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EXPERIMENTAL THERAPEUTICS

Tetrahydrophthalazinone Inhibitor of Phosphodiesterase with *In Vitro* Activity against Intracellular Trypanosomatids

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ABSTRACT The phosphodiesterase inhibitor tetrahydrophthalazinone NPD-008 was explored by phenotypic *in vitro* screening, target validation, and ultrastructural approaches against *Trypanosoma cruzi*. NPD-008 displayed activity against different forms and strains of *T. cruzi* (50% effective concentration [EC_{50}], 6.6 to 39.5 μM). NPD-008 increased cAMP levels of *T. cruzi* and its combination with benznidazole gave synergistic interaction. It was also moderately active against intracellular amastigotes of *Leishmania amazonensis* and *Leishmania infantum*, confirming a potential activity profile as an antitrypanosomatid drug candidate.

KEYWORDS *L. amazonensis*, *L. infantum*, *T. cruzi*, cAMP, *in vitro* activity, phosphodiesterase inhibitor

Intracellular trypanosomatids are the causative agents of two major neglected tropical diseases (NTDs), i.e., leishmaniasis (*Leishmania* spp.) and Chagas disease (*Trypanosoma cruzi*). Both diseases place high social and economic burdens on developing countries. The currently available drugs are old and have several drawbacks, and no vaccine is available, highlighting the need for novel therapeutic strategies (1, 2). Previous studies demonstrated that phthalazinones are promising antitrypanosomatid drug chemotypes through the inhibition of cAMP phosphodiesterases (PDEs) (3–5). The activity of tetrahydrophthalazinone NPD-008 was first reported on *Trypanosoma brucei*, characterizing this compound as a selective TbrPDEB1 inhibitor (6). Considering the high structural conservation of PDEs among kinetoplastids (7), the present *in vitro* study evaluated the phenotypic effects of NPD-008 on *T. cruzi*, *Leishmania amazonensis*, and *Leishmania infantum*, as well as its effects on mammalian cells, parasite ultrastructure, and *T. cruzi* cAMP metabolism. Synergism with the reference drug benznidazole was investigated.

NPD-008 was synthesized as described by Blaazer and colleagues (6). Assays involving animal procedures performed at Fundação Oswaldo Cruz (FIOCRUZ) were carried out in accordance with the guidelines established by the Committee of Ethics for the Use of Animals (CEUA L038/2017).

NPD-008 was active against the three parasitic forms of *T. cruzi* belonging to distinct strains (6) and discrete typing units (DTUs) relevant for human infection (8) (Table 1). Incubation for 96 h of *T. cruzi*-infected L929 cell cultures (Tulahuen strain transfected with β-galactosidase gene, DTU VI) with NPD-008 produced a 50% effective concentration (EC_{50}) of 9.7 $\pm$ 1.3 μM. Epimastigotes (Y strain, DTU II), the multiplying form present in the insect vector gut, were less susceptible (EC_{50}, 39.5 μM; 48 h exposure). However, bloodstream trypomastigotes (BT) (Y strain) were highly susceptible to NPD-008,

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TABLE 1 *In vitro* phenotypic screening of NPD-008 and reference drugs for Chagas disease (benznidazole) and leishmaniasis (pentamidine)

	NPD-008		Benznidazole		NPD-008		Pentamidine	
Activity	EC_{50} (mean ± SD) μM	SI[a]	EC_{50} (mean ± SD) μM	SI	EC_{50} (mean ± SD) μM	SI	EC_{50} (mean ± SD) μM	SI
Anti-*T. cruzi*								
Intracellular amastigotes	9.7 ± 1.3	20.6	2.7 ± 0.4	>370				
Bloodstream trypomastigotes	6.6 ± 1.1	>30.3	12.9 ± 1.9	>77				
Epimastigotes	39.5 ± 13.2							
Anti-*Leishmania*								
Ex vivo amastigotes (*L. amazonensis*)					14.9 ± 2	>4.3	1.25 ± 0.09	ND[b]
Intracellular amastigotes (*L. infantum*)					12.5 ± 10.6	>5.1	ND	ND

[a]SI, selectivity index.
[b]ND, not done.

showing an EC_{50} of 6.6 ± 1.1 μM after 24 h incubation, approximately twice as active as benznidazole (EC_{50}, 12.9 ± 1.9 μM). Next, the combination of benznidazole with NPD-008 was tested using a fixed-ratio method (9, 10), and its fractional inhibitory concentration index (FICI) and sum (ΣFICI) were calculated and classified as reported (11). Our results showed ΣFICI values of 0.51 and 1.19 for amastigotes and BT, respectively, with the first value indicating borderline synergism (Fig. 1), a profile already reported for *T. cruzi* isolates incubated with the phthalazinone PDE inhibitor NPD-040 (5). These data represent a desirable characteristic, since combined therapy is a valuable tool in improving treatment efficacy while reducing dose levels and toxicity and preventing the development of drug resistance (12).

NPD-008 did not exert toxicity on mammalian host cells obtained from different sources, including cell lineages and primary cultures of cardiac cells (50% lethal concentration [LC_{50}], >200 μM; Table 2), leading to selectivity indexes of >20, as recommended for anti-*T. cruzi* drug candidates (13). On-target inhibition of phosphodiesterases was confirmed by cAMP measurements, determined by immunoassay as described (4, 6), showing a significant (unpaired Student's t test, $P \leq 0.05$) and dose-dependent intracellular increase in cAMP in NPD-008-treated amastigotes and BT (Y strain) exposed for 2.5 h at 37°C using 2× and 5× their respective EC_{50} concentrations (Fig. 2). For trypomastigotes, the extracellular cAMP concentration was also increased ($P < 0.001$), with the increased cAMP efflux partially compensating for PDE inhibition. These findings validate PDEs as *T. cruzi* targets of NPD-008, as reported for other PDE inhibitors (5, 14). Our present results align with findings in *T. brucei* isolates of dose-dependent increases in intracellular cAMP levels after incubation with NPD-008 (6) and other TbrPDEB1 inhibitors (3, 4, 15).

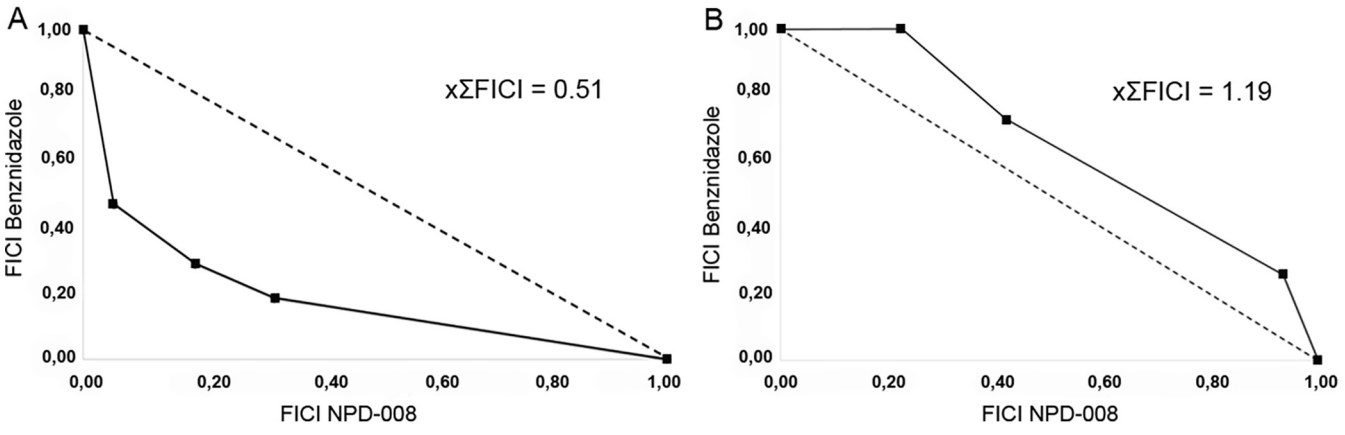

FIG 1 *In vitro* combined therapy of NPD-008 plus benznidazole on *T. cruzi* intracellular forms of Tulahuen strain (A) and bloodstream trypomastigotes of Y strain (B).

TABLE 2 Mammalian cell toxicity

	LC_{50} (μM)	
Source	**NPD-008**	**Benznidazole**
L929 fibroblasts	199.8	>1,000
Primary cardiac cultures	>200	>1,000
MRC-5	>64	ND

To detect primary cellular damages triggered by exposure to NPD-008 (1× EC_{50}; 15 min), BT and epimastigotes (Y strain) were examined by scanning electron microscopy. Epimastigotes showed body shortening and BT displayed an altered, rounded morphology (Fig. 3), suggestive of osmotic distress, an outcome already described during PDE inhibition in *T. cruzi* isolates (16). Ultrastructural alterations due to osmotic imbalance have been suggested in *T. cruzi* isolates exposed to imidazole derivatives (14) and phthalazinones (5), both inhibitors of one or more *T. cruzi* PDEs that caused autophagy-like cell death outcomes. The remarkable speed of the morphological changes is especially promising for this compound series.

To more broadly investigate the potential activity of NPD-008 on obligate intracellular kinetoplastid parasites, assays were performed on amastigotes of *L. amazonensis* (LTB0016) and *L. infantum* (MHOM/MA/67/ITMAP263). The *L. amazonensis* amastigotes were isolated from mouse skin lesions as reported previously (17) and incubated with NPD-008 for 48 h, revealing leishmanicidal activity with an EC_{50} of 14.9 ± 2 μM. Spleen-

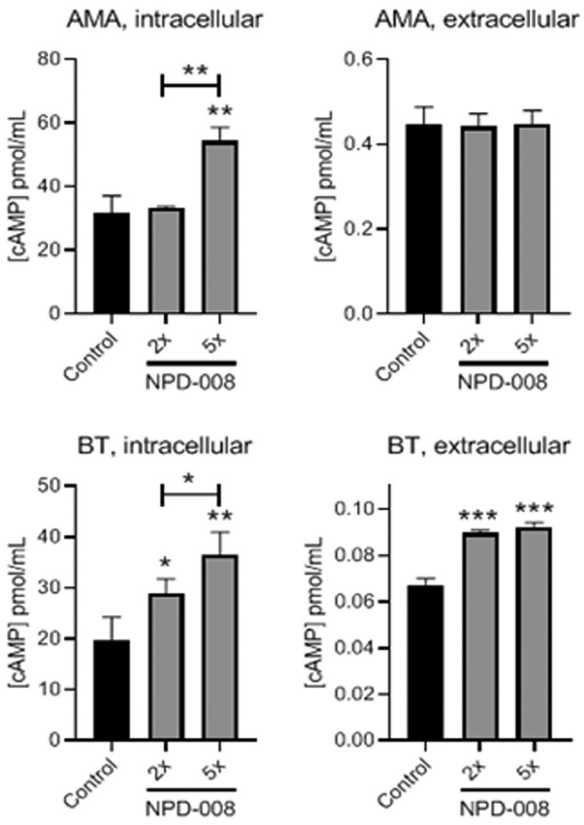

FIG 2 Intracellular contents of cAMP in *T. cruzi* free amastigotes (AMA) and trypomastigotes (BT) and in their respective culture media: untreated (control) and after exposure for 2.5 h with 2× and 5× EC_{50} of NPD-008. Control: untreated parasites, 2× and 5× = treated parasites using 2× and 5× the EC_{50} values. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus control or lower concentration, as indicated.

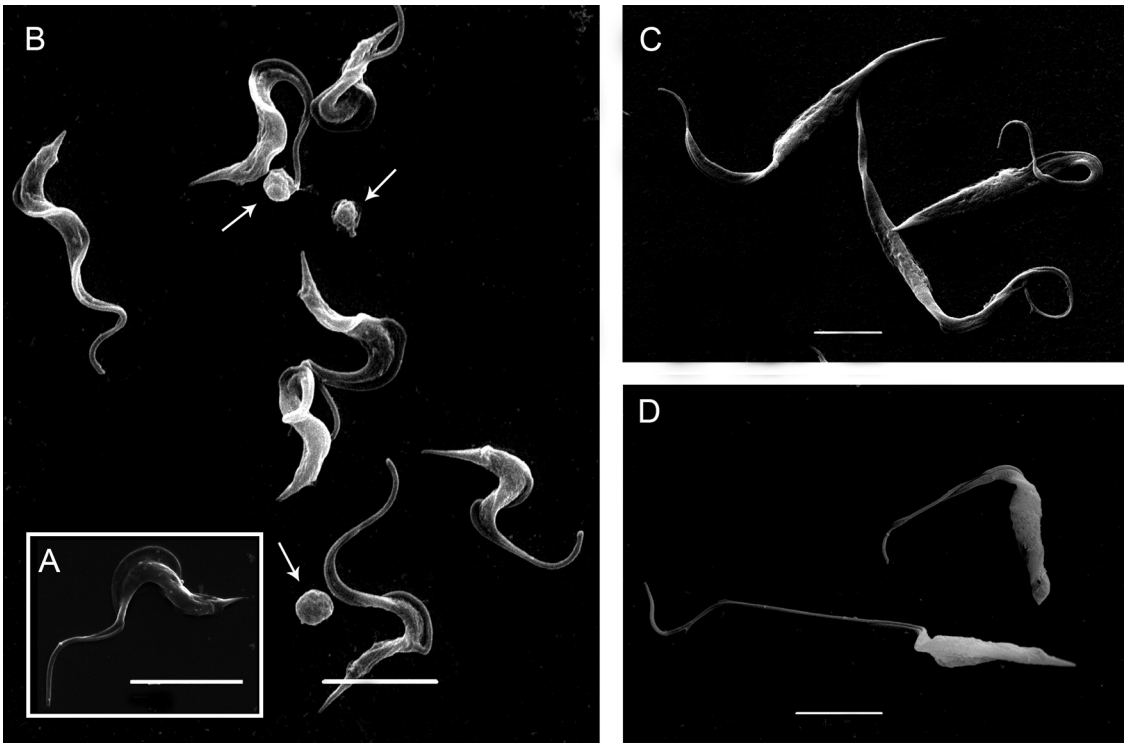

FIG 3 Scanning electron microscopy of bloodstream trypomastigotes and epimastigotes untreated (A, C) and treated with NPD-008 for 15 min (B, D). (B) Arrows, rounded parasites. Bar, 5 μM.

derived amastigotes of *L. infantum* in primary peritoneal mouse macrophages were exposed to drug for 72 h for microscopic examination of the cellular amastigote burdens (18), revealing a mean EC_{50} of 12.5 $\pm$ 10.6 μM (Table 1). Although less active than the reference drug pentamidine (1.25 $\pm$ 0.09 μM), the low cytotoxicity profile of NPD-008 against L929 (LC_{50}, 199.8 μM) and MRC-5 (LC_{50}, $\geq$64 μM) fibroblast cell lines and cardiac cells may argue in favor of further study and optimization of phthalazinone PDE inhibitors toward alternative therapeutic approaches for patients affected by Chagas disease and leishmaniasis (Table 2).

We report promising activity of the tetrahydrophthalazinone NPD-008 against amastigotes of *L. amazonensis*, *L. infantum*, and different strains and DTUs of *T. cruzi*. NPD-008 displays superior potency against *T. cruzi* trypomastigotes over benznidazole and is on target, not cardiotoxic, and synergistic with benznidazole, all favorable indicators and consistent with the existing target product profile (19, 20). However, its activity against the other relevant form of *T. cruzi* for mammalian cell infection, the intracellular amastigotes, although $<$10 μM, was less than that of benznidazole; therefore, NPD-008 does not match all characteristics necessary to move to animal experimental models (13). Although it is not an endpoint in drug development for Chagas disease and leishmaniasis, NPD-008 represents an important staging post for further lead optimization via Medicinal Chemistry aiming to provide preclinical and clinical candidates for both NTDs. While the manuscript was in preparation, we published a study with TbrPDEB1, which showed that specific further modification of the scaffold allows for superior targeting of the parasite-specific PDE P-pocket, improving antiparasite efficacy and selectivity (4, 21). Thus, the next proposed steps are to evaluate this latest compound series on *T. cruzi* amastigotes and trypomastigotes *in vitro* to select a final PDE inhibitor candidate for future *in vivo* testing in a murine Chagas disease model as monotherapy and in combination with benznidazole.

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