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Corresponding Author	Prof. Cordeiro-da-Silva Anabela (IBMC)
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Abstract	Current treatments for African trypanosomiasis are either toxic, costly, difficult to administer or prone to elicit resistance. This study evaluates the activity of bisnaphthalimidopropyl (BNIP) derivatives against Trypanosoma brucei. BNIPDabut, the most active compound, showed in vitro inhibition in the single unit nanomolar range, similar to the reference drug pentamidine, and presented low toxicity and adequate metabolic stability. Additionally, using a murine model of acute infection and live imaging, significant decrease of parasite load in BNIPDabut-treated mice was observed. However, cure was not achieved. BNIPDabut constitutes a new scaffold for antitrypanosomal drugs that deserves further consideration.
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1 **Activity of Bisnaphthalimidopropyl Derivatives Against**

2 ***Trypanosoma brucei***

3
4 Running title: BNIP derivatives against *T. brucei*

5
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24 **ABSTRACT**

25 Current treatments for African trypanosomiasis are either toxic, costly, difficult to
26 administer or prone to elicit resistance. This study evaluates the activity of
27 bisnaphthalimidopropyl (BNIP) derivatives against *Trypanosoma brucei*. BNIPDabut, the
28 most active compound, showed *in vitro* inhibition in the single unit nanomolar range,
29 similar to the reference drug pentamidine, and presented low toxicity and adequate
30 metabolic stability. Additionally, using a murine model of acute infection and live imaging,
31 significant decrease of parasite load in BNIPDabut-treated mice was observed. However,
32 cure was not achieved. BNIPDabut constitutes a new scaffold for antitrypanosomal drugs
33 that deserves further consideration.

34 **MAIN TEXT**

35 African trypanosomiasis is an infectious disease caused by parasites of the species
36 *Trypanosoma brucei*. The parasite is transmitted by an insect vector, the tsetse fly
37 (*Glossina spp.*). The disease is mainly distributed in the African continent, with distinct
38 subspecies causing different forms of human disease: *T. brucei gambiense* infection
39 produces a chronic form that may last for years and was responsible for nearly 98% of the
40 cases in the past decade; the acute form is caused by *T. brucei rhodesiense* and usually kills
41 the host within weeks, accounting for the remaining 2% of the reported cases (1, 2).

42 Since vaccination remains elusive and vector control strategies are frequently
43 insufficient, chemotherapy is still the most efficient option to control the disease (2-5).
44 However, the drugs in use have many drawbacks, mostly related with cost, effectiveness,
45 toxicity, difficult administration and the appearance of resistance (6). Therefore, the
46 development of new drugs is urgently needed.

47 Bisnaphthalimidopropyl (BNIP) derivatives have previously been shown to possess both
48 anticancer activity (7-11) and also have been shown to be antiparasitic against a related
49 trypanosomatid *Leishmania infantum* (12-14). The potential activity of three BNIP
50 derivatives previously synthesized (10, 11, 15, >96% pure), namely BNIPDiaminobutane
51 (BNIPDabut), BNIPDiaminoheptane (BNIPDahep) and BNIPDiaminooctane (BNIPDaoct)
52 (FIG 1A), against *T. brucei brucei* Lister 427 bloodstream forms (BSF) was investigated.
53 These were selected from a series of compounds based on preliminary studies of
54 bioavailability and *in vitro* and *in vivo* activity against both *T. brucei* and *L. infantum* (12
55 and unpublished data). The *in vitro* antiparasitic activity was assessed using a resazurin
56 assay, as previously described, with minor modifications (incubation with 10^3
57 parasites/well, in 200 μ L, 16). All three BNIPs demonstrated a potent inhibitory effect on

58 the parasites' growth, with IC_{50} within the nanomolar range (FIG 1B, TABLE 1).
59 BNIPDabut was the most active compound with an $IC_{50} \pm SD$ of 2.4 ± 1.0 nM similar to the
60 reference drug pentamidine with 2.9 ± 0.7 nM (TABLE 1). Since this class of compounds
61 has previously been described as inhibitors of the *L. infantum* Silent information regulator 2
62 related protein 1 (*LiSir2rp1*, accession: AAN39039.1) (15), we evaluated whether
63 inhibition of the *T. brucei* orthologue, *TbSir2rp1* (accession: AAX70528.1) would be a
64 possible mechanism of action. Whereas BNIPDabut was shown to inhibit the NAD^{+} -
65 dependent deacetylase activity of *TbSir2rp1* with an $IC_{50} \pm SD$ of 155 ± 42 μ M, suggesting
66 that this is not the major mechanism of action (data not shown), *LiSir2rp1* was inhibited
67 with an $IC_{50} \pm SD$ of 35.0 ± 5.8 μ M (15). The 47% identity between *LiSir2rp1* and
68 *TbSir2rp1* obtained by protein sequence alignment (Clone Manager 9, BLOSUM 62
69 scoring matrix) might explain the differences observed (17). Moreover, no correlation
70 between the enzymatic inhibition and activity towards *T. brucei* parasites was observed. To
71 evaluate *in vitro* toxicity towards mammalian cells, all the compounds were studied with
72 the MTT assay (18) in THP1-derived macrophages and two primary cell cultures: rat
73 cortical neurons and mouse hepatocytes (TABLE 2). The CC_{50} values for these molecules
74 translate into selectivity indexes ($SI=CC_{50}/IC_{50}$) higher than 100. All BNIP derivatives
75 exhibited high SIs, with BNIPDabut in particular being at least 800 times more selective
76 towards *T. brucei* parasites. All BNIPs had potency and selectivity that warranted
77 additional characterization (TABLE 2). To further evaluate the potential toxic effects of
78 BNIPs in host cells, a set of *in vitro* assays was performed in hepatocytes and neuronal
79 primary cells. These assays evaluated different possible mechanisms of toxicity based on:
80 a) reactive oxygen species determination (CM-H2DCFDA probe, by High Content

81 Analysis – HCA); b) mitochondrial dysfunction (TMRM probe, by HCA); c) membrane
82 integrity (lactate dehydrogenase quantification); d) apoptosis (caspase 3/7 activation); e)
83 either DNA damage for hepatocytes (H2AX antibody, by HCA) or neurite outgrowth for
84 neurons (anti-tubulin III antibody, by HCA); f) cell viability as measured by WST-8 probe
85 and g) Hoechst staining for nuclear detection. Nimesulide (400 μ M) was included as a
86 positive control and the vehicle as a neutral control (19-21). The relative percentage of
87 deviation from the neutral control was quantified and assigned with a number from 0 to 5
88 according to the following criteria: 0 (0-20% deviation), 1 (20-40%), 2 (40-60%), 3 (60-
89 100%), 4 (100-1000%), or 5 (>1000% deviation). The sum of these values was posteriorly
90 ranked to create a combined injury criteria that varied from no injury (0), low injury (1 to
91 <5), moderate injury (\geq 5-to <12) to high injury (\geq 12). All BNIPs showed a dose-dependent
92 injury score close to pentamidine in both cell types (FIG 2A-B). BNIPDabut had a toxicity
93 profile indistinguishable from the reference drug pentamidine.

94 To infer metabolic stability, mouse microsomes were incubated over 45 minutes
95 with 5 μ M of each compound and the drug was quantified by LC-MS/MS. Similarly to
96 pentamidine, BNIPDabut was more stable than both BNIPDahep and BNIPDaoct, with 95
97 to 100% of the drug not being metabolized (TABLE 3). This high metabolic stability is an
98 indicator that the molecule is not easily subjected to common inactivation or loss of
99 potency by reactions catalized by liver enzymes, and is kept intact in circulation for longer
100 periods.

101 To determine the pharmacokinetics of BNIPDabut, a 10 mg/kg dose was
102 administered to BALB/c mice by intravenous injection. Five minutes after, a concentration
103 of 58 nM was achieved and during the following 24 hours remained higher than 41 nM
104 (data not shown), thus approximately 8 times higher than the calculated IC₉₀.

105 Taking in consideration the previous results, BNIPDabut was chosen for *in vivo* efficacy
106 studies. All the experiments involving animals were carried out in accordance with the
107 IBMC Animal Ethics Committees and the Portuguese and European Authorities for Animal
108 Health guidelines. *T. b. brucei* Lister 427 parasites were transfected with a construct kindly
109 provided by M. Taylor, in which the red-shifted luciferase gene (PpyRE9H) is flanked by
110 5'VSG/3'tubulin (22). Upon transfection, clones were screened for bioluminescent signal
111 and the ones expressing the highest levels were selected. Their *in vitro* growth was
112 compared to wild type parasites and found to be similar (data not shown). *In vitro* detection
113 limits were also analyzed for BSF in a 96-well plate and determined to be about 2500 cells
114 (data not shown). BALB/c female mice were inoculated intraperitoneally with 10^4 BSF.
115 Three days post-infection, five groups of mice (n=4) were treated intravenously with:
116 saline, pentamidine at 2.5 mg/kg/day, DMSO at 16.7%, or BNIPDabut both at a 10
117 mg/kg/day and a 20 mg/kg/day dose. Pentamidine was administered for 4 days, while
118 BNIPDabut and the respective vehicle (DMSO 16.7%) were administered for 6 days (FIG
119 3A). No adverse effects were observable following any administration regimen. Treatment
120 efficacy was followed through whole animal live imaging using an IVIS Lumina LT
121 (Perkin Elmer). Parasitaemias were also assessed, and animals were euthanized after
122 reaching a parasitaemia of 10^8 parasites/mL. Similarly to pentamidine, two administrations
123 of BNIPDabut both at 10 and 20 mg/kg efficiently reduced parasitaemia below detection
124 limit (5×10^4 /mL) (FIG 3B). However, whole mice imaging reveals that reduction of
125 bioluminescent signal to the background level (obtained with non-infected mice) is only
126 achieved in mice treated with 20 mg/kg of BNIPDabut during at least 5 consecutive days as
127 opposed to 2 days treatment for the pentamidine group. Although treatment with 20mg/kg
128 BNIPDabut apparently cleared the infection, cure was not achieved as the parasite burden

129 relapsed when treatment was stopped (FIG 3B). Indeed, BNIPDabut (10 and 20 mg/kg)
130 treatment increases mice survival, but in contrast with pentamidine, animals were not cured
131 (FIG 3C). A hypothesis is that BNIPDabut, although highly trypanocidal cannot reach and
132 clear all parasites, either due to potency, distribution, or both. A frequent observation was
133 that recurrence of parasitaemia was preceded by imaging of parasite loads in the peritoneal
134 zone, where animals were originally injected with the parasites. It has been demonstrated
135 that trypanosomes invade extravascular tissues as a defense mechanism against host
136 immunity, and that this process may be related with relapses after treatment interruption
137 (23, 24). Indeed, the presence of parasites in the extravascular tissues might explain the
138 discrepancy of radiance values between days 4 and 12 while average parasitaemia remain
139 similar in mice treated with BNIPDabut (10 and 20 mg/kg) (FIG 3C). Nonetheless, it
140 remains to be elucidated whether BNIPDabut is active on a mouse model of the late stage
141 of the disease as this is the central objective in drug discovery against human African
142 trypanosomiasis. Additional chemical modifications to BNIPDabut may improve potency
143 and/or distribution of the drug, while maintaining or improving the toxicity and metabolism
144 profile.

145 In conclusion, this work demonstrates that BNIPDabut has potent *in vitro* and *in*
146 *vivo* antitrypanosomal activity with acceptable toxicity and high metabolic stability.
147 However, chemical modifications are needed in order to improve its pharmacodynamic
148 and/or pharmacokinetic properties.

149

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166

167 **REFERENCES**

168

- 169 1. **Franco JR, Simarro PP, Diarra A, Jannin JG.** 2014. Epidemiology of human
170 African trypanosomiasis. *Clin Epidemiol* **6**:257-275.
- 171 2. **Franco JR, Simarro PP, Diarra A, Ruiz-Postigo JA, Jannin JG.** 2014. The
172 journey towards elimination of gambiense human African trypanosomiasis: not far,
173 nor easy. *Parasitology* **141**(6):748-760.
- 174 3. **Horn D.** 2014. Antigenic variation in African trypanosomes. *Mol Biochem*
175 *Parasitol* **195**(2):123-129.

- 176 4. **Radwanska M, Guirnalda P, De Trez C, Ryffel B, Black S, Magez S.** 2008.
177 Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite
178 antibody responses and abolishment of vaccine-induced memory responses. *PLoS*
179 *Pathog* **4(5):e1000078**. doi:10.1371/journal.ppat.1000078.
- 180 5. **Schofield CJ, Kabayo JP.** 2008. Trypanosomiasis vector control in Africa and
181 Latin America. *Parasit Vectors* **1(1):24**.
- 182 6. **Brun R, Blum J, Chappuis F, Burri C.** 2010. Human African trypanosomiasis.
183 *Lancet* **375(9709):148-159**.
- 184 7. **Ralton LD, Bestwick CS, Milne L, Duthie S, Kong Thoo Lin P.** 2009.
185 Bisnaphthalimidopropyl spermidine induces apoptosis within colon carcinoma cells.
186 *Chem Biol Interact* **177(1):1-6**.
- 187 8. **Barron GA, Bermano G, Gordon A, Kong Thoo Lin P.** 2010. Synthesis,
188 cytotoxicity and DNA-binding of novel bisnaphthalimidopropyl derivatives in
189 breast cancer MDA-MB-231 cells. *Eur J Med Chem* **45(4):1430-1437**.
- 190 9. **Bestwick CS, Ralton LD, Milne L, Kong Thoo Lin P, Duthie SJ.** 2011. The
191 influence of bisnaphthalimidopropyl polyamines on DNA instability and repair in
192 Caco-2 colon epithelial cells. *Cell Biol Toxicol* **27(6):455-463**.
- 193 10. **Oliveira J, Ralton L, Tavares J, Codeiro-da-Silva A, Bestwick CS, McPherson**
194 **A, Thoo Lin PK.** 2007. The synthesis and the in vitro cytotoxicity studies of
195 bisnaphthalimidopropyl polyamine derivatives against colon cancer cells and
196 parasite *Leishmania infantum*. *Bioorg Med Chem* **15(1):541-545**.
- 197 11. **Dance AM, Ralton L, Fuller Z, Milne L, Duthie S, Bestwick CS, Lin PK.** 2005.
198 Synthesis and biological activities of bisnaphthalimido polyamines derivatives:

- 199 cytotoxicity, DNA binding, DNA damage and drug localization in breast cancer
200 MCF 7 cells. *Biochem Pharmacol* **69**(1):19-27.
- 201 12. **Tavares J, OuaiSSI A, Silva AM, Lin PK, Roy N, Cordeiro-da-Silva A.** 2012.
202 Anti-leishmanial activity of the bisnaphthalimidopropyl derivatives. *Parasitol Int*
203 **61**(2):360-363.
- 204 13. **Tavares J, OuaiSSI A, Lin PK, Tomas A, Cordeiro-da-Silva A.** 2005. Differential
205 effects of polyamine derivative compounds against *Leishmania infantum*
206 promastigotes and axenic amastigotes. *Int J Parasitol* **35**(6):637-646.
- 207 14. **Costa Lima S, Rodrigues V, Garrido J, Borges F, Kong Thoo Lin P, Cordeiro**
208 **da Silva A.** 2012. In vitro evaluation of bisnaphthalimidopropyl derivatives loaded
209 into pegylated nanoparticles against *Leishmania infantum* protozoa. *Int J*
210 *Antimicrob Agents* **39**(5):424-430.
- 211 15. **Tavares J, OuaiSSI A, Kong Thoo Lin P, Loureiro I, Kaur S, Roy N, Cordeiro-**
212 **da-Silva A.** 2010. Bisnaphthalimidopropyl derivatives as inhibitors of *Leishmania*
213 SIR2 related protein 1. *ChemMedChem* **5**(1):140-147.
- 214 16. **Bowling T, Mercer L, Don R, Jacobs R, Nare B.** 2012. Application of a
215 resazurin-based high-throughput screening assay for the identification and
216 progression of new treatments for human African trypanosomiasis. *Int J Parasitol*
217 *Drugs Drug Resist* **2**:262-270.
- 218 17. **Eddy SR.** 2004. Where did the BLOSUM62 alignment score matrix come from?
219 *Nat Biotech* **22**(8):1035-1036.
- 220 18. **van Meerloo J, Kaspers GL, Cloos J.** 2011. Cell Sensitivity Assays: The MTT
221 Assay, p 237-245. *In* Cree IA (ed), *Cancer Cell Culture*, vol 731. Humana Press.

- 222 19. **Mingatto FE, Rodrigues T, Pigoso AA, Uyemura SA, Curti C, Santos AC.**
223 2002. The critical role of mitochondrial energetic impairment in the toxicity of
224 nimesulide to hepatocytes. *J Pharmacol Exp Ther* **303**(2):601-607.
- 225 20. **Tripathi R, Tripathi P, Pancholi SS, Patel CN.** 2014. The genotoxic and
226 cytotoxic effects of nimesulide in the mouse bone marrow. *Drug Chem Toxicol*
227 **37**(3):255-260.
- 228 21. **Borkotoky D, Panda SK, Sahoo GR, Parija SC.** 2014. Genotoxicity of
229 nimesulide in Wistar rats. *Drug Chem Toxicol* **37**(2):178-183.
- 230 22. **McLatchie AP, Burrell-Saward H, Myburgh E, Lewis MD, Ward TH,**
231 **Mottram JC, Croft SL, Kelly JM, Taylor MC.** 2013. Highly sensitive in vivo
232 imaging of *Trypanosoma brucei* expressing "red-shifted" luciferase. *PLoS Negl*
233 *Trop Dis* **7**(11):e2571. doi:10.1371/journal.pntd.0002571.
- 234 23. **Whitelaw DD, Gardiner PR, Murray M.** 1988. Extravascular foci of
235 *Trypanosoma vivax* in goats: the central nervous system and aqueous humor of the
236 eye as potential sources of relapse infections after chemotherapy. *Parasitology*
237 **97**(01):51-61.
- 238 24. **Claes F, Vodnala SK, van Reet N, Boucher N, Lunden-Miguel H, Baltz T,**
239 **Goddeeris BM, Buscher P, Rottenberg ME.** 2009. Bioluminescent imaging of
240 *Trypanosoma brucei* shows preferential testis dissemination which may hamper
241 drug efficacy in sleeping sickness. *PLoS Negl Trop Dis* **3**(7):e486.

242

243 **FIGURE LEGENDS**

244

245 **FIG 1** *In vitro* antitrypanosomal activity of BNIP compounds.

246 A) Chemical structures of pentamidine and BNIP derivatives BNIPDabut, BNIPDahep,
247 BNIPDaoct. B) Growth inhibition curves of *Trypanosoma brucei brucei* BSF incubated *in*
248 *vitro* with the indicated concentrations of pentamidine, BNIPDabut, BNIPDahep or
249 BNIPDaoct for 72h. Parasite density was evaluated using resazurin. Dots and error bars
250 represent the mean + standard deviation of antiparasitic activity. Data of 3 independent
251 experiments.

252

253 **FIG 2** *In vitro* toxicity of BNIP derivatives.

254 A) Hepatotoxicity injury score. The score was calculated as the sum of individual scores
255 obtained from a panel of *in vitro* cytotoxicity assays that include: measurement of reactive
256 oxygen species using CM-H2DCFDA and cells imaging by high content analysis;
257 assessment of mitochondrial dysfunction measured by TMRM probe dynamics in cells and
258 image by high content analysis; membrane integrity assayed by lactate dehydrogenase
259 quantification; DNA damage by imaging with H2AX antibody and high content analysis;
260 and apoptosis by caspase 3/7 activation; Hoechst staining for nuclear detection; and cell
261 viability by WST-8 probe. Nimesulide (400 μ M), an approved drug with a mild
262 toxicological profile, was included as a toxicity control. Individual scores are calculated
263 based on the relative percentage of deviation from the negative control quantified and
264 assigned with a number from 0 to 5 according to the following criteria: 0 (0-20%
265 deviation), 1 (20-40%), 2 (40-60%), 3 (60-100%), 4 (100-1000%), or 5 (>1000%
266 deviation). The data represent the mean sum of these values + standard deviation B)
267 Neurotoxicity Injury Score. The score was calculated similarly to the hepatotoxicity score
268 but instead of DNA damage by H2AX antibody, an assay to test neurite outgrowth as

269 imaged with an anti-tubulin III antibody and high content analysis was performed. The data
270 represent the mean sum of these values + standard deviation. Data of 2 independent
271 experiments.

272

273 **FIG 3** BNIPDabut *in vivo* efficacy against *T. b. brucei*.

274 A) Schematic of the experimental design to evaluate the *in vivo* efficacy of BNIPDabut. B)

275 Mice were infected with 10^4 LUC⁺ BSF by intraperitoneal injection and initiated the

276 different treatments 3 days post-infection. Whole mice bioluminescence imaging was done

277 at days 3, 4, 9 and 12 using an IVIS LUMINA LT and upon injection of 2.1mg luciferin.

278 Bioluminescence average radiance (p/sec/cm²/sr) of whole mice was quantified and the

279 mean + standard deviation (n=4) is shown in bars. Parasitemia was determined using a

280 haemocytometer and the mean + standard deviation is represented by red dots. Red crosses

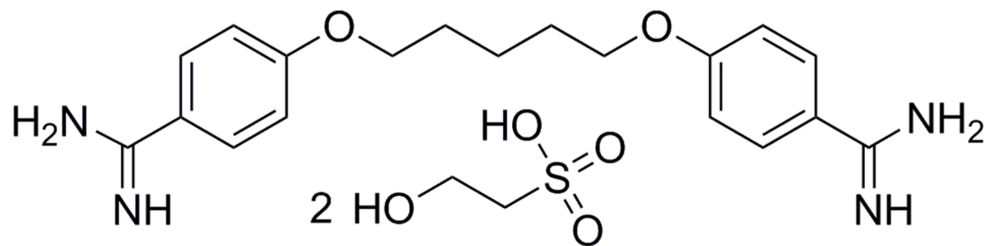
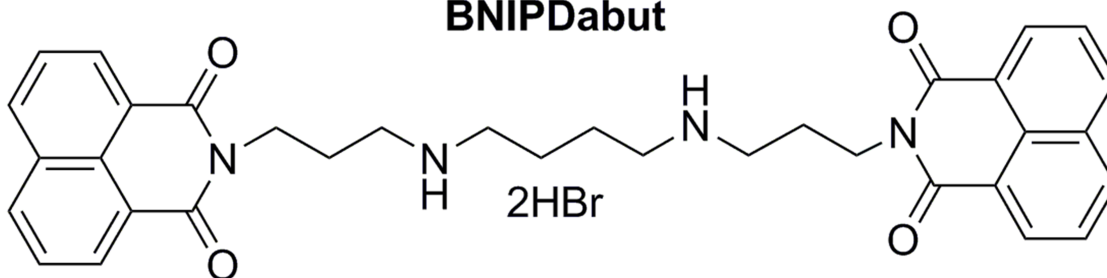
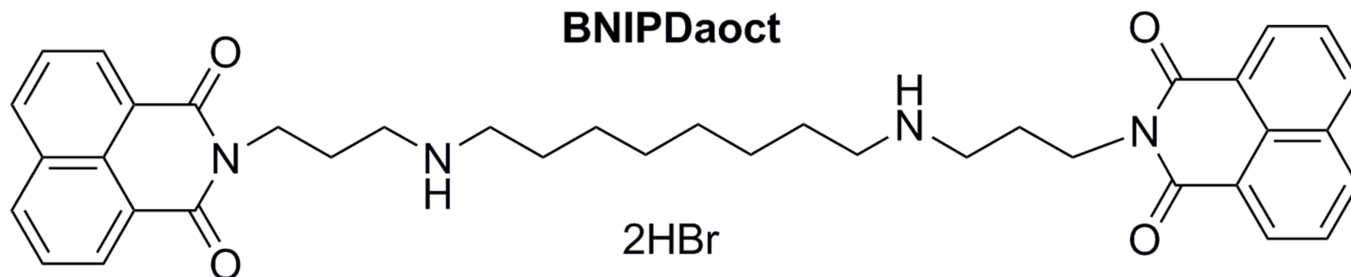
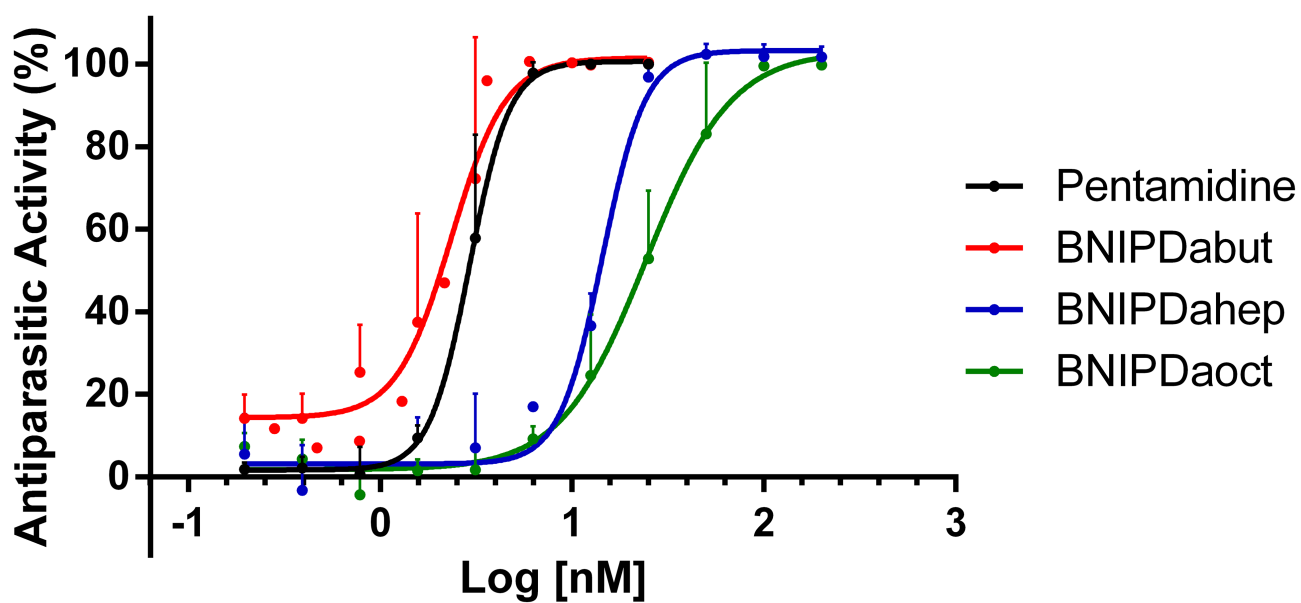
281 represent the parasitaemia of the only animal where parasites could be detected and

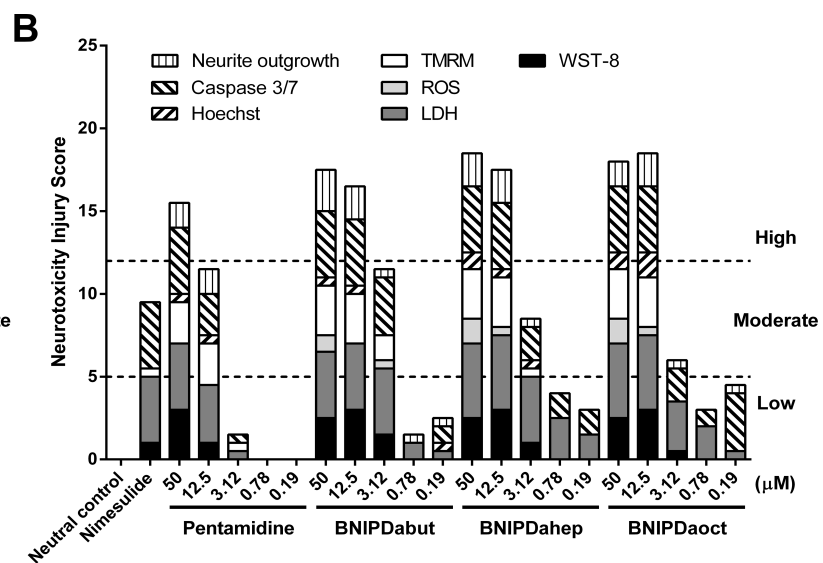
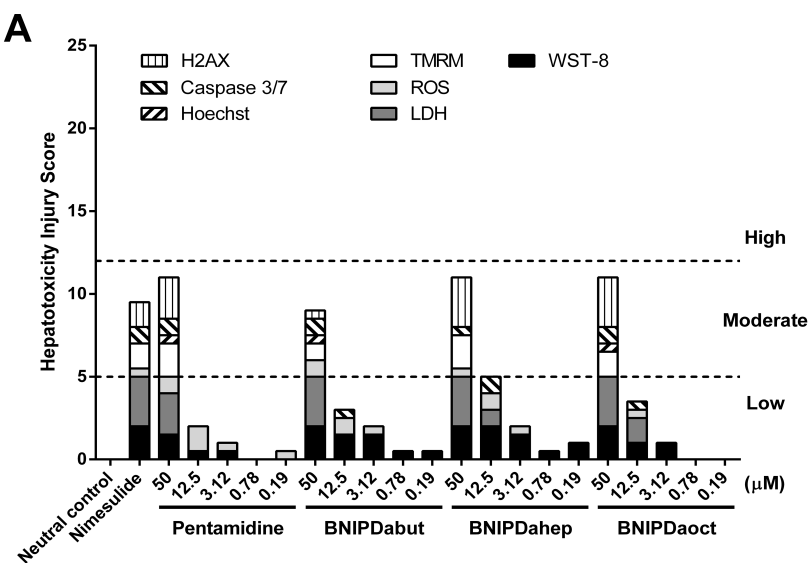
282 quantified. Parasitaemia detection limit is 5×10^4 parasites/mL. C) Kaplan-Meyer survival

283 curves of the infected mice treated with controls and experimental doses of BNIPDabut. B-

284 C) The data is representative of 3 independent experiments.

285

A**Pentamidine****BNIPDabut****BNIPDahep****BNIPDaoct****B**



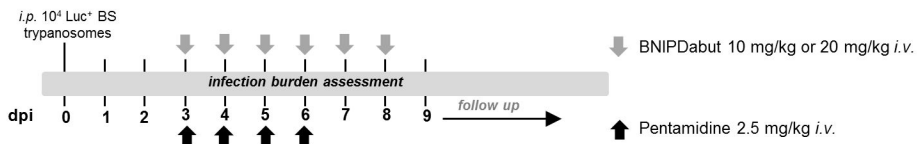
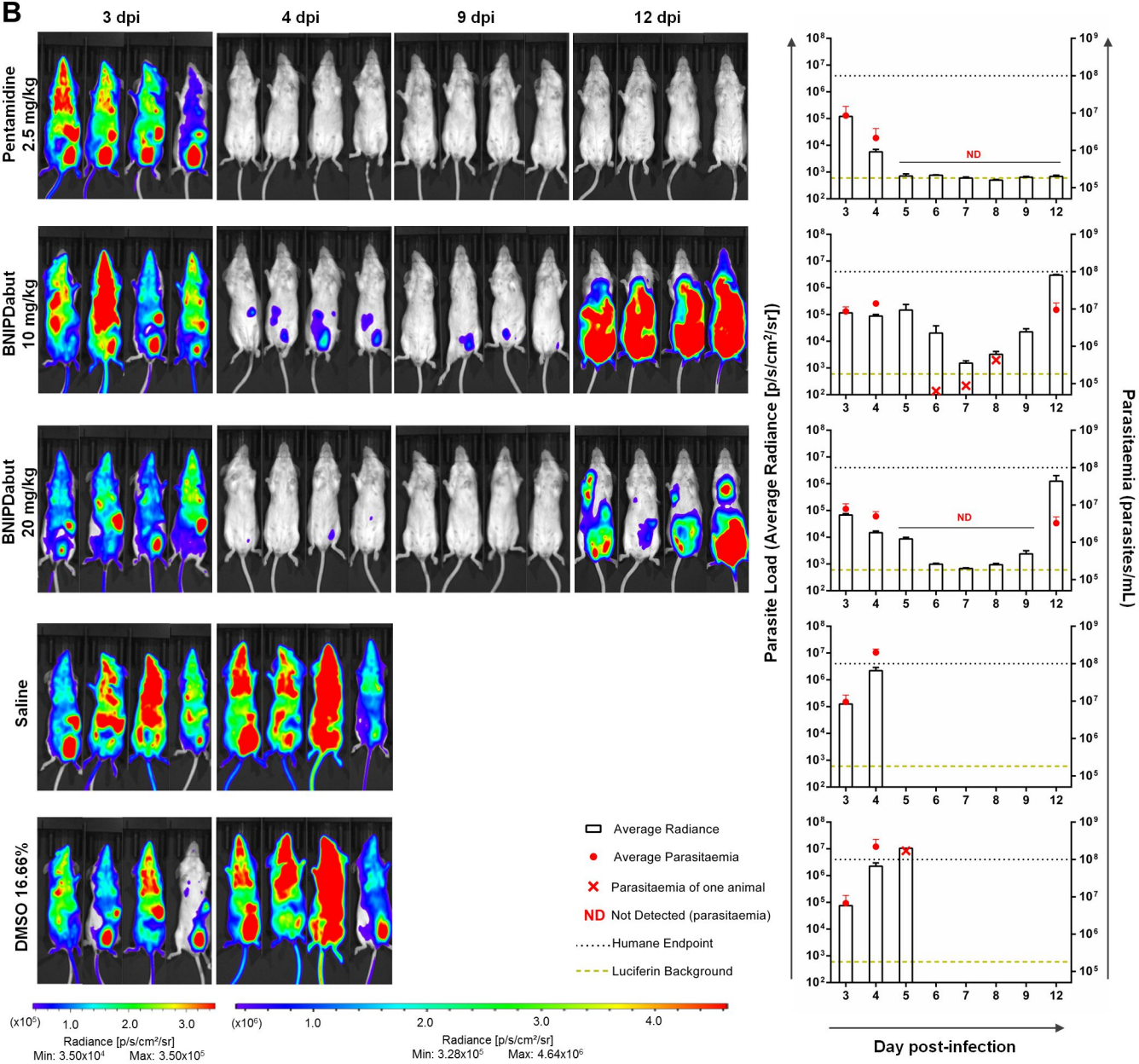
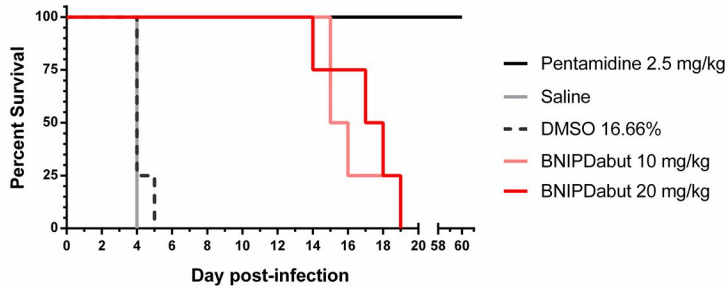
A**B****C**

TABLE 1 *In vitro* activity of BNIP derivatives against *Trypanosoma brucei* L427 bloodstream forms

	<u>IC₅₀ ± SD (nM)</u>	<u>IC₉₀ ± SD (nM)</u>
Pentamidine	2.94 ± 0.74	5.26 ± 0.58
BNIPDabut	2.35 ± 0.99	3.83 ± 1.40
BNIPDahep	14.32 ± 1.21	23.07 ± 1.01
BNIPDaoct	26.15 ± 10.43	63.36 ± 21.19

TABLE 2 *In vitro* cytotoxicity of BNIP derivatives in different cell types

	CC ₅₀ ± SD (µM)			Selectivity Index*		
	THP1	Hepatocytes	Neurons	THP1	Hepatocytes	Neurons
Pentamidine	47.73 ± 3.32	18.21 ± 0.66	8.23 ± 0.88	16259	6203	2803
BNIPDabut	5.90 ± 0.40	9.19 ± 0.06	2.06 ± 1.69	2514	3916	878
BNIPDahep	3.34 ± 0.11	4.23 ± 0.48	2.31 ± 1.65	233	295	161
BNIPDaoct	3.88 ± 0.59	18.35 ± 4.58	3.97 ± 1.30	148	702	152

*Selectivity Index = CC₅₀ cell line/IC₅₀ *T. brucei*

TABLE 3 Mouse microsomal stability

	Metabolic stability (%)	<i>In vitro</i> Intrinsic Clearance Cl_{int} (μ L/min/mg protein)	Degradation non NADPH-dependent (%)
Pentamidine	95 - 100	-	< 5
BNIPDabut	95 - 100	-	< 5
BNIPDahep	85	7	< 5
BNIPDaoct	64	20	9