A new non-polar \(N\)-Hydroxy Imidazoline lead compound with improved activity in a murine model of late stage \(T. b. brucei\) infection is not cross-resistant with diamidines

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Abbreviations:

BBB, blood–brain barrier

BSA, bovine serum albumine

clogP, calculated log of octanol-water partition coefficient

CNS, central nervous system

HAPT, high affinity pentamidine transporter

HAT, human African trypanosomiasis

hCMEC/D3, human cerebral microvessel endothelial cell line

LAPT, low affinity pentamidine transporter

LY, lucifer yellow

HSA, human serum albumin

P-gp, P-glycoprotein

SPR, surface plasmon resonance

WT, wild type
ABSTRACT

Treatment of late-stage sleeping sickness requires drugs that can cross the blood-brain barrier (BBB) to reach the parasites located in the brain. We report here the synthesis and evaluation of four new N-hydroxy and twelve new N-alkoxy derivatives of bisimidazoline leads as potential agents for the treatment of late-stage sleeping sickness. These compounds, which have reduced basicity compared to the parent leads (i.e., are less ionized at physiological pH), were evaluated in vitro against *T. brucei rhodesiense* and in vivo in murine models of first- and second-stage sleeping sickness. Resistance profile, physicochemical parameters, in vitro BBB permeability, and microsomal stability were also determined. The N-hydroxy imidazoline analogues were the most effective in vivo with 4-(((1-hydroxy-4,5-dihydro-1H-imidazol-2-yl)amino)-N-((1-hydroxy-4,5-dihydro-1H-imidazol-2-yl)amino)phenyl)benzamide (**14d**) showing 100% cures in the first-stage disease, whilst **15d**, **16d** and **17d** appeared to slightly improve survival. In addition, **14d** showed weak activity in the chronic model of CNS infection in mice. No evidence of reduction of this compound with hepatic microsomes and mitochondria was found in vitro suggesting that N-hydroxy imidazoles are metabolically stable and have intrinsic activity against *T. brucei*. In contrast to its unsubstituted parent compound, uptake of **14d** in *T. brucei* was independent of known drug transporters (i.e. *TbAT1/P2* and HAPT) indicating a lower predisposition to cross-resistance with other diamidines and arsenical drugs. Hence, the N-hydroxy bisimidazolines (**14d** in particular) represent a new class of promising antitrypanosomal agents.

**Keywords:** *Trypanosoma brucei*, chemotherapy, imidazoline, blood–brain barrier, hCMEC/D3 cell line, pKₘ, protein binding, mitochondrial amidoxime reducing component (mARC).
INTRODUCTION

African trypanosomes (Trypanosoma brucei spp.) are protozoan parasites that cause human African trypanosomiasis (HAT or sleeping sickness) and the corresponding animal disease (called nagana) in cattle. Despite the great harm it causes at human and socioeconomical levels in sub-Saharan Africa, sleeping sickness is still one of the most neglected tropical diseases (1). This is evidenced by the lack of acceptable treatment options for the CNS-stage of T. b. rhodesiense infection which still relies solely on the highly toxic arsenical drug melarsoprol (2). Indeed, the only available drug for the treatment of early stage rhodesiense sleeping sickness, suramin, was introduced in the early 1920s and also exhibits unacceptable side effects. Thus, new drugs that are safe and effective for early (haemolymphatic) and late (CNS) stages of HAT are needed.

Several antiparasitic dicationic drugs have been in clinical use for decades (3, 4). For example, pentamidine remains the standard drug to treat early stage T. b. gambiense infection whereas diminazene aceturate is used for animal trypanosomiasis. In the last years, the diamidine pafuramidine reached phase III clinical trials as oral treatment for HAT. However, its development was stopped recently due to unexpected renal toxicity discovered in an extended phase I safety trial (5). Nevertheless, other CNS-permeable diamidines such as DB829 are still being studied as possible clinical candidates for second stage gambiense disease (5).

In recent years, our group has been involved in the study of diphenyl dicationic antitrypanosomal agents that hold 2-aminoimidazolinium moieties (6, 7). This class of molecules has produced very active compounds in mouse models of stage 1 trypanosome infection; some compounds were curative in a mouse model of acute HAT (T. b. rhodesiense STIB900) but were devoid of activity in a mouse model of CNS-
stage infection owing probably to poor brain permeation (6). Low BBB permeability was confirmed in vitro with a humanized model of brain endothelial cells (i.e., human cerebral microvessel endothelial cell line, hCMEC/D3) (8). Due to their dicationic nature these compounds diffuse poorly across the BBB. To reduce the ionization state of the compounds at physiological pH, a strategy consisting of derivatizing the basic nitrogens of the imidazoline ring with O-alkyl or OH substituents was tested, and showed that, in this series, the N-hydroxy derivative had enhanced BBB permeability (8). However, this compound was only moderately active in a mouse model of first-stage HAT and was not studied further as trypanocide.

In the current work, we have broadened the scope of our study to recently discovered bisaminoimidazoline leads (9) that are even more effective antitrypanosomal agents in an acute mouse model of infection (Fig. 1). We previously reported that these compounds (I–V) cured *T. b. rhodesiense* infected mice by intraperitoneal administration at 20 mg/kg ip. An in vivo follow up study (Table 3) showed that this activity was retained at low dosage (5–10 mg/kg ip) and by oral administration for I and V (50 mg/kg po). However, no activity was obtained in the GVR35 mouse model of late-stage disease (Table 4), indicating that the compounds needed further optimization to attain curative concentrations in the brain.

Here, a series of non polar N-hydroxy and N-alkoxy derivatives of these new leads was synthesized to incorporate one OR group (R = H, OMe, OEt, OBn) on the N1-nitrogen of each imidazoline ring (Fig. 1, 14–17a-e). Our intention was to reduce the pKₐ of these leads to decrease the proportion of ionized form of the compounds in biological fluids. The in vitro antiprotozoal activities of the compounds as well as their in vivo antitrypanosomal effects in mouse models of stage 1 and CNS-stage HAT were studied. In addition, the ionization constants (pKₐ) of these derivatives were measured, important
physicochemical parameters relevant to membrane permeation (e.g. clogP, logD) were calculated, binding to human serum albumin (HSA) was measured, and in vitro BBB permeability was determined using the hCMEC/D3 model. One compound, the \( N \)-hydroxy derivative 14d, was 100% curative in the mouse model of first-stage HAT and weakly active (i.e. increased parasite-free survival time vs control) by ip dosage in the GVR35 chronic model, showing no overt toxicity and a higher safety profile than the corresponding parent compound I. We also investigated whether 14d was a substrate for \( N \)-hydroxylamine reductases occurring in hepatic fractions to give the corresponding imidazoline parent compound I, in a similar way as amidoxime prodrugs generate amidine active drugs.

[Insert Fig 1 here]

MATERIALS AND METHODS

Chemistry. All dry solvents were purchased from Aldrich or Fluka 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) or HPLC–MS. Chromatography was performed with Isolute SI prepacked columns. \(^1\)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\(_2\)O (\( \delta \) 4.6 ppm), CD\(_3\)OD (3.49 ppm) and DMSO (\( \delta \) 2.49 ppm). \( J \) values are given in Hz. Signal splitting patterns
are described as: singlet (s), broad singlet (br s), doublet (d), triplet (t), quadruplet (q), multiplet (m), or combination thereof. Melting points were determined in open capillary tubes with a SMP3–Stuart Scientific apparatus or Mettler Toledo MP70 melting point system, and are uncorrected. 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 a Xbridge C18–3.5 µm (2.1×100 mm) 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 = 5 min, H₂O/CH₃CN (10:90 → 90:10) (HCO₂H 0.1 %), flow rate = 1 mL/min, UV detection: diode array (λ = 190–400 nm). Accurate mass were measured with an Agilent Technologies Q–TOF 6520 spectrometer using electrospray ionization. Starting material diamines 1 and 3 were commercially available. Diamine 2 was obtained by reduction of the commercially available 1,3-bis(4-nitrophenyl)urea as described (9).

9,10-dihydroanthracene-2,6-diamine (4). Diamine 4 was synthesized using a modification of the procedure described by Takimiya et al. (10). A Kimax tube was charged with 2,6-diamino-9,10-anthraquinone (1.08 g, 4.5 mmol), activated zinc‡ (4.5 g, 68 mmol) and 30% aqueous ammonia (10 mL). The tube was stoppered and heated at 100 ºC for 23 h. The green precipitate was collected by filtration on celite. The filter cake was rinsed thoroughly with acetone (250 mL) to extract the product (yellow) out of

‡ Activation of zinc: zinc powder was stirred with 10% aqueous HCl solution for 2 min. The powder was collected on fritted filter and rinsed with H₂O and acetone, successively. The powder was transferred to a flask and vigorously shaken with Et₂O for 10 min. The activated zinc powder was drained, rinsed with Et₂O and used in the reaction.
the zinc salts. The yellow (fluorescent) acetone filtrate was dried (Na$_2$SO$_4$) and the solvent was evaporated to give 4 as yellow solid (830 mg, 88%). The product was >90% pure and used without further purification. Alternatively, the product can be recrystallized in MeOH. $^1$H NMR data were consistent with the reported ones (10).

1. Synthesis of isothiocyanates 5–8. Thiophosgene (2.5 equiv.) [CAUTION: highly toxic reagent that must be handled with adequate protecting clothes in a well ventilated fumehood] was added with a syringe to a stirred suspension of diamine 1–4 (1 equiv) in Et$_2$O/H$_2$O (3:1, v/v). The reaction mixture was stirred at room temperature overnight. The precipitate was filtered, rinsed with water, and dried under vacuum to yield the isothiocyanates 5–8 in excellent yields and adequate purity.

**4-isothiocyanato-N-(4-isothiocyanatophenyl)benzamide (5).** Diamine 1 (3.42 g, 15 mmol) and thiophosgene (2.51 mL, 33 mmol) were reacted according to the general protocol to yield 5 as grey solid (92%). HPLC (UV) > 95%; mp 199–200 ºC. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.88 (br s, 1H, NH), 7.85 (d, $J = 8.7$, 2H, ArH), 7.63 (d, $J = 8.8$, 2H, ArH), 7.31 (d, $J = 8.7$, 2H, ArH), 7.22 (d, $J = 8.8$, 2H, ArH). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 164.7, 138.6, 137.1, 135.9, 135.5, 133.2, 129.0, 127.8, 127.0, 126.5, 121.5. LRMS (ESI$^+$) $m/z$ = 312.27 (M+H).

**1,3-bis(4-isothiocyanatophenyl)urea (6).** Diamine 2 (2.02 g, 8.84 mmol) and thiophosgene (1.69 mL, 22.1 mmol) were reacted according to the general protocol to yield 6 as grey solid (78%). HPLC (UV) > 95%; mp > 235 ºC. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.29 (br s, 2H, NH), 7.40 (d, $J = 8.8$, 4H, ArH), 7.12 (d, $J = 8.8$, 2H, ArH). LRMS (ESI$^+$) $m/z$ = 327.19 (M+H).

**1,2-bis(4-isothiocyanatophenyl)ethane (7).** Diamine 3 (3.23 g, 15.2 mmol) and thiophosgene (2.55 mL, 33.5 mmol) were reacted according to the general protocol to
yield 7 as off-white solid (84%). HPLC (UV) = 95%; mp 128–129 °C. $^1$H NMR (300 MHz, CDCl$_3$) δ 7.13 (m, 4H, ArH), 7.06 (m, 4H, ArH), 2.89 (s, 4H, CH$_2$).

**2,6-diisothiocyanato-9,10-dihydroanthracene (8).** Diamine 4 (2.46 g, 11.7 mmol) and thiophosgene (1.96 mL, 26 mmol) were reacted according to the general protocol to yield 8 as beige solid (89%). HPLC (UV) > 90%; mp > 300 °C. $^1$H NMR (300 MHz, DMSO) δ 7.42 (d, $J$ = 1.9, 2H, ArH), 7.40 (d, $J$ = 8.0, 2H, ArH), 7.27 (dd, $J$ = 1.9, 8.0, 2H, ArH), 3.94 (s, 4H, CH$_2$). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 137.9, 136.0, 132.9, 128.5, 127.7, 124.5, 123.6, 34.4.

**2. General procedure for the synthesis of 1-alkoxy-2-arylaminoimidazolines 14a–b, 15b, 15e, 16a, 17a.** A suspension of isothiocyanate (5–8, 1 equiv.) in dry DMF (5 mL) was added dropwise to a stirred solution of 9a–d, (2.5 equiv.) in dry DMF (5 mL) under argon atmosphere. The flask containing the isothiocyanate suspension was rinsed with approximately 20 mL of dry DMF that were subsequently added to the reaction mixture. The reaction mixture was stirred at room temperature and the formation of the intermediate thiourea (10–13) was checked by HPLC/MS. Nosyl groups removal was performed in the same flask by addition of thiophenol (10 equiv.) and K$_2$CO$_3$ (20 equiv.). The reaction mixture was warmed to 65 °C and stirred until complete cyclization to the final 1-alkoxy-2-arylaminoimidazolines 14a, 14b, 15b, 15e, 16a, and 17a. The crude reaction mixture was filtered on Celite and the filter cake was rinsed with CH$_2$Cl$_2$. The organic phase was washed successively with saturated NaHCO$_3$ solution (30 mL), brine (30 mL), and dried over MgSO$_4$. The solvents were removed under vacuum and the crude residue was purified by silica chromatography.

**4-((1-methoxy-4,5-dihydro-1H-imidazol-2-yl)amino)-N-(4-((1-methoxy-4,5-dihydro-1H-imidazol-2-yl)amino)phenyl)benzamide (14a).** The reaction was carried
out following the general procedure with 5 (420 mg, 1.32 mmol) and 9a (900 mg, 3.3 mmol). Purification by silica chromatography (10 g prepacked cartridge) eluting with CH₂Cl₂/MeOH (9:1) with 0.1% of Et₃N yielded 14a as brown solid (420 mg, 75%). HPLC (UV) > 91%; mp 165–170 ºC. ¹H NMR (300 MHz, CDCl₃) δ 8.41 (br s, 1H, CONH), 7.68 (d, J = 8.5, 2H, ArH), 7.41 (d, J = 8.5, 2H, ArH), 7.27 (d, J = 8.5, 2H, ArH), 7.16 (d, J = 8.5, 2H, ArH), 6.13 – 5.25 (br s, 2H, NH), 3.70 (s, 6H, OCH₃) 3.42 (m, 4H, NCH₂), 3.33 (m, 4H, NCH₂). ¹³C NMR (75 MHz, CDCl₃) δ 165.8, 158.5, 158.0, 138.9, 133.3, 128.6(2×C), 128.4, 121.8 (2×C), 120.4 (2×C), 119.5 (2×C), 115.5, 63.1 (2×C), 51.8 (2×C), 45.8 (2×C). LRMS (ESI⁺) m/z = 424.23 (M+H). HRMS (ESI⁺) C₂₁H₂₅N₇O₃ requires 423.2019 (found: 423.2023).

4-((1-ethoxy-4,5-dihydro-1H-imidazol-2-yl)amino)-N-(4-((1-ethoxy-4,5-dihydro-1H-imidazol-2-yl)amino)phenyl)benzamide (14b). The reaction was carried out following the general procedure with 5 (680 mg, 2.26 mmol) and 9b (1.63 g, 5.6 mmol). In this case the cyclization process was very slow and required 24 days stirring at 65 ºC. Purification by silica chromatography (20 g prepacked cartridge) eluting with CH₂Cl₂/MeOH (100/0→90/10) yielded 14b as brown solid (50 mg, 5%). HPLC (UV) > 90%; mp 135–136 ºC. ¹H NMR (300 MHz, CDCl₃) δ 8.85 (br s, 1H, CONH), 7.75 (d, J = 8.5, 2H, ArH), 7.49 (d, J = 8.2, 2H, ArH), 7.30 (d, J = 8.2, 2H, ArH), 7.17 (d, J = 8.5, 2H, ArH), 5.88 (br s, 2H, NH), 3.93 (q, J = 7.0, 4H, OCH₂CH₃), 3.53 – 3.28 (m, 8H, NCH₂), 1.24 (t, J = 7.0, 6H, OCH₂CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 165.9, 159.0, 158.4, 146.0, 138.0, 133.7, 128.6 (2×C), 128.3, 121.8 (2×C), 120.4 (2×C), 119.3 (2×C), 70.90, 70.88, 52.6 (2×C), 45.7 (2×C), 14.3 (2×C). LRMS (ESI⁺) m/z = 452.12 (M+H). HRMS (ESI⁺) C₂₃H₂₉N₇O₃ requires 451.2332 (found: 451.2335).

1,3-bis(4-((1-ethoxy-4,5-dihydro-1H-imidazol-2-yl)amino)phenyl)urea (15b). The reaction was carried out following the general procedure with 6 (737 mg, 2.26 mmol)
and 9b (1.63 g, 5.6 mmol). In this case the cyclization process required 11 days stirring at 65 °C. Purification by silica chromatography (20 g prepacked cartridge) eluting with CH$_2$Cl$_2$/MeOH (100/0→90/10) yielded 15b as brown solid (253 mg, 24%). HPLC (UV) > 95%; mp 144–146 ºC. $^1$H NMR (300 MHz, CDCl$_3$) δ 8.53 (br s, 2H, NH), 7.21 (d, J = 8.0, 4H, ArH), 7.04 (d, J = 8.0, 4H, ArH), 4.40 (br s, 2H, NH) 3.96 (q, J = 7.0, 4H, OCH$_2$CH$_3$), 3.40 (m, 8H, NCH$_2$), 1.26 (t, J = 7.0, 6H, OCH$_2$CH$_3$). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 159.5 (2×C), 154.4, 136.7 (2×C), 135.2, 128.8, 124.4, 121.4 (4×C), 120.4 (4×C), 71.1 (2×C), 52.6 (2×C), 44.8 (2×C), 14.3 (2×C). LRMS (ESI$^+$) m/z = 467.14 (M+H).

1,3-bis(4-((1-(benzyloxy)-4,5-dihydro-1H-imidazol-2-yl)amino)phenyl)urea (15e). The reaction was carried out following the general procedure with 6 (300 mg, 0.92 mmol) and 9d (418 mg, 1.19 mmol) (1.63 g, 5.6 mmol). In this case the cyclization process required 22 days stirring at 65 °C. Purification by silica chromatography (10 g prepacked cartridge) eluting with CH$_2$Cl$_2$/MeOH (100/0→90/10) yielded 15e as brown solid (60 mg, 11%). HPLC (UV) = 91%; mp 60–65 ºC. $^1$H NMR (300 MHz, CDCl$_3$) δ 8.54 (br s, 2H, NH), 7.44 – 7.25 (m, 10H, ArH), 7.15 (d, J = 8.6, 4H, ArH), 6.88 (d, J = 8.6, 4H, ArH), 4.90 – 4.73 (s, 4H, OCH$_2$), 4.56 (br s, 2H, NH), 3.29 (m, 4H, NCH$_2$), 3.17 (m, 4H, NCH$_2$). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 159.5 (2×C), 153.9, 136.2 (2×C), 135.2 (2×C), 134.9 (2×C), 129.3 (4×C), 128.7(2×C), 128.6 (4×C), 121.3 (4×C), 120.2 (4×C), 77.8 (2×C), 52.4 (2×C), 44.4 (2×C). LRMS (ESI$^+$) m/z = 591.49 (M+H).

$N,N'$-(ethane-1,2-diylbis(4,1-phenylene))bis(1-methoxy-4,5-dihydro-1H-imidazol-2-amine) (16a). The reaction was carried out following the general procedure with 7 (420 mg, 1.32 mmol) and 9a (900 mg, 3.3 mmol). Purification by silica chromatography (10 g prepacked cartridge) eluting with CH$_2$Cl$_2$/MeOH (9:1) and 0.1% Et$_3$N yielded 16a as brown solid (470 mg, 87%). HPLC (UV) > 90%; mp 106–108 ºC.
H NMR (300 MHz, CDCl$_3$) δ 8.05 (br s, 2H, NH), 7.32 (d, J = 8.2, 4H, ArH), 7.10 (d, J = 8.2, 4H, ArH), 3.82 (s, 6H, OCH$_3$), 3.62 (t, J = 6.8, 4H, NCH$_2$), 3.46 (t, J = 6.8, 4H, NCH$_2$), 3.10 – 2.77 (m, 4H, CH$_2$). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 158.8 (2×C), 136.3 (2×C), 129.3 (4×C), 127.2 (2×C), 119.7 (4×C), 63.2 (2×C), 52.0 (2×C), 46.8 (2×C), 37.5 (2×C). LRMS (ESI$^+$) m/z = 409.20 (M+H). HRMS (ESI$^+$) C$_{22}$H$_{28}$N$_6$O$_2$ requires 408.2274 (found: 408.2278).

$N^2,N^6$-bis(1-methoxy-4,5-dihydro-1H-imidazol-2-yl)-9,10-dihydroanthracene-2,6-diamine (17a). The reaction was carried out following the general procedure with 8 (392 mg, 1.32 mmol) and 9a (900 mg, 3.3 mmol). Purification by silica chromatography (10 g prepacked cartridge) eluting with CH$_2$Cl$_2$/MeOH (9:1) and 0.1% Et$_3$N yielded the product as 8:2 mixture of 17a and 18a. Brown solid (389 mg, 72%). HPLC (UV) > 90%; mp 110–112 ºC. H NMR (300 MHz, CDCl$_3$) δ 7.36 (s, 2H, ArH), 7.12 (s, 4H, ArH), 3.82 (s, 4H, CH$_2$), 3.78 (s, 6H, OCH$_3$), 3.63 – 3.51 (m, 4H, NCH$_2$), 3.50 – 3.34 (m, 4H, NCH$_2$). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 158.6 (2×C), 137.6 (2×C), 131.3 (2×C), 127.8 (2×C), 124.9 (2×C), 118.8 (2×C), 117.7 (2×C), 63.0 (2×C), 51.5 (2×C), 46.0 (2×C), 35.5 (2×C). LRMS (ESI$^+$) m/z = 407.11 (M+H).

3. General procedure for the synthesis of 1-(2-(alkoxyamino)ethyl)-3-arylthioureas 10c, 11a, 11c, 12b–c, and 13b–c. A suspension of isothiocyanate 5–8 (2.4 mmol, 1 equiv.) in dry DMF (5 mL) was added dropwise to a stirred solution of 9a–c (6 mmol, 2.5 equiv.) in dry DMF (5 mL) under argon atmosphere. The flask containing the isothiocyanate suspension was rinsed with approximately 20 mL of dry DMF that were subsequently added to the reaction mixture. The reaction mixture was stirred at room temperature for 12h. The solvent was removed under high-vacuum and the crude product was treated with methanol. Trituration with a spatula yielded a crude solid that was filtered and washed with MeOH. The compounds were purified as specified below.

The reaction was carried out following the general procedure with 5 (750 mg, 2.4 mmol) and 9c (2.07 g, 6.0 mmol). The product was purified by precipitation from MeOH. Compound 10c was obtained as light yellow solid (1.18 g, 41%). HPLC (UV) > 95%; mp 132–133 ºC.

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.01-7.78 (m, 6H), 7.74 (m, 4H), 7.62 (m, 2H), 7.35 (d, $J = 8.2$, 2H), 7.21 (d, $J = 8.2$, 2H), 4.96 (d, $J = 6.8$, 1H, OCH), 4.88 (d, $J = 7.2$, 1H, OCH), 4.37 – 4.19 (m, 2H, OCH$_2$), 3.99 – 3.83 (m, 2H, NCH$_2$), 3.73 – 3.56 (m, 2H, NCH$_2$), 3.50 – 3.32 (m, 2H, NCH$_2$), 3.20 – 3.02 (m, 2H, NCH$_2$), 2.76 (m, 2H, OCH$_2$), 1.94 – 1.61 (m, 4H, CH$_2$), 1.56 – 1.28 (m, 8H, CH$_2$). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 180.3, 179.9, 165.2, 149.8, 149.7, 140.3, 137.2, 135.8, 135.6, 132.54, 132.51, 132.4, 132.0 (2×C), 131.5, 131.4, 129.2 (2×C), 126.2 (2×C), 125.4, 124.24, 124.21, 123.7 (2×C), 121.9, 105.9, 105.8, 65.8, 65.6, 52.8, 52.6, 41.6, 41.5, 28.9 (2×C), 24.6 (2×C), 21.2 (2×C). LRMS (ESI$^+$) $m/z$ = 1002.45 (M+H).

1,3-bis(4-(3-(2-N-methoxy-2-nitrophenoxy)sulfonamido)ethyl)thiourea (11a). The reaction was carried out following the general procedure with 6 (750 mg, 2.4 mmol) and 9a (2.07 g, 6.0 mmol). Purification by silica chromatography (10 g prepacked cartridge) eluting with hexane/EtOAc (100/0 → 0/100) yielded 11a as light green solid (774 mg, 91%).

HPLC (UV) > 95%; mp > 182 ºC (dec.). $^1$H NMR (400 MHz, DMSO) $\delta$ 9.60 (br s, 2H, NH), 8.69 (br s, 2H, NH), 8.14 – 7.98 (m, 6H, ArH), 7.97 – 7.87 (m, 2H, ArH), 7.73 (br s, 2H, NH), 7.42 (d, $J = 8.6$, 4H, ArH), 7.24 (d, $J = 8.6$, 4H, ArH), 3.83 (s, 6H, OCH$_3$), 3.75 (m, 4H, NCH$_2$), 3.29 – 3.17 (m, 4H, NCH$_2$). $^{13}$C NMR (101 MHz, DMSO) $\delta$ 180.6 (2×C), 152.5, 149.3 (2×C), 136.8 (2×C), 136.5 (2×C), 132.6, 132.0, 131.9 (2×C), 124.7
1,3-bis(4-(3-(2-(N-tetrahydro-2H-pyran-2-yl)oxy-2-nitrophenylsulfonamido)ethyl)thioureido)phenylurea (11c). The reaction was carried out following the general procedure with 6 (722 mg, 2.44 mmol) and 9c (2.1 g, 6.26 mmol). Purification by silica chromatography eluting with hexane/EtOAc (100/0 → 0/100) yielded 11c as light yellow solid (806 mg, 32%). HPLC (UV) > 95%; mp 164–166 ºC. $^1$H NMR (400 MHz, DMSO) $\delta$ 9.68 (br s, 2H, NH), 8.70 (br s, 2H, NH), 8.12 – 8.00 (m, 6H, ArH), 7.97 – 7.89 (m, 2H, ArH), 7.43 (d, $J$ = 8.8, 4H, ArH), 7.31 (br s, 2H, NH), 7.21 (d, $J$ = 8.8, 4H, ArH), 5.11 – 4.75 (m, 2H, OCH$_2$), 4.13 – 3.93 (m, 2H, OCH$_2$), 3.79 – 3.62 (m, 4H, NCH$_2$), 3.59 – 3.46 (m, 2H, OCH$_2$), 3.42 – 3.35 (m, 2H, OCH$_2$), 3.35 – 3.22 (m, 4H, NCH$_2$), 3.25 (m, 4H, NCH$_2$), 2.84 (s, 4H, PhCH$_2$), 1.18 (t, $J$ = 7.0, 6H, OCH$_2$CH$_3$).

$^1$C NMR (101 MHz, DMSO) $\delta$ 180.3 (2×C), 152.5, 148.9 (2×C), 137.0 (2×C), 136.6 (2×C), 132.2 (2×C), 124.9 (2×C), 124.2 (4×C), 123.9 (2×C), 118.7 (4×C), 104.9 (2×C), 64.0 (2×C), 52.0 (4×C), 40.6 (4×C), 28.3 (2×C), 24.3 (2×C), 19.7 (2×C). LRMS (ESI$^+$) $m/z$ = 1017.91 (M+H).

$N,N'$-(((ethane-1,2-diylbis(4,1-phenylene))bis(azanediyl))bis(carbonothioyl))bis(azanediyl))bis(ethane-2,1-diyl)bis(N-ethoxy-2-nitrobenzenesulfonamide) (12b). The reaction was carried out following the general procedure with 7 (740 mg, 2.5 mmol) and 9b (1.81 g, 6.23 mmol). The product was purified by precipitation from MeOH. Light yellow solid (1.98 g, 91%). HPLC (UV) = 95%, mp 198–200 ºC. $^1$H NMR (400 MHz, DMSO) $\delta$ 9.66 (br s, 2H, NH), 8.05 (m, 6H, ArH), 7.97 – 7.89 (m, 2H, ArH), 7.80 (br s, 2H, NH), 7.29 (d, $J$ = 8.5, 4H, ArH), 7.22 (d, $J$ = 8.5, 4H, ArH), 4.09 (q, $J$ = 7.0, 4H, OCH$_2$CH$_3$), 3.75 (m, 4H, NCH$_2$), 3.25 (m, 4H, NCH$_2$), 2.84 (s, 4H, PhCH$_2$), 1.18 (t, $J$ = 7.0, 6H, OCH$_2$CH$_3$).
$^{13}\text{C}\text{ NMR (101 MHz, DMSO)}\  \delta \ 180.5\ (2\times\text{C}),\ 149.2\ (2\times\text{C}),\ 137.8\ (2\times\text{C}),\ 136.7\ (2\times\text{C}),\ 136.4\ (2\times\text{C}),\ 132.0\ (2\times\text{C}),\ 132.0\ (2\times\text{C}),\ 128.6\ (4\times\text{C}),\ 124.1\ (2\times\text{C}),\ 123.7\ (2\times\text{C}),\ 123.3\ (4\times\text{C}),\ 73.4\ (2\times\text{C}),\ 51.8\ (2\times\text{C}),\ 41.0\ (2\times\text{C}),\ 36.5\ (2\times\text{C}),\ 13.3\ (2\times\text{C}).\ LRMS\ (ESI^+)\ m/z = 875.69\ (M+H).

$\text{N,N'}-(((\text{ethane-1,2-diylbis(4,1-phenylene)})\text{bis(azanediyl)})\text{bis(carbonothioyl)})\text{bis(azanediyl)})\text{bis(ethane-2,1-diyl)})\text{bis(2-nitro-N-((tetrahydro-2H-pyran-2-yl)oxy)benzenesulfonamide)}$ (12c). The reaction was carried out following the general procedure with 7 (722 mg, 2.44 mmol) and 9c (2.1 g, 6.26 mmol). The product was purified by precipitation from cold CH$_2$Cl$_2$. Recrystallization in cold CH$_2$Cl$_2$ yielded 12c as white solid (1.25 g, 52 %). HPLC (UV) = 92 %; mp 104–105 °C. $^1\text{H}\text{ NMR (400 MHz, DMSO)}\  \delta \ 9.76\ (\text{br s, 2H, NH}),\ 8.13 – 8.00\ (\text{m, 6H, ArH}),\ 7.98 – 7.90\ (\text{m, 2H, ArH}),\ 7.39\ (\text{br s, 2H, NH}),\ 7.25\ (\text{s, 8H, ArH}),\ 5.01 – 4.97\ (\text{m, 2H, OCH}),\ 4.08 – 3.98\ (\text{m, 2H, OCH$_2$}),\ 3.76 – 3.65\ (\text{m, 4H, NCH$_2$}),\ 3.58 – 3.49\ (\text{m, 2H, NCH$_2$}),\ 3.34 – 3.30\ (\text{m, 2H, NCH$_2$}),\ 2.84\ (\text{s, 4H, PhCH$_2$}),\ 2.83 – 2.76\ (\text{m, 2H}),\ 1.88 – 1.77\ (\text{m, 2H}),\ 1.67\ (\text{s, 2H}),\ 1.56 – 1.34\ (\text{m, 8H}).\ ^{13}\text{C}\text{ NMR (101 MHz, DMSO)}\  \delta \ 180.2\ (2\times\text{C}),\ 149.0\ (2\times\text{C}),\ 138.3\ (2\times\text{C}),\ 136.6\ (2\times\text{C}),\ 136.3\ (2\times\text{C}),\ 132.2\ (2\times\text{C}),\ 128.9\ (2\times\text{C}),\ 124.3\ (2\times\text{C}),\ 123.9\ (2\times\text{C}),\ 123.8,\ 104.9\ (2\times\text{C}),\ 64.0\ (2\times\text{C}),\ 52.0\ (2\times\text{C}),\ 40.6\ (2\times\text{C}),\ 36.6\ (2\times\text{C}),\ 28.3\ (2\times\text{C}),\ 24.3\ (2\times\text{C}),\ 19.7\ (2\times\text{C}).\ LRMS\ (ESI^+)\ m/z = 988\ (M+H).

$\text{N,N'}-(((\text{9,10-dihydroanthracene-2,6-diyl)})\text{bis(azanediyl)})\text{bis(carbonothioyl)})\text{bis(azanediyl)})\text{bis(ethane-2,1-diyl)})\text{bis(N-ethoxy-2-nitrobenzenesulfonamide)}$ (13b). The reaction was carried out following the general procedure with 8 (0.61 g, 2.08 mmol) and 9b (1.49 g, 5.2 mmol). The product was purified by silica chromatography with hexanes/EtOAc (80/20 → 0/100) yielded 13b as light brown solid (562 mg, 32%). HPLC (UV) = 95%; mp 126–127 °C. $^1\text{H}\text{ NMR
(400 MHz, DMSO) δ 9.67 (br s, 2H), 8.16 – 7.98 (m, 6H), 7.98 – 7.90 (m, 2H), 7.77 (br s, 2H), 7.33 (m, 2H), 7.27 (d, J = 8.0, 2H), 7.14 (d, J = 8.0, 2H), 4.11 (q, J = 7.1, 4H), 3.87 (s, 4H), 3.79 – 3.67 (m, 4H), 3.30 – 3.17 (m, 4H), 1.18 (t, J = 7.1, 6H). 13C NMR (101 MHz, DMSO) δ 180.6 (2×C), 149.2 (2×C), 137.2, 136.7, 136.4 (2×C), 132.9, 132.25, 132.19, 127.5 (2×C), 124.1 (2×C), 123.8 (2×C), 122.5 (2×C), 121.5 (2×C), 73.4 (2×C), 51.8 (2×C), 41.0 (2×C), 35.0 (2×C), 13.3 (2×C). (ESI+) m/z = 873.7 (M+H).

N,N'-((((9,10-dihydroanthracene-2,6-diyl)bis(azanediyl))bis(carbonothioyl))bis(azanediyl))bis(ethane-2,1-diyl))bis(2-nitro-N-((tetrahydro-2H-pyran-2-yl)oxy)benzenesulfonamide) (13c). The reaction was carried out following the general procedure with 8 (0.722 g, 2.44 mmol) and 9c (2.1 g, 6.26 mmol). Purification by silica chromatography with hexanes/EtOAc (100/0 → 0/100) yielded 13c as brown solid (846 mg, 35%). HPLC (UV) > 90%; mp 116–118 ºC. 1H NMR (400 MHz, DMSO) δ 9.79 (br s, 2H), 8.14 – 8.01 (m, 6H), 7.99 – 7.91 (m, 2H), 7.37 – 7.25 (m, 4H), 7.17 – 7.05 (m, 2H), 4.92 – 4.88 (m, 2H), 4.14 – 4.06 (m, 2H), 3.89 (s, 4H), 3.80 – 3.64 (m, 2H), 3.61 – 3.44 (m, 4H), 3.14 – 3.06 (m, 2H), 2.81 (m, 2H), 1.76 (m, 2H), 1.61 (m, 2H), 1.32 (m, 8H). 13C NMR (101 MHz, DMSO) δ 180.2 (2×C), 149.8 (2×C), 137.5, 136.6, 136.2, 133.6, 132.25, 132.19, 127.7 (2×C), 124.2 (2×C), 123.8 (2×C), 122.9 (2×C), 122.0 (2×C), 104.9 (2×C), 64.0 (2×C), 52.0 (2×C), 40.5 (2×C), 35.0 (2×C), 28.3, 24.2 (2×C), 19.7 (2×C), 14.1 (2×C). LRMS (ESI+) m/z = 985.75 (M+H).

4. General procedure for the synthesis of 1-alkoxy-2-arylaminoimidazolines 14c, 15a, 15c, 16b, 16c, 17b and 17c from the corresponding thioureas. To a stirred solution of thiourea in dry DMF (100 mL) was added thiophenol (6 equiv.) and K2CO3 (12 equiv.). After removal of the nosyl protecting groups (checked by HPLC–MS) the reaction mixture was diluted with dry DMF (100 mL). The reaction mixture was stirred
for several days at room temperature (time indicated for each compound) until formation of the final products (checked by HPLC–MS). Solvents were removed under vacuum and the crude residue was dissolved in CH$_2$Cl$_2$. The precipitate was filtered off and the filtrate was washed with 5% aqueous NaHCO$_3$ solution (1 × 50 mL) and brine (1 × 50 mL). The organic phase was dried (MgSO$_4$) and evaporated to yield the crude 1-alkoxy-2-arylaminoimidazoline. Compounds were purified by silica chromatography (10 g SI cartridge) using CH$_2$Cl$_2$/MeOH: 100/0 → 90/10 (14c, 15c, 16c and 17c) or CH$_2$Cl$_2$/ NH$_3$-saturated MeOH: 100/0 → 90/10 (15d). Compounds 15a and 16b were purified by neutral alumina chromatography using CH$_2$Cl$_2$/ NH$_3$-saturated MeOH: 100/0 → 90/10.

$N$-$(4-((1$-hydroxy$-4,5$-dihydro$-1H$-imidazol$-2$-yl)$amino$)phenyl$)-4-((1$-((tetrahydro$-2H$-pyran$-2$-yl)$oxy$)-4,5$-dihydro$-1H$-imidazol$-2$-yl)$amino$)benzamide (14c). The reaction was carried out following the general procedure with 10c (570 mg, 0.57 mmol), thiophenol (0.349 mL, 3.41 mmol) and K$_2$CO$_3$ (943 mg, 6.38 mmol) at room temperature for 5 days. Purification by silica chromatography as described above yielded 14c as brown solid (147 mg, 46%). HPLC (UV) = 95%; mp 121–123 ºC. $^1$H NMR (300 MHz, CDCl$_3$) δ 8.03 (br s, 1H, CO$_2$NH), 7.80 (d, $J = 8.7$, 2H, ArH), 7.57 (dd, $J = 2.4$, 8.7, 4H, ArH), 7.42 (d, $J = 8.7$, 2H, ArH), 4.95 – 4.80 (m, 2H, OCH$_2$), 4.21 – 4.07 (m, 2H, OCH$_2$), 3.71 – 3.54 (m, 8H, NCH$_2$), 3.39 – 3.25 (m, 2H, OCH$_2$), 2.01 – 1.73 (m, 4H, CH$_2$), 1.69 – 1.64 (m, 8H, CH$_2$). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 165.4, 159.6, 159.1, 143.8, 136.5, 133.1, 128.3 (2×C), 127.9, 121.3 (2×C), 119.2 (2×C), 117.7 (2×C), 105.1, 104.9, 66.4, 66.0, 53.8 (2×C), 49.8, 48.9, 29.4, 29.4, 25.0 (2×C), 21.7, 21.5. LRMS (ESI$^+$) m/z = 564.47 (M+H).

$1,3$-bis(4-((1$-methoxy$-4,5$-dihydro$-1H$-imidazol$-2$-yl)$amino$)phenyl$)urea (15a). The reaction was carried out following the general procedure with 11a (1.59 g, 1.8
mmol), thiophenol (0.49 mL, 4.8 mmol) and K$_2$CO$_3$ (1.32 g, 9.6 mmol) at room temperature for 7 days. Purification by alumina chromatography as described above yielded 15a as brown solid (83 mg, 10%). HPLC (UV) = 97%; mp > 135 ºC (dec.). $^1$H NMR (400 MHz, DMSO) δ 9.54 (br s, 2H, NH), 7.45 (d, $J$ = 8.8, 4H, ArH), 7.37 (d, $J$ = 8.8, 4H, ArH), 3.77 (s, 6H, OCH$_3$), 3.58 (t, $J$ = 7.1, 4H, NCH$_2$), 3.48 (t, $J$ = 7.1, 4H, NCH$_2$). $^{13}$C NMR (101 MHz, DMSO) δ 159.0, 152.8 (2×C), 136.7 (2×C), 132.1 (2×C), 122.6 (4×C), 118.4 (4×C), 63.1 (2×C), 50.6 (2×C), 43.5 (2×C). LRMS (ES$^+$) m/z = 439.45 (M+H).

1,3-bis(4-((1-((tetrahydro-2H-pyran-2-yl)oxy)-4,5-dihydro-1H-imidazo-2-yl)amino)phenyl)urea (15c). The reaction was carried out following the general procedure with 11c (570 mg, 0.8 mmol), thiophenol (0.348 mL, 3.41 mmol) and K$_2$CO$_3$ (943 mg, 6.83 mmol) at room temperature for 5 days. Purification by silica chromatography as described above yielded 15c as brown solid (53 mg, 11%). HPLC (UV) > 90%; mp 108–110 ºC. $^1$H NMR (300 MHz, CDCl$_3$) δ 7.87 (br s, 2H, NH), 7.26 – 7.14 (m, 8H, ArH), 4.88 (d, $J$ = 5.0, 2H, OCH), 4.19 – 3.98 (m, 2H, OCH$_2$), 3.66 – 3.46 (m, 8H, NCH$_2$), 3.45 – 3.19 (m, 2H, OCH$_2$), 2.07 – 1.71 (m, 4H, CH$_2$), 1.70 – 1.38 (m, 8H, CH$_2$). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 159.8 (2×C), 153.9, 135.9 (2×C), 134.0 (2×C), 120.7 (4×C), 120.0 (4×C), 104.5 (2×C), 65.5 (2×C), 53.7 (2×C), 48.1 (2×C), 29.1 (2×C), 24.9 (2×C), 21.8 (2×C). LRMS (ESI$^+$) m/z = 579.52 (M+H).

$N,N'$-(ethane-1,2-diylbis(4,1-phenylene))bis(1-ethoxy-4,5-dihydro-1H-imidazol-2-amine) (16b). The reaction was carried out following the general procedure with 12b (900 mg, 1.1 mmol), thiophenol (0.68 mL, 6.6 mmol) and K$_2$CO$_3$ (1.82 g, 13.2 mmol) at room temperature for 7 days. Purification by alumina chromatography as described above yielded 16b as brown solid (45 mg, 5.6%). HPLC (UV) = 97%; mp 65–70 ºC. $^1$H NMR (400 MHz, CDCl$_3$) δ 7.28 (d, $J$ = 8.4, 4H, ArH), 7.05 (d, $J$ = 8.4, 4H, ArH), 5.28
(br s, 2H, NH), 3.98 (q, J = 7.0, 4H, OCH₂CH₃), 3.57 (t, J = 7.2, 4H, NCH₂), 3.39 (t, J = 7.2, 4H, NCH₂), 2.81 (s, 4H, PhCH₂), 1.31 (t, J = 7.0, 6H, OCH₂CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 159.1 (2×C), 138.4 (2×C), 136.1 (2×C), 129.1 (4×C), 127.1 (4×C), 119.4 (2×C), 70.9 (2×C), 53.0 (2×C), 47.2 (2×C), 37.4 (2×C), 14.4 (2×C). LRMS (ESI⁺) m/z = 437.49 (M+H).

N₂N'-(ethane-1,2-diylbis(4,1-phenylene))bis(1-((tetrahydro-2H-pyran-2-yl)oxy)-4,5-dihydro-1H-imidazol-2-amine) (16c). The reaction was carried out following the general procedure with 12c (448 mg, 0.45 mmol), thiophenol (0.275 mL, 2.7 mmol) and K₂CO₃ (745 mg, 5.4 mmol) at room temperature for 13 days. Purification by silica chromatography as described above yielded 16c as brown solid (86 mg, 33%). HPLC (UV) > 99%; mp 200–201 ºC. ¹H NMR (400 MHz, CDCl₃) δ 7.36 (d, J = 8.2, 4H, ArH), 7.05 (d, J = 8.2, 4H, ArH), 4.86 (s, 2H, OCH), 4.14 (m, 2H, OCH₂), 3.86 – 3.71 (m, 2H, NCH₂), 3.70 – 3.55 (m, 6H, NCH₂), 3.37 – 3.24 (m, 2H, OCH₂), 2.80 (s, 4H, PhCH₂), 1.98 – 1.73 (m, 4H, CH₂), 1.68 – 1.46 (m, 8H, CH₂). ¹³C NMR (101 MHz, CDCl₃) δ 159.6 (2×C), 135.5 (2×C), 129.0 (2×C), 118.3 (2×C), 104.9 (2×C), 66.1 (2×C), 54.0 (2×C), 49.7 (2×C), 37.6 (2×C), 29.5 (2×C), 25.0 (2×C), 21.6 (2×C). LRMS (ESI⁺) m/z = 549.56 (M+H).

N²N⁶-bis(1-ethoxy-4,5-dihydro-1H-imidazol-2-yl)-9,10-dihydroanthracene-2,6-diamine (17b). The reaction was carried out following the general procedure with 13b (615 mg, 0.64 mmol), thiophenol (0.39 mL, 3.85 mmol) and K₂CO₃ (1.06 g, 7.72 mmol) at room temperature for 7 days. Purification by silica chromatography as described above yielded 17b as brown solid (30 mg, 11%). HPLC (UV) = 92%; mp 125–127 ºC. ¹H NMR (300 MHz, CDCl₃) δ 7.38 (s, 2H, ArH), 7.13 (s, 4H, ArH), 5.72 (m, 2H, NH), 3.96 (q, J = 7.0, 4H, OCH₂), 3.83 (s, 4H, CH₂), 3.57 (m, 4H, CH₂), 3.39 (m, 4H, CH₂), 1.27 (d, J = 7.0, 6H, CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 159.2 (2×C=N), 138.7 (2×Ar-
$N^2,N^6$-bis(1-((tetrahydro-2H-pyran-2-yl)oxy)-4,5-dihydro-1H-imidazol-2-yl)-9,10-dihydroanthracene-2,6-diamine (17c). The reaction was carried out following the general procedure with 13c (800 mg, 0.81 mmol), thiophenol (0.5 mL, 4.87 mmol) and K$_2$CO$_3$ (1.34 g, 9.74 mmol) at room temperature for 10 days. Purification by silica chromatography as described above yielded 17c as brown solid (188 mg, 43%). HPLC (UV) = 90%; mp 130–132 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.7 (s, 2H), 7.34 (s, 4H, ArH), 7.11 (s, 2H, ArH), 5.1 (m, 2H, OCH), 4.0 (m, 2H, OCH$_2$), 3.70 (s, 4H, CH$_2$), 1.8 (m, 4H) 1.6 (m, 8H, CH$_2$). $^{13}$C NMR (101 MHz, DMSO) $\delta$ 160.0 (2×C=N), 155.0 (2×C), 137.9 (Ar-C), 135.1 (Ar-C), 132.4 (2×Ar-CH), 128.8 (Ar-C), 128.4 (2×Ar-CH), 127.7 (Ar-C), 127.2 (Ar-CH), 124.0 (Ar-CH), 103.4 (2×CH), 62.2 (2×CH$_2$), 52.8 (2×CH$_2$), 41.1 (2×CH$_2$), 34.8 (2×CH$_2$), 27.3 (2×CH$_2$), 24.5 (2×CH$_2$), 18.5 (2×CH$_2$). LRMS (ESI$^+$) m/z = 547.41 (M+H).

5. General Procedure for THP group removal. A stirred solution of 14c–17c in MeOH (1 mL) was treated with HCl$_g$-saturated dioxane solution (3 mL). The reaction mixture was stirred at room temperature overnight and the volatiles were evaporated under vacuum. Compounds 14d, 15d, and 17d were recrystallized from MeOH/acetone and rinsed with Et$_2$O. Compound 16d was recrystallized from tPrOH and rinsed with Et$_2$O.

4-((1-hydroxy-4,5-dihydro-1H-imidazol-2-yl)amino)-N-(4-((1-hydroxy-4,5-dihydro-1H-imidazol-2-yl)amino)phenyl)benzamide (14d). Compound 14c (130 mg, 0.238 mmol) was treated as described above to yield 14d as white solid (91.6 mg, 97%). HPLC (UV) > 99%; mp 164–165 °C. Dihydrochloride salt of 14d: $^1$H NMR (400 MHz,
DMSO) δ 11.15 (s, 1H, NH), 11.09 (s, 1H, OH), 11.01 (s, 1H, NH), 10.77 (s, 1H, NH), 10.72 (s, 1H, NH), 9.36 (s, 1H, OH), 9.02 (s, 1H, NH), 8.16 (d, J = 8.7, 2H, ArH), 7.96 (d, J = 8.9, 2H, ArH), 7.51 (d, J = 8.7, 2H, ArH), 7.32 (d, J = 8.9, 2H, ArH), 3.82 – 3.70 (m, 4H, NCH$_2$), 3.65 – 3.52 (m, 4H, NCH$_2$).

$^{13}$C NMR (101 MHz, DMSO) δ 164.6 (C=O), 160.1 (C=N), 159.5 (C=N), 138.22 (Ar-C), 132.19 (Ar-C) 132.2 (Ar-C), 130.2 (Ar-C), 129.3 (2 × Ar-CH), 124.8 (2 × Ar-CH), 123.2 (2 × Ar-CH), 121.3 (2 × Ar-CH), 52.0 (NCH$_2$), 51.8 (NCH$_2$), 40.6 (NCH$_2$), 40.4 (NCH$_2$). LRMS (ESI$^+$) m/z = 396.33 (M+H). HRMS (ESI$^+$) C$_{19}$H$_{21}$N$_7$O$_3$ requires: 395.1706 (found: 395.1720).

1,3-bis(4-((1-hydroxy-4,5-dihydro-1H-imidazol-2-yl)amino)phenyl)urea  (15d).

Compound 15c (10 mg, 0.017 mmol) was treated as described above to yield 15d as brown solid (6 mg, 84%). HPLC (UV) = 96 %; mp 170–175 ºC. Dihydrochloride salt of 15d: $^1$H NMR (300 MHz, DMSO) δ 10.79 (s, 2H, NH), 10.64 (s, 2H, NH), 9.65 (s, 2H, OH), 8.89 (s, 2H, NH), 7.55 (d, J = 8.6, 4H, ArH), 7.25 (d, J = 8.6, 4H, ArH), 7.38 – 3.62 (m, 4H, NCH$_2$), 3.60 – 3.45 (m, 4H, NCH$_2$). $^{13}$C NMR (101 MHz, DMSO) δ 160.3 (C=O), 152.7 (2 × C=N), 139.0 (2 × Ar-C), 128.2 (2 × Ar-C), 125.5 (2 × Ar-CH), 118.4 (2 × Ar-CH), 52.1 (2 × CH$_2$), 48.6 (2 × CH$_2$). LRMS (ESI$^+$) m/z = 411.31 (M+H). HRMS (ESI$^+$) C$_{19}$H$_{22}$N$_8$O$_3$ requires: 410.1815 (found: 410.1807).

2,2'-(ethane-1,2-diylbis(4,1-phenylene))bis(azanediyl)bis(4,5-dihydro-1H-imidazol-1-ol)  (16d). Compound 16c (50 mg, 0.091 mmol) was treated as described above to yield 16d as brown solid (23.7 mg, 68%). HPLC (UV) = 95 %; mp > 224 ºC (dec.). Dihydrochloride salt of 16d: $^1$H NMR (400 MHz, DMSO) δ 11.04 (s, 2H, OH), 10.77 (s, 2H, NH), 9.00 (s, 2H, NH), 7.35 (d, J = 7.8, 4H, ArH), 7.26 (d, J = 7.8, 4H, ArH), 3.73 (t, J = 7.7, 4H, NCH$_2$), 3.54 (t, J = 7.7, 4H, NCH$_2$), 2.91 (s, 4H, CH$_2$). $^{13}$C NMR (101 MHz, DMSO) δ 159.9 (2 × C=N), 140.3 (2 × Ar-C), 132.9 (2 × Ar-C), 129.5 (4 × Ar-CH), 124.1 (4 × Ar-CH), 51.9 (2 × NCH$_2$), 40.5 (2 × NCH$_2$), 36.2 (2 × CH$_2$).
LRMS (ESI⁺) m/z = 381.43 (M+H). HRMS (ESI⁺) C_{20}H_{24}N_{6}O_{2} requires: 381.1961 (found: 381.1585).

2,2′-((9,10-dihydroanthracene-2,6-diyl)bis(azanediyl))bis(4,5-dihydro-1H-imidazol-1-ol) (17d). Compound 17c (96 mg, 0.18 mmol) was treated as described above to yield 17d as light brown solid (45.9 mg, 69%). HPLC (UV) = 97%; mp > 130 °C (dec.). Dihydrochloride salt of 17d: ¹H NMR (300 MHz, DMSO) δ 10.80 (br s, 2H, NH), 10.77 (s, 2H, NH), 8.92 (br s, 2H, OH), 7.43 (d, J = 8.0, 2H, ArH), 7.33 (d, J = 2.0, 2H, ArH), 7.17 (dd, J = 8.0, 2.0, 2H, ArH), 3.96 (s, 4H, CH₂), 3.77 – 3.67 (m, 4H, NCH₂), 3.60 – 3.51 (m, 4H, NCH₂). ¹³C NMR (101 MHz, DMSO) δ 159.6 (2×C=N), 148.7 (2 × Ar-C), 138.6 (Ar-C), 136.1 (Ar-C), 132.7 (2 × Ar-CH), 129.2 (Ar-C), 128.1 (2 × Ar-CH), 125.4 (Ar-C), 123.5 (Ar-CH), 122.7 (Ar-CH), 50.3 (2 × NCH₂), 44.0 (2 × NCH₂), 36.2 (2 × CH₂). LRMS (ESI⁺) m/z = 379.33 (M+H). HRMS (ESI⁺) C_{20}H_{22}N_{6}O_{2} requires: 378.1804 (found: 378.1792).

**Biology.** (i) *Cultivation of parasites, in vitro activity, and cytotoxicity.* For all the susceptibility assays with parasites and L6-cells, each compound was tested in duplicate and each assay was repeated at least once.

**(a) Activity against *T. brucei.*** The in vitro trypanocidal and cytotoxic activities were determined using an Alamar blue-based assay (11, 12). Detailed experimental protocols for these assays with *T. b. rhodesiense* STIB900 and rat skeletal myoblast L6-cells (ATCC: CRL-1458) have been reported before (13). A slight modification of this protocol (i.e., 5-fold higher parasite load and longer –24 h– incubation time with Alamar blue before reading) was used for the assays with wild type and resistant *T. b. brucei* strains, as described (14). The TbAT1-KO strain is derived from the wild type strain *T. b. brucei* Lister 427 (s427) by deletion of the *TbAT1* gene (15). The B48 strain
is a mutant derived from TbAT1-KO by in vitro selection to high levels of pentamidine, and does not express a functional High Affinity Pentamidine Transporter (HAPT1) (16). The STIB900 strain was isolated in 1982 from a human patient in Tanzania and, after several mouse passages, was cloned and adapted to axenic culture conditions (17, 18).

(b) Activity against *T. cruzi*, *L. donovani*, and *P. falciparum*. IC\textsubscript{50} values against amastigotes of *L. donovani* strain MHOM/ET/67/L82 were determined using an Alamar blue-based assay (11, 12). IC\textsubscript{50} values against erythrocytic stages of *P. falciparum* was determined by a [\textsuperscript{3}H]hypoxanthine incorporation assay (19) using the chloroquine- and pyrimethamine-resistant strain K1, which originated from Thailand (20). IC\textsubscript{50} values against amastigote 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 (21). Detailed experimental protocols for all of these assays have been reported before (13).

(ii) In vivo antityranosomal activity. (a) *T. b. rhodesiense* (STIB900) acute mouse model. This mouse model mimics the first stage of the disease. Four female NMRI mice were used per experimental group. Each mouse was inoculated i.p. with 10\textsuperscript{4} bloodstream forms of STIB900, respectively. Heparinized blood from a donor mouse with approximately 5 × 10\textsuperscript{6} /mL parasitaemia was suspended in PSG to obtain a trypanosome suspension of 1 × 10\textsuperscript{5} /mL. Each mouse was injected with 0.25 mL. Compounds were formulated in 100% DMSO and diluted 10-fold in distilled water. Compound treatment was initiated 3 days post-infection on four consecutive days for all administration routes (i.p., p.o.) in a volume of 0.1 mL/10 g. Four mice served as infected-untreated controls. They were not injected with the vehicle alone since we have established in our laboratory, over many years, that these vehicles do not affect
parasitaemia nor the mice (results not shown). Parasitaemia was monitored using smears of tail-snip blood twice a week after treatment for two weeks followed by once a week until 60 days post-infection. Mice were considered cured when there was no parasitaemia relapse detected in the tail blood over the 60-day observation period. Mean relapse days (MRD) were determined as day of relapse post-infection of mice.

(b) *T. b. brucei* (GVR35) Mouse CNS Model. The GVR35 strain was isolated from a wildebeest in the Serengeti in 1966 (primary isolate S10) (22). The GVR35 mouse CNS model mimics the second stage of the disease. Five female NMRI mice per experimental group were used. Each mouse was inoculated i.p. with $2 \times 10^4$ bloodstream forms. The treatment was i.p. in a volume of 10 mL kg$^{-1}$ at five consecutive days from day 17 to 21 post-infection. A control group was treated on day 17 with a single dose of diminazene aceturate at 40 mg/kg of body weight i.p., which is subcurative since it clears the trypanosomes only in the haemolymphatic system and not in the CNS, leading to a subsequent reappearance of trypanosomes in the blood (22). Parasitaemia was monitored twice in the first week after treatment followed by once a week until 180 days postinfection. Mice were considered cured when there was no parasitemia relapse detected in the tail blood over the 180-day observation period. Surviving mice were euthanized on day 180, and the day of death of the animals recorded (including the cured mice, as >180) to calculate the MRD.

All the in vivo efficacy studies in mice (STIB900 and GVR35 models) were conducted at the Swiss Tropical and Public Health Institute (Basel) according to the rules and regulations for the protection of animal rights ("Tierschutzverordnung") of the Swiss "Bundesamt für Veterinärwesen". They were approved by the veterinary office of Canton Basel-Stadt, Switzerland.
(iii) **In vitro BBB permeability studies. (a) Cell line culture.** The human cerebral endothelial monolayers hCMEC/D3 were grown in EBM-2 medium (Lonza, Basel, Switzerland) supplemented with 5% fetal bovine serum ‘Gold,’ 10 mM hydroxyethyl-piperazineethane sulfonic acid (HEPES) (PAA Laboratories GmbH, Pasching, Austria), 1% Penicillin–Streptomycin, 1% chemically defined lipid concentrate (Invitrogen Ltd, Paisley, UK), 1.4 mM hydrocortisone, 5 mg/mL ascorbic acid, and 1 ng/mL basic fibroblast growth factor (bFGF) (Sigma-Aldrich, St Louis, MO, USA). Cells were seeded at a density of 50 000 cells/cm² in precoated, with rat type I collagen (R&D Systems, Minneapolis, MN, USA), Transwell® culture insert (0.4 mm pore size; Corning, Lowell, MA, USA) and grown 6 days at 37 °C in a humidified incubator in 5% CO₂. The medium was changed 3 days after seeding.

(b) **Permeability assays.** The compounds were dissolved in distilled water (10 mM stock solution) and the permeability assays were performed with the hCMEC/D3 human brain endothelial monolayer as described earlier (8). Prior to compound permeability studies, the working concentration of the compounds (i.e., concentration that does not disturb the cellular complex between endothelial cells) was determined using the fluorescent dye lucifer yellow [LY, Sigma, St Louis, MO, USA] as a marker of tight junction integrity. The working concentrations used were 100 µM for pentamidine, I, 14d, and III, and 50 µM for V. Briefly, the experiments were run as follows: after 6 days of culture, coated culture inserts with and without endothelial cell were transferred to 6-well plates containing 2.6 mL of transport buffer (Hanks buffer saline solution with CaCl₂ and MgCl₂, 10 mM HEPES and 1 mM sodium pyruvate (Invitrogen Ltd, Paisley, UK), and when required 0.1% BSA) 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 occurred 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 I, 14d, III, V, and pentamidine). Endothelial permeability was calculated from the clearance rate and the surface area, as previously described (8, 23, 24). Data represent means of three independent culture inserts per condition.

To temporarily destroy tight-junctions between cerebral endothelial cells, a hyperosmotic solution of D-mannitol (1.4 M in EBM-2) was applied to cells 30 min prior permeability studies.

**(c) 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 defrosted and stored at 4 °C the day before the HPLC analysis. The samples were shaken with an orbital shaker for 40 min and the analytical HPLC was run with a Waters Sunfire C18–3.5 µm (4.6×50mm) column on a Waters 2690 separation module with Waters 996 photodiode array detector. The following HPLC conditions were used: column temperature = 30 °C, flow rate = 1 mL/min, volume of injection = 10 µL. The solvent mixture was H$_2$O (+0.05% CF$_3$CO$_2$H):CH$_3$CN (+0.05% CF$_3$CO$_2$H) with the following proportions: pentamidine (isocratic 20:80, gradient time = 10 min), I (isocratic 10:90, gradient time = 5 min), 14d (isocratic 7:93, gradient time = 5 min), III (isocratic 15:85, gradient time = 5 min), and V (isocratic 10:20, gradient time = 5 min). The analytical wavelength was 262 nm (pentamidine), 275 nm (compounds I and 14d), 234 nm (compound III), and 280 nm (compound V). 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 obtained by serial double dilution in the assay buffer starting from 150 µM (pentamidine, I, III, and V), or 120 µM for 14d. 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.

Physicochemical studies. (i) pKa determination by UV-vis spectrophotometry. Ionization constants were measured by UV-spectrophotometry using 96-well microtitre plates as described elsewhere (25). The method consists in the simultaneous determination of the compound’s UV-spectra as a function of pH. The compounds in stock solution in DMSO are dissolved in different aqueous buffer solutions directly in the microtitre plate (the maximum amount of DMSO in the final solution is 2% v/v). Further treatment of the data generates the pKa values in a medium-throughput manner. All the pKa values were measured at 30 ºC, at a concentration of 0.2 mM and constant ionic strength (I = 0.1 M).

(ii) SPR–biosensor measurements of HSA binding. Surface plasmon resonance (SPR) experiments were performed at 25 ºC with a Biacore X–100 apparatus (GE Healthcare, Biacore AB, Uppsala, Sweden). (a) Immobilization of HSA. Essentially fatty acid and globulin free HSA (Sigma-Aldrich) was used without further purification. A stock solution was prepared in phosphate buffered saline (PBS, pH 7.4) and stored at -20 ºC. Immediately prior to use, this solution was diluted to a concentration of 100 µg/mL in 10 mM sodium acetate, pH 5.0. HSA was immobilized on CM5 sensor chips (Biacore) by the use of amine-coupling chemistry. The surface was blocked by 7-min
injection with 1 M ethanolamine, pH 8.0. The immobilization level ranged between 8000 and 9000 RU.

**(b) Ranking experiments.** Control drugs (warfarin, phenytoin, prednisone, and sulfanilamide) and compounds I, 14a, 14d, 15e, 16b, 16d, IV, and V were prepared as 10 mM stock solutions in 100% dimethyl sulfoxide (DMSO). The stock solutions were diluted in PBS containing DMSO to reach a final concentration of 40 µM in PBS containing 3% DMSO. Binding studies were performed at a flow rate of 90 µL/min and 40 s association and 60 s dissociation times. Regeneration was not required between injections cycles. To clean the flow system, an extra wash with 50% DMSO was performed between each injection. Binding responses were corrected for solvent (DMSO bulk differences) by using the software available on the Biacore X-100. Several measurements of warfarin binding to HSA were carried out over the course of the experiment as a mean of control of the HSA binding efficiency throughout the assay. The dose-response curves were obtained by plotting the RU/Da*100 against the drug concentrations. At working concentration (40 µM), all of the compounds gave measurable responses. Positive controls were used ranking from warfarin to prednisone and the graph of HSA binding levels could be divided into regions of low, intermediate, and high binding responses (Fig. 3). Sulfanilamide was a negative control as it hardly binds to HSA

**RESULTS**

**Chemistry.** The lead compounds I–V (Fig. 1) were synthesized as previously reported (9, 26). Their spectroscopic and analytical data were in agreement with the reported ones. The N-alkoxy derivatives 14–17a-e were synthesized in 3 steps from the
corresponding diamines 1–4 following a protocol developed previously in our laboratory (Fig. 2) (8, 27). Diamines 1–4 were converted to the isothiocyanates 5–8 in high yield using thiophosgene in H$_2$O/Et$_2$O. Addition of 2 equivalents of the N-(2-aminoethyl)-N-alkoxy-2-nitrobenzenesulfonamide reagent 9 (27) to 5–8 gave the thioureas 10–13. Some of these intermediate thioureas were isolated by crystallization or silica chromatography (compounds 10c, 11a, 11c, 12b–c, and 13b–c) whereas the others (10a–b, 11b, 11d, 12a, and 13a) were detected by HPLC–MS in the reaction and the crude product was used as such in the next steps. Nosyl groups removal and intramolecular cyclization to generate the imidazoline target compounds 14–17a–e were performed either in a one pot procedure (compounds 14a–b, 15b, 15d, 16a, and 17a) or in two steps (compounds 14c, 15a, 15c, 16b, 16c, and 17c) with isolation of the intermediate thiourea (vide infra). Compound 17a was contaminated with approximately 20% of anthracene 18a which probably results from the competitive oxidation of the intermediates (13a and/or 17a) during the deprotection-cyclization steps in presence of sulfur reagent (28). Since we were not able to separate both compounds 17a was assayed as a mixture. The N-hydroxy derivatives 14d–17d were obtained smoothly as hydrochloride salts by acidic hydrolysis of the THP-protected precursors 14c–17c.

[Insert Fig 2 here]

**In Vitro Antiprotozoal Activity.** The N-alkoxy and N-hydroxy derivatives 14–17a–e were assayed in vitro against *T. brucei rhodesiense* using an Alamar blue-based assay (11). Their cytotoxicity against mammalian cells (i.e., rat L6-cells) was also evaluated.
In addition the compounds were screened against other related parasites (T. cruzi, L. donovani, and P. falciparum). The new compounds showed little activity against T. cruzi or L. donovani amastigotes and moderate activities (IC\(_{50}\) in the range 0.6–6.3 µM) against the chloroquine/pyrimethamine-resistant strain of P. falciparum. These values represent a > 30-fold loss of activity against P. falciparum as compared to the leads I–V (Table 1). In contrast, eight compounds (14d, 15d, 15e, 16a–16d, and 17c) showed low micromolar IC\(_{50}\) values (≤ 10 µM) against T. brucei trypomastigotes, four compounds (14a–14c, and 15c) had IC\(_{50}\)s in the range 10–20 µM, and two compounds (15a and 15b) displayed poor activity (> 20 µM) (Table 1). These values represent a 20 to 160-fold loss of activity against T. b. rhodesiense vs the lead compounds I–V. Some general trends were observed regarding the in vitro anti-T. brucei activity of the N-OR derivatives; the influence of the N-substituent in order of decreasing activity was: OBn > OH > OTHP > OEt > OMe. Importantly, compounds with OH substituents were less cytotoxic than the rest of the molecules, including the parent lead compounds, and showed the best selectivity indices among the new compounds. In contrast, the N-OTHP analogues (14c–17c) were the most cytotoxic from each series. The N-hydroxy derivative 14d displayed the best in vitro efficacy against T. brucei with submicromolar IC\(_{50}\) value (0.89 µM) and excellent selectivity (SI > 240) for the parasite.

[Insert TABLE 1 Here]

In vitro activity against drug resistant T. brucei strains. Cross-resistance with existing trypanocidal drugs such as pentamidine or melarsoprol is a drawback that is commonly observed with diamidine-like compounds. Diamidines are substrates of the
TbAT1/P2 aminopurine transporter (29), and of the high and low affinity pentamidine transporters (HAPT and LAPT, respectively) (30). Loss of one or more of these transporters has been linked to cross-resistance between arsenicals and diamidine drugs (31). Since our compounds are diamidine-like and hold the P2-transporter recognition motif (32) we tested their activity against two different drug resistant strains of trypanosomes (i.e., TbAT1-KO and B48). Hence, the most active analogue, 14d, and its parent compound I were tested against wild type (WT) and drug-resistant strains of T. brucei to establish their resistance profile. The parent compound I was approximately 5-fold more active against wild type than against either of the drug-resistant T. b. brucei strains (Table 2) indicating that cellular uptake of this compound is partly dependent on the TbAT1 transporter (compare WT vs TbAT1-KO), whereas the expression of HAPT1 did not appear to influence sensitivity to these compounds (compare TbAT1-KO vs B48). In contrast, 14d showed similar activity against the three T. brucei strains indicating that these transporters are not essential for uptake of this compound in vitro. We conclude that I is dependent on the presence of transporters for uptake across biological membranes, as the absence of TbAT1 in trypanosomes reduces its activity. In contrast, 14d diffuses transporter-independently across the same membranes.

[Insert TABLE 2 here]

In vivo antitrypanosomal activity. Mouse model of acute HAT. The in vivo efficacy of the new derivatives displaying the best in vitro activities and selectivity (i.e. IC₅₀ < 10 µM and SI >10) was checked in the STIB900 murine model of sleeping sickness (Table 3). In this stringent stage 1 model, mice infected with T. b. rhodesiense
parasites develop an acute haemolymphatic infection that is difficult to cure. Each compound was administered to groups of 4 mice in a 4-day schedule either by oral (po) or intraperitoneal (ip) route. The mean day of relapse of parasitaemia and the number of animals cured (i.e., animals that survived and were parasite-free for 60 days) was calculated for each group (Table 3). The lead compounds were very effective in this model as they were curative with ip dosage as low as 5 mg/kg (I, II, and V) and 10 mg/kg (III, IV). Moreover, compounds I and V were also curative by oral route at 50 mg/kg/day po. Among the new compounds, the methoxy (14a, 16a, and 17a), ethoxy (14b and 16b), and benzyloxy (15e) derivatives were inactive both by intraperitoneal and oral routes. The methoxy analogue 16a appeared to be toxic by ip administration at the dose tested. No toxicity was observed after oral treatment, but the compound was inactive by this route. In this series, the most active molecules were the hydroxy derivatives (14d, 17d, 15d, and 16d) that were able to increase moderately the mean day of relapse of parasitaemia at 5, 20, 40, and 50 mg/kg/day ip, respectively. The N-hydroxy analogue 14d was 100% curative in this model with a dosage as low as 20 mg/kg ip. Oral administration was only weakly effective at the dose tested (50 mg/kg).

[Insert TABLE 3 here]

**Mouse model of chronic (CNS stage) HAT.** The capacity of the lead compounds I–V and the most active derivative, 14d, to cure the CNS stage of the disease was investigated using the GVR35 mouse model. In this model of chronic infection, trypanosomes have invaded the brain of the animals and are present in the cerebrospinal fluid (22). To cure this stage of trypanosomiasis, the drugs must be able to cross the
BBB. In these experiments diminazene aceturate, a very efficient trypanocidal drug for stage 1 animal trypanosomiasis, was used as a negative control, being unable to penetrate into the brain. In contrast, melarsoprol cures this mouse model at a dose of 15 mg/kg given on the intraperitoneal route on 5 consecutive days (33). As shown in Table 4, the lead compounds I–IV were inactive in this model (as a dosage of 50 mg/kg ip for 5 days) gave no better protection than diminazene aceturate. In contrast, the lead compound V increased by 131% the mean parasite-free survival time, $T_S$, compared to control animals treated with diminazene aceturate ($P < 0.01$), possibly indicating modest brain permeation.

The hydroxy derivative 14d was inactive in this model of CNS infection when administered at a dosage of 5×40 mg/kg/day ip. However, in another experiment, using a higher dosage and different treatment regimen (100 mg/kg/day ip given as twice daily dose of 50 mg/kg), 14d increased $T_S$ to 143% of control ($P < 0.01$) although no cures were obtained (Table 4). Altogether the in vivo results indicate that the $N$-hydroxy derivative 14d is safer than I (i.e. a higher dosage is tolerated) and exhibits improved activity against brain infection with trypanosomes.

[Insert TABLE 4 here]

**Physicochemical properties. $pK_a$ determination.** The $pK_a$ of a drug is an essential physicochemical parameter that gives information on the ionization state of the molecule at physiological pH. The knowledge of this parameter is important because the bis-2-aminoimidazoline lead compounds I–V are dibasic molecules ($pK_a$ in the range 9.29–10.71) that will be dications at pH 7.4. This may result in poor diffusion across the
BBB which may explain in part their poor efficacy in the CNS-stage of the disease compared to the early-stage model. Our intention when synthesizing $N$-alkoxy and $N$-hydroxy derivatives was to reduce the $pK_a$ of these leads to decrease the proportion of the ionized form of the compounds in biological fluids.

The $pK_a$ values of these dibasic compounds were measured by UV-spectrophotometry in a medium throughput manner using a 96-wells microtitre plate-based protocol recently developed in our group (25). The results of experimental $pK_a$ as well as other calculated physicochemical properties are shown in Table 5. The introduction of O-alkoxy substituents on the imidazoline nitrogen reduced the basicity of the lead compounds by approximately 2 $pK_a$ units as expected. However, the nature of the O-alkyl substituent did not alter the $pK_a$ within the homologous series (e.g., compare 14a–d). The $pK_a$ of $N$-OR substituted $N$-phenylbenzamide derivatives (14a–d) is in the range 7.2–7.4. Hence these compounds will be approximately 50% ionized at physiological pH (Table 5) whereas the $pK_a$ of the urea and ethylene derivatives (15a–b and 16a–d, respectively) is about 0.5 $pK_a$ units higher, which means approximately 80% ionization under physiological conditions.

[Insert TABLE 5 here]

**SPR–biosensor analysis of serum protein binding.** Human Serum Albumin (HSA) is one of the most relevant plasma proteins affecting drug distribution in the body (34). HSA plays a critical role in transporting drugs, metabolites and endogenous ligands (35). Since high binding to plasma protein reduces the free drug concentration, decreased antiparasitic activity is expected for high level HSA binders. Some classes of
antibiotics, for instance, provide a well-documented examples of this relationship between antimicrobial activity and the unbound concentration at the site of infection (36). Hence, knowing the binding levels of our compounds to HSA should provide essential information to anticipate their pharmacokinetic behaviour in man. In the present study, the amount of compound binding to HSA at a single concentration (40 µM) was determined by SPR–biosensor binding experiments using previously reported protocols (37, 38). Four drugs with different binding levels to HSA were used as controls: warfarin, phenytoin, prednisone, and sulfanilamide. This allowed the classification of the compounds into regions of high, intermediate, and low binding to HSA, respectively (Fig. 3). All compounds tested showed reversible binding to HSA. A representative set of compounds (i.e. lead compounds, N-OH, N-OMe, N-OEt, and N-OBn derivatives) including the most interesting one (14d) was assayed. The dicationic lead compounds (I, IV, and V) and analogue 15e exhibited intermediate binding (in the range of prednisone and phenytoin), whereas N-alkoxy (14a, 16b) and N-hydroxy (14d, 16d) derivatives displayed low binding to HSA. This low binding level to plasma proteins is expected to positively influence the antiparasitic activity of these compounds by increasing their free concentration in the blood.

[Insert Fig. 3 here]

**In vitro study of hepatic metabolism by amidoxime reductases.** Since the most active compound (14d) in the late-stage disease is a N-hydroxy imidazoline derivative that is structurally related to N-hydroxyguanidines and amidoximes, we investigated whether this compound could be reduced to the parent compound (I) by amidoxime
reductases under the same conditions described for amidoxime (e.g. benzamidoxime, \(N\)-hydroxypentamidine) and \(N\)-hydroxyguanidine prodrugs (e.g. guanoxabenz, \(N\)-hydroxydebrisoquine) (39-41). This is important because if 14d is metabolized by amidoxime reductases, it may work as a prodrug of compound I.

Incubations of 14d with human liver microsomes or human liver mitochondria in the presence of NADH as cofactor were carried out in two different buffers (pH 6 and 7.4) for 60 min. No reduction of 14d was observed by RP-HPLC when compared with the corresponding controls analyzed under the same conditions but without enzymatic fractions (a chromatogram is presented as Supporting Information, Figure S2). The absence of reduction of 14d to the double \(N\)-dehydroxylated compound I under the conditions investigated was confirmed by HPLC-MS. No intermediate metabolites corresponding to a single \(N\)-dehydroxylation were observed in those incubations when compared with controls. In contrast, benzamidoxime, which is commonly used as a reference substrate of \(N\)-hydroxylamine or amidoxime reductases, was reduced to benzamidine by the same hepatic subcellular fractions. The metabolic rate obtained for the formation of benzamidine from benzamidoxime was 0.86 and 0.26 nmoles/min/mg of protein for human liver microsomes and human liver mitochondria, respectively (details of these experiments can be found in Supporting Information). We therefore conclude that 14d does not work as a prodrug of I and its efficacy against \textit{T. brucei} is most probably due to its intrinsic activity against the parasite (IC\(_{50}\) = 0.89 \(\mu\)M, SI > 240).

**In vitro determination of compound’s permeability across the blood–brain barrier.**

The capacity of compounds 14d and V (which showed significant activity in the mouse model of CNS infection) to cross the BBB was measured in vitro using the immortalized human brain endothelial cell line hCMEC/D3 (42) which has proved
useful as an in vitro BBB model (43). This cellular model is able to discriminate between compounds with low, medium and high permeability across the BBB. The permeability of the reference drug pentamidine, which is known to have low CNS bioavailability and does not cure late-stage HAT (44), and III, were also measured as a means of comparison.

In order to measure distribution across a hCMEC/D3 layer, cells were cultured in a Boyden chamber-like system, with the luminal and abluminal compartments representing the blood and cerebral compartments respectively (32). Test compounds were loaded in the luminal compartment and, after a preset time, their amounts in both compartments were determined by HPLC, yielding the mass balance. Then, the clearance principle (i.e., the slope of the volume of drug cleared from the luminal compartment to the abluminal one versus time) was used to calculate the permeability coefficients (23, 45). The small hydrophilic compound Lucifer Yellow (LY), diffusion of which is strongly restricted at the BBB level, was used as marker of paracellular permeation and as control for low permeability. LY permeability (1.63 +/- 0.09 × 10^{-3} cm/min) was attributed the value of 100%; pentamidine permeability was very similar (Supplementary material, Fig. S3a, 91% vs 100%).

The lead compounds I, III, and V all displayed similar permeability values (133%, 142% and 138%, respectively; Figure S3a). Although slightly higher than that of LY, these values represent a low BBB permeability in this model when compared to phenytoin (5.62 × 10^{-3} cm/min, 345%) and diazepam (120 × 10^{-3} cm/min, 7362%), used as markers for medium and high BBB permeability, respectively (46). The permeability value of 14d was significantly lower than that of the lead I in this assay. Possible metabolism of the compounds by hCMEC/D3cells was disregarded as the mass balance for all of the compounds was ~100% in all the experiments.
To check whether our assay was able to detect the passage of 14d despite its low permeability, the endothelial layer was hyperosmotically challenged with a 1.4 M solution of mannitol, temporarily disrupting tight-junctions, allowing free paracellular diffusion. Hyperosmotic shock robustly increased LY and 14d permeabilities (Fig. S3b, 692% vs 100% for LY alone, and 217% vs 49% for 14d alone, respectively). However, 14d may reach the brain by different routes than the paracellular pathway or free diffusion across both endothelial plasma membranes (e.g. transcytosis or active transport) (47). Bovine serum albumin (BSA) was added to the buffer in order to increase any transcytosis of 14d, but if anything reduced permeability for this compound (Fig. S3b, 28% compared to 49% for 14d alone).

**DISCUSSION**

New N-alkoxy and N-hydroxy derivatives of bisimidazoline lead compounds were synthesized to reduce their polarity and potentially improve their in vivo antitrypanosomal activity against late-stage sleeping sickness. To treat this stage of the disease, which is characterized by the presence of trypanosomes into the brain and cerebrospinal fluid, drugs with the ability to cross the BBB are required. Since bisimidazolines are dicationic molecules at physiological pH their BBB permeability may be compromised as previously observed with other dicationic compounds. In the case of the diamidine drugs pentamidine and furamidine, which are carried into the brain through influx transporters, limited CNS availability is the result of the concomitant sequestration within the capillary endothelium and rapid efflux by P-glycoproteins (44, 48). In the present study, we have shown that N-OR substituted imidazoline derivatives have lower pKa values compared to their unsubstituted
analogues (approximately 2 $pK_a$ units) and are therefore less ionized at physiological pH. Compound 14d, which is only 50% ionized at pH 7.4, was the most active compound of the series, being curative in the haemolymphatic stage and weakly active against the CNS-stage of the infection in mice. In contrast, derivatives 14a–b, which have $pK_a$ values similar to that of 14d, were inactive probably due to their low in vitro activity against the parasite. Alternatively, this may be the result of different interactions with the putative diamidine transporters and/or P-gp efflux pumps expressed at the BBB (44).

Compound 14d showed similar susceptibilities towards wild type and drug resistant strains of trypanosomes lacking the $Tb$AT1/P2 aminopurine transporter and the HAPT transporter ($Tb$AT1-KO and B48, respectively). This indicates that these transporters are not essential for uptake of this compound in vitro. Hence, 14d should not be prone to cross-resistance with other trypanocidal drugs such as diamidines and arsenicals (31).

The more active antitrypanosomal compounds in the chronic model of HAT (14d and V) presented a low in vitro permeability value in the hCMEC/D3 model of BBB. However, differential expression of transporters and metabolizing enzymes at the BBB is one of the main factors that significantly contribute to the inter-individual variation in the bioavailability and vulnerability to drugs and xenobiotics (49, 50). Hence, species differences could account for the divergences observed between in vitro BBB permeability assays with human cells and in vivo results in murine models of HAT. It is possible that, in contrast to the passive diffusion hypothesis, brain uptake of 14d happens mainly through a specific transporter that is expressed in mice but not in the human hCMEC/D3 cell line (51). In fact, slight differences regarding ABC transporters expression have been observed between this cell line and human brain isolated microvessels (52). Moreover, the di-cationic diamidine DB829 is curative in the same
murine model of late-stage trypanosomiasis, although its close analogues DB75 and
diminazene have no activity at all against the cerebral infection (53). This is clear proof
that highly specific transporters for diamidine analogues exist on the murine BBB, with
sufficient capacity to facilitate curative levels of such compounds into the brain (54).
Moreover, the fact that 14d hardly binds to plasma proteins (< 20%) would give rise to
a higher free plasma concentration of the compound, which in turn would make it more
available to such solute transporters and lead to higher activity in vivo in the mouse
model of stage 2 sleeping sickness.

Altogether, these data show that the hydroxy derivative 14d is more effective in the
CNS-stage of HAT than the unsubstituted lead I and it has larger therapeutic index in
vivo in mice. Apparently, 14d is able to reach the brain in a specific manner, across an
intact BBB but it does not cross the BBB in sufficient amounts to cure the CNS
infection with the dosage/administration scheme used in this study. Further
investigations to elucidate the nature of the transporter(s) involved will be needed, as
well as a complete pharmacokinetic analysis to determine the levels of 14d in the mouse
brain.

The observations with 14d appear similar to those with the N-methoxyamidine prodrug
pafuramidine (DB289) which, in contrast to the active diamidine DB75, penetrates the
BBB unaltered and is subject to biotransformation within the brain (48). However,
unlike DB289, cerebral uptake of 14d is not believed to be the result of simple diffusion
across the BBB, and we have further shown here that 14d was not metabolized in vitro
by amidoxime reductases that are present in human hepatic microsomal and
mitochondrial fractions. Hence, 14d is likely to be the active molecule that inhibits
trypanosome growth within the brain.
Among the new compounds reported here, the N-hydroxy derivatives 14d–17d have shown the best activity/selectivity profiles in vitro and higher activity in vivo compared to other N-alkoxy derivatives. On the one hand, these results confirm previous findings on similar series of N-hydroxy imidazolines (8). On the other hand, this is different from a series of diamidoxime (i.e., di-N-hydroxy amidines) metabolites which were found to be less active in vivo than the monoamidoxime/monomethoxyamidine metabolites of the dimethoxyamidine prodrugs (53).

Regarding the mode of action of the antitrypanosomal lead compounds I–V, earlier studies have shown that this class of dicationic compounds binds to the DNA minor groove at AT-rich sequences (55-58). In trypanosomes, the kinetoplast DNA (kDNA) has a high content of AT-rich regions that could be a site of selective action of this kind of compound (59). In fact some correlation between DNA binding and in vitro antitrypanosomal activity was observed suggesting a mode of action due in part to the formation of a DNA complex (9). Interestingly, we have observed that the N-alkoxy (14a-c–16a-c) and N-hydroxy derivatives (14d–16d) did also bind selectively to AT-rich DNA, some of them, as 14d, with affinity in the same range as the unsubstituted, dicationic lead compound (C. H. Ríos Martínez and C. Dardonville, unpublished data). Further work to elucidate the interaction of these compounds with DNA will be the subject of a different paper.

**Conclusion.** We have shown here that introducing alkoxy and hydroxy substituents (–OR; R = H, Me, Et) at position N1 of the imidazoline rings reduces the basicity of aminoimidazoline compounds by approximately 2 pKₐ units. While the nature of the R substituent does not alter significantly the pKₐ in a homologous series (ΔpKₐ ≤ 0.2 for R
H ≈ Me ≈ Et), its impact on in vivo activity is significant. The N-hydroxy substituted compound showed efficacy in models of first- and second-stage *T. brucei* infection in mice whereas N-OMe or N-OEt analogues did not. Besides, N-hydroxy derivatives were less cytotoxic and better tolerated in vivo. The activity profile demonstrated by 14d in vitro and in vivo in both experimental models of HAT, and the absence of hepatic metabolism observed in vitro (i.e. N-OH reduction by amidoxime reductases) indicate that 14d, and possibly other N-hydroxy imidazoline derivatives, represent a new class of promising antitrypanosomal agents. This class of compounds appears to have activity against *T. brucei*, favorable drug-resistance profile, more favorable physicochemical properties (e.g. lower pKa, low level of protein binding) and a higher therapeutic index than the unsubstituted parent compounds, indicating a good progress towards the discovery of new drugs for late-stage sleeping sickness. Hence, further optimization of these compounds in an effort to obtain CNS-curative trypanocidal agents is warranted.

Finally, it is noteworthy that the bisimidazolinium fluorene lead compound V was curative in the STIB900 mouse model for acute African trypanosomiasis whereas its guanidine analogue was inactive in vivo (60). This finding illustrates once again the superiority of the (4,5-dihydro-1H-imidazol-2-yl)amino group, compared to guanidine, as useful cationic moiety for the design of antitrypanosomal compounds.

**ACKNOWLEDGEMENTS**

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This work was awarded the “GlaxoSmithKline prize” from the Spanish Society of Medicinal Chemistry to C.R. (“XV Convocatoria de Premios para Investigadores Noveles en el Campo de la Búsqueda y Desarrollo de Nuevos Fármacos”). Thanks are given to Dr. Pierre-Olivier Couraud for logistical collaboration. A.A.E. was supported by a British Commonwealth Scholarship. The contributions of Elsa Berthaut to pKₐ measurements, and Eddysson Jamir Flores Pérez to the synthesis of 15a and 16b are gratefully acknowledged.

**Supporting Information Available.** Detailed description of the protocols used to assess the metabolism of compound 14d and benzamidoxime by amidoxime reductases. Figure S3: Permeability values in the human brain endothelial cell line hCMEC/D3 of pentamidine, I, 14d, III, and V compared to lucifer yellow (LY).

**REFERENCES**


40. **Clement B, Mau S, Deters S, Havemeyer A.** 2005. Hepatic, extrahepatic, microsomal, and mitochondrial activation of the N-hydroxylated prodrugs benzamidoxime, guanoxabenz, and Ro 48-3656 (\([1\-\{(2s)\-2\-\{(4\-\{(hydroxyamino)iminomethyl\}benzoyl}amino\}-1\-oxopropyl\}-4\-piperidinyloxy\}acetic acid). Drug Metab. Dispos. 33:1740-1747.


TABLE 1. In Vitro antiprotozoal activity of N-alkoxy and N-hydroxy analogues of the lead compounds I–V.

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>IC₅₀ (µM) for the following parasiteᵃᵇ</th>
<th>Cytotoxicity [CC₅₀ (µM)] for L6-cellsᶜ</th>
<th>SIᶜᵈ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T. b. rhodesiense</td>
<td>T. cruzi</td>
<td>L. donovani</td>
</tr>
<tr>
<td>Iᵉ</td>
<td>0.025 ± 0.002</td>
<td>ndᶠ</td>
<td>nd</td>
</tr>
<tr>
<td>14a</td>
<td>18.4 ± 4.3</td>
<td>152 ± 9</td>
<td>41.6 ± 1.4</td>
</tr>
<tr>
<td>14b</td>
<td>11.6 ± 1</td>
<td>130 ± 8</td>
<td>15.9 ± 2.7</td>
</tr>
<tr>
<td>14c</td>
<td>16.7 ± 7.6</td>
<td>37.7 ± 3.3</td>
<td>92 ± 2.3</td>
</tr>
<tr>
<td>14d</td>
<td>0.89 ± 0.07</td>
<td>161 ± 29</td>
<td>&gt;213</td>
</tr>
<tr>
<td>IIᵉ</td>
<td>0.122 ± 0.004</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>15a</td>
<td>150 ± 2</td>
<td>&gt;228</td>
<td>171 ± 81</td>
</tr>
<tr>
<td>15b</td>
<td>45.9 ± 0.9</td>
<td>&gt;192</td>
<td>96 ± 10</td>
</tr>
<tr>
<td>15c</td>
<td>19.5 ± 12.8</td>
<td>131 ± 41</td>
<td>88.3 ± 7.4</td>
</tr>
<tr>
<td>15d</td>
<td>7.9 ± 3.8</td>
<td>108 ± 3</td>
<td>&gt;206</td>
</tr>
<tr>
<td>15e</td>
<td>2.4 ± 0.3</td>
<td>13.7 ± 7.3</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>IIIᵉ</td>
<td>0.054 ± 0.004</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>16a</td>
<td>10.9 ± 2.5</td>
<td>32 ± 7</td>
<td>138 ± 57</td>
</tr>
<tr>
<td>16b</td>
<td>4.4 ± 0.1</td>
<td>41.5 ± 2.5</td>
<td>123 ± 60</td>
</tr>
<tr>
<td>16c</td>
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<td>7.8 ± 3.0</td>
<td>98 ± 41</td>
</tr>
<tr>
<td>16d</td>
<td>5.4 ± 0.4</td>
<td>111 ± 14</td>
<td>179 ± 59</td>
</tr>
<tr>
<td>IVᵉ</td>
<td>0.060 ± 0.025</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>17c</td>
<td>3.5 ± 1</td>
<td>26 ± 0.3</td>
<td>45.6 ± 12.1</td>
</tr>
<tr>
<td>Vᵉ</td>
<td>0.005 ± 0.001</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>melarsoprol</td>
<td>0.005 ± 0.003</td>
<td></td>
<td></td>
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<tr>
<td>benznidazole</td>
<td>1.35 ± 0.28</td>
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<td></td>
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<tr>
<td>miltefosine</td>
<td>0.53 ± 0.17</td>
<td></td>
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<tr>
<td>chloroquine</td>
<td>0.13 ± 0.04</td>
<td></td>
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<tr>
<td>podophyllotoxin</td>
<td>0.012 ± 0.005</td>
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</tr>
</tbody>
</table>

ᵃ IC₅₀ ± SD (average of two independent assays).ᵇ T. brucei rhodesiense STIB900 trypomastigotes, T. cruzi Tulahuen strain C2C4 intracellular amastigotes, axenically
grown *L. donovani* strain MHOM/ET/67/L82 amastigotes, and *P. falciparum* K1 strain erythrocytic stages were used.

c Rat skeletal myoblast L-6 cells.

d Selectivity index calculated as (CC$_{50}$ for L6-cells) / (IC$_{50}$ for *T. b. rhodesiense*).

e Data previously reported in reference (9) and included here for comparative purposes.

f Not determined.

**TABLE 2.** In vitro activity against WT and drug-resistant *T. brucei* strains

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µM)</th>
<th>WT$^a$</th>
<th>TbAT1-KO$^b$</th>
<th>RF$^c$</th>
<th>B48$^d$</th>
<th>RF</th>
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<tr>
<td></td>
<td></td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5.70 ± 0.96</td>
<td>3</td>
<td>25.5 ± 3.5</td>
<td>3</td>
<td>4.5</td>
<td>3</td>
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<tr>
<td>14d</td>
<td>15.5 ± 2.0</td>
<td>3</td>
<td>17.1 ± 1.9</td>
<td>3</td>
<td>1.1</td>
<td>3</td>
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<tr>
<td>pentamidine</td>
<td>0.006 ± 0.002</td>
<td>3</td>
<td>0.011 ± 0.002</td>
<td>3</td>
<td>1.8</td>
<td>3</td>
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<tr>
<td>diminazene</td>
<td>0.148 ± 0.011</td>
<td>3</td>
<td>0.738 ± 0.066</td>
<td>3</td>
<td>5.0</td>
<td>3</td>
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</table>

$^a$ *T. b. brucei* s427 trypomastigotes. Values represent IC$_{50}$ ± SEM (n ≥ 3).

$^b$ *T. b. brucei* knockout strain lacking a functional P2-transporter and resistant to diminazene aceturate (15).

$^c$ Resistance factor compared to WT.

$^d$ The B48 strain is a mutant derived from the TbAT1-KO strain with a nonfunctional High Affinity Pentamidine transporter (HAPT). This strain is resistant to diminazene, pentamidine and melaminophenyl arsenicals (16).
TABLE 3. In vivo antitypanosomal activity of lead compounds and N-substituted analogues in the *T. b. rhodesiense* (STIB900) mouse model

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>R</th>
<th>Dosage route&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Dosage (mg/kg)</th>
<th>Cured&lt;sup&gt;c&lt;/sup&gt;/Infected</th>
<th>Mean day of relapse&lt;sup&gt;d&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>0/4</td>
<td>7&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>I</strong></td>
<td>H</td>
<td>ip</td>
<td>4 × 20&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4/4</td>
<td>&gt; 60</td>
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<td></td>
<td></td>
<td>ip</td>
<td>4 × 10</td>
<td>4/4</td>
<td>&gt; 60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ip</td>
<td>4 × 5</td>
<td>4/4</td>
<td>&gt; 60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>po</td>
<td>4 × 50</td>
<td>4/4</td>
<td>&gt; 60</td>
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<tr>
<td>14d</td>
<td>OH</td>
<td>ip</td>
<td>4 × 50</td>
<td>4/4</td>
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</tr>
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<td>4/4</td>
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<td>0/4</td>
<td>7</td>
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<td></td>
<td>po</td>
<td>4 × 50</td>
<td>0/4</td>
<td>7.7 ± 1.5</td>
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<td>4 × 50</td>
<td>0/4</td>
<td>14 ± 0</td>
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<td></td>
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<td><strong>III</strong></td>
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<td>&gt; 60</td>
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<td>0/4</td>
<td>7</td>
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<tr>
<td>16d</td>
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<td>4 × 50</td>
<td>0/4</td>
<td>14 ± 0</td>
</tr>
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<td></td>
<td></td>
<td>ip</td>
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<td>4/4</td>
<td>&gt; 60</td>
</tr>
<tr>
<td></td>
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<td>ip</td>
<td>4 × 10</td>
<td>3/4</td>
<td>&gt; 60</td>
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<td><strong>IV</strong></td>
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<td>4 × 20</td>
<td>0/4</td>
<td>7.7 ± 1.5</td>
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</table>
response experiments at lower dosages (ip and po) are reported here for the first time.

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<td></td>
<td>4 × 20</td>
<td>0/4</td>
<td>18.3 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>po</td>
<td></td>
<td>4 × 50</td>
<td>0/4</td>
<td>7</td>
</tr>
<tr>
<td>V</td>
<td>H</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ip</td>
<td></td>
<td>4 × 20</td>
<td>4/4</td>
<td>&gt; 60</td>
<td></td>
</tr>
<tr>
<td>ip</td>
<td></td>
<td>4 × 10</td>
<td>3/3</td>
<td>&gt; 60</td>
<td></td>
</tr>
<tr>
<td>ip</td>
<td></td>
<td>4 × 5</td>
<td>4/4</td>
<td>&gt; 60</td>
<td></td>
</tr>
<tr>
<td>ip</td>
<td></td>
<td>4 × 50</td>
<td>3/4</td>
<td>&gt; 50</td>
<td></td>
</tr>
<tr>
<td>Melarsorpol&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ip</td>
<td>4 × 2</td>
<td>4/4</td>
<td>&gt; 60</td>
<td></td>
</tr>
<tr>
<td>Pentamidine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ip</td>
<td>4 × 5</td>
<td>1/4</td>
<td>&gt; 38</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> See experimental section for details of STIB900 (T. b. rhodesiense) model.

<sup>b</sup> ip = intraperitoneal, po = per os.

<sup>c</sup> Number of mice that survive and are parasite free for 60 days.

<sup>d</sup> Average day of relapse of parasitaemia ± SD.

<sup>e</sup> Control mice were always positive and were euthanized on day 7.

<sup>f</sup> The in vivo activity at 20 mg/kg ip was reported previously in ref. (9); the dose-response experiments at lower dosages (ip and po) are reported here for the first time.

<sup>g</sup> One mouse died during treatment (day 4).

<sup>h</sup> Data reported in reference (61).
TABLE 4. In vivo antitrypanosomal activity in the chronic (CNS-stage) phase of infection

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dosage (mg/kg)</th>
<th>Cured/Infected</th>
<th>Mean day of relapse (control group)</th>
<th>T_S (% of control)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>diminazene aceturate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 × 40</td>
<td>0/5</td>
<td>46.6 ± 5.1 (control 1)</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 × 50</td>
<td>T_b</td>
<td>46.6 ± 5.1 (control 1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5 × 20</td>
<td>0/5</td>
<td>51.2 ± 6.7 (1)</td>
<td>108%</td>
<td>0.26</td>
</tr>
<tr>
<td>14d</td>
<td>5 × 40</td>
<td>1/4 c</td>
<td>45.0 ± 5.6 (1)f</td>
<td>98%</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>5 × 100</td>
<td>0/4 e</td>
<td>52.2 ± 9.5 (2)f</td>
<td>143%</td>
<td>0.0052</td>
</tr>
<tr>
<td>II</td>
<td>5 × 20</td>
<td>0/5</td>
<td>42.8 ± 7.9 (1)</td>
<td>91%</td>
<td>0.39</td>
</tr>
<tr>
<td>III</td>
<td>5 × 50</td>
<td>0/5 g</td>
<td>40 ± 1.7 (1)</td>
<td>85%</td>
<td>0.081</td>
</tr>
<tr>
<td>IV</td>
<td>5 × 50</td>
<td>0/5 g</td>
<td>43 ± 5.3 (1)</td>
<td>91%</td>
<td>0.38</td>
</tr>
<tr>
<td>V</td>
<td>5 × 50</td>
<td>0/4 e</td>
<td>49 ± 0 (3)</td>
<td>131%</td>
<td>0.0035</td>
</tr>
</tbody>
</table>

Summary of three experiments using the GVR35 mouse model of late-stage trypanosomiasis. All administrations were by the intraperitoneal route; diminazene treatment was used as a control and the controls for the three experiments are given separately, representing average days of relapse of parasitaemia ± SD for 5 (control 1) or 6 mice (controls 2 and 3). All treatments were initiated on day 17 post infection (p.i.). Mice that survived and remained parasite-free after treatment up to 180 days p.i. were considered cured. Statistical significance was assessed for each group against their control group using an unpaired Student’s t-test. n/a, not applicable.

a The number between brackets identifies the control group for each experiment.

b All mice died after 4th treatment (T, terminated).

c In this experiment, 3 mice relapsed, on day 39, 46 and 50 (not statistically different from control 1), and one mouse survived negative 180 days but was discarded in the calculation of the MDR as a probable outlier.

d Compound given as twice daily dose of 50 mg/kg (b.i.d.).

e One mouse died during treatment and was excluded from experiment.
In this experiment, 4 mice relapsed on day 51, 51, 42 and 65.

Two mice died during treatment, assumed to be due to toxicity of the compound.
**TABLE 5.** Calculated physicochemical parameters, and experimental ionization constants.

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>MW&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HBD</th>
<th>clogP&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>log D&lt;sub&gt;7.4&lt;/sub&gt;&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>PSA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>exp. pK&lt;sub&gt;a&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>% ionization at pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>363</td>
<td>6</td>
<td>1.67</td>
<td>-1.2</td>
<td>101.94</td>
<td>9.29 ± 0.07&lt;sup&gt;d&lt;/sup&gt;</td>
<td>98.7</td>
</tr>
<tr>
<td>14a</td>
<td>423</td>
<td>4</td>
<td>2.20</td>
<td>1.24</td>
<td>102.82</td>
<td>7.27 ± 0.09&lt;sup&gt;d&lt;/sup&gt;</td>
<td>42.8</td>
</tr>
<tr>
<td>14b</td>
<td>451</td>
<td>4</td>
<td>2.91</td>
<td>1.94</td>
<td>102.82</td>
<td>7.34 ± 0.03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>46.8</td>
</tr>
<tr>
<td>14d</td>
<td>395</td>
<td>6</td>
<td>1.44</td>
<td>0.50</td>
<td>124.82</td>
<td>7.43 ± 0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>51.8</td>
</tr>
<tr>
<td>II</td>
<td>378</td>
<td>7</td>
<td>1.73</td>
<td>-1.25</td>
<td>113.97</td>
<td>10.34 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>99.9</td>
</tr>
<tr>
<td>15a</td>
<td>438</td>
<td>5</td>
<td>2.25</td>
<td>1.26</td>
<td>114.85</td>
<td>7.95 ± 0.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>78.2</td>
</tr>
<tr>
<td>15b</td>
<td>466</td>
<td>5</td>
<td>2.97</td>
<td>1.96</td>
<td>114.85</td>
<td>8.27 ± 0.07&lt;sup&gt;d&lt;/sup&gt;</td>
<td>88.2</td>
</tr>
<tr>
<td>15d</td>
<td>410</td>
<td>7</td>
<td>1.50</td>
<td>0.52</td>
<td>136.85</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>15e</td>
<td>590</td>
<td>5</td>
<td>5.70</td>
<td>4.68</td>
<td>114.85</td>
<td>nd&lt;sup&gt;e&lt;/sup&gt;</td>
<td>nd</td>
</tr>
<tr>
<td>III</td>
<td>348</td>
<td>5</td>
<td>3.12</td>
<td>-0.27</td>
<td>72.84</td>
<td>10.71 ± 0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>99.95</td>
</tr>
<tr>
<td>16a</td>
<td>408</td>
<td>3</td>
<td>3.64</td>
<td>2.55</td>
<td>73.72</td>
<td>8.01 ± 0.12&lt;sup&gt;d&lt;/sup&gt;</td>
<td>80.4</td>
</tr>
<tr>
<td>16b</td>
<td>436</td>
<td>3</td>
<td>4.36</td>
<td>3.23</td>
<td>73.72</td>
<td>8.01 ± 0.21&lt;sup&gt;d&lt;/sup&gt;</td>
<td>80.4</td>
</tr>
<tr>
<td>16d</td>
<td>380</td>
<td>5</td>
<td>2.89</td>
<td>1.83</td>
<td>95.72</td>
<td>7.97 ± 0.13&lt;sup&gt;d&lt;/sup&gt;</td>
<td>78.7</td>
</tr>
<tr>
<td>IV</td>
<td>346</td>
<td>5</td>
<td>2.79</td>
<td>-060</td>
<td>72.84</td>
<td>9.82 ± 0.26</td>
<td>99.6</td>
</tr>
<tr>
<td>17a</td>
<td>406</td>
<td>3</td>
<td>3.32</td>
<td>2.22</td>
<td>73.72</td>
<td>7.53 ± 0.14</td>
<td>57.4</td>
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<tr>
<td>17d</td>
<td>378</td>
<td>5</td>
<td>2.56</td>
<td>1.50</td>
<td>95.72</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>V</td>
<td>332</td>
<td>5</td>
<td>2.35</td>
<td>-1.09</td>
<td>72.84</td>
<td>10.12 ± 0.17</td>
<td>99.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> The molecular weight (g/mol), clogP values, polar surface area (Å<sup>2</sup>) and logD<sub>7.4</sub> were calculated using ChemAxon software MarvinSketch v.5.11.1.

<sup>b</sup> Calculated with the weighted method considering tautomerization/resonance; electrolyte concentration = 0.1 mol/dm<sup>3</sup> and using a training set containing the experimental pK<sub>a</sub> values of 21 related compounds.

<sup>c</sup> Measured by UV-spectrophotometry in H<sub>2</sub>O/DMSO (2% v/v) at 30 ºC as described previously (25). Values are the mean of 3 independent determinations ± SD. Only one pK<sub>a</sub> could be calculated for both imidazoline rings in the molecule.

<sup>d</sup> Taken from reference (25).

<sup>e</sup> Not determined due to solubility problems in the buffers used for pK<sub>a</sub> determination.
FIG. 1. Lead compounds (R = H, dihydrochloride salts) with in vivo antitrypanosomal activity in the acute *T. b. rhodesiense* mouse model (9) and new N-alkoxy and N-hydroxy analogues [R = OMe (a), OEt (b), OTHP (c), OH (d), OBn (e)].
FIG. 2. Synthesis of 1-alkoxy-2-arylaminomidazolines.

Reagents and conditions. (i) H₂, Pd-C 5%, MeOH, rt; (ii) Activated Zn, NH₃, sealed tube, ∆; (iii) Thiophosgene, H₂O-ether, rt; (iv) 9 (2 equiv.), DMF, rt; (v) 1) PhSH (6 equiv.), K₂CO₃ (12 equiv.), DMF, rt; 2) 50 ºC; (vi) HCl/dioxane, MeOH, rt.
FIG. 3. Plot of HSA binding levels determined by SPR for the lead compounds (I, IV, and V), N-alkoxy (14a, 15e, and 16b), and N-hydroxy (14d and 16d) derivatives. The compounds were ranked as high level HSA binders (black), intermediate binders (deep grey), and low binders (pale grey) based on the binding affinity of four control drugs: warfarin, phenytoin, prednisone, and sulfanilamide, respectively.