for all of these assays have been reported before.[37]

In vivo antitrypanosomal activity. The STIB900 acute mouse model mimics the first stage of the disease. Experiments were performed as previously reported,[16] with minor modifications. Briefly, female NMRI mice were infected intraperitoneally (i.p.) with 2 x 10^4 bloodstream forms of *T. b. rhodesiense* (strain STIB900). Experimental groups of four mice were treated i.p. or orally (per os [p.o.]) with compounds on four consecutive days from day 3 to 6 postinfection. A control group was infected but remained untreated. The tail blood of all mice was checked for parasitemia until 60 days after infection. Surviving and aparasitemic mice at day 60 were considered cured and then euthanized. The day of death of the animals was recorded (including the cured mice, as >60) to calculate the mean survival time in days (MSD). Mean relapse days were determined as day of relapse post-infection of mice. All protocols and procedures used in the current study were reviewed and approved by the local veterinary authorities of the Canton Basel-Stadt.

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Table of Contents graphic