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# *In vitro* activity and mode of action of Distamycin analogues against African trypanosomes

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# 32 Graphical abstract





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# 35

# 36 Highlights

- 37 tri-thiazoles resembling distamycin present anti-*Trypanosoma brucei* activity
- the most lipophilic derivative shows higher potency and selectivity than nifurtimox
- 39 cytocidal action involves lysosomal damage, release of free-iron and oxidation
- 40 subtle substitutions confer selectivity for the molecular target of tri thiazoles

#### 41 Abstract

42 Distamycin, a natural polyamide containing three heterocycle rings with a polar end, has inspired several groups to prepare synthetic analogues, which proved to have anti-43 44 trypanosomal and anti-tumoral activity. We describe the synthesis of bi and tri thiazoles 45 amides that harbor different substitutions at their ends and the evaluation of their anti-Trypanosoma brucei activity. The most active compound 10b showed better biological 46 activity (EC<sub>50</sub> 310 nM and selectivity index 16) than the control drug nifurtimox (EC<sub>50</sub> 15  $\mu$ M 47 48 and selectivity index 10). Studies on the mode of action show that the parasiticidal activity of 49 10b originates from disruption of lysosomal homeostasis, which is followed by release of 50 redox active iron, an increase in oxidizing species and collapse of cell membrane integrity. In 51 this respect, our study suggests that non-charged lipophylic distamycins destabilize cell 52 membranes.

- 53 54
- 55 Keywords: thiazole, *Trypanosoma brucei*, lysosome, iron, membrane damage
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#### 58 **1. Introduction**

59 Human African Trypanosomiasis (HAT or sleeping sickness) is caused by the hemoflagellated parasites Trypanosoma brucei rhodesiense and Trypanosoma brucei gambiense whereas 60 61 Trypanosoma brucei brucei is the etiological agent of the cattle disease Nagana. The parasites 62 are transmitted by the bite of an infected tsetse fly (Glossina genus) that infests 37 sub-Saharan countries. Human and cattle infections by African trypanosomes are either fatal or 63 64 highly disabling if left untreated [1]. Although control programs implemented years ago 65 contributed to reduce the number of HAT cases to less than 10000 per year, Nagana remains a 66 major obstacle for the nutritional and economical development of the rural communities from 67 endemic areas [2]. For instance, per year about 3 million cattle dye, 35 million doses of trypanocidal drugs are administered and direct losses in cattle production alone account for 6 68 69 to 12 billion US\$ [3].

70 Present-day chemotherapy for HAT includes: suramin (1916), pentamidine (1937), 71 melarsoprol (1949), effornithine (1990) and nifurtimox-effornithine combined therapy (NECT, 72 2009), all of them suffering from poor efficacy, undesirable route of administration, 73 unacceptable toxicity and emergence of drug resistance [4-6]. Nagana treatment includes: 74 isometamidium chloride, diminazene aceturate, homidium bromide and chloride, both proven 75 mutagenic agents [7-9]. The easy accession to these drugs and lack of administration controls 76 are considered the main causes for the increasing drug resistance reported in 17 African 77 countries [10]. Thus, the significant limitations of the current chemotherapy prompt the urgent 78 discovery of safer and effective anti-trypanosomal drugs.

79 Natural products are widely recognized for playing important roles in drug development, 80 particularly as anticancer, antibiotic and antiparasitic drugs [11,12]. Furthermore, natural 81 polyamides containing heterocycles as distamycin and netropsin analogues have been 82 investigated as molecules with helical topology that form strong complexes with the minor 83 groove of DNA [13]. Many research groups have developed methodologies to obtain 84 distamycin (1, Figure 1) analogues and study their DNA binding affinity and sequence selectivity as anti-infective agents [14-17]. Recently, Suckling's group reported a series of 85 oligoamides of 2-amino-5-alkylthiazole 4-carboxylic acids (2, Figure 1), synthesized as 86

distamycin and netropsin analogues, with interesting anti-trypanosomal activities [18]. Although this work did not address the mode of action of the most active compounds, possible mechanisms of action were proposed based on a structure-activity relationship analysis and the structural/physicochemical properties of the compounds. Similarly to diamidines, these compounds may intercalate and disrupt the mitochondrial DNA (kinetoplast) [13] and/or, as certain anti-microbial peptides called  $\alpha$ - and  $\beta$ -defensins, may permeabilize cell membranes due to the presence of ionizable groups [19].

As part of our search for candidate compounds to develop novel antiparasitic drugs employing molecular simplification [20-22], here we present the results on the synthesis of thiazoles linked by amide bonds as distamycin analogues (**3**, figure 1) and their biological activity against bloodstream *T. brucei brucei* and murine macrophages (cell line J774). Furthermore, we investigated the potential antitrypanosomal mode of action of the most potent and selective analogue identified.





Distamycin (1)





R<sub>1</sub>= OH, OEt R<sub>2</sub>= NH<sub>2,</sub> NHBoc, NHAc, CH<sub>3</sub>

100

101 Figure 1. Distamycin and oligoamide analogues

102

## 103 2. Results and Discussion

104 2.1. Synthesis of distamycin analogues

105 The synthetic strategy started with Hantszch's thiazoles synthesis, obtained from ethyl bromo

106 piruvate and acetylthioamide to obtain thiazole 4a or thiourea to obtain thiazole 4b (Scheme

107 1). Then, ethyl ester hydrolysis of thiazole 4a allowed us to obtain thiazole acid 4e. The amine
108 group in position 5 of thiazole 4b was protected with di-*tert*-butyl dicarbonate (4c) and the
109 ethyl ester group was hydrolyzed to obtained thiazole acid 4d.

110 Bis-thiazole ester **5a** was obtained from thiazole amine **4b** and thiazole acid **4e**, employing 111 HBTU (O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) as 112 coupling reagent, in 90% yield (Scheme 1).

113 Boger's and Brucoli's groups reported bis-thiazole **5b** synthesis employing EDCI (N-(3-114 Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) as coupling reagent with 58% 115 and 71% yield, respectively [23, 24]. In our hands, this coupling reagent resulted in 5b with 116 44% yield from both thiazole amine 4b and thiazole acid 4d. However, employing HBTU as 117 coupling reagent, the yield was increased to 70%. The bis-thiazoles analogues 6a and 6b were 118 obtained quantitatively by ester hydrolysis of thiazole esters 5a and 5b, respectively. N-Boc cleavage of bis-thiazole 5b gave bis-thiazole 7 (quantitative) and allowed us to prepare the N-119 120 acetyl derivate 8 (90% yield). Compound 8 was converted quantitatively into the bis-thiazole 121 **9** by classical ester hydrolysis.

122 Following the same serie of reactions we prepared tri-thiazoles 10a and 10b. Despite the well 123 known poor reactivity of the amine thiazole, we were able to obtain a 66% yield for the 124 synthesis of 10a from 4b and 6a. For preparation of the tri-thiazole 10b (synthesis reported in 125 reference [25] with EDCI as coupling reagent in 52% yield) two strategies were explored: 1) 126 from thiazole acid 4d and amine bithiazole 7 with HBTU, we obtained tri-thiazole 10b in 33% 127 yield and 2) from amine thiazole 4b and bithiazole acid 6b with HBTU, we obtained tri-128 thiazole 10b in 45% yield. Coupling reagents as EDCI and DEPBT resulted in a poor 129 formation of tri-thiazole 10b. Then, analogues 11 and 12 were obtained in good yields by N-130 deprotection and N-acetylation reactions, respectively. Tri-thiazole 13 and 14 were prepared 131 by ester hydrolisis of tri-thiazole 10b and 12, respectively.



i- (Boc)<sub>2</sub>O, Et<sub>3</sub>N, THF:CH<sub>2</sub>Cl<sub>2</sub> (1:1), reflux, 3d, 74%. ii- KOH aq 10%, THF:H<sub>2</sub>O (1:1). rt, 2h, quant. iii- HBTU, DIPEA, 4-DMAP, CH<sub>2</sub>Cl<sub>2</sub>, rt, 24h. iv- TFA, CH<sub>2</sub>Cl<sub>2</sub>, rt, 24h, quant. v- Ac<sub>2</sub>O, Et<sub>3</sub>N, Py, CH<sub>2</sub>Cl<sub>2</sub>, rt, 48h.

- 133 Scheme 1. Synthesis of distamycin analogues.
- 134

135 2.2. Biological activity

Bi- and tri-thiazoles linked by amide bond were evaluated *in vitro* against the infective form of *T. b. brucei*, which is also a suitable laboratory model of the human pathogen *T. b. rhodesiense*. An initial screening was performed at 5 and 30  $\mu$ M compound followed by the

139 assessment of the  $EC_{50}$  for compounds displaying a cytotoxicity against the parasite > 40% at

140 5  $\mu$ M. In addition, the selectivity of their biological activity was tested towards murine 141 macrophages (cell line J774). The results are summarized in Table 1.

142

143	Table 1	. Biological	activity of	of distamyci	n analogues.
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	Compound <sup>a</sup>	Infective T. b. brucei <sup>b</sup>		Selectivity
Entry		$EC_{50}(\mu M)$	growth inhibition at 5 μM (%)	index <sup>c</sup>
1	BocHN-Tz-Tz-COOEt (5b)	$13.7 \pm 1.4$	0	3.5
2	BocHN-Tz-Tz-COOH (6b)		$10.8 \pm 7.3$	ND
3	H <sub>2</sub> N-Tz-Tz-COOEt (7)		$25.6\pm7.4$	ND
4	AcHN-Tz-Tz-COOEt (8)	9.6 ± 5.6	$\sim$	7.4
5	AcHN-Tz-Tz-COOH (9)		$20.0\pm2.5$	ND
6	BocHN-Tz-Tz-COOEt (10b)	$0.31\pm0.07$		16.5
7	BocHN-Tz-Tz-Tz-COOH (13)		$22.7\pm8.4$	ND
8	H <sub>2</sub> N-Tz-Tz-COOEt (11)	5.5 ± 0.1		19.6
9	AcHN-Tz-Tz-Tz-COOEt (12)	5.4 ± 1.2		29.1
10	AcHN-Tz-Tz-Tz-COOH (14)		no effect	ND
11	H <sub>3</sub> C-Tz-Tz-COOEt (5a)		no effect	ND
12	H <sub>3</sub> C-Tz-Tz-COOH (6a)		$8.1\pm2.0$	ND
13	H <sub>3</sub> C-Tz-Tz-Tz-COOEt (10a)	≅ 5		10.5
14	Nifurtimox	$15.0\pm2.5$		10.0

144 ND, not determined.

<sup>a</sup> Tz, thiazole.

146 <sup>b</sup> the values are expressed as mean  $\pm$  SD (n=3).

<sup>c</sup> Ratio EC<sub>50</sub> murine macrophage/EC<sub>50</sub> *T. b. brucei* 

- 148 Overall, the tri-thiazole derivatives presented higher anti-trypanosomal activity (4 out of 6
- 149 with  $EC_{50} \le 10 \ \mu\text{M}$ ) and selectivity (SI  $\ge 10$ ) than the bi-thiazole analogues (1 out of 7 with
- 150  $EC_{50} \le 10 \ \mu M$  and SI <10). For instance, the addition of a heterocycle to **5a** (entry 11, not
- 151 active) and 7 (entry 3, 26% growth inhibition at 5  $\mu$ M) produced the more potent analogues
- 152 **10a** (entry 13, EC<sub>50</sub>  $\approx$  5 µM) and **11** (entry 8, EC<sub>50</sub> = 5.5 µM) respectively, and conversion of
- 153 the bi-thiazole **5b** (entry 1,  $EC_{50} = 13.7 \mu M$ ) into the tri-thiazole **10b** increased near 40 times
- 154 its anti-trypanosomal activity (entry 6,  $EC_{50} = 0.31 \mu M$ ).
- For the bi-thiazoles, there appear to be a need for fulfilling an steric demand on the molecular target since the 2-amino substituted (position  $R_2$ ) thiazole **5b** and **8** (entries 1 and 4, respectively, average EC<sub>50</sub> of 11 µM) were more active than the 2-methyl analogue **5a** (entry 11, no cytotoxic at 5 µM) and the *N*-unsubstituted bis-thiazole **7** (entry 3, 26% growth inhibition at 5 µM). In contrast, the nature of the substituent at  $R_2$  does not affect significantly the activity of bi-thiazoles when the structure contains a carboxylic acid at  $R_1$  (compounds **6a**, **6b** and **9** entries 12, 2 and 5, average growth inhibition of 15% at 5 µM).
- 162 The most active compound against T. b. brucei, the N-Boc trithiazole ester 10b (entry 6), 163 displayed a potency almost 50-fold (EC<sub>50</sub> =  $0.31 \mu$ M) higher than that of the control drug 164 nifurtimox (EC<sub>50</sub> = 15  $\mu$ M) and a similar selectivity index (SI) (SI = 16.5 for **10b** vs. SI = 10 165 for nifurtimox). Interestingly, compound 14 (entry 10), the closest analogue to the most active 166 tri-heterocycle derivative reported by Lang *et al.* (MIC = 64 nM) [18] is inactive towards 167 trypanosomes when tested at 30  $\mu$ M. The major differences between these compounds are: a) 168 thiazole moiety (14) vs. N-methyl pyrrole, and b) 5-unsubstituted (14) vs. 5-isopropyl 169 thiazoles. This suggests that hydrophobicity may play an important role in molecular 170 recognition of the cellular target and, hence, in the biological activity of distamycin analogues. 171 In line with this, all the molecules that can be charged at physiological pH (compounds **6a**, **6b**, 172 9, 13 and 14) presented null or marginal activity against the parasite (average 10% growth 173 inhibition at 5  $\mu$ M). Furthermore, almost all esters derivatives exhibited higher activities than 174 the corresponding compounds with a free carboxylate moiety: i.e. compare 5b with 6b (entries 1 and 2), 8 with 9 (entries 4 and 5), 10b with 13 (entries 6 and 7) and 12 with 14 (entries 9 and 175 176 10). In addition, compounds with blocked amino groups presented higher activity than their

177 congeners with a free amine – i.e. compare the tri-thiazoles **10b** and **11** (entries 6 and 8) and 178 the bi-thiazoles **5b** and **8** – suggesting that increased lipophilicity may contribute to their 179 activity. In fact, **10b** presented the highest log *P* values among the different analogues tested 180 here (Suplementary Table S1). Interestingly, a similar correlation between lipophilicity and 181 anti-*T. brucei* activity was reported for related bisimidazolines [26].

182 The SAR data shows that both the number of heterocycles rings and the lack of polarity183 modulate the biological activity of the distamycin analogues.

184

## 185 2.3. Studies on the mechanism of action

186 Distamycin and netropsin analogues were proposed as DNA minor groove binders that disrupt 187 parasite cell cycle and, consequently, inhibit parasite proliferation. Because of their chemical 188 nature and peptide like-structure these compounds were also suggested to act as  $\alpha$ - and  $\beta$ -189 defensins that interact and destabilize cell membranes (*e.g.* pore formation). While the first 190 mechanism is expected to exert a cytostatic effect, the second one should trigger cell lysis 191 (cytotoxic effect).

As a first approach to investigate the mode of action of the most active distamycin analogue identified in this work, compound **10b**, the integrity of the parasite membrane was monitored using the membrane impermeant dye propidium iodide (PI).

195 Membrane integrity assays were performed exposing the parasites for short times (0.5 - 4 h)196 to 10b at  $\frac{1}{2}$ - and 1-fold its EC<sub>50</sub> value (310 nM) or to the detergent Triton X-100 (0.001%) 197 v/v; positive control), and adding PI immediately prior to sample analysis by flow cytometry. 198 The assay shows a time-dependent increase in the number of cells PI positive, which, in 199 general, correlated also with the concentration of **10b** (Figure 2). For instance, added at its 200  $EC_{50}$ , the compound triggered membrane damage in about 10% of the cell population after 1 h 201 of treatment, a value that increased to 80-90% for the subsequent 2-4 h exposure. At this time 202 point, the degree of membrane permeabilization produced by 10b (310 or 155 nM) was almost 203 identical to that exerted by the detergent used as control. The mode of action of nifurtimox 204 involves its reduction to a non-saturated open-chain nitrile that acts as potent electrophile, 205 which modifies different biomolecules [27]. As expected, nifurtimox tested at its  $EC_{50}$  (15  $\mu$ M)

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did not affect membrane permeability in parasites treated for 0.5-4 h. It is worth to note that except for a single data point (77.5 nM compound for 1 h), the effect exerted by **10b** on membrane integrity was statistically significant (p < 0.05) for all concentrations and time points tested compared to parasites treated with the vehicle alone (DMSO) or nifurtimox (NFX).

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Figure 2. Membrane permeability of bloodstream *T. brucei* treated with compound 10b. Five x10<sup>5</sup> parasites/mL were treated with different concentrations (310, 155 and 77.5 nM) of 10b for different times: A) 0.5 h, B) 1 h, C) 2 h and D) 4 h. Control treatments included Triton X-100 at 0.001% v/v, nifurtimox (NFX) at 15  $\mu$ M and DMSO at 1% v/v. Values are expressed as mean ± SD (n=3) and the asterisks denote the probability indexes of 10b-treated parasites *vs.* DMSO control (\* *p* < 0.05; \*\* *p* < 0.01; \*\*\* *p* < 0.001), calculated applying the ONE WAY ANOVA test and the Dunnets's multiple comparision post test.

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221 Different molecular mechanisms may lead to the disruption of parasite membrane integrity, 222 from the insertion of the compound into the cell membrane, as shown for different mammalian 223 antimicrobial peptides that kill African trypanosomes (e.g. a- and \beta-defensins and 224 cathelicidins) [28], to the inhibition of components from the parasite anti-oxidant system. In 225 this respect, it is well documented that down-regulation or gene knockout of key enzymes 226 from the parasite redox system triggers the oxidation of unsaturated lipids in lysosomal or 227 mitochondrial membranes, which releases iron that enhances the peroxidation of membranes 228 (via Fenton reaction) resulting in a rapid cell lysis [29-32].

A *T. b. brucei* transgenic cell line expressing a GFP-based redox biosensor was used to investigate whether the mode of action of **10b** implies a deregulation of the intracellular redox milieu. The biosensor, hGrx-roGFP2, is able to rapidly (nano second) translate changes in the pool of reduced and oxidized glutathione (GSH/GSSG) into changes in its fluorescence intensity at 488 nm [33].

234 The redox reporter T. brucei cell line was exposed to different concentrations of 10b, 235 menadione, NFX, the membrane permeant reducing agent dithiothreitol (DTT) or the vehicle 236 DMSO for 1 h, and the fluorescence intensity of the biosensor was analyzed by flow 237 cytometry. As shown in Figure 3 A, at all concentrations tested (77.5-310 nM) 10b lowered 238 by ~ 10% (p < 0.001) the fluorescence intensity of the biosensor, which indicates a higher 239 GSSG/GSH ratio or a more oxidative millieu, compared to parasites treated or not with 240 DMSO. As expected, the potent redox cycler menadione added at 1 mM caused a 21% 241 oxidation of the biosensor whereas 15 µM NFX or 1 mM DTT did not elicit redox changes 242 after 1 h treatment.

In order to confirm the redox basis of the changes in biosensor fluorescence triggered by **10b**, the cells exposed to 77.5-310 nM compound for 1 h were treated with 1 mM DTT for additional 30 min. This treatment was able to restore the redox state of the biosensor to levels similar to the non-treated or vehicle-treated cells (Figure 3 B), supporting the idea that **10b** alters the intracellular redox balance. Similarly, the oxidation of the biosensor in parasites exposed to menadione was reverted by treatment with DTT. For the control conditions (NFX or DMSO), the reducing agent did not modify the fluorescence intensity of the biosensor.



Figure 3. Evaluation of the intracellular redox state of infective T. b. brucei treated 252 253 with 10b. Parasites (1x10<sup>6</sup> cells/mL) expressing the redox biosensor hGrx-roGFP2 were 254 treated for 1 h with 10b (77.5, 155 and 310 nM) or different control compounds such as 255 menadione (1 mM), nifurtimox (NFX, 15 µM), dithiothreitol (DTT, 1 mM), DMSO 1% v/v 256 and 1 mM DTT. After the corresponding treatments the cells were analyzed by flow 257 cytometry. Redox analysis was performed only for PI negative cells. The results are presented 258 as percentage biosensor reduction relative to DMSO for: A) parasites treated for 1 h with 259 different compounds, and **B**) followed by a 30 min treatment with 1 mM DTT. Values are expressed as mean SD (n=3) and the asterisks indicate difference (p < 0.001) with respect to 260 the DMSO control according to the ONE WAY ANOVA test and the Dunnets's multiple 261 262 comparison post test.

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In principle, the intracellular oxidation of parasites treated with **10b** may be consequence of 264 265 the direct inhibition of specific component(s) from the antioxidant system. In order to test this 266 hypothesis, we first analyzed the potential of **10b** to inhibit trypanothione synthetase (TryS), 267 the enzyme producing the major redox cosubstrate in trypanosomatids [29, 34], namely trypanothione. At 30 µM 10b, as well as none of the distamycin analogues synthesized in this 268 269 work, did not inhibit to a significant level trypanothione synthetase from T. brucei, 270 Trypanosoma cruzi and Leishmania infantum (Supplementary Table 2). Next, mammalian 271 cells (cell line CHO-K1) expressing the redox biosensor rxYFP, which also detects changes in the GSSG/GSH ratio [34], were exposed to concentrations of 10b around the EC<sub>50</sub> determined 272 273 for macrophages (5 µM) for 1 h and analyzed by flow cytometry (Fig 4 A and B). Irrespective 274 of the concentration tested (1-10 µM), **10b** produced about 10% oxidation of the biosensor in

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275 CHO-K1 cells compared to cells treated with vehicle (p < 0.01). Control treatments with 276 menadione or H<sub>2</sub>O<sub>2</sub>, both added at 1 mM, induced a marked oxidation (i.e. 70% and 25%, 277 respectively) of the biosensor (p < 0.001). Addition of 1 mM DTT for 30 min to cells treated 278 with **10b**, menadione or H<sub>2</sub>O<sub>2</sub> (10 µM) restored the fluorescence intensity of rxYFP to basal 279 levels, confirming thus the redox origin of the changes in biosensor fluorescence.

280



281

Figure 4. Evaluation of intracellular redox state of a mammalian cell line treated with 282 283 **10b.** Cells (8x10<sup>5</sup> cells/mL) CHO-K1 expressing the redox biosensor rxYFP were treated for 1 h with **10b** (1, 5 and 10 µM) or different control compounds such as menadione (1 mM), 284 hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, 1 mM), dithiotreitol (DTT, 1 mM), DMSO 1% v/v and 1 mM 285 DTT. After the corresponding treatments the samples were analyzed by flow cytometry and 286 the redox changes quantified only for PI negative cells. The results are presented as 287 288 percentage rxYFP reduction relative to DMSO for: A) cells treated for 1 h with different 289 compounds, and **B**) followed by a 30 min treatment with 1 mM DTT. Values are expressed as mean SD (n=3) and the asterisks denote difference (\*\* = p < 0.01 and \*\*\* = p < 0.001) 290 291 with respect to the DMSO control according to the ONE WAY ANOVA test and the 292 Dunnets's multiple comparison post test.

293

Taken together, these results led us to conclude that the intracellular oxidation induced by **10b** cannot be ascribed to the inhibition of a specific component of the redox metabolism of the parasite.

Alternatively, the redox changes triggered by **10b** may be consequence of a secondary process

- associated to a functional or structural impairment of organelles (i.e. lysosome, endoplasmic
- reticulum and/or mitochondria) that host (pro)oxidant molecules [31, 32].

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300 In this regard, we evaluated the integrity of the mitochondrion and lysosome, both single copy 301 organelles in trypanosomes, using the organelle-specific fluorophores MitoTracker and 302 LysoTracker, respectively. Parasites pre-treated with 310 nM 10b for 30 min were further 303 incubated with the mitochondrial marker for additional 30 min at 37°C and then fixed with 304 paraformaldehyde. Mitotracker is incorporated to the single parasite's mitochondrion via 305 active transport; therefore, a functional or structural impairment of this organelle will result in 306 a lower MitoTracker signal. The morphology and staining (fluorescence intensity) of the 307 mitochondrion of parasites exposed to 10b was similar to that of cells treated with DMSO 1% 308 v/v (Supplementary Figure 1), indicating that the compound does not exert mitochondrial 309 damage and loss of function.

310 In trypanosomes, the lysosome is located between the nuclear and mitochondrial (kinetoplast) 311 DNA. Most parasites incubated at 37°C for 30 min with DMSO 1% v/v and Lysotracker 312 showed a discrete lysosomal staining (Figure 5A). In contrast, parasites exposed for 30 min to 313 10b showed a non-discrete staining with the acidotropic probe that included the labeling of 314 several vesicular compartments (Figure 5B) and a uniform staining of the cytosol (Figure 5C), 315 which is reminiscent of an early and advanced damage of the lysosome. The quantitative 316 analysis (Figure 5D) shows that the number of parasites presenting a discrete (single) 317 lysosomal signal is more than 3-fold lower in the culture treated with 10b (23  $\pm$  4%) 318 compared to the DMSO-treated control ( $77 \pm 3\%$ ). Although our experiment does not allow to 319 discriminate whether the dispersed LysoTracker staining is due to an enlargement of the 320 lysosome or to the lost of its integrity, it is important to recall that similar phenotypic changes 321 were reported for parasites defective in the expression of a cytosolic peroxidase that protects 322 against lipoperoxidation of the lysosomal membrane [30, 31] or of Rab4, a protein involved in 323 lysosomal trafficking [35]. In summary, our finding indicates that the rapid and lethal 324 cytotoxic action of **10b** is probably triggered by disruption of lysosomal homeostasis.

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- 326





328 Figure 5. Compound 10b disrupts the lysosomal homeostasis of bloodstream *T. brucei*. *T.* brucei brucei (2.5x10<sup>6</sup> cells/mL) was treated with 10b (0.31 µM) or DMSO 1% v/v and 329 LysoTracker Red DND-99 25 µM. After 30 min incubation at 37°C, the cells were fixed with 330 331 paraformaldehyde and the slides observed under a fluorescence microscope. Representative 332 images of parasites treated with A) DMSO and showing discrete (single red dot) lysosome 333 staining, B) 10b and showing early (multiple red dots) or C) late lysosomal damage (dispersed 334 signal). D) Percentage of parasites showing discrete lysosomal staining. A total of 90 cells 335 were analyzed per treatment. Values are expressed as mean  $\pm$  SD (n=3), with the asterisk 336 denoting statistical difference with a p < 0.0001 compared to DMSO (one-tailed Student's t-337 test). For each condition are shown bright field and fluorescence (Lysotracker: red color, and DAPI staining for nucleus: N and mitochondrial DNA (kinetoplast): K) images from the samecell.

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341 In African trypanosomes, the lysosome plays a central role in iron metabolism because in this 342 organelle the transition metal is freed from host transferrin, stored or further mobilized to the 343 cytosol [36, 37]. Free iron is able to undergo Fenton reaction with the concomitant generation 344 of highly oxidizing species that cause irreversible damage in different macromolecules, such 345 as cell membranes [31]. In order to test whether the morphological alteration of the lysosome 346 caused by 10b involves the release of iron, the content of intracellular free-iron was analyzed 347 using the fluorescent probe PhenGreen SK diacetate. This reagent is a phenanthroline derivative conjugated to FITC ( $\lambda_{ex}$  488 nm), whose fluorescence is quenched by iron or 348 349 increased if the metal is sequestered by a chelator. All treatments were performed for 1 h at 350 37°C and the results are shown in Figure 6. First, parasites exposed to 10 µM deferoxamine (DFO), a chelator of Fe<sup>3+</sup> that following endocytic uptake localizes almost exclusively in 351 lysosomes [38], showed a 32% increase in probe fluorescence compared to the DMSO control 352 353 (p < 0.01; Figure 6A). In contrast, cells treated with 10b at 3.1  $\mu$ M showed a marked 354 reduction (50%, p < 0.001) of probe fluorescence, which is indicative of an increase in the 355 pool of free-iron at intracellular level (Figure 6A). Although not statistically significant, co-356 incubation with 10 µM DFO reduced by 15% fluorescence quenching triggered by 10b 357 (Figure 6A). As expected, a 10-fold higher concentration of DFO (100 µM) yielded a higher 358 unquenching effect of the iron probe in **10b**-treated cells (p < 0.01; Figure 6B). Compared to DFO, bipyridine (BiPy), a membrane-permeant Fe<sup>2+</sup>-chelator, added at 10 or 100 µM proved 359 more effective (p < 0.01 or p < 0.001, respectively) in preventing iron probe quenching in 360 361 cells exposed to 10b (Figure 6A and 6B). Altogether, these results confirm that 10b promotes 362 the release of free-iron in the cells and that the parasite's lysosome is a major source of 363 intracellular iron.

364



Figure 6. Evaluation of free-iron in bloodstream T. brucei treated with 10b. The 367 infective form of T. b. brucei ( $2x10^6$  cells/mL) was treated with 3.1  $\mu$ M 10b, 368 deferoxamine (DFO) and bipyridine (BiPy) at 10 µM (A) or 100 µM (B). Treatment 369 370 with DMSO 1% v/v was included as control. After incubation for 1 h at 37°C the 371 cells were analyzed by flow cytometry. Values are expressed as mean  $\pm$  SD (n=3) relative 372 fluorescence units (urf). The probability indexes for the different treatments are indicated with asterisks when compared to DMSO control (\* p = 0.1, \*\* = p < 0.01373 and \*\*\* = p < 0.001) or with hashtags when compared to **10b alone**-treated parasites (## 374 375 = p < 0.01 and ### = p < 0.001), respectively. The ONE WAY ANOVA test and the 376 Dunnets's multiple comparison post test were applied for statistical calculations.

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379 If the release of lysosomal iron triggered by **10b** is responsible for the collapse of the cell 380 membrane, the sequestration of free-iron should, at least in part, counteract this effect. In 381 order to confirm this hypothesis, parasites were treated for 2 h at 37°C with 0.31  $\mu$ M **10b** or 382 different concentrations of the iron chelators BiPy and DFO (Figure 7). As shown in Fig. 7A, 383 **10b** added alone increased to 87% the percentage of parasites with membrane damage (PI (+)), 384 whereas in the presence of BiPy 100  $\mu$ M the amount of PI(+) cells was significantly lower

(47%, p < 0.001). Although to a minor extent, also the co-incubation with DFO 100  $\mu$ M 385 386 reduced by 7% the number of PI (+) cells (p < 0.001). As expected, control treatments with 387 BiPy or DFO (100 µM) alone did not induce membrane permeabilization. As stated above, BiPy is a lipophilic molecule that rapidly permeates eukaryotic cell membranes through 388 389 passive diffusion, whereas DFO is endocyted slowly by the parasite, which can explain why 390 the last was unable to reduce parasite death caused by 10b under this assay condition. Indeed, 391 pre-treating cultures with different concentrations of DFO (125-500 µM) 3 h prior to a 3 h-392 exposure to 10b, reduced significantly (p < 0.001) and in a dose-response manner the number 393 of non-viable parasites (40-67% PI(+) cells) compared to cultures treated with 10b alone (80% PI(+) cells; Figure 7B). Interestingly, under the assay conditions tested here (Figure 6 394 and 7) and given the preferences ( $Fe^{2+}$  vs.  $Fe^{3+}$ ) and capacity (bidentate vs. hexadentate, for 395 BiPy vs. DFO) in iron binding, BiPy appeared to be more effective than DFO in counteracting 396 the effects triggered by **10b**, which suggest that  $Fe^{2+}$  is the major species at intracellular level. 397 Our findings strongly suggest that cell death triggered by 10b starts with the lost of lysosomal 398 399 homeostasis, accompanied by release of iron from this organelle, an increase in the 400 intracellular oxidative milieu and damage of cell membranes. In this respect, it is important to 401 note that the lysosome is a highly oxidizing and acidic organelle that host proteolytic activities. 402 Therefore, the collapse of the lysosome will lead to the release of these toxic components into 403 the cytosol, which will further exacerbate cellular damage and death.

404



Compound 10b

405



Figure 7. Iron chelators protect bloodstream T. brucei against membrane damage by 10b. 407 T. b. brucei (1x10<sup>6</sup> cells/mL) was treated with: A) 0.31  $\mu$ M 10b and 100  $\mu$ M deferoxamine 408 409 (DFO) or bipyridine (BiPy) for 2 h at 37°C, or B) different concentrations of DFO (125-500 410  $\mu$ M) for 3 h and then for additional 3 h with 0.31  $\mu$ M **10b**. After the corresponding treatments, membrane integrity was analyzed using PI staining and flow cytometry. Values are expressed 411 412 as mean  $\pm$  SD (n=3) and the asterisks denote the probability indexes of **10b**-treated parasites 413 vs. DMSO control (\*\*\*p < 0.001). Hashtags denote the probability indexes for DFO +/- 10b 414 or BiPy +/- 10b treated parasites vs. 10b alone (### = p < 0.001). The ONE WAY ANOVA 415 and the Dunnets's multiple comparison post tests were used for statistical calculations. 416

417

### 418 **3. Conclusion**

Novel distamycin analogues were synthesized in good yields, with several trithiazoles 419 420 displaying low to sub-µM and >10-fold cytotoxic selectivity towards the bloodstream form of 421 African trypanosomes. SAR analysis highlights the relevance of the number of heterocycles 422 linked by amide bonds and the hydrophobic character of the compound ends, as major 423 determinants of biological activity. In line with this, investigation of the mechanism of action 424 for the most active and lipophilic derivative (10b) suggests that the compound kills the 425 parasite by affecting the integrity of the lysosomal membrane, which is accompanied by 426 release of labile iron, and likely proteases, that upon redox cycling shift the intracellular redox 427 milieu to a more oxidative state both cuasing massive membrane damage. Our study provides 428 the first evidence that the most potent and non-polar trithiazole identified here behaves as a 429 defensin- or short hydrophobic peptide-like molecule that destabilize membrane structures

430 (citocidal), rather than as DNA-intercalating agent (citostatic), as recently reported for a

- 431 different series of distamycin analogues [39]. It also highlights the versatility and suitability of
- 432 distamycin-like structures to be modified in order to gain selectivity for a particular molecular
- 433 target.
- 434

# 435 **4. Experimental**

436 *4.1. Chemical methods* 

437 IR spectra were recorded on a Shimadzu FTIR 8101A spectrophotometer. <sup>1</sup>H NMR and <sup>13</sup>C
438 NMR spectra were recorded on Bruker Avance DPX- 400. Chemical shifts are related to TMS
439 as an internal standard. High resolution mass spectra (HRMS) were obtained on a micro Q440 TOF (ESI) (Bruker Daltonics).

441 Melting points were measured using a Fisher-Johns Melting Point Apparatus. Flash column 442 chromatography was carried out with Silica gel 60 (J.T. Baker, 40 μm average particle 443 diameter). All reactions and chromatographic separations were monitored by TLC, conducted 444 on 0.25 mm Silica gel plastic sheets (Macherey/Nagel, Polygram\_ SIL G/UV 254). TLC 445 plates were analyzed under 254 nm UV light, iodine vapor, p-hydroxybenzaldehyde spray or 446 ninhydrine spray. Yields are reported for chromatographically and spectroscopically (<sup>1</sup>H and 447 <sup>13</sup>C NMR) pure compounds.

- 448 All solvents were purified according to literature procedures [40]. All reactions were carried449 out in dry, freshly distilled solvents under anhydrous conditions unless otherwise stated.
- 450

4.1.1. General procedure for amide bond formation: HBTU (1.2 eq.), DIPEA (2.2 eq.) and 4-451 452 DMAP (0.2 eq.) were added to a stirred solution of the respective amine (1.0 eq.) and acid 453 (1.0 eq.) under N<sub>2</sub> atmosphere in dry CH<sub>2</sub>Cl<sub>2</sub> at 0° C. The resulting mixture was stirred at 454 room temperature for 24-72 h. Then it was filtered through celite, washed with CHCl<sub>3</sub> and 455 evaporated in vacuo. The crude was redissolved in EtOAc, washed with 5% v/v HCl and then 456 with a saturated solution of NaHCO<sub>3</sub>, dried with MgSO<sub>4</sub>, filtered and evaporated in vacuo. 457 The crude was purified by flash chromatography using the corresponding eluent to give the 458 amide.

460 *4.1.1.1*  $H_3C$ - $T_z$ - $T_z$ -COOEt (**5a**, Supp. Fig. S3): Compound **5a** was prepared following the 461 general procedure for amide bond formation from **4e** and **4b**. Yield 90%. White solid. M.p. 462 166-169°C. **R**<sub>f</sub> = 0.33 (EtOAc:hexane, 1:1), <sup>1</sup>**H NMR** (CDCl<sub>3</sub>, 400 MHz):  $\delta$  1.43 (t, 3H, J= 463 7.1Hz), 2.75 (s, 3H), 4.44 (q, 2H, J= 7.1Hz), 7.91 (s, 1H), 8.16 (s, 1H), 10,73 (s, 1H). <sup>13</sup>**C** 464 **NMR** (100 MHz, CDCl<sub>3</sub>):  $\delta$  14.4, 19.2, 61.5, 122.5, 125.8, 142.2, 147.0, 157.3, 158.6, 161.5, 465 167.0. **HRMS** m/z calcd for C<sub>11</sub>H<sub>11</sub>N<sub>3</sub>NaO<sub>3</sub>S<sub>2</sub> ([M+Na]<sup>+</sup>) 320.0134, found 320.0183. **IR film** 466 v(cm<sup>-1</sup>) 3109, 2924, 1716, 1666, 1624, 1535, 1234, 1211, 1173, 1096.

467

468 *4.1.1.2 BocHN-Tz-Tz-COOEt* (5b): Compound 5b was prepared following the general 469 procedure for amide bond formation from 4b and 4d. Yield 70%. White solid. M.p. 249-470 250°C. <sup>1</sup>H NMR ((CD<sub>3</sub>)<sub>2</sub>CO, 400 MHz):  $\delta$  1.35 (t, 3H, *J* = 7.3 Hz), 1.55 (s, 9H), 4.32 (q, 2H, 471 *J* = 7.3 Hz), 8.04 (s, 1H), 8.10 (s, 1H), 10.68 (s, 2H). <sup>13</sup>C NMR ((CD<sub>3</sub>)<sub>2</sub>CO, 100 MHz):  $\delta$  14.6, 472 28.3, 61.4, 83.0, 121.0, 123.4, 143.1, 143.3, 153.8, 158.1, 159.7, 161.3, 161.8.

473

474 *4.1.1.3*  $H_3C$ - $T_z$ - $T_z$ - $T_z$ -COOEt (**10a**, Supp. Fig. S5): Compound **10a** was prepared following 475 the general procedure for amide bond formation from **6a** and **4b**. Yield 66%. Solid. M.p. 225-476 229°C. **R**<sub>f</sub> = 0.34 (EtOAc:hexane, 2:1), <sup>1</sup>**H NMR** (400 MHz, CDCl<sub>3</sub>)  $\delta$  1.44 (t, 3H, J= 7.1Hz), 477 2.86 (s, 3H), 4.45 (q, 2H, J = 7.1Hz), 7.92 (s, 1H), 8.04 (s, 1H), 8.22 (s, 1H), 10.41 (s, 1H), 478 10.54 (s. 1H). <sup>13</sup>**C NMR** (100 MHz, CDCl<sub>3</sub>)  $\delta$  14.4, 19.2, 61.4, 121.2, 122.5, 126.2, 142.1, 479 142.3 146.7, 157.3, 157.4, 158.5, 158.7, 161.4, 167.4. **HRMS** m/z calcd for C<sub>15</sub>H<sub>13</sub>N<sub>5</sub>NaO<sub>4</sub>S<sub>3</sub> 480 ([M+Na]<sup>+</sup>) 446.0022, found 446.0025. **IR film** v(cm<sup>-1</sup>) 3421, 2928, 1624, 1539, 1230, 1092.

482 4.1.1.4 BocHN-Tz-Tz-Tz-COOEt (10b, Supp. Fig. S2): Compound 10b was prepared 483 following the general procedure for amide bond formation from 6b and 4b. Yield 45%. White 484 solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): δ1.42 (t, 3H, J = 7.07 Hz), 1.59 (s, 9H), 4.42 (q, 2H, J =485 7.07 Hz), 7.91 (s, 1H), 7.96 (s, 1H), 8.02 (s, 1H), 8.46 (s, 1H), 10.14 (s, 1H), 10.51 (s, 1H). 486 <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): δ14.4, 28.1 , 61.5, 83.7, 120.8, 121.4, 122.6, 141.9, 142.1, 487 142.2, 157.5 , 158.7 , 159.9 , 161.5.

489 4.1.2. General procedure for deprotection of the NH-Boc group: Trifluoro acetic acid (TFA) 490 was added on an ice bath, to a solution of NH-Boc compound in dry  $CH_2Cl_2$  under nitrogen 491 atmosphere. The mixture was stirred at room temperature 24 h. Saturated solution of NaHCO<sub>3</sub> 492 was added until pH 8, extracted exhaustively in AcOEt, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and the 493 solvent was removed under reduced pressure.

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495 4.1.2.1  $H_2N$ - $T_z$ - $T_z$ -COOEt (7): Compound 7 was prepared following the general procedure for 496 NH-Boc deprotection from **5b**. Yield 100%. <sup>1</sup>H NMR ((CD<sub>3</sub>)<sub>2</sub>CO, 400 MHz):  $\delta$  1.35 (t, 3H, J 497 = 7.19 Hz), 4.32 (q, 2H, J = 7.19 Hz), 6.85 (s, 2H), 7.61 (s, 1H), 8.0 (s, 1H), 10.54 (s, 1H). 498 <sup>13</sup>C-NMR ((CD<sub>3</sub>)<sub>2</sub>CO, 100 MHz):  $\delta$  14.6, 61.4, 116.0, 123.3, 143.1, 144.2, 158.1, 159.7, 499 161.8, 169.7.

500

501 *4.1.2.2*  $H_2N$ - $T_z$ - $T_z$ - $T_z$ -COOEt (**11**, Supp. Fig. S8): Compound **11** was prepared following the 502 general procedure for NH-Boc deprotection from **11b**. White solid. Yield 90 %. M.p. 295 °C 503 decomp. <sup>1</sup>H NMR (DMSO- $d_6$ , 400 MHz):  $\delta$  1.29 (t, 3H, J = 7.12 Hz), 4.28 (q, 2H, J = 7.12504 Hz), 7.69 (s, 1H), 8.11 (s, 1H), 8.36 (s, 1H), 11.58 (s, 1H), 12.51 (s, 1H). <sup>13</sup>C NMR (DMSO-505  $d_6$ , 100 MHZ):  $\delta$  14.2, 61.7, 115.5, 121.4, 123.3, 141.2, 142.2, 142.9, 157.7, 158.0, 159.2, 506 159.5, 161.0, 168.5. HRMS m/z calcd for C<sub>14</sub>H<sub>12</sub>N<sub>6</sub>NaO<sub>4</sub>S<sub>3</sub> ([M+Na]<sup>+</sup>) 446,9980, found 507 446.9977. IR KBr v(cm<sup>-1</sup>): 3350, 3211, 3118, 1720, 1680, 1369, 1288, 1242, 1159.

508

4.1.3. General procedure for ethyl ester hydrolysis: An aqueous KOH (10% w/v) solution was
added to an ester solution in THF. The reaction mixture was stirred at room temperature until
reagent disappearance was confirmed by TLC. HCl 1 M was added until pH 4 and the solution
was extracted with EtOAc. The organic layer was dried over MgSO<sub>4</sub> and concentrated *in vacuo* to afford the acid.

514

515 *4.1.3.1*  $H_3C$ - $T_z$ - $T_z$ -COOH (**6a**, Supp. Fig. S4): Compound **6a** was prepared following the 516 general procedure for ethyl ester hydrolysis from **5a**. Yield 100%. <sup>1</sup>H NMR (DMSO- $d_6$ , 400

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517 MHz):  $\delta$  2.77 (s, 3H), 8.09 (s, 1H), 8.59 (s, 1H), 12.44 (s, 1H), 12.95 (s, 1H). <sup>13</sup>C-NMR 518 (DMSO-*d*<sub>6</sub>, 100 MHz):  $\delta$  19.3, 123.4, 127.6, 142.7, 147.2, 158.1, 159.7, 162.8, 167.2. **HRMS** 519 *m*/*z* calcd for C<sub>9</sub>H<sub>7</sub>N<sub>3</sub>NaO<sub>3</sub>S<sub>2</sub> ([M+Na]<sup>+</sup>) 291.9821, found 291.9820. **IR film** v(cm<sup>-1</sup>) 3362, 520 3159, 3040, 2613, 2459, 1750, 1674, 1518, 1388, 1269, 1240, 1157.

521

522 4.1.3.2 BocHN-Tz-Tz-COOH (6c): Compound 6c was prepared following the general
523 procedure for ethyl ester hydrolysis from 5c. White solid. Yield 100%. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400
524 MHz): d 1.50 (s, 9H), 8.03 (s, 1H), 8.26 (s, 1H), 11.84 (s, 1H), 12.21 (bp, 2H). <sup>13</sup>C NMR
525 (CDCl<sub>3</sub>, 100 MHz): δ 28.7, 83.0, 121.1, 123.4, 142.9, 143.1, 153.4, 158.1, 159.7, 161.1, 162.6,
526 172.2.

527

528 *4.1.3.3 AcHN-Tz-Tz-COOH* (9, Supp. Fig. S7): Compound 9 was prepared following the 529 general procedure for ethyl ester hydrolysis from 8. White solid. Yield 100%. M.p. 278 °C 530 decomp. <sup>1</sup>H NMR (DMSO- $d_6$ , 400 MHz): δ 1.29 (s, 3H), 7.15 (s, 1H), 7.42 (s, 1H), 11.5 (s, 531 1H), 11.59 (bp, 2H). <sup>13</sup>C NMR (DMSO- $d_6$ , 100 MHz): δ 22.4, 120.9, 122.6, 142.1, 142.3, 532 157.8, 158.1, 159.4; 162.4; 169.2; 170.6. HRMS m/z calcd for C<sub>120</sub>H<sub>8</sub>N<sub>4</sub>NaO<sub>4</sub>S<sub>2</sub> ([M+Na]<sup>+</sup>) 533 334.9879, found 334.9844. IR KBr v(cm<sup>-1</sup>): 3442, 3180, 3109, 2974, 1669, 1550, 1371, 534 1298, 1222, 1122.

535

536 *4.1.3.4 BocHN-Tz-Tz-Tz-COOH* (**13**, Supp. Fig. S10): Compound **13** was prepared following 537 the general procedure for ethyl ester hydrolysis from **10b**. White solid. Yield 100 %. <sup>1</sup>H NMR 538 (DMSO- $d_6$ , 400 MHz):  $\delta$  1.51 (s, 9H), 8.04 (s, 1H), 8.25 (s, 1H), 8.37 (s, 1H), 11.83 (s, 1H), 539 12.19(s, 1H), 12.43 (s, 1H). <sup>13</sup>C-NMR (DMSO- $d_6$ , 100 MHz):  $\delta$  27.9, 79.1, 120.6, 121.3, 540 122.8,142.2, 142.3, 157.7, 157.9, 159.3, 159.4, 160.4, 162.3, 162.8. HRMS *m*/*z* calcd for 541 C<sub>17</sub>H<sub>16</sub>N<sub>6</sub>NaO<sub>6</sub>S<sub>3</sub> ([M+Na]<sup>+</sup>) 519.0186, found 519.0179. IR KBr v(cm<sup>-1</sup>): 3358, 3190, 3113, 542 2980, 1720, 1678, 1288, 1269, 1246, 1157.

543

544 4.1.3.5 AcHN-Tz-Tz-COOH (14, Supp. Fig. S11): Compound 14 was prepared following
545 the general procedure for ethyl ester hydrolysis from 12. White solid. Yield 40 %. M.p. 290 °C

546 decomp. <sup>1</sup>**H NMR** (DMSO- $d_6$ , 400 MHz): 2.06 (s, 3H), 7.92 (s, 1H), 8.16 (s, 1H), 8.26 (s, 1H), 547 12.33 (s, 1H), 12.36 (s, 1H), 12.6 (s, 1H). <sup>13</sup>**C NMR** (DMSO- $d_6$ , 100 MHz):  $\delta$  22.5, 121.3, 548 122.8, 123.3, 141.9, 142.3, 158.0, 158.2, 159.4, 159.5, 162.3, 169.3. **HRMS** m/z calcd for 549 C<sub>14</sub>H<sub>10</sub>N<sub>6</sub>NaO<sub>5</sub>S<sub>3</sub> ([M+Na]<sup>+</sup>) 460.9746, found 460.9767. **IR KBr** v(cm<sup>-1</sup>): 3387, 3111, 1672, 550 1637, 1431m 1369, 1286, 1273, 1201, 1118.

551

552 4.1.4. BocHN-Tz-COOEt (4c): Thiazole 4b (0.439 g 2.5 mmol) was dissolved in a mixture of 553 CH<sub>2</sub>Cl<sub>2</sub>:THF (1:1) (10 mL). (Boc)<sub>2</sub>O (0.577 g, 2.65 mmol) and TEA (0.744 g, 7.36 mmol) 554 were added. The mixture was refluxed for 72 h and then concentrated under reduced pressure. 555 The residue was disolved in AcOEt, washed with HCl 5% v/v (3x 15 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, 556 filtered and concentrated under reduced pressure. The residue was purified by silica flash cromatography AcOEt:EP (3:7). White solid. Yield 74%. <sup>1</sup>H NMR ((CD<sub>3</sub>)<sub>2</sub>CO, 400 MHz): δ 557 1.33 (t, 3H, J = 7.2 Hz), 1.54 (s, 9H), 4.30 (q, 2H, J = 7.2 Hz), 7.22 (s, 1H), 10.33 (s, 1H).  $^{13}$ C 558 **NMR** ((CD<sub>3</sub>)<sub>2</sub>CO, 100 MHz): δ 14.6, 28.2, 61.1, 82.2, 122.4, 143.0, 160.4, 161.8. 559

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4.1.5 AcHN-Tz-Tz-COOEt (8, Supp. Fig. S6): To a solution of 5b (100 mg, 0.33 mmol) in dry 561 CH<sub>2</sub>Cl<sub>2</sub> (5 mL) under a nitrogen atmosphere, once placed on an ice bath, an excess of pyridine 562 563 and acetic anhydre was added. Reaction mixture was stirred for 48 h. Then, H<sub>2</sub>O (15 mL) was added and extracted in CH<sub>2</sub>Cl<sub>2</sub> (3 x 15 mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered 564 565 and concentrated under vaccum. The residue was purified by silica flash cromatography 566 AcOEt:EP (1:1). Pale yellow solid. Yield 90%. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  1.41 (t, 3H, J = 7.1 Hz), 2.36 (s, 3H), 4.42 (q, 2H, J = 7.1 Hz), 7.89 (s, 1H), 7.95 (s, 1H), 9.72 (s, 1H), 10.51 567 (s, 1H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): δ 14.4, 23.2, 61.5, 121.1, 122.7, 141.8, 141.9, 157.6, 568 158.1, 159.1, 161.7, 168.4. **HRMS** m/z calcd for C<sub>12</sub>H<sub>12</sub>N<sub>4</sub>NaO<sub>4</sub>S<sub>2</sub> ([M+Na]<sup>+</sup>) 363.0192, 569 found 320.0190. **IR KBr** v(cm<sup>-1</sup>): 3342, 3261, 3080, 1708, 1662, 1570, 1371, 1281, 1271, 570 571 1244, 1099. Mp: 203 °C

- 572
- 573 *4.1.6 AcHN-Tz-Tz-Tz-COOEt* (**12**, Supp. Fig. S9): Compound **10b** (100 mg, 0.18 mmol) was 574 disolved in dry CH<sub>2</sub>Cl<sub>2</sub> (4 mL) under a nitrogen atmosphere on an ice bath and an excess of

575 pyridine and Ac<sub>2</sub>O was added to the mixture. The reaction is stirrred 48 h and filtered. White 576 solid. Yield 40%. M.p. 283 °C decomp. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHZ): δ 1.23 (t, 3H, J =577 7.2 Hz), 2.13 (s, 3H), 4.22 (q, 2H, J = 7.2 Hz), 8.06 (s, 1H), 8.26 (s, 1H), 8.32 (s, 1H), 12.39 (s, 578 1H), 12. 47 (bp, 2H). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz): δ 14.2, 22.6, 61.0, 120.8, 121.5, 123.4, 579 141.1, 142.0, 142.3, 142.7, 158.1, 158.3, 159.5, 159.6, 161.1, 169.4. HRMS *m/z* calcd for 580  $C_{16}H_{14}N_6NaO_5S_3$  ([M+Na]<sup>+</sup>) 489.0080, found 489.0071. IR KBr v(cm<sup>-1</sup>): 3373, 3309, 3111, 581 1691, 1523, 1288, 1255, 1192, 825.

582

583 *4.2. Biology* 

584 4.2.1 Materials

585 Chemical reagents were purchased from Sigma-Aldrich or ROCHE and were of analytical 586 grade. The media and the consumables for cell cultures were purchased from Invitrogen and 587 Greiner, respectively.

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589 4.2.2 Viability assays for T. brucei and murine macrophages

590 The bloodstream form of T. b. brucei strain 427 was grown in HMI-9 medium complemented 591 with 10% (v/v) Fetal Bovine Serum (FBS; GIBCO®), 10 U/mL penicillin and 10 µg/mL streptomycin. Cells were incubated aerobically in a humidified incubator containing 5% CO<sub>2</sub> 592 593 at 37 °C. The cytotoxic effect of the distamycin analogues was evaluated as described by Maiwald *et al.* [41]. Briefly, 200  $\mu$ L of a cell suspension containing 5x10<sup>5</sup> parasites/mL in 594 595 exponential growth phase were seeded per well in a 96-well culture plate and 2 µL each 596 compound prepared at different concentrations in 100% v/v DMSO were added. For the screening and  $EC_{50}$  assays the final concentrations of compounds tested were 5 and 30  $\mu$ M, 597 598 and between 0.005 to 150  $\mu$ M, respectively. Control wells included cells treated with 1% v/v 599 DMSO or 15  $\mu$ M nifurtimox. After addition of the test and control compounds, the culture 600 plates were incubated at 37°C, 5% CO<sub>2</sub> for 24 h. Then, 100 µL from each well was transferred 601 to a 2 mL tube or to a 96 U bottom well plate containing 200 µL of sterile phosphate-buffered saline (PBS) 1% glucose (m/v) (pH 7.4), 2 µg/mL propidum iodide (PI) and analyzed by flow 602 cytometry using a CyAn<sup>TM</sup> ADP (Beckman Coulter) or an Accuri<sup>TM</sup> C6 (BD) devices with 603

604 laser  $\lambda_{ex}$ =488 nm and filter  $\lambda_{em}$ =613/20 nm for the CyAn<sup>TM</sup> and  $\lambda_{em}$ =540/85 for the Accuri<sup>TM</sup> 605 C6. The data were analyzed with the Summit (Dako) software or Accuri C6 software (BD).

606 The murine macrophages (cell line J774) were cultivated in DMEM medium supplemented

607 with 10% (v/v) FCS (GIBCO®), 10 U/mL penicillin and 10  $\mu$ g/mL streptomycin, at 37°C, 5% 608 CO<sub>2</sub> in a humidified incubator. The EC<sub>50</sub> against macrophages was assayed only for

- 609 compounds for which the corresponding  $EC_{50}$  towards parasites was previously determined.
- 610 Cell viability was determined at 6-point concentrations of compound tested in triplicate, using
- 611 the WST-1 reagent and the protocol described by Demoro *et* al. [42].
- 612

For all assays, cell viability was calculated as follows: viability (%) =  $100 \times (number of cells for compound Y at concentration X/ number of cells in the DMSO-treated control).$ 

615 All  $EC_{50}$  values were obtained from dose/response curves fitted to a sigmoidal Boltzmann

616 equation (errors calculated using errors propagation) or extrapolated from non-linear fitting

617 plots. The error is expressed as 2.S.D and estimated as  $2\sigma$  (n-1).

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- 619 4.2.3 Assays with redox reporter cell lines

Transgenic cell lines of bloodstream *T. b. brucei* strain 427 and Chinese hamster ovary cells (CHO-K1) expressing the redox biosensor hGrx-roGFP2 [33] and rxYFP [34], respectively, were employed to monitor, by a non-invasive manner, intracellular redox changes induced by tests compounds. The generation and characterization of the cell lines will be described elsewhere.

The T. b. brucei cell line hGrx-roGFP2 was grown as described above except that phleomycin 625 626  $(0.2 \,\mu\text{g/mL})$  and hygromycin (5  $\mu\text{g/mL})$  were added to select for the constitutive expression of 627 the tetracycline repressor protein and for the hGrx-roGFP2 gene, respectively, whereas the 628 expression of the last was induced by supplementing the medium with 1 µg/mL oxytetracyclin. 629 Exponentially growing parasites were resuspended in fresh medium added of oxytetracycline at a density of  $5 \times 10^5$  cells/mL, 200 µL of this cell suspension was seeded per well (96-well 630 631 culture microplate) and incubated aerobically with 5% CO<sub>2</sub> in a humidified incubator at 37°C for 24 h. From this culture plate, a new plate containing  $1 \times 10^6$  parasites/200 µL per well in 632

633 culture medium without oxytetracylin was prepared. Next, 2  $\mu$ L of the different compounds 634 were added in triplicates at final concentrations of 78, 156 and 310 nM for **10b**, 1 mM for 635 menadione, 15  $\mu$ M for nifurtimox, 1 mM for DTT or 1% v/v for DMSO. After 1 h incubation 636 (5% CO<sub>2</sub> and 37°C), 100  $\mu$ L from each well were transferred to o a 96 U bottom well plate 637 containing 200  $\mu$ L of sterile PBS 1% glucose (m/v) while the remaining parasites in the plate 638 were incubated for additional 30 min in the presence of 1 mM DTT.

- 639 Transgenic cells CHO-K1-rxYFP were cultured in DMEM medium supplemented with 10% (v/v) FCS (GIBCO®), 10 U/mL penicillin and 10 µg/mL streptomycin, at 37°C, 5% CO<sub>2</sub> in a 640 641 humidified incubator. Forty eight h prior to the assay, zeocin (Invitrogen) was added at a final 642 concentration of 400 µg/mL. Cells were trypsinized for 5 min at 37°C, centrifuged at 800 RPM for 5 min, resuspended in fresh medium and 200  $\mu$ L/well of the cell suspension (8x10<sup>5</sup>) 643 644 cells/mL) was seeded on a 96-well plate. Then, 2 µL of the different compounds were added 645 in triplicates at final concentrations of 10, 5 and 1 µM for 10b, 1 mM for menadione, 646 hydrogen peroxide and DTT or 1% v/v for DMSO.
- 647 After 1 h incubation (5%  $CO_2$  and 37°C), 100  $\mu$ L from each well were transferred to a 96 U 648 bottom well plate containing 200  $\mu$ L of sterile PBS 1% glucose (m/v) while the remaining 649 cells in the plate were incubated for additional 30 min in the presence of 1 mM DTT.

For both assays, PI was added at a final concentration of 2  $\mu$ g/mL and then samples were analyzed with an Accuri<sup>TM</sup> C6 flow cytometer using the following laser/filter pairs:  $\lambda_{ex}$ =488 nm/ $\lambda_{em}$ =530/33 nm for hGrx-roGFP2 or rxYFP and  $\lambda_{ex}$ =488 nm/ $\lambda_{em}$ =540/85 nm for PI. The data were processed and analyzed with the Accuri C6 software.

- 654
- 655 4.2.4 Membrane permeability assay

The bloodstream form of *T. b. brucei* strain 427 was grown as previously described. Five hundred thousand parasites/mL were seeded on a 96-well plate and 2  $\mu$ L of the different compounds were added in triplicates at final concentrations of 310, 155 and 77.5 nM for **10b**, 0.001% v/v for Triton X-100, 15  $\mu$ M for nifurtimox or 1 % v/v for DMSO. After incubation for 0.5, 1, 2 and 4 h, 100  $\mu$ L from each well were transferred to a 96 U bottom well plate containing 200  $\mu$ L of sterile PBS 1% glucose (m/v). PI was then added at a final concentration 662 of 2  $\mu$ g/mL and data acquisition was performed with an Accuri<sup>TM</sup> C6 device as described 663 above.

664

#### 665 4.2.5 Intracellular iron

666 The bloodstream form of T. b. brucei strain 427 was grown as previously described. Two million parasites/mL were seeded on a 96-well plate and 2 µL of the different compounds 667 668 were added in triplicates at final concentrations of 3.1  $\mu$ M for 10b, 10 and 100  $\mu$ M for bipyridine or deferoxamine mesylate, or 1% v/v for DMSO. After 40 min incubation (5% CO<sub>2</sub> 669 and 37°C), PhenGreen SK diacetate (Invitrogen) was added at a final concentration of 10 µM 670 671 and incubated for additional 20 min. Then, 100  $\mu$ L from each well were transferred to a 96 U 672 bottom well plate containing 200 µL of sterile PBS 1% glucose (m/v). The samples were 673 analyzed using a CyAn<sup>TM</sup> ADP Flow Cytometer (Beckman Coulter, USA) with the following laser/filter band-pass pair:  $\lambda_{ex}$ =488 nm/ $\lambda_{em}$ = 530-40. Summit 4.3 software was used for data 674 675 acquisition and analysis.

676

#### 677 4.2.6 Iron-chelators assays

The bloodstream form of T. b. brucei strain 427 was grown as previously described. One 678 679 million parasites/mL were seeded on a 96-well plate and 2 µL of the different compounds 680 were added in triplicates at final concentrations of 310 nM for 10b, 500, 250, 125 or 100  $\mu$ M for deferoxamine mesylate, 100  $\mu$ M for bipyridine or 1% v/v for DMSO and incubated for 2 681 or 3 h at 37°C. Then, 100 µL from each well were transferred to a tube containing 200 µL of 682 683 sterile PBS 1% glucose (m/v) and added of 2 µg/mL PI. Samples were analyzed with a Accuri<sup>TM</sup> C6 flow cytometer using the following laser/filter-band pass pair:  $\lambda_{ex}$ =488 684  $nm/\lambda_{em}=613-30$  nm for PI. The data were processed and analyzed with the C6Accuri software. 685

- 686
- 687 4.2.7 Fluorescence microscopy assays

For assays involving mitochondrial staining,  $1 \times 10^6$  bloodstream *T. b. brucei* (strain 427)/mL were grown as previously described in the presence of 0.31 µM **10b** for 30 min. Mitotracker® Red CM-H<sub>2</sub>XRos (Invitrogen) was then added at a final concentration of 250 nM and

- 691 incubation resumed for further 30 min. Parasites were recovered by centrifugation (2000 g, 10 692 min at room temperature: RT) and washed with sterile PBS.
- 693 For assays involving lysosomal staining, 2.5x10<sup>6</sup> bloodstream T. b. brucei (strain 427)/mL
- 694 were grown as previously described in the presence of 0.31 μM **10b** and 25 μM LysoTracker
- Red DND-99 (Invitrogen) for 30 min. Parasites were recovered by centrifugation (2000 g, 10
- 696 min at RT) and washed twice with HMI-9 medium.
- 697 For both staining protocols, the cell pellet was homogeneously resuspended in paraformaldehyde 4% v/v in PBS (1x10<sup>6</sup> parasites/50 µL) and incubated for 18 min at RT. 698 699 After three consecutive washing steps with PBS, the parasites were resuspended in PBS at a density of  $4 \times 10^4$  parasites/µL. Cells were then plated on a cover slide and incubated overnight 700 at 4°C, protected from light. The slides were then mounted with Fluoroshield<sup>TM</sup> containing 701 DAPI to allow labeling of DNA (nuclei and kinetoplasts). Parasites were analyzed by 702 703 epifluorescence microscopy using 60X or 100X oil-objectives and an OLYMPUS IX81 704 microscope.
- 705

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- 846 **Figure legends**
- 847
- 848 Figure 1. Distamycin and oligoamide analogues
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Figure 2. Membrane permeability of bloodstream *T. brucei* treated with compound 10b. Five x10<sup>5</sup> parasites/mL were treated with different concentrations (310, 155 and 77.5 nM) of 10b for different times: A) 0.5 h, B) 1 h, C) 2 h and D) 4 h. Control treatments included Triton X-100 at 0.001% v/v, nifurtimox (NFX) at 15  $\mu$ M and DMSO at 1% v/v. Values are expressed as mean  $\pm$  SD (n=3) and the asterisks denote the probability indexes of 10b-treated parasites *vs.* DMSO control (\* *p* < 0.05; \*\* *p* < 0.01; \*\*\* *p* < 0.001), calculated applying the ONE WAY ANOVA test and the Dunnets's multiple comparision post test.

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858 Figure 3. Evaluation of the intracellular redox state of infective T. b. brucei treated 859 with 10b. Parasites (1x10<sup>6</sup> cells/mL) expressing the redox biosensor hGrx-roGFP2 were treated for 1 h with 10b (77.5, 155 and 310 nM) or different control compounds such as 860 menadione (1 mM), nifurtimox (NFX, 15 µM), dithiothreitol (DTT, 1 mM), DMSO 1% v/v 861 and 1 mM DTT. After the corresponding treatments the cells were analyzed by flow 862 cytometry. Redox analysis was performed only for PI negative cells. The results are presented 863 as percentage biosensor reduction relative to DMSO for: A) parasites treated for 1 h with 864 different compounds, and **B**) followed by a 30 min treatment with 1 mM DTT. Values are 865 866 expressed as mean SD (n=3) and the asterisks indicate difference (p < 0.001) with respect to 867 the DMSO control according to the ONE WAY ANOVA test and the Dunnets's multiple 868 comparison post test.

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870 Figure 4. Evaluation of intracellular redox state of a mammalian cell line treated with 10b. Cells (8x10<sup>5</sup> cells/mL) CHO-K1 expressing the redox biosensor rxYFP were treated 871 872 for 1 h with 10b (1, 5 and 10 µM) or different control compounds such as menadione (1 mM), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, 1 mM), dithiotreitol (DTT, 1 mM), DMSO 1% v/v and 1 mM 873 874 DTT. After the corresponding treatments the samples were analyzed by flow cytometry and the redox changes quantified only for PI negative cells. The results are presented as 875 876 percentage rxYFP reduction relative to DMSO for: A) cells treated for 1 h with different 877 compounds, and **B**) followed by a 30 min treatment with 1 mM DTT. Values are expressed 878 as mean SD (n=3) and the asterisks denote difference (\*\* = p < 0.01 and \*\*\* = p < 0.001) 879 with respect to the DMSO control according to the ONE WAY ANOVA test and the

880 Dunnets's multiple comparison post test.

882 Figure 5. Compound 10b disrupts the lysosomal homeostasis of bloodstream *T. brucei*. *T.* brucei brucei (2.5x10<sup>6</sup> cells/mL) was treated with **10b** (0.31 µM) or DMSO 1% v/v and 883 LysoTracker Red DND-99 25 µM. After 30 min incubation at 37°C, the cells were fixed with 884 885 paraformaldehyde and the slides observed under a fluorescence microscope. Representative 886 images of parasites treated with A) DMSO and showing discrete (single red dot) lysosome 887 staining, B) 10b and showing early (multiple red dots) or C) late lysosomal damage (dispersed 888 signal). D) Percentage of parasites showing discrete lysosomal staining. A total of 90 cells were analyzed per treatment. Values are expressed as mean  $\pm$  SD (n=3), with the asterisk 889 890 denoting statistical difference with a p < 0.0001 compared to DMSO (one-tailed Student's t-891 test). For each condition are shown bright field and fluorescence (Lysotracker: red color, and 892 DAPI staining for nucleus: N and mitochondrial DNA (kinetoplast): K) images from the same 893 cell.

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Figure 6. Evaluation of free-iron in bloodstream T. brucei treated with 10b. The 895 infective form of T. b. brucei ( $2x10^6$  cells/mL) was treated with 3.1  $\mu$ M 10b, 896 deferoxamine (DFO) and bipyridine (BiPy) at 10 µM (A) or 100 µM (B). Treatment 897 898 with DMSO 1% v/v was included as control. After incubation for 1 h at 37°C the 899 cells were analyzed by flow cytometry. Values are expressed as mean  $\pm$  SD (n=3) relative 900 fluorescence units (urf). The probability indexes for the different treatments are 901 indicated with asterisks when compared to DMSO control (\* p = 0.1, \*\* = p < 0.01and \*\*\* = p < 0.001) or with hashtags when compared to **10b alone**-treated parasites (## 902 903 = p < 0.01 and ### = p < 0.001), respectively. The ONE WAY ANOVA test and the 904 Dunnets's multiple comparison post test were applied for statistical calculations.

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906 Figure 7. Iron chelators protect bloodstream T. brucei against membrane damage by 10b. T. b. brucei (1x10<sup>6</sup> cells/mL) was treated with: A) 0.31  $\mu$ M 10b and 100  $\mu$ M deferoxamine 907 (DFO) or bipyridine (BiPy) for 2 h at 37°C, or **B**) different concentrations of DFO (125-500 908 909  $\mu$ M) for 3 h and then for additional 3 h with 0.31  $\mu$ M **10b**. After the corresponding treatments, 910 membrane integrity was analyzed using PI staining and flow cytometry. Values are expressed as mean  $\pm$  SD (n=3) and the asterisks denote the probability indexes of **10b**-treated parasites 911 912 vs. DMSO control (\*\*\*p < 0.001). Hashtags denote the probability indexes for DFO +/- 10b 913 or BiPy +/- 10b treated parasites vs. 10b alone (### = p < 0.001). The ONE WAY ANOVA 914 and the Dunnets's multiple comparison post tests were used for statistical calculations.

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