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Antiprotozoal activity and DNA binding of *N*-Substituted *N*-phenylbenzamide and 1,3-Diphenylurea Bisguanidines

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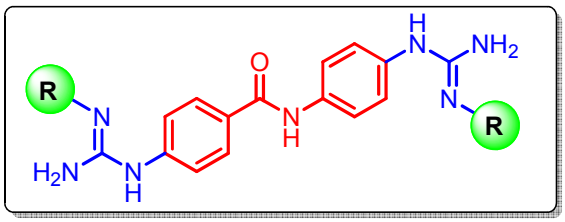
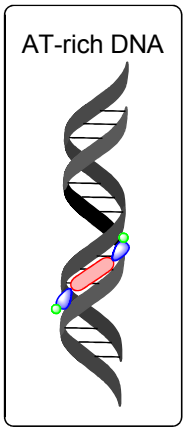
‡

‡ Abbreviations: BBB, blood-brain barrier; CNS, central nervous system; DIPEA, diisopropylethylamine; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HAT, human African trypanosomiasis; MW, microwave; NECT, nifurtimox-eflornithine combination therapy; SAR, structure-activity relationships; SI, selectivity index; SPR, surface plasmon resonance; TMSCl, trimethylsilylchloride.

Graphical abstract





	T. b. rhodesiense	*P. falciparum*
R = Et	9 nM (SI > 13000)	232 nM (SI = 500)
R = ^{i}Pr	11 nM (SI > 9000)	446 nM (SI = 230)

4/4 cures at 20 mg/kg ip

Abstract

Two series of *N*-alkyl, *N*-alkoxy, and *N*-hydroxy bisguanidines derived from the *N*-phenylbenzamide and 1,3-diphenylurea scaffolds were synthesised in three steps from the corresponding 4-amino-*N*-(4-aminophenyl)benzamide and 1,3-bis(4-aminophenyl)urea, respectively. All of the new compounds were evaluated in vitro against *T. b. rhodesiense* (STIB900) trypomastigotes and *P. falciparum* NF54 parasites (erythrocytic stage). *N*-alkoxy and *N*-hydroxy derivatives showed weak micromolar range IC_{50} values against *T. b. rhodesiense* and *P. falciparum* whereas the *N*-alkyl analogues displayed submicromolar and low nanomolar IC_{50} values against *P. falciparum* and *T. brucei*, respectively. Two compounds, 4-(2-ethylguanidino)-*N*-(4-(2-ethylguanidino)phenyl)benzamide dihydrochloride (**7b**) and 4-(2-isopropylguanidino)-*N*-(4-(2-isopropylguanidino)phenyl)benzamide dihydrochloride (**7c**), which showed favourable drug-like properties and in vivo efficacy (100% cures) in the STIB900 mouse model of acute human African trypanosomiasis represent interesting leads for further in vivo studies. The binding of these compounds to AT-rich DNA was confirmed by surface plasmon resonance (SPR) biosensor experiments.

Highlights

- *N*-alkoxy, *N*-hydroxy and *N*-alkyl bisguanidine derivatives were synthesized.
- The compounds were tested against *T. b. rhodesiense* and *P. falciparum.*
- *N*-alkylated compounds **7b** and **7c** were 100% curative in the STIB900 mouse model of HAT.
- DNA binding affinity was measured by SPR with AATT, $(AT)_4$ and $(CG)_4$ hairpin duplexes.
- **7b** and **7c** bind selectively to AT-rich DNA.

Keywords: *Trypanosoma brucei*, *Plasmodium falciparum*, parasite chemotherapy, guanidine, minor groove binder, surface plasmon resonance (SPR) biosensor.

1. Introduction

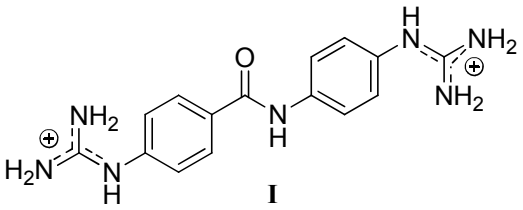
Human African trypanosomiasis (HAT) and malaria are parasitic diseases that are endemic in sub-Saharian Africa where they cause great morbidity and high mortality rates (especially for malaria). Two subspecies of trypanosomes (*Trypanosoma brucei gambiense* and *T. b. rhodesiense*) lead to chronic and acute forms of HAT, respectively [1]. The apicomplexan parasite *Plasmodium falciparum*, which is the cause of the most severe and deadly form of malaria, is mainly prelavent in Africa being responsible of over 75% of cases in that continent [2].

New drugs are required to complete and/or improve the chemotherapeutic arsenal available for both diseases. On the one hand, drugs for the treatment of HAT are scarce (i.e. pentamidine, suramin, melarsoprol, eflornithine and nifurtimox-eflornithine combination therapy – NECT –), stage and species specific, very toxic, and not orally active [3, 4]. On the other hand, malaria chemotherapy is suffering from extended resistance to conventional drugs (e.g. chloroquine) and the rapid development of *Plasmodium* strains that are resistant to "newly" marketed drugs (e.g. artemisinin) [5, 6]. In a word, new drugs are urgently needed to fight against these deadly diseases.

Dicationic compounds such as diamidines [7-10] and bis(2-aminoimidazolines) [11-13] have a track record of success as antiprotozoan agents. These compounds bind strongly to the DNA minor groove at AT-rich sites which is one of the known targets of these molecules. Accordingly dicationic diamidines were shown to be localized to the DNA-containing nucleus and/or kinetoplast of trypanosomes [14]. Bisguanidine related molecules are also very active against *T. brucei* and *P. falciparum* [11-13, 15-19]. However, low nanomolar in vitro activities do not always result in potent in vivo activity in murine models of HAT and malaria [16, 17]. Besides, this class of dibasic

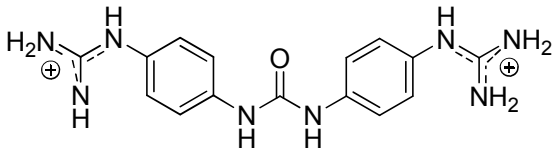
compounds is highly polar (i.e. dicationic at physiological pH) and, like pentamidine, mostly fails in curing CNS-stage *T. brucei* infections.

In recent years, we have discovered a set of bisguanidinium hits that displayed excellent activities in vitro against *T. b. rhodesiense* and *P. falciparum* [11]. Two hit compounds derived from the *N*-phenylbenzamide scaffold (**I**) and the 1,3-diphenylurea scaffold (**II**) that presented nanomolar IC_{50}s, adequate selectivities, and promising in vivo activity in the STIB900 mouse model of acute HAT were chosen as templates for further development (Figure 1). The choice of these scaffolds as antitrypanosomal templates was also supported by the excellent results obtained with bis(2-aminoimidazoline) analogues that proved to be 100% curative in vivo in the STIB900 mouse model [11].



I

IC_{50} (*T. b. rhodesiense)* = 36 nM (SI = 319)
IC_{50} (*P. falciparum* K1) = 55 nM (SI = 209)



II

IC_{50} (*T. b. rhodesiense)* = 187 nM (SI > 1256)
IC_{50} (*P. falciparum* K1) = 96 nM (SI > 2547)

Figure 1. Structure, In Vitro Activity and Selectivity Index of the *N*-Phenylbenzamide (**I**) and 1,3-Diphenylurea (**II**) Bisguanidinium Leads [11]

In previous work, Arafa *et al* [15] showed that *N*-alkyl derivatives of diguanidino fused ring systems had improved in vivo potency in the STIB900 mouse model of acute HAT

compared with the unsubstituted diguanidino parent compounds. In the present study, we tested a similar strategy with the hits **I** and **II** to check if the antiprotozoan activity of these hits could be enhanced in that way. In fact, the addition of *N*-alkyl and *N*-alkoxy substituents in the structure should increase the lipophilicity of the compounds whereas *N*-alkoxy substituents should decrease their basicity with respect to **I** and **II**. All of these modifications were expected to enhance the antiprotozoan activity of the compounds as well as to improve their membrane permeation (e.g. blood-brain barrier). Hence, six *N*-alkyl (**7a-c**, **8a-c**), four *N*-alkoxy (**7d-g**), and one hydroxy derivatives of the hits **I** and **II** were synthesised and tested against *T. b. rhodesiense* and *P. falciparum*. The most active compounds in vitro were further evaluated in vivo in the STIB900 mouse model of acute HAT. This allowed drawing preliminary structure-activity relationships (SAR) for antimalarial and antitrypanosomal activity. Besides, the DNA binding affinity of the compounds was assessed by surface plasmon resonance (SPR) biosensor experiments with three different hairpin oligonucleotides.

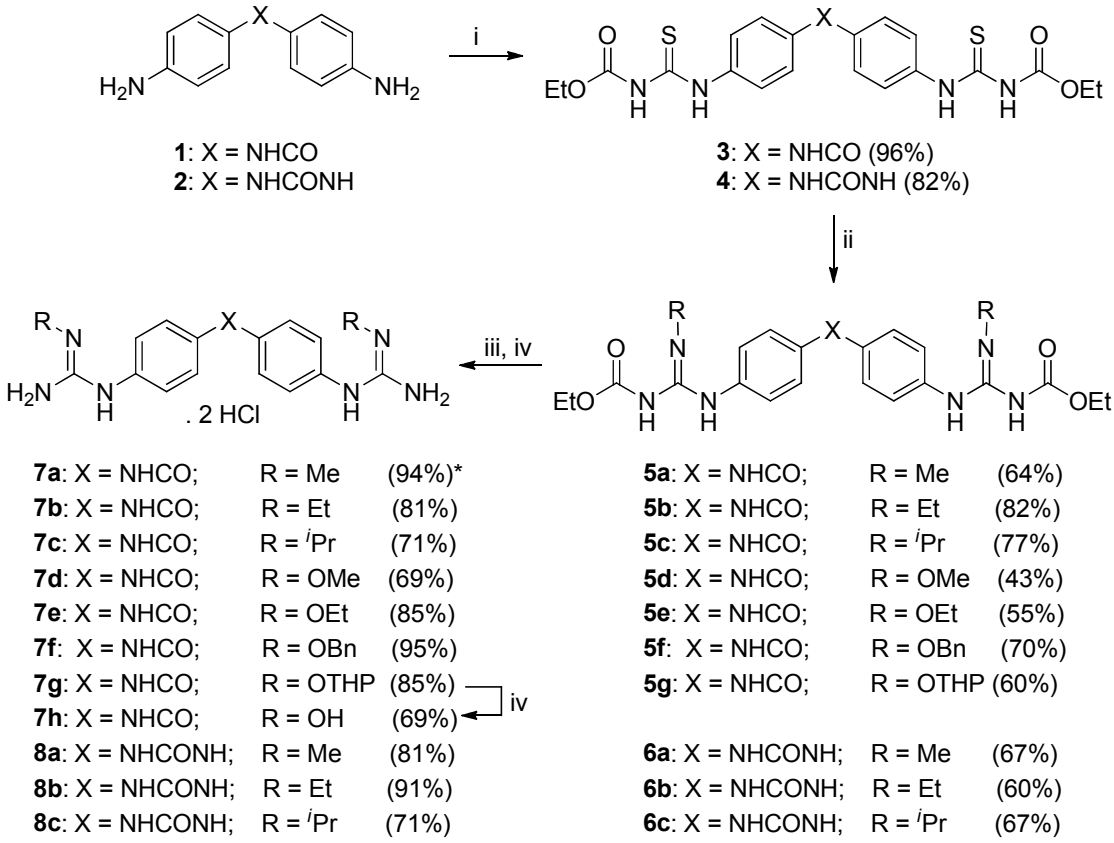
2. Results

2.1. Chemistry

N-substituted guanidines **7a-g** and **8a-c** were synthesized in three steps from diamines **1** and **2**, respectively, following the Manimala and Anslyns's methodology (Scheme 1) [20]. The synthesis started with the preparation of the bis(ethoxycarbonylthioureas **3** and **4** from 4-amino-*N*-(4-aminophenyl)benzamide and 1,3-bis(4-aminophenyl)urea, respectively. These thioureas were reacted with an excess (4 equiv.) of *N*-alkyl (R = Me, Et, ^{i}Pr) and *N*-alkoxy amines (R = OMe, OEt, OBn, OTHP) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and *N*,*N*-diisopropylethylamine

(DIPEA) to give the corresponding *N*-substituted bis(ethoxycarbonyl) guanidines **5a-g** and **6a-c**. Two strategies were tried to remove the ethoxycarbonyl groups to obtain the target guanidines **7a-h** and **8a-c**.

Scheme 1.[a] Synthesis of *N*-alkyl and *N*-alkoxy guanidines



1: X = NHCO
2: X = NHCONH

3: X = NHCO (96%)
4: X = NHCONH (82%)

7a: X = NHCO; R = Me (94%)*
7b: X = NHCO; R = Et (81%)
7c: X = NHCO; R = ^{i}Pr (71%)
7d: X = NHCO; R = OMe (69%)
7e: X = NHCO; R = OEt (85%)
7f: X = NHCO; R = OBn (95%)
7g: X = NHCO; R = OTHP (85%)
7h: X = NHCO; R = OH (69%)
8a: X = NHCONH; R = Me (81%)
8b: X = NHCONH; R = Et (91%)
8c: X = NHCONH; R = ^{i}Pr (71%)

5a: X = NHCO; R = Me (64%)
5b: X = NHCO; R = Et (82%)
5c: X = NHCO; R = ^{i}Pr (77%)
5d: X = NHCO; R = OMe (43%)
5e: X = NHCO; R = OEt (55%)
5f: X = NHCO; R = OBn (70%)
5g: X = NHCO; R = OTHP (60%)

6a: X = NHCONH; R = Me (67%)
6b: X = NHCONH; R = Et (60%)
6c: X = NHCONH; R = ^{i}Pr (67%)

[a] Reagents and conditions. (i) Ethoxycarbonyl isothiocyanate, CH_2Cl_2, rt; (ii) R–NH_2, CH_2Cl_2, DIPEA, EDAC, rt; (iii) KOH, MeOH, 80 ºC; (iv) HCl_g–saturated 1,4-dioxane solution.* Yields for step (iii).

The first strategy using Lewis acids such as trimethylsilyl (TMSCl) chloride [21, 22] and bromide (TMSBr) [20] to generate directly the guanidinium salts was mostly unsuccessful in our hands, leading to complex mixtures of partially and fully

deprotected products together with 2-alkylamino(3*H*)quinazolin-4-one by-products as previously reported [22]. Apparently, the success of this deprotection protocol is very dependent on the R group present on the guanidine moiety [e.g. compound **5c** with R = ^{i}Pr was cleanly deprotected using TMSCl (10 equiv) / DMF / 80 ºC / MW / 60 min] and was not applicable to most of our compounds. Optimal conditions for the removal of the ethoxycarbonyl groups took place under basic conditions (0.1 M aqueous KOH in MeOH at 80 ºC in a sealed tube) to yield **7a-g** and **8a-c** as free bases. Hydrochloride salts of **7a-f**, **8a-c**, and **7h** [23] were obtained from the free base guanidines **7a-f**, **8a-c**, and **7g** with HCl_{gas}–saturated 1,4-dioxane solution, respectively.

2.2. In Vitro and In Vivo Antiprotozoal Activity

The target compounds **7a-h** and **8a-c** were tested in vitro against *T. brucei rhodesiense* (STIB900 strain) by means of an Alamar blue-based assay [24]. The antimalarial activity against erythrocytic forms of *P. falciparum* was evaluated with a [^{3}H]hypoxanthine incorporation assay [25] using the chloroquine-sensitive strain NF54. The cytotoxicity of the compounds against mammalian L6-cells was also evaluated to determine their selectivity index. The results are shown in Table 1.

All of the guanidines with *N*-alkyl substituents (**7a-c** and **8a-c**) displayed submicromolar range IC_{50} values against *T. b. rhodesiense*. Compounds with ethyl (**7b**, **8b**) and isopropyl groups (**7c**, **8c**) were particularly effective with low nanomolar IC_{50} values, in the range of the reference drug melarsorprol (IC_{50} = 8 nM), and high selectivities (SI > 1000). These values represent a 2- to 3-fold increase in antitrypanosomal activity compared with the unsubstituted parent compounds **II** and **I**, respectively. In contrast, *N*-hydroxy (**7h**) and *N*-alkoxy (**7d**, **7g**) guanidines showed

weak activity. As far as the linker of the diphenyl scaffold is concerned, the amide gave 5- to 10-fold higher potencies than the urea linker. In general, most of these guanidines presented low cytotoxicity (IC_{50} > 100 μM against L6-cells) even though some differences could be observed between both series. Compound **I** derivatives (**7a-d** and **7g-h**) in particular were approximately 10-times less cytotoxic than the parent guanidine **I**. On the contrary, derivatives **8a-c** were somewhat more cytotoxic than **II**.

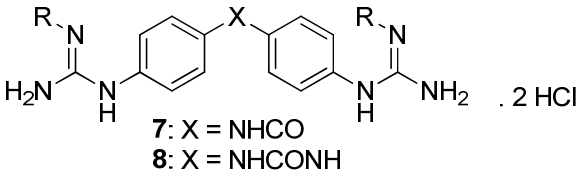
As with the anti-*T. brucei* activity, the best antimalarial compounds were the *N*-alkyl derivatives **7b-c** and **8a-c** that displayed submicromolar IC_{50} values with selectivities for the parasite > 200. Again, *N*-hydroxy and *N*-alkoxy derivatives were less potent with IC_{50}s in the low micromolar range.

To evaluate the in vivo potential of these new *N*-alkyl guanidines, the best trypanocidal compounds in vitro (**7b**, **7c**, **8b**, and **8c**) were tested in a mouse model of acute (stage 1) HAT. Groups of four mice infected with *T. b. rhodesiense* (strain STIB900) received a four days treatment (days 3, 4, 5 and 6 post-infection) with the tested compounds (4×20 mg/kg ip). One control group (3 mice) remained untreated. The blood parasitemia was checked periodically over the 60 days of the experiment and the mean day of relapse of parasitemia was calculated. The mice that survived 60 days free of parasites were considered as cured (Table 1).

The 1,3-diphenylurea analogues (**8b**, **8c**) were poorly active in vivo and showed acute toxicity at the dose tested. The lead compound **I** showed high efficacy in this model, giving 4/4 cures by intraperitoneal (4×5 mg/kg ip) and oral administration (4×50 mg/kg po). Unfortunately, it was inactive in the GVR35 model of late-stage disease (i.e. 5×20 mg/kg ip, data not shown), which is probably due to poor BBB permeation. The *N*-phenylbenzamide derivatives **7b** and **7c** were 100% curative in the STIB900 model

showing that the introduction of *N*-ethyl and *N*-isopropyl substituents is well tolerated in vivo. Altogether, the results indicate that compounds **7b** and **7c** are promising candidates for further in vivo studies (e.g. CNS stage).

Table 1. In Vitro and In Vivo Antitrypanosomal and Antimalarial Activity



Cmpd	R	*T. b. rhodesiense*[a]	*P. falciparum*[b]	Cytotoxicity[c]	STIB900 mouse model of acute HAT[d]		
		IC_{50} (Selectivity index)[e] µM		CC_{50} µM	Dosis (mg/kg)	Cured/infected	Mean day of relapse[f]
I	H	0.036[g] (319)	ND[h]	11.5[g]	4×5 ip	4/4	>60
					4×50 po	4/4	>60
II	H	0.187[g] (>1256)	ND	> 235[g]		ND	ND
7a	Me	0.177 (977)	1.02 (169)	173		ND	ND
7b	Et	0.009 (13111)	0.232 (509)	118	4×20 ip	4/4	>60
7c	^{i}Pr	0.011 (9364)	0.446 (231)	103	4×20 ip	4/4	>60
7d	OMe	56.0	7.49	141		ND	ND
7f	OBn	3.13	2.83	6.6		ND	ND
7g	OTHP	51.4	42.6	160		ND	ND
7h	OH	4.88	3.58	100		ND	ND
8a	Me	0.264 (670)	0.192 (922)	177		ND	ND
8b	Et	0.090 (1289)	0.397 (292)	116	4×20 ip	0/3[i]	18.3
8c	^{i}Pr	0.049 (2571)	0.437 (288)	126	4×20 ip	0/2[j]	10

IC_{50} values reported are the average of two independent assays and vary less than ± 50%. [a] *T. b. rhodesiense* STIB900 trypomastigotes; reference drug: melarsoprol, IC_{50} = 0.008 µM. [b] *P. falciparum* NF54, intraerythrocytic stage: this strain is susceptible to chloroquine, IC_{50} = 0.006 µM. [c] Rat skeletal myoblast L6-cells; reference drug: podophyllotoxin, IC_{50} = 0.014 µM. [d] Compounds were administrated intraperitoneally (ip) at 20 mg/kg/day (4 days). [e] SI = [CC_{50} /IC_{50} (parasite)]. [f] Untreated control mice were all positive at day 7. [g] Data previously reported in reference [11] and shown here

for comparative purpose. [h] Not determined. [i] One mouse died after first application. [j] Two mice died after 4th application.

2.3. DNA Binding Affinity. SPR–Biosensor Experiments

Since *N*-phenylbenzamide and 1,3-diphenylurea guanidine and imidazoline derivatives are strong DNA minor groove binders [11, 26-28], we were interested in quantitatively evaluating the binding affinity of the new compounds with DNA. Binding to DNA and resultant inhibition of transcription and activity of other DNA-dependent enzymes is thought to contribute to the antiprotozoal activity of dicationic compounds [29]. This was done by biosensor–SPR experiments with different DNA hairpin duplexes [i.e., AATT, $(AT)_4$, $(CG)_4$] immobilized on a chip biosensor surface [30, 31]. All of the compounds were first screened for binding to the three oligonucleotide sequences at a single concentration (25 μM). This allowed the ranking of the compounds as binders/non binders (data not shown). Then, the binding constants of the most interesting compounds with the three different DNA hairpins were determined (Table 2). Compounds **7b** and **7c** bound selectively to AT-rich DNAs (*K* values in the range $4.0 – 9.8 \times 10^5$ M^{-1}) with a slight preference for the AATT (approximately 1.5 – 2-fold) over the $(AT)_4$ sequence. The stoichiometry of binding was 2 for both AT-sequences (Figure 2). These *K* values were approximately 7 to 9-fold lower (for AATT) and 3 to 4-fold lower (for $(AT)_4$) than the values reported for the unsubstituted bisguanidine **I** [26]. Hence, in this series, the alkylation of the guanidine moieties resulted in a decrease in binding affinity to AT sequences. Besides, no important differences in binding were observed between ^{i}Pr and Et groups. These findings agree with the data reported earlier with a series of linear triaryl bisguanidines [17].

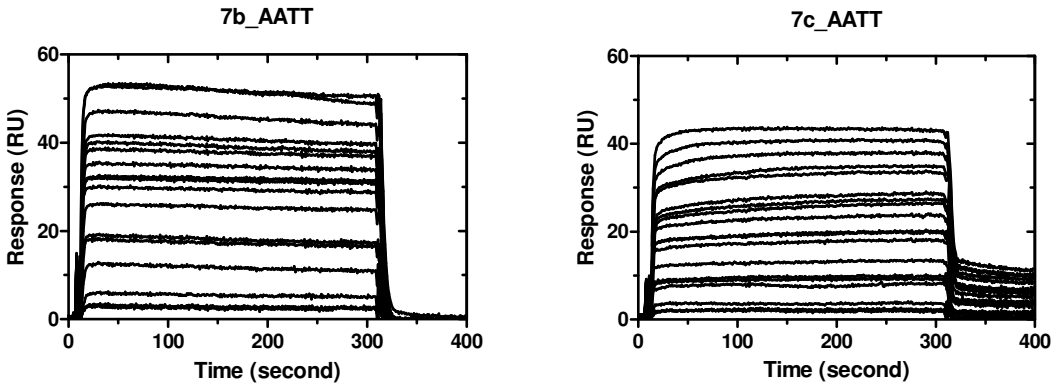
On the one hand, none of the compounds tested bound significantly to the $(CG)_4$ sequence (Figure 2B) suggesting a specific AT-rich minor groove mode of binding for **7b** and **7c**. On the other hand, binding of the urea derivative **8b** was approximately 30-times weaker than that of **7b** and **7c** ($K < 1\times 10^5$ M^{-1}) suggesting unspecific binding to DNA.

Table 2. DNA Binding Constants Determined by SPR

	K ($\times 10^5$ M^{-1})		
Compound	AATT[a]	$(AT)_4$[b]	$(CG)_4$[c]
I	71.0[d]	18.0[d]	<1[d]
7b	8.1[e]	5.2[e]	<1[g]
7c	9.8[e]	4.0[e]	<1[g]
8b	0.19[f]	0.15[e]	[h]

5'-biotin labelled DNA sequences used (the hairpin loop is underlined): [a] 5'-biotin-CGAATTCGTCTCCGAATTCG-3'. [b] 5'-biotin-CATATATATCCCCATATATATG-3'. [c] 5'-biotin-CGCGCGCGTTTTCGCGCGCG-3'. [d] Taken from reference [26]. [e] Primary binding constant for fitting to a two-site binding model. [f] Binding constant for fitting to a one-site binding model. [g] Non-specific binding. [h] There is not enough signal to noise ratio to get a binding constant for this hairpin oligonucleotide.

(A)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7c_AATT
Response (RU)
Time (second)
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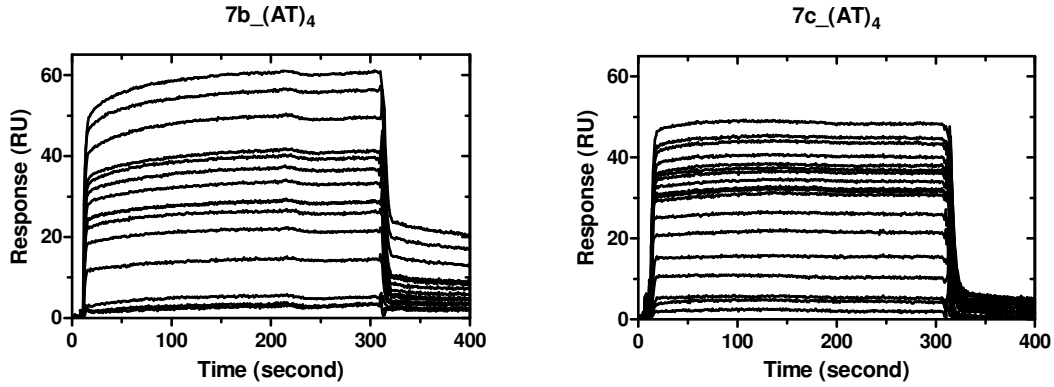
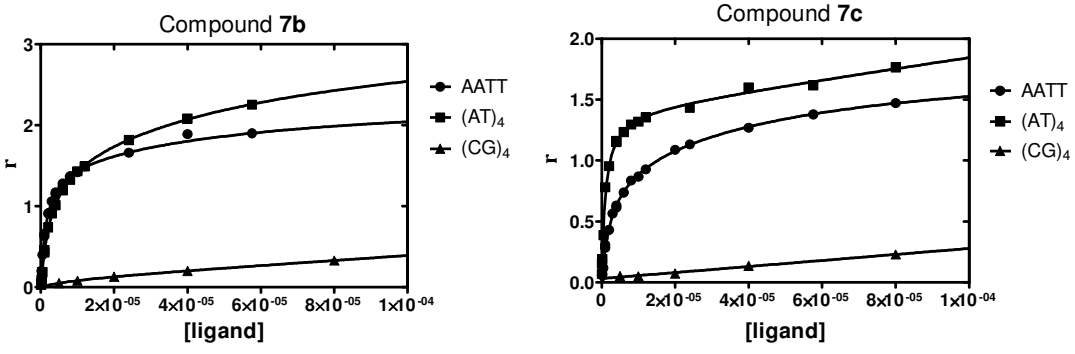
20
40
60
0
100
200
300
400


7b_(AT)4
7c_(AT)4
Response (RU)
Time (second)
0
20
40
60
0
100
200
300
400

(B)


Compound 7b
Compound 7c
r
[ligand]
AATT
(AT)4
(CG)4
0
0.5
1.0
1.5
2.0
2
1
3
2x10-05
4x10-05
6x10-05
8x10-05
1x10-04

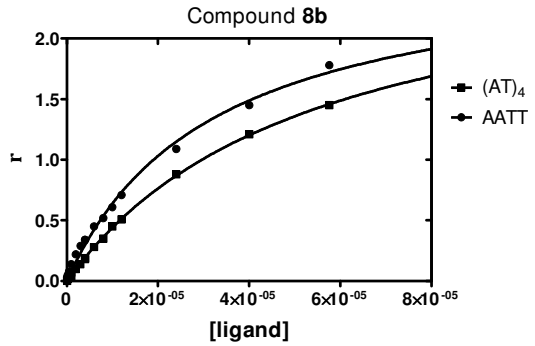

Compound 8b
r
[ligand]
(AT)4
AATT
0.0
0.5
1.0
1.5
2.0
2x10-05
4x10-05
6x10-05
8x10-05

Figure 2. (A) Sensorgrams for the interaction of **7b** (left panel) and **7c** (right panel) with the AATT and $(AT)_4$ hairpin oligonucleotides using increasing concentrations of ligand (from bottom to top) in the range 5×10^{-8} M to 5.76×10^{-5} M [**7b**_AATT and **7c**_$(AT)_4$] and 2.5×10^{-7} M to 8×10^{-5} M [**7b**_$(AT)_4$ and **7c**_AATT]. (B) Binding curves of **7b**, **7c**, and **8b** to AATT (●), $(AT)_4$ (■), and $(CG)_4$ (▲) DNA hairpins. The SPR response (RU) from the steady-state region of the sensorgrams was converted to r (moles of bound compound per mole of DNA hairpin duplex; $r = RU / RU_{max}$) and was plotted against the unbound compound concentration ([ligand] = flow solution). The data were fitted to a two-site binding model (except **8b**_AATT which was fitted to a one-site binding model) using equation (1) (see experimental section).

3. Discussion

Two series of *N*-substituted bisguanidines derived from the *N*-phenylbenzamide and the 1,3-diphenylurea scaffolds (**7a-h** and **8a-c**, respectively) were synthesized and evaluated as antitrypanosomal and antimalarial agents. The aim of this research was to improve the antiprotozoal activity of the leads **I** and **II** by introducing *N*-alkyl and *N*-alkoxy substituents on the guanidine moieties. As far as *T. brucei* is concerned, this was achieved successfully with the introduction of Et and ^{i}Pr substituents to get low nanomolar range IC_{50}s (**7b** and **7c**, respectively). A similar effect, although less pronounced, was obtained with the 1,3-diphenylurea derivatives **8b** and **8c**. Interestingly, the trypanocidal activity enhancement effect due to alkyl substitution was dependant on the size of the alkyl group. Hence, methyl groups were detrimental to the activity (e.g. compare **7a** vs. **I** and **8a** vs. **II**) whereas the larger isopropyl substituent gave lower IC_{50} values in comparison with the leads. These results indicate that, in this series, big and/or lipophilic alkyl substituents are favourable for antitrypanosomal activity (Figure 3). Remarkably, these SAR results are opposite to the findings reported by Arafa *et al* with a series of linear triaryl bisguanidines [17]. In contrast, small alkyl

groups appeared to be favourable to increase the antimalarial activity of the urea (Me > Et > ^{i}Pr) and the benzamide (Et > ^{i}Pr) derivatives as previously reported [17]. The introduction of alkoxy substituents on the guanidine groups (**7d**, **7f**, **7g**) was detrimental to both antitrypanosomal and antimalarial activities although *N*-hydroxy substituents were somewhat better tolerated (IC_{50} < 5 μM). These results are consistent with those observed for fused ring bisguanidines [15]. In general, the difference in SAR between antitrypanosomal and antimalarial action probably reflects the presence of different cellular targets for both antiparasitic activities (Figure 3).

The excellent in vivo efficacy of **7b** and **7c** in the mouse model of first stage HAT is linked to favourable drug-like properties (i.e. bioavailability, lead likeness, Lipinski rule of 5) as observed with **I** (Table 3). Considering that compound **I** was 100% curative by oral dosage in this mouse model, further in vivo studies with **7b** and **7c** are clearly warranted. In contrast, urea derivatives **8b** and **8c** were weakly active in the STIB900 model and showed acute toxicity issues in vivo. This was somewhat unexpected as both compounds displayed low cytotoxicity against rat L6-cells (> 100 μM) resulting in high selectivity indices in vitro (SI > 1200). However, the drug-like properties calculated for **8b** and **8c** are clearly less than optimum (Table 3) and may explain the poor efficacy of these compounds in vivo.

Among the new compounds reported here, two molecules derived from the *N*-phenylbenzamide scaffold, **7b** and **7c**, bound strongly to AT-rich DNA. No correlation between DNA binding and in vitro activity was observed which was not unexpected as this is a general trend that has been reported previously with related bisguanidines and other dicationic minor groove binders [11]. However, it was also noted in these reports that weak DNA binding results in reduced antitrypanosomal activity [15, 29, 32, 33].

Hence, the level of DNA binding displayed by **7b** and **7c** is consistent with the existence of a DNA target (even though this may not be the sole target).

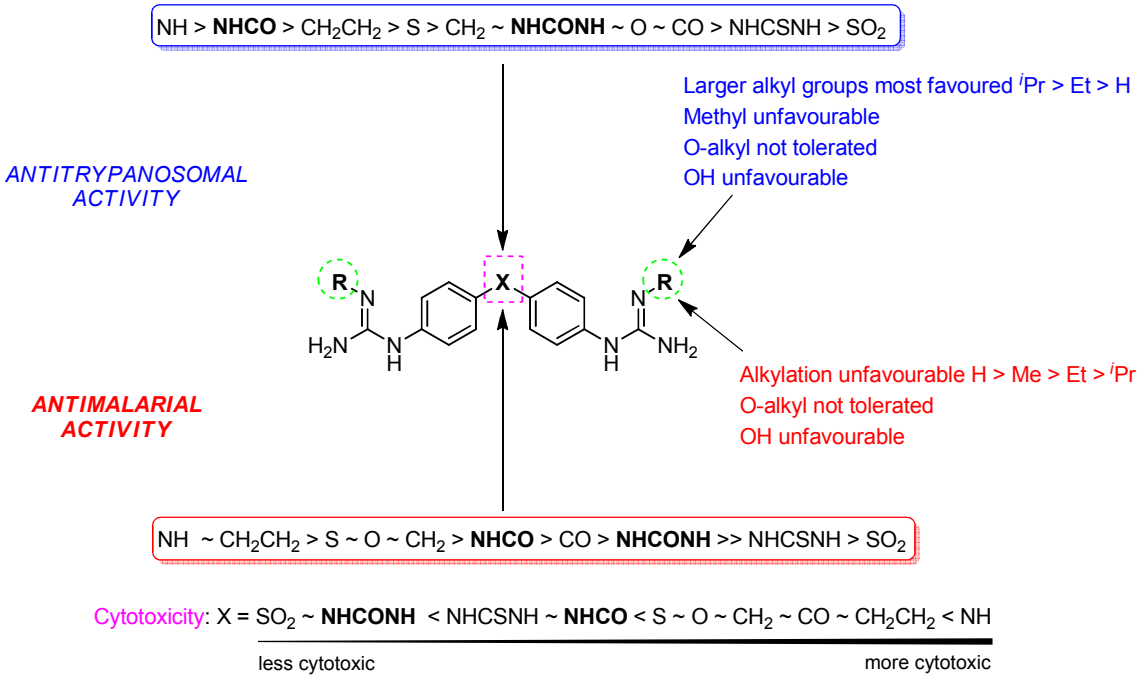


Figure 3. SAR Results for Antitrypanosomal and Antimalarial In Vitro Activities of Bisguanidine Derivatives. The linker of the compounds reported in this study are indicated as bold faces (X = NHCO, NHCONH). Other linkers are shown here for comparative purpose (data reported in reference [11]).

Table 3. Calculated Physical and Chemical Properties and Estimation of Solubility and Permeability of Orally Active Compounds.

Cmpd	TPSA	cLogP	LogD	Bioavail.[a]	Lead likeness[b]	Lipinski rule of 5[c]	In vivo efficacy[d]
I	157.9	0.28	-2.16	■	■	■	● [e]
II	169.9	0.33	-2.19	■	■	■	nd[f]
7a	129.9	0.83	-1.77	■	■	■	nd
7b	129.9	1.55	-1.08	■	■	■	● [g]
7c	129.9	2.38	-0.27	■	■	■	● [g]
7d	148.4	1.16	0.90	■	■	■	nd
7e	148.4	1.87	1.58	■	■	■	nd
7f	148.4	4.60	4.26	■	■	■	nd
7g	166.8	2.96	2.67	■	■	■	nd
7h	170.4	0.40	0.19	■	■	■	nd
8a	141.9	0.89	-1.80	■	■	■	nd
8b	141.9	1.60	-1.11	■	■	■	● [g]
8c	141.9	2.43	-0.30	■	■	■	● [g]

Calculations were performed with ChemAxon software Instant JChem 6.1.5 using Chemical Terms expressions (http://www.chemaxon.com). [a] Bioavailability (at least 6 subresults are satisfied): mass ≤ 500 (+), logP ≤ 5 (+), H-donor count ≤ 5 (+), H-acceptor count ≤ 10 (+), rotatable bond count ≤ 10 (+), PSA ≤ 200 (+), fused aromatic ring count ≤ 5. [b] Lead likeness: mass ≤ 450 & $logD_{7.4}$ ≥ -4 & $logD_{7.4}$ ≤ 4 & ring count ≤ 4 & rotatable bond count ≤ 10 & H-donor count ≤ 5 & H-acceptor count ≤ 8. [c] Lipinski Ro5 (4 of 4): mass ≤ 500 & logP ≤ 5 & H-donor count ≤ 5 & H-acceptor count ≤ 10. [d] STIB900 mouse model of acute HAT. [e] Oral administration. [f] Not determined. [g] ip administration.

4. Conclusions

In this study we have shown that the *N*-alkylation of the guanidine moiety is an effective strategy to enhance the antiparasitic activity of leads **I** and **II** against *T. b. rhodesiense*, whilst it is detrimental to the antimalarial action and DNA binding affinity. Low nanomolar IC_{50} values against *T. b. rhodesiense* were obtained with ethyl and isopropyl substituents (**7b**, **7c**, **8b**, and **8c**). However, only **7b** and **7c** showed in vivo efficacy (100% cures) in the STIB900 mouse model of acute HAT. Hence, *N*-alkylated bisguanidines derived from the *N*-phenylbenzamide scaffold are very promising DNA binders with in vivo antitrypanosomal activities that outperform the triaryl class of bisguanidines [16, 17]. Because of their favourable drug-like properties, further studies of these molecules are warranted.

Interestingly, several SAR differences against *T. brucei* were observed (i.e. guanidine substituent effect) with respect to other bisguanidine classes indicating that no general trend can be drawn for this class of dicationic compounds. It seems that potent antitrypanosomal activity depends on the complementary match between the central diphenyl scaffold and the substituted (or not) guanidine moieties.

5. Experimental

5.1. Chemistry

5.1.1. General

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 argon. All reactions were monitored by HPLC–MS or Thin Layer

Chromatography (TLC) using silica gel 60 F_{254} plates (Merck). Chromatography was performed with Isolute SI prepacked columns. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Advance 300, Varian Inova 400, or Varian Inova 500 spectrometer. Chemical shifts of the ^{1}H NMR spectra were internally referenced to TMS (δ 0 ppm) for $CDCl_3$ or the residual proton resonance of the deuterated solvents: D_2O (δ 4.79 ppm), CD_3OD (δ 3.31 ppm) and DMSO (δ 2.50 ppm). Signal splitting patterns are described as: singlet (s), broad singlet (br s), doublet (d), triplet (t), quadruplet (q), multiplet (m), or combination thereof. *J* values are given in Hz. Melting points were determined in open capillary tubes with a SMP3–Stuart Scientific apparatus or Mettler Toledo MP70 melting point system, and are uncorrected. All compounds are >95% pure by HPLC or combustion analysis otherwise noted. Elemental analysis was performed on a Heraeus CHN–O Rapid analyser. Analytical results were within ± 0.4 % of the theoretical values unless otherwise noted. Analytical HPLC–MS was run with an Xbridge C18–3.5 μm (2.1×100 mm) column on a Waters 2695 separation module coupled with a Waters Micromass ZQ spectrometer using electrospray ionization (ESI^+). The following HPLC conditions were used: column temperature = 30 ºC, gradient time = 5 min, H_2O/CH_3CN (10:90 → 90:10) (HCO_2H 0.1 %), flow rate = 1 mL/min, UV detection: photodiode array (λ = 190−400 nm). Accurate mass were measured with an Agilent Technologies Q–TOF 6520 spectrometer using electrospray ionization.

*5.1.2. General procedure for the synthesis of bis(ethoxycarbonylthioureas) (**3, 4**)*

To a solution of diamine **1** or **2** (1 equiv.) in dry CH_2Cl_2 (15 mL) cooled to 0 ºC and under argon atmosphere, was added slowly an excess of ethoxycarbonyl isothiocyanate (2.2 equiv.). The reaction mixture was stirred at room temperature overnight and the

precipitate was collected by filtration. The precipitate was rinsed with hexane to yield the ethoxycarbonylthioureas **3** and **4** with good yields.

5.1.2.1. Ethyl N-((4-((4-

((((ethoxycarbonyl)amino)methanethioyl)amino)phenyl)carbamoyl)phenyl)carbamothio yl)carbamate (**3**). The reaction of **1** (1.0 g, 4.4 mmol) with ethoxycarbonyl isothiocyanate (1.1 mL, 9.7 mmol) following the general procedure yielded **3** as white solid (2.1 g, 96%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.72 (s, 1H, *NH*), 11.49 (s, 1H, *NH*), 11.36 (s, 1H, *NH*), 11.22 (s, 1H, *NH*), 10.33 (s, 1H, *NH*), 7.97 (d, *J* = 9.0, 2H, Ar*H*), 7.80 (m, 4H, Ar*H*), 7.54 (d, *J* = 9.0, 2H, Ar*H*), 4.22 (q, *J* = 7.1, 2H, O-*CH_2*-), 4.21 (q, *J* = 7.1, 2H, O-*CH_2*-), 1.26 (t, *J* = 7.1, 3H, *CH_3*), 1.25 (t, *J* = 7.1, 3H, *CH_3*). ^{13}C NMR (75 MHz DMSO-d_6) δ 178.6 (*C*=O), 164.8 (2 × *C*=O), 153.6 (*C*=S), 153.5 (*C*=S), 141.0, 137.2, 133.5, 132.0, 128.2, 125.0, 123.7, 120.3, 62.2 (O-*CH_2*-), 62.1 (O-*CH_2*-), 14.2 (2 × *CH_3*). Mp > 300 °C. HPLC (UV) > 95%. LRMS (ES^+) *m/z* = 490.11 (M+H).

5.1.2.2. Ethyl N-((4-(((4-

((((ethoxycarbonyl)amino)methanethioyl)amino)phenyl)carbamoyl)amino)phenyl)carba mothioyl)carbamate (**4**). The reaction of **2** (1.17 g, 5.76 mmol) with ethoxycarbonyl isothiocyanate (1.2 mL, 10.6 mmol) following the general procedure yielded **4** as white solid (2 g, 82%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.44 (s, 2H, *NH*), 11.20 (s, 2H, *NH*), 8.78 (s, 2H, *NH*), 7.47 (s, 8H, Ar*H*), 4.21 (q, *J* = 7.1, 4H, O-*CH_2*), 1.26 (t, *J* = 7.1, 6H, *CH_3*). ^{13}C NMR (101 MHz, DMSO-d_6) δ 178.5 (*C*=O), 153.6 (*C*=S), 152.5 (*C*=S), 137.7 (2 × Ar-*C*), 132.0 (2 × Ar-*C*), 125.3 (Ar, 4 × *CH*), 118.1 (Ar, 4 × *CH*), 62.0 (2 × O-*CH_2*-), 14.2 (2 × *CH_3*). Mp > 300 °C. HPLC (UV) > 95%. LRMS (ES^+) *m/z* = 505.39 (M+H).

*5.1.3. General procedure for the synthesis of N-alkylguanidines (**5a-c, 6a-c**) and N-alkoxy guanidines (**5d-h**).*

To a solution of 4 equivalents of alkylamine (i.e., methylamine, ethylamine, isopropylamine) or hydroxylamine (i.e., *O*-methylhydroxylamine hydrochloride, *O*-ethylhydroxylamine, *O*-benzylhydroxylamine) in dry CH_2Cl_2 (5 mL) under argon atmosphere, was added diisopropylethylamine (DIPEA, 6 equiv.). The reaction mixture was stirred 15 minutes at room temperature. The reaction mixture was cooled with an ice-bath and bis(ethoxycarbonylthiourea) **3** or **4** (1 equiv.) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC, 4 equiv.) were added successively. The reaction mixture (which turned from white to light brown color) was stirred at room temperature for 12 h. The reaction mixture was diluted with CH_2Cl_2 (10 mL) and washed successively with water (3 × 10 mL) and brine (10 mL). The organic phase was dried ($MgSO_4$) and the solvent evaporated under vacuum. The product was purified as specified in each case.

*5.1.3.1. 4-(3-Ethoxycarbonyl-2-methylguanidino)-N-(4-(3-ethoxycarbonyl-2-methylguanidino)phenyl)benzamide (***5a***)*

Compound **5a** was obtained following the general procedure with **3** (800 mg, 1.6 mmol), DIPEA (1.71 mL, 9.84 mmol), EDAC (1.26 g, 6.56 mmol) and methylamine (2 M solution in THF, 3.28 mL, 6.56 mmol). The product was purified by precipitation with EtOAc and washed with Et_2O to yield **5a** as white solid (495 mg, 64%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.55 (br s, 1H, *NH*), 8.67 (br s, 1H, *NH*), 7.86 (d, *J* = 8.6, 2H, Ar*H*), 7.69 (d, *J* = 8.3, 2H, Ar*H*), 7.18 (m, 2H, Ar*H*), 7.13 (d, *J* = 8.6, 2H, Ar*H*), 4.85 (br s, 2H, *NH*), 4.14 (q, *J* = 7.1, 4H, O-*CH*$_2$-), 2.94 (s, 3H, N-*CH*$_3$), 2.89 (s, 3H, N-*CH*$_3$), 1.29 (m, 6H, -*CH*$_3$). ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.8 (*C*=O), 163.4 (*C*=O), 163.2

(*C*=O), 159.1 (*C*=N), 158.6 (*C*=N), 141.4 (*Ar*-*C*), 136.3 (Ar-*C*), 132.8 (Ar-*C*), 129.7 (Ar-*C*), 128.2 (Ar-*CH*), 124.9 (Ar-*CH*), 122.4 (Ar-*CH*), 120.6 (Ar-*CH*), 59.7 (-O*CH*$_2$-), 59.6 (-O*CH*$_2$-), 28.4 (-N*CH*$_3$), 28.1 (-N*CH*$_3$), 14.7 (*CH*$_3$), 14.6 (*CH*$_3$). Mp 127–130 °C. HPLC (UV) = 96 %. LRMS (ES$^+$) *m/z* = 484.46 (M+H).

*5.1.3.2. 4-(2-Ethyl-3-ethoxycarbonylguanidino)-N-(4-(2-ethyl-3-ethoxycarbonylguanidino)phenyl)benzamide (**5b**)*

Compound **5b** was obtained following the general procedure with **3** (500 mg, 1.0 mmol), DIPEA (1.1 mL, 6.1 mmol), EDAC (784 mg, 4.1 mmol) and ethylamine hydrochloride (331 mg, 4.1 mmol). The product was purified by silica chromatography (10 g SI prepacked column) with CH_2Cl_2/EtOAc (9:1) to yield **5b** as white solid (419 mg, 81%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.66 (br s, 1H, N*H*), 8.49 (br s, 1H, N*H*), 7.86 (d, *J* = 7.5, 2H, Ar*H*), 7.68 (d, *J* = 8.5, 2H, Ar*H*), 7.14 (m, 5H, Ar*H* + N*H*), 4.76 (br s, 1H, N*H*), 4.14 (q, *J* = 7.1, 4H, -O*CH*$_2$-), 3.39 (m, 4H, -N*CH*$_2$-), 1.28 (t, *J* = 7.1, 6H, *CH*$_3$), 1.20 (t, *J* = 6.9, 3H, *CH*$_3$), 1.11 (t, *J* = 7.1, 3H, *CH*$_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.5 (*C*=O), 136.9 (2 × Ar-*C*), 129.1 (Ar, 2 × *CH*), 126.6 (Ar, 2 × *CH*), 124.0 (Ar, 2 × *CH*), 122.1 (Ar, 2 × *CH*), 61.6 (-O*CH*$_2$-), 61.2 (-O*CH*$_2$-), 36.4 (2 × -N*CH*$_2$), 15.0 (*CH*$_3$), 14.8 (*CH*$_3$), 14.6 (2 × *CH*$_3$). Mp 170–171 °C. HPLC (UV) > 90%. HRMS (ES$^+$) required for $C_{25}H_{33}N_7O_5$: 511.2541 (found: 511.2543).

*5.1.3.3. 4-(3-Ethoxycarbonyl-2-isopropylguanidino)-N-(4-(3-ethoxycarbonyl-2-isopropylguanidino)phenyl)benzamide (**5c**).*

Compound **5c** was obtained following the general procedure with **3** (800 mg, 1.6 mmol), DIPEA (1.7 mL, 9.8 mmol), EDAC (1.26 g, 6.56 mmol) and isopropylamine (0.57 mL, 6.56 mmol). The product was purified by silica chromatography (10 g SI prepacked column) with CH_2Cl_2/EtOAc (100/0→90/10) to yield **5c** as white solid (690 mg, 77%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.57 (br s, 1H, N*H*), 8.48 (br s, 1H, N*H*),

7.89 (d, *J* = 7.9, 2H, Ar*H*), 7.70 (d, *J* = 8.3, 2H, Ar*H*), 7.13 (m, 4H, Ar*H*), 4.59 (br s, 1H, N*H*), 4.27 (br m, 2H, -*CH*-), 4.15 (q, *J* = 7.1, 4H, -O*CH*$_2$-), 1.30 (m, 6H, *CH*$_3$), 1.20 (d, *J* = 6.4, 6H, *CH*$_3$), 1.11 (d, *J* = 6.4, 6H, *CH*$_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.5 (*C*=O), 164.7 (2 × *C*=O), 158.2 (2 × *C*=N), 137.0 (2 × Ar-*C*), 132.2 (2 × Ar-*C*), 129.2 (Ar, 2 × *CH*), 126.6 (Ar, 2 × *CH*), 124.0 (Ar, 2 × *CH*), 122.2 (Ar-*CH*), 61.6 (-O*CH*$_2$-), 61.1 (-O*CH*$_2$-), 43.1 (-*CH*-), 43.0 (-*CH*-), 23.1 (2 × *CH*$_3$), 23.0 (2 × *CH*$_3$), 14.8 (*CH*$_3$), 14.6 (*CH*$_3$). Mp 136–138 °C. HPLC (UV) > 95%. HRMS (ES^+) required for $C_{27}H_{37}N_7O_5$: 539.2856 (found: 539.2849).

*5.1.3.4. 4-(3-Ethoxycarbonyl-2-methoxyguanidino)-N-(4-(3-ethoxycarbonyl-2-methoxyguanidino)phenyl)benzamide (**5d**)*

Compound **5d** was obtained following the general procedure with **3** (500 mg, 1.0 mmol), DIPEA (1.1 mL, 6.1 mmol), EDAC (784 mg, 4.1 mmol) and methoxyamine hydrochloride (341 mg, 4.1 mmol). The product was purified by silica chromatography (5 g SI prepacked column) with CH_2Cl_2/EtOAc (9:1) to yield **5d** as white solid (226 mg, 43%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.17 (br m, 1H, N*H*), 8.84 (br s, 1H, N*H*), 8.02 (br s, 2H, N*H*), 7.80 (d, *J* = 8.7, 2H, Ar*H*), 7.76 (br s, 1H, N*H*), 7.53 (d, *J* = 8.7, 4H, Ar*H*), 7.43 (d, *J* = 8.7, 2H, Ar*H*), 4.23 (q, *J* = 7.0, 2H, O-*CH*$_2$-), 4.20 (q, *J* = 7.0, 2H, O-*CH*$_2$-), 3.83 (s, 3H, O-*CH*$_3$), 3.77 (s, 3H, O-*CH*$_3$), 1.39 (m, 6H, *CH*$_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.1 (*C*=O), 153.5 (2 × *C*=O), 145.2 (Ar-*C*), 144.2 (*C*=N), 142.2 (*C*=N), 135.4 (Ar-*C*), 132.6 (Ar-*C*), 127.8 (Ar-*C*), 128.2 (Ar, 2 × *CH*), 121.0 (Ar, 2 × *CH*), 119.7 (Ar, 2 × *CH*), 118.4 (Ar, 2 × *CH*), 62.7 (2 × *O-CH*$_2$), 62.1 (2 × *O-CH*$_3$), 14.4 (2 × *CH*$_3$). Mp 190–191 °C. HPLC (UV): 97%. HRMS (ES^+) required for $C_{23}H_{29}N_7O$: 515.2128 (found: 515.2134).

*5.1.3.5. 4-(2-Ethoxy-3-ethoxycarbonylguanidino)-N-(4-(2-ethoxy-3-ethoxycarbonylguanidino)phenyl)benzamide (**5e**)*

Compound **5e** was obtained following the general procedure with **3** (500 mg, 1 mmol), DIPEA (1.1 mL, 6.1 mmol), EDAC (784 mg, 4.1 mmol) and ethoxyamine hydrochloride (397 mg, 4.1 mmol). The product was purified by silica chromatography (5 g SI prepacked column) with CH_2Cl_2/EtOAc (9:1) to yield **5e** as white solid (299 mg, 55%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.17 (br s, 1H, N*H*), 8.84 (br s, 1H, N*H*), 8.04 (br s, 2H, N*H*), 7.79 (d, 2H, *J* = 8.8, Ar*H*), 7.78 (s, 1H, N*H*), 7.53 (d, *J* = 8.8, 4H, Ar*H*), 7.44 (d, *J* = 8.6, 2H, Ar*H*), 4.24 (m, 4H, O-*CH_2*-), 4.05 (m, 4H, O-*CH_2*-), 1.32 (m, 12H, *CH_3*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.2 (*C*=O), 153.5 (2 × *C*=N), 144.6 (Ar-*C*), 143.9 (*C*=O), 142.3 (*C*=O), 135.6 (Ar-*C*), 132.4 (Ar-*C*), 128.2 (Ar, 2 × *CH*), 127.7 (Ar-*C*), 121.0 (Ar, 2 × *CH*), 119.4 (Ar-*CH*), 118.2 (Ar-*CH*), 69.7 (-O*CH_2*-), 69.5 (-O*CH_2*-), 62.6 (-O*CH_2*-), 62.4 (-O*CH_2*-), 14.7 (*CH_3*), 14.4 (*CH_3*). Mp 181–183 °C. HPLC (UV): 95%. LRMS (ES^+) *m/z* = 544.17 (M+H).

*5.1.3.6. 4-(2-Benzyloxy-3-ethoxycarbonylguanidino)-N-(4-(2-benzyloxy-3-ethoxycarbonylguanidino)phenyl)benzamide (**5f**)*

Compound **5f** was obtained following the general procedure with **3** (800 mg, 1.6 mmol), DIPEA (1.7 mL, 9.8 mmol), EDAC (1.26 g, 6.56 mmol) and benzyloxyamine (0.76 mL, 6.56 mmol). The product was purified by silica chromatography (5 g SI prepacked column) with EtOAc/MeOH (100/0→90/10) to yield **5f** as white solid (875 mg, 80%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.18 (br s, 1H, N*H*), 8.84 (br s, 1H, N*H*), 8.00 (br m, 2H, N*H*), 7.78 (d, *J* = 8.7, 2H, Ar*H*), 7.67 (br s, 1H, N*H*), 7.52 (m, 4H, Ar*H*), 7.47 – 7.30 (m, 12H, Ar*H*), 5.03 (s, 2H, -O*CH_2*-), 5.00 (s, 2H, -O*CH_2*-), 4.23 (q, *J* = 7.1, 4H, -O*CH_2*-), 1.32 (t, *J* = 7.1, 3H, *CH_3*), 1.31 (t, *J* = 7.1, 3H, *CH_3*). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.9 (*C*=O), 153.3 (2 × *C*=O), 144.8 (*C*=N), 144.0 (*C*=N), 143.9 (Ar-*C*), 142.1 (Ar-*C*), 137.5 (Ar-*C*), 137.3 (Ar-*C*), 135.4 (Ar-*C*), 132.3 (Ar-*C*), 128.8 (Ar, 2 × *CH*), 128.8 (Ar, 2 × *CH*), 128.43 (Ar, 2 × *CH*), 128.37 (Ar, 2 × *CH*), 128.2 (Ar, 2 ×

CH), 127.6 (Ar-*CH*), 120.8 (Ar, 2 × *CH*), 119.5 (Ar-*CH*), 118.2 (Ar-*CH*), 76.3 (O-CH_2-), 76.2 (O-CH_2-), 62.5 (O-CH_2-), 62.3 (O-CH_2-), 14.2 (CH_3). Mp 156–158 °C. HPLC (UV) = 95%. LRMS (ES^+) *m/z* = 668.68 (M+H).

*5.1.3.7. 4-(3-Ethoxycarbonyl-2-((tetrahydro-2H-pyran-2-yl)oxy)guanidino)-N-(4-(3-ethoxycarbonyl-2-((tetrahydro-2H-pyran-2-yl)oxy)guanidino)phenyl)benzamide (**5g**)*

Compound **5g** was obtained following the general procedure with **3** (650 mg, 1.3 mmol), DIPEA (1.5 mL, 8.6 mmol), EDAC (1.1 g, 5.7 mmol) and *O*-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine [34] (670 mg, 5.7 mmol). The product was purified by precipitation in EtOAc to yield **5g** as white solid (560 mg, 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.21 (br s, 1H, N*H*), 8.88 (br s, 1H, N*H*), 7.97 (s, 2H, N*H*), 7.88 (s, 1H, N*H*), 7.79 (d, *J* = 8.8, 2H, Ar*H*), 7.54 (m, 4H, Ar*H*), 7.44 (d, *J* = 9.0, 2H, Ar*H*), 5.14 (dd, *J* = 5.8, 2.2, 1H, O-*CH*-O), 5.10 (dd, *J* = 6.0, 2.1, 1H, O-*CH*-O), 4.24 (m, 4H, O-CH_2-), 3.94 (m, 2H, -CH_2-), 3.63 (m, 2H, -CH_2-), 2.48 (m, 2H, -CH_2-), 1.86 (m, 4H, -CH_2-), 1.61 (m, 6H, -CH_2-), 1.34 (m, 6H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.3 (*C*=O), 153.5 (2 × *C*=O), 146.0 (*C*=N), 145.3 (*C*=N), 142.1 (Ar-*C*), 135.5 (Ar-*C*), 132.7 (Ar-*C*), 128.3 (Ar, 2 × *CH*), 128.0 (Ar-*C*), 121.1 (Ar, 2 × *CH*) , 119.8 (Ar, 2 × *CH*), 118.6 (Ar, 2 × *CH*), 101.8 (O-*CH*-O), 101.7 (O-*CH*-O), 64.3 (O-CH_2-), 64.2 (O-CH_2-), 62.9 (O-CH_2-), 62.7 (O-CH_2-), 29.6 (-CH_2-), 29.5 (-CH_2-), 25.4 (-CH_2-), 21.0 (-CH_2-), 20.9 (-CH_2-), 14.5 (2 × CH_3). Mp 116–118 °C. HPLC (UV) = 95%. LRMS (ES^+) *m/z* = 656.59 (M+H).

*5.1.3.8. 1,3-Bis(4-(3-ethoxycarbonyl-2-methylguanidino)phenyl)urea (**6a**)*

Compound **6a** was obtained following the general procedure with **4** (800 mg, 1.6 mmol), DIPEA (1.66 mL, 9.52 mmol), EDAC (1.21 g, 6.36 mmol) and methylamine (2 M solution in THF, 3.18 mL, 6.36 mmol). The crude product was triturated in Et_2O to

yield **6a** as white solid (707 mg, 73%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.63 (br s, 2H, N*H*), 7.45 (d, *J* = 8.4, 4H, Ar*H*), 7.19 (d, *J* = 8.4, 4H, Ar*H*), 3.94 (q, *J* = 7.1, 4H, O-*CH_2*-), 2.78 (m, 6H, N-*CH_3*), 1.15 (t, *J* = 7.1, 6H, *CH_3*) [35]. ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.7 (*C*=O), 159.6 (2 × *C*=O), 153.1 (2 × *C*=N), 137.9 (2 × Ar-*C*), 131.2 (2 × Ar-*C*), 126.0 (Ar, 4 × *CH*), 118.5 (Ar, 4 × *CH*), 59.9 (2 × O-*CH_2*-), 28.4 (2 × N-*CH_3*), 15.0 (2 × *CH_3*). Mp > 280 °C (dec.). HPLC (UV) = 98%. LRMS (ES^+) *m/z* = 499.44 (M + H).

*5.1.3.9. 1,3-Bis(4-(2-ethyl-3-ethoxycarbonylguanidino)phenyl)urea (**6b**)*

Compound **6b** was obtained following the general procedure with **4** (800 mg, 1.6 mmol), DIPEA (1.6 mL, 9.5 mmol), EDAC (1.23 g, 6.4 mmol) and ethylamine hydrochloride (518 mg, 6.4 mmol). The product was purified by silica chromatography (5 g SI prepacked column) with CH_2Cl_2/MeOH (100/0→95/5) to yield **6b** as white solid (418 mg, 60%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.69 (s, 2H, N*H*), 7.44 (d, *J* = 8.2, 4H, Ar*H*), 7.20 (d, *J* = 8.2, 4H, Ar*H*), 3.94 (q, *J* = 7.0, 4H, -O*CH_2*-), 3.28 (m, 4H, -N*CH_2*-), 1.15 (t, 6H, *J* = 7.0, *CH_3*), 1.10 (t, 6H, *J* = 7.0, *CH_3*) [34]. ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.5 (*C*=O), 158.4 (2 × *C*=O), 152.5 (2 × *C*=N), 137.0 (2 × Ar-*C*), 131.2 (2 × Ar-*C*), 125.5 (Ar, 4 × *CH*), 118.6 (Ar, 4 × *CH*), 59.6 (2 × -O*CH_2*-) , 35.6 (2 × -N*CH_2*-), 14.8 (2 × *CH_3*), 14.7 (2 × *CH_3*). Mp 190–191 °C. HPLC (UV) > 95%. LRMS (ES^+) *m/z* = 527.58 (M+H).

*5.1.3.10. 1,3-Bis(4-(3-ethoxycarbonyl-2-isopropylguanidino)phenyl)urea (**6c**)*

Compound **6c** was obtained following the general procedure with **4** (300 mg, 0.6 mmol), DIPEA (0.64 mL, 3.69 mmol), EDAC (472 mg, 2.5 mmol) and isopropylamine (0.21 mL, 2.46 mmol). The product was purified by silica chromatography (5 g SI prepacked column) with CH_2Cl_2/MeOH (100/0→95/5) to yield **6c** as white solid (228

mg, 67%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.52 (br s, 1H, N*H*), 8.41 (br s, 2H, N*H*), 7.43 (brm, 4H, Ar*H*), 7.03 (d, *J* = 8.0, 4H, Ar*H*), 4.70 – 4.38 (br s, 2H, N*H*), 4.25 – 4.22 (br s, 2H, -*CH*-), 4.18 (q, *J* = 7.0, 4H, O-CH_2), 1.28 (t, *J* = 7.0, 6H, CH_3), 1.11 (m, 12H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.4 (*C*=O), 158.4 (2 × C=O), 153.2 (2 × *C*=N), 138.5 (2 × Ar-*C*), 129.9 (2 × Ar-*C*), 126.8 (Ar, 4 × *CH*), 120.5 (Ar, 4 × *CH*), 61.6 (2 × O-CH_2-), 43.2 (2 × -*CH*-), 23.1 (4 × CH_3), 14.8 (2 × CH_3). Mp > 130 °C (dec.). HPLC (UV) > 95%. LRMS (ES^+) *m/z* = 555.58 (M+H).

*5.1.4. General procedure for the synthesis of diguanidines **7a-7h** and **8a-8c***

The reaction was performed in a Kimax glass tube sealed with a screw stopper. To a solution of bis-3-ethoxycarbonyldiguanidine (**5a-g**, **6a-c**) in MeOH (1 mL) was added 0.1 M aqueous KOH (2 mL). The tube was stoppered and the reaction mixture was stirred at 80 ºC for 3–12 h. The reaction mixture was transferred to a round-bottomed flask and the solvents were evaporated under vacuum. The product was precipitated from water and triturated with a spatula to give the diguanidine as free base. The guanidine product was collected by filtration on a fritted plate (Nº 3).

Formation of the diguanidinium dihydrochloride salts. To a solution of the diguanidine in MeOH (1 mL) was added HCl_g-saturated 1,4-dioxane solution (3 mL). The reaction mixture was stirred at room temperature for 1 h and the volatiles were removed under vacuum. The residue was triturated in Et_2O to give the diguanidinium dihydrochloride salts as colorless solids.

*5.1.4.1. 4-(2-Methylguanidino)-N-(4-(2-methylguanidino)phenyl)benzamide (**7a**)*

Following the general procedure, the reaction of **5a** (136 mg, 0.28 mmol) with 1 M KOH gave the free base of **7a** as white solid (90 mg, 94%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.91 (s, 1H, N*H*), 7.84 (d, *J* = 8.0, 2H, Ar*H*), 7.69 (d, *J* = 8.0, 2H, Ar*H*), 7.13 (br s, 2H, N*H*), 6.89 (m, 4H, Ar*H*), 5.96 (br s, 4H, *NH*$_2$), 2.70 (s, 3H, -*CH*$_3$), 2.68 (s, 3H, -*CH*$_3$). ^{13}C NMR (75 MHz, DMSO-d_6) δ 165.3 (*C*=O), 154.2 (*C*=N), 153.1 (*C*=N), 134.9 (2 × Ar-*C*), 129.0 (Ar, 2 × *CH*), 126.2 (2 × Ar-*C*), 123.9 (Ar, 2 × *CH*), 122.6 (Ar, 2 × *CH*), 121.6 (Ar-*CH*), 28.2 (N-*CH*$_3$), 28.1 (N-*CH*$_3$). Mp > 300 °C. HPLC (UV) = 93%. LRMS (ES$^+$) *m/z* = 340.45.

*5.1.4.2. 4-(2-Ethylguanidino)-N-(4-(2-ethylguanidino)phenyl)benzamide (7**b**)*

Following the general procedure, the reaction of **5b** (350 mg, 0.68 mmol) with 1 M KOH gave the free base of **7b** (205 mg, 81%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.89 (s, 1H, N*H*), 7.83 (d, *J* = 8.2, 2H, Ar*H*), 7.67 (d, *J* = 8.4, 2H, Ar*H*), 6.88 (m, 4H, Ar*H*), 3.16 (m, 4H, N-*CH*$_2$-), 1.09 (m, 6H, *CH*$_3$). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.1 (*C*=O), 160.1 (Ar-*C*), 154.3 (Ar-*C*), 152.9 (*C*=N), 152.0 (*C*=N), 134.7 (Ar-*C*), 128.7 (Ar, 2 × *CH*), 125.8 (Ar-*C*), 123.6 (Ar, 2 × *CH*), 122.4 (Ar, 2 × *CH*), 121.3 (Ar, 2 × *CH*), 35.6 (N-*CH*$_2$-), 35.4 (N-*CH*$_2$-), 15.0 (*CH*$_3$), 14.7 (*CH*$_3$). Mp 134–135 °C. HPLC (UV) = 92%. LRMS (ES$^+$) *m/z* = 368.49 (M+H).

Dihydrochloride salt of **7b**: white solid (182 mg). HPLC (UV) = 92%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.51 (s, 1H, N*H*), 10.31 (s, 1H, N*H*), 9.83 (s, 1H, N*H*), 8.32 (s, 1H, N*H*), 8.10 (d, *J* = 8.4, 2H, Ar*H*), 8.00 (br s, 3H, N*H*), 7.92 (d, *J* = 8.1, 2H, Ar*H*), 7.68 (br s, 2H, N*H*), 7.35 (d, *J* = 8.1, 2H, Ar*H*), 7.21 (d, *J* = 8.4, 2H, Ar*H*), 3.30 (m, 4H, N-*CH*$_2$-), 1.16 (t, *J* = 7.3, 3H, *CH*$_3$), 1.13 (t, *J* = 7.3, 3H, *CH*$_3$). ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.6 (*C*=O), 154.9 (*C*=N), 154.3 (*C*=N), 139.5 (Ar-*C*), 137.7 (Ar-*C*),

130.9 (Ar-*C*), 130.6 (Ar-*C*), 129.3 (Ar, 2 × *CH*), 125.3 (Ar, 2 × *CH*), 122.4 (Ar, 2 × *CH*), 121.4 (Ar, 2 × *CH*), 36.5 (N-CH_2), 36.2 (N-CH_2), 14.2 (-CH_3), 14.1 (-CH_3).

*5.1.4.3. 4-(2-Isopropylguanidino)-N-(4-(2-isopropylguanidino)phenyl)benzamide (7**c**)*

Following the general procedure, the reaction of **5c** (300 mg, 0.56 mmol) with 1 M KOH gave the free base of **7c** (157 mg, 71%) which was transformed into the dihydrochloride salt of **7c**. Dihydrochloride of **7c**: white solid (106 mg). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.51 (s, 1H, N*H*), 10.28 (s, 1H, N*H*), 9.80 (s, 1H, N*H*), 8.35 (br s, 1H, N*H*), 8.10 (d, *J* = 8.5, 2H, Ar*H*), 8.03 (br s, 3H, N*H*), 7.91 (d, *J* = 8.5, 2H, Ar*H*), 7.64 (br s, 2H, N*H*), 7.34 (d, *J* = 8.0, 2H, Ar*H*), 7.20 (d, *J* = 8.0, 2H, Ar*H*), 3.94 (br m, 2H, -*CH*-), 1.19 (t, *J* = 6.2, 12H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.5 (*C*=O), 154.2 (*C*=N), 153.5 (*C*=N), 139.6 (Ar-*C*), 137.5 (Ar-*C*), 130.7 (Ar-*C*), 130.7 (Ar-*C*), 129.3 (Ar, 2 × *CH*), 125.1 (Ar-*CH*), 122.2 (Ar-*CH*), 121.3 (Ar-*CH*), 43.8 (-*CH*-), 43.4 (-*CH*-), 22.3 (2 × CH_3), 22.2 (2 × CH_3). Mp 206–208 °C. HPLC (UV) = 96%. LRMS (ES$^+$) *m/z* = 396.47 (M + H).

*5.1.4.4. 4-(2-Methoxyguanidino)-N-(4-(2-methoxyguanidino)phenyl)benzamide (7**d**)*

Following the general procedure, the reaction of **5d** (200 mg, 0.39 mmol) with 1 M KOH gave the free base of **7d** as white solid (100 mg, 69%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.78 (br s, 1H, N*H*), 7.81 (d, *J* = 7.1, 2H, Ar*H*), 7.73 – 7.56 (br s, 1H, N*H*), 7.53 (d, *J* = 7.2, 2H, Ar*H*), 7.40 (d, *J* = 7.2, 2H, Ar*H*), 7.28 (d, *J* = 7.1, 2H, Ar*H*), 5.40 (s, 2H, NH_2), 5.21 (s, 2H, NH_2), 3.62 (s, 3H, O-CH_3), 3.59 (s, 3H, O-CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.6 (*C*=O), 151.5 (*C*=N), 150.7 (*C*=N), 144.6 (Ar-*C*), 137.4 (Ar-*C*), 131.8 (Ar-*C*), 128.4 (Ar, 2 × *CH*), 125.5 (Ar-*C*), 121.0 (Ar, 2 × *CH)*, 117.2 (Ar-*CH*), 115.9 (Ar-*CH*), 60.6 (O-CH_3), 60.5 (O-CH_3). Mp 205–208 °C. HPLC (UV) > 95%. LRMS (ES$^+$) *m/z* = 372.48 (M+H).

Dihydrochloride of **7d**: white solid (79 mg). ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.61 (br s, 1H), 10.63 (s, 1H), 10.58 (s, 1H), 10.24 (s, 1H), 8.40 (s, 2H), 8.14 (s, 2H), 8.11 (d, *J* = 8.4, 2H, Ar*H*), 7.93 (d, *J* = 8.3, 2H, Ar*H*), 7.40 (d, *J* = 8.1, 2H, Ar*H*), 7.25 (d, *J* = 8.1, 2H, Ar*H*), 3.74 (s, 3H, O-*CH*$_3$), 3.71 (s, 3H, O-*CH*$_3$). ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.6 (*C*=O), 156.3 (*C*=N), 155.5 (*C*=N), 138.7 (Ar-*C*), 138.0 (Ar-*C*), 131.3 (Ar-*C*), 129.7 (Ar-*C*), 129.3 (Ar, 2 × *CH*), 125.4 (Ar, 2 × *CH*), 122.6 (Ar, 2 × *CH*), 121.3 (Ar, 2 × *CH*), 64.5 (O-*CH*$_3$), 64.4 (*O*-*CH*$_3$). HPLC (UV) > 95%.

*5.1.4.5. 4-(2-Ethoxyguanidino)-N-(4-(2-ethoxyguanidino)phenyl)benzamide (**7e**)*

Following the general procedure, the reaction of **5e** (30 mg, 0.05 mmol) with 1 M KOH gave the free base of **7e** as white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.75 (s, 1H, N*H*), 8.09 (s, 1H, N*H*), 7.80 (d, *J* = 8.9, 2H, Ar*H*), 7.58 (s, 1H, N*H*), 7.52 (d, *J* = 8.9, 2H, Ar*H*), 7.39 (d, *J* = 8.9, 2H, Ar*H*), 7.27 (d, *J* = 8.9, 2H, Ar*H*), 5.31 (s, 2H, N*H*$_2$), 5.14 (s, 2H, N*H*$_2$), 3.83 (m, 4H, O-*CH*$_2$), 1.20 (m, 6H, *CH*$_3$). ^{13}C NMR (126 MHz, DMSO-d_6) δ 164.5 (*C*=O), 151.3 (*C*=N), 150.5 (*C*=N), 144.7 (Ar-*C*), 137.5 (Ar-*C*), 131.7 (Ar-*C*), 128.4 (Ar, 2 × *CH*), 125.4 (Ar-*C*), 121.0 (Ar, 2 × *CH*) , 117.1 (Ar, 2 × *CH*), 115.8 (Ar, 2 × *CH*), 67.6 (O-*CH*$_2$), 67.4 (O-*CH*$_2$), 14.7 (2 × *CH*$_3$). Mp 184–186 °C. HPLC (UV) > 95%. LRMS (ES$^+$) *m/z* = 400.39 (M + H).

Dihydrochloride of **7e**: white solid (19 mg, 85% for 2 steps). ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.41 (br s, 1H), 10.50 (s, 1H), 10.42 (br s, 1H), 10.09 (s, 1H), 8.23 (br m, 2H), 8.09 (d, *J* = 8.6, 4H), 7.91 (d, *J* = 8.8, 2H), 7.40 (d, *J* = 8.6, 2H), 7.25 (d, *J* = 8.8, 2H), 3.94 (d, *J* = 7.0, 4H), 1.26 (t, *J* = 7.0, 6H).

*5.1.4.6. 4-(2-(Benzyloxy)guanidino)-N-(4-(2-(benzyloxy)guanidino)phenyl)benzamide (**7f**)*

Following the general procedure, the reaction of **5f** (200 mg, 0.39 mmol) with 1 M KOH gave the free base of **7f** as white solid (140 mg, 95%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.75 (s, 1H, N*H*), 8.10 (s, 1H, N*H*), 7.78 (d, *J* = 8.8, 2H, Ar*H*), 7.60 (s, 1H, N*H*), 7.50 (d, *J* = 9.0, 2H, Ar*H*), 7.45 – 7.40 (m, 4H, Ar*H*), 7.39 – 7.31 (m, 6H, Ar*H*), 7.32 – 7.25 (m, 2H, Ar*H*), 7.22 (d, *J* = 9.0, 2H, Ar*H*), 5.43 (s, 2H, NH_2), 5.26 (s, 2H, NH_2), 4.88 (s, 2H, O-CH_2-), 4.84 (s, 2H, O-CH_2-). ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.5 (*C*=O), 151.5 (*C*=N), 150.7 (*C*=N), 144.6 (Ar-*C*), 139.25 (Ar-*C*), 139.16 (Ar-*C*), 137.4 (Ar-*C*), 131.7 (Ar-*C*), 128.4 (Ar, 2 × *CH*), 128.1 (Ar, 2 × *CH*), 128.0 Ar, 2 × *CH*), 127.95 (Ar, 2 × *CH*), 127.91 (Ar-*CH*), 127.3 (Ar-*CH*), 127.2 (Ar-*C*), 125.4 (Ar, 2 × *CH*), 121.0 (Ar, 2 × *CH*), 117.1 (Ar, 2 × *CH)*, 115.9 (Ar, 2 × *CH*), 74.32 (O-CH_2), 74.25 (O-CH_2-). Mp 110–113 °C. HPLC (UV) = 95%. LRMS (ES$^+$) *m/z* = 524.56 (M + H).

Dihydrochloride of **7f**: white solid (95 mg). HPLC (UV) = 98%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.54 (s, 2H), 10.55 (s, 1H), 10.53 (s, 2H), 10.20 (s, 1H), 8.26 (s, 2H), 8.08 (d, *J* = 8.6, 2H, Ar*H*), 7.91 (d, *J* = 8.8, 2H, Ar*H*), 7.52 (m, 4H, Ar*H*), 7.41 (m, 6H, Ar*H*), 7.29 (d, *J* = 8.6, 2H, Ar*H*), 7.15 (d, *J* = 8.8, 2H, Ar*H*), 4.96 (s, 2H, O-CH_2), 4.92 (s, 2H, O-CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.6 (*C*=O), 156.6 (*C*=N), 155.6 (*C*=N), 139.1 (Ar-*C*), 137.9 (Ar-*C*), 135.2 (Ar-*C*), 135.0 (Ar-*C*), 129.8 (Ar-*C*), 129.53 (Ar, 4 × *CH*) , 129.48 (Ar, 4 × *CH*), 129.2 (Ar, 2 × *CH*), 128.7 (Ar, 2 × *CH*), 128.4 (Ar, 2 × *CH*), 125.1 (Ar, 2 × *CH*), 122.0 (Ar-*C*), 121.3 (Ar, 2 × *CH*), 78.1 (O-CH_2-), 77.8 (O-CH_2).

*5.1.4.7. 4-(2-(Tetrahydro-2H-pyran-2-yl)oxy)guanidino-N-((4-(2-(tetrahydro-2H-pyran-2-yl)oxy)guanidino)phenyl)benzamide (**7g**)*

Following the general procedure, the reaction of **5g** (200 mg, 0.39 mmol) with 1 M KOH gave the free base of **7g** as white solid (170 mg, 85%). ^{1}H NMR (400 MHz,

DMSO-d_6) δ 9.78 (s, 1H, N*H*), 8.31 (s, 1H, N*H*), 7.79 (d, *J* = 8.8, 2H, Ar*H*), 7.73 (s, 1H, N*H*), 7.52 (d, *J* = 9.0, 2H, Ar*H*), 7.38 (d, *J* = 8.8, 2H, Ar*H*), 7.26 (d, *J* = 9.0, 2H, Ar*H*), 5.48 (s, 2H, NH_2), 5.27 (s, 2H, NH_2), 4.96 (t, *J* = 3.7, 1H, O-*CH*-O), 4.92 (t, *J* = 3.8, 1H, O-*CH*-O), 3.81 (m, 2H, -CH_2-), 3.43 (m, 2H, -CH_2-), 1.86 (m, 2H, -CH_2-), 1.65 (m, 4H, -CH_2-), 1.49 (m, 6H, -CH_2-). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.2 (*C*=O), 152.8 (*C*=N), 152.1 (*C*=N), 145.4 (Ar-*C*), 138.1 (Ar-*C*), 132.4 (Ar-*C*), 129.1 (Ar, 2 × *CH*), 126.1 (Ar-*C*), 121.7 (Ar, 2 × *CH*), 117.9 (Ar, 2 × *CH*), 116.6 (Ar, 2 × *CH*), 99.9 (2 × O-*CH*-O), 62.0 (2 × O-CH_2), 29.9 (-CH_2-), 29.8 (-CH_2-), 25.9 (2 × -CH_2-), 20.1 (-CH_2-), 20.0 (-CH_2-). Mp > 300 °C. HPLC (UV) > 95%. LRMS (ES^+) *m/z* = 512.67 (M + H).

*5.1.4.8 4-(2-Hydroxyguanidino)-N-(4-(2-hydroxyguanidino)phenyl)benzamide dihydrochloride (**7h**)*

A solution of **7g** (100 mg, 0.2 mmol) in MeOH (1 mL) and HCl_g-saturated 1,4-dioxane solution (2 mL) was stirred at room temperature for 12 h. The volatiles were removed under vacuum, Et_2O was added and the solid was triturated with a spatula to give the dihydrochloride of **7h** as white powder (69 mg, 69%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.25 (s, 1H), 10.83 (s, 1H), 10.49 (s, 1H), 10.36 (s, 2H), 10.12 (s, 1H), 9.95 (s, 1H), 8.20 (s, 2H), 8.08 (d, *J* = 8.3, 2H, Ar*H*), 7.89 (d, *J* = 8.4, 2H, Ar*H*), 7.34 (d, *J* = 8.2, 2H, Ar*H*), 7.20 (d, *J* = 8.4, 2H, Ar*H*). ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.9 (*C*=O), 157.4 (*C*=N), 156.4 (*C*=N), 139.6 (Ar-*C*), 137.9 (Ar-*C*), 130.6 (Ar-*C*), 129.6 (Ar, 4 × *CH*), 125.3 (Ar-*C*), 121.7 (Ar, 4 × *CH*). Mp > 300 °C. HPLC (UV) > 95%. LRMS (ES^+) *m/z* = 344.57 (M + H).

*5.1.4.9. 1,3-Bis(4-(2-methylguanidino)phenyl)urea (**8a**)*

Following the general procedure, the reaction of **6a** (132 mg, 0.4 mmol) with 1 M KOH gave the free base of **8a** (107 mg, 81%) which was transformed into the dihydrochloride

of **8a**. Dihydrochloride of **8a**: white solid (95 mg). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.8 (s, 2H, N*H*), 9.6 (br s, 2H, N*H*), 7.7 (m, 6H, NH_3), 7.5 (d, *J* = 8.8, 4H, Ar*H*), 7.1 (d, *J* = 8.8, 4H, Ar*H*), 2.8 (d, *J* = 4.9, 6H, NCH_3). ^{13}C NMR (126 MHz, DMSO-d_6) δ 155.9 (2 × *C*=O), 152.7 (2 × *C*=N), 138.7 (2 × Ar-*C*), 128.5 (Ar, 4 × *CH*), 126.3 (2 × Ar-*C*), 118.6 (Ar, 4 × *CH*), 28.2 (N-CH_3). Mp 277–278 °C. HPLC (UV) = 95%. LRMS (ES$^+$) *m/z* = 383.60 (M + H).

*5.1.4.10. 1,3-Bis(4-(2-ethylguanidino)phenyl)urea (**8b**)*

Following the general procedure, the reaction of **6b** (200 mg, 0.38 mmol) with 1 M KOH gave the free base of **8b** as white solid (132 mg, 91%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.25 (br s, 2H, N*H*), 7.23 (d, *J* = 8.7, 4H, Ar*H*), 6.65 (d, *J* = 8.7, 4H, Ar*H*), 5.25 (br s, 2H), 4.76 (br s, 4H), 3.12 (d, *J* = 7.2, 4H, O-CH_2-), 1.06 (t, *J* = 7.2, 6H, CH_3). ^{13}C NMR (101 MHz, DMSO) δ 152.9 (*C*=O), 151.3 (2 × *C*=N), 145.5 (2 × Ar-*C*), 132.7 (2 × Ar-*C*), 123.0 (Ar, 4 × *CH*), 119.3 (Ar, 4 × *CH*), 35.2 (2 × N-CH_2-), 15.1 (2 × CH_3). Mp 211–212 °C. HPLC (UV) > 98%. LRMS (ES$^+$) *m/z* = 383.53 (M+H).

Dihydrochloride of **8b**: white solid (79 mg). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.81 (s, 2H, N*H*), 9.57 (s, 2H, N*H*) 7.83 (s, 2H, N*H*), 7.60 (br s, 4H) 7.53 (d, *J* = 8.7, 4H, Ar*H*), 7.14 (d, *J* = 8.7, 4H, Ar*H*), 3.25 (m, 4H, N-CH_2-), 1.12 (t, *J* = 7.1, 6H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 155.0 (2 × *C*=N), 152.7 (*C*=O), 138.7 (2 × Ar-*C*), 128.6 (Ar, 4 × *CH*), 126.3 (2 × Ar-*C*), 118.7 (Ar, 4 × *CH*), 36.3 (2 × N-CH_2), 14.3 (2 × CH_3). HPLC (UV) = 98%.

*5.1.4.11. 1,3-Bis(4-(2-isopropylguanidino)phenyl)urea (**8c**)*

Following the general procedure, the reaction of **6c** (300 mg, 0.56 mmol) with 1 M KOH gave the free base of **8c** as white solid (157 mg, 71%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.30 (m, 2H), 7.23 (d, *J* = 8.7, 4H, Ar*H*), 6.65 (d, *J* = 8.7, 4H, Ar*H*), 4.98

(br s, 6H), 3.83 (hept, *J* = 6.4, 2H, -*CH*-), 1.09 (d, *J* = 6.4, 12H, -*CH*$_3$). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ 152.9 (*C*=O), 150.7 (2 × *C*=N), 145.2 (2 × Ar-*C*), 132.8 (Ar-*C*), 123.0 (Ar, 4 × *CH*), 119.3 (Ar, 4 × *CH*), 41.4 (2 × N-*CH*), 23.0 (4 × *CH*$_3$). Mp 206–207 ºC. HPLC (UV) = 96 %. LRMS (ES$^+$) *m/z* = 396.47 (M + H).

Dihydrochloride salt of **8c**: White solid (105 mg). ^{1}H NMR (300 MHz, DMSO-d$_6$) δ 9.72 (br s, 2H), 9.44 (br s, 2H), 7.82 (m, 2H), 7.53 (d, *J* = 8.5, 4H, Ar*H*), 7.48 (br s, 4H) 7.14 (d, *J* = 8.5, 4H, Ar*H*), 3.87 (m, 2H), 1.17 (d, *J* = 6.2, 12H). ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 154.2 (*C*=O), 152.7 (2 × *C*=N), 138.6 (2 × Ar-*C*), 128.7 (2 × Ar-*C*), 126.0 (Ar, 4 × *CH*) 118.7 (Ar, 4 × *CH*), 43.4 (2 × N-*CH*), 22.3 (4 × *CH*$_3$). HPLC (UV) = 97%.

5.2. Biology

5.2.1. In vitro antiprotozoal activity

Susceptibility assays against *T. b. rhodesiense* (strain STIB900) and cytotoxicity assays against rat myoblast L6-cells were performed using an Alamar blue-based assay [24, 36]. Detailed experimental procedures were described in a previous paper [37]. The in vitro antimalarial activity against the chloroquine sensitive strain of *P. falciparum* NF54 was determined using the [^{3}H]hypoxanthine incorporation assay [25] as previously reported [37].

5.2.2. In vivo activity against T. b. rhodesiense: STIB900 mouse model of stage 1 HAT

Four female NMRI mice were used per experimental group. Each mouse was inoculated i.p. with 10^4 bloodstream forms of STIB900, respectively. Heparinized blood from a donor mouse with approximately 5 × 10^6 /mL parasitaemia was suspended in PSG to obtain a trypanosome suspension of 1 × 10^5 /mL. Each mouse was injected with 0.25

mL. Compounds were formulated in 100% DMSO, diluted 10-fold in distilled water or as specified by the supplier. 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. Three mice served as infected-untreated controls. They were not injected with the vehicle alone since we have established in our laboratory that these vehicles do not affect parasitaemia nor the mice (data 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.

All the in vivo efficacy studies in mice 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.

5.3. DNA Binding: Surface Plasmon Resonance Studies

5.3.1. Compounds, DNA and buffers

SPR experiments were performed at 25 ºC with a Biacore X–100 apparatus (GE Healthcare, Biacore AB, Uppsala, Sweden) in MES buffer (10 mM 2-(*N*-morpholino)ethanesulfonic acid, 1 mM EDTA, 100 mM NaCl, 0.005% surfactant P20, pH 6.25). The 5'-biotin labeled DNA hairpins were purchased from Sigma-Aldrich with HPLC purification. The DNA hairpin sequences included 5'-biotin-CGAATTCGTCTCCGAATTCG, 5'-biotin-CATATATATCCCCATATATATG, and 5'-biotin-CGCGCGCGTTTTCGCGCGCG (the loop region is underlined) referred as

AATT, $(AT)_4$, and $(CG)_4$, respectively. The hydrochloride salts of compounds **7b**, **7c**, and **8b** were dissolved in MES buffer and these stock solutions (C = 5 mM) were used to prepare the different dilutions for the binding experiments.

5.3.2. DNA Immobilization

The DNA hairpins were immobilized independently on three streptavidin-derivatized gold chips (SA chip from Biacore containing 2 flow cells) by injection of a 25 nM hairpin DNA solution with a flow rate of 1 μL/min until a response of about 400 RU was reached. Flow cell 1 was used as reference while flow cell 2 was immobilized with the different DNA hairpins.

5.3.3. Binding Experiments

Typically, a series of different concentrations of compounds **7b**, **7c**, and **8b** was injected onto the chip at 25 ºC with a flow rate of 20 μL/min for a period of 5 min followed by a dissociation period of 5 min. After the dissociation process, the chip surface was regenerated with a 20 μL injection of 200 mM NaCl and 10 mM NaOH solution, injection tube rinsing, and multiple 1 min buffer injections. The number of binding sites and the binding constants at equilibrium were obtained by fitting SPR results to a one site ($K_2 = 0$) or two-site binding model according to equation 1:

$$r = RU/RU_{max} = (K_1C_f + 2K_1K_2C_f^2) / (1 + K_1C_f + K_1K_2C_f^2) \qquad (1)$$

where r is the moles of bound compound per mole of DNA hairpin duplex, K_1 and K_2 are microscopic binding constants, and C_f is the free compound concentration at equilibrium [30, 38].

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