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Short Communication

Prodrugs as new therapies against Chagas disease: in vivo synergy between *Trypanosoma cruzi* proline racemase inhibitors and benznidazole



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ABSTRACT

Objectives: : Chagas disease, caused by the parasitic protozoan *Trypanosoma cruzi*, affects approximately 6–7 million people worldwide. There are limited available therapies and they exhibit low efficacy, often high toxicity in chronic cases and some drug resistance. In this study, our objective was to develop ester prodrugs that inhibit proline racemase (TcPRAC), a parasitic enzyme previously identified and characterised as a promising target because of its essential role in the parasite's life cycle and virulence, and to test their activity against *T. cruzi*.

Methods: : Using structural bioinformatics, we modelled several functional intermediates of the catalytic site between the opened and closed conformations of TcPRAC based on its crystal structures in complex with its competitive inhibitor, pyrrole-2-carboxylic acid. Guided by these intermediates, which were later validated in cocrystals, we designed and evaluated numerous compounds and tested them enzymatically on live parasites and in mice with our quick and straightforward drug screening method, which is based on state-of-the-art bioluminescent *T. cruzi* parasites injected subcutaneously.

Results: : Some of our novel compounds specifically inhibited racemase activity, as determined through biochemical assays, and covalently bound to TcPRAC. Furthermore, the corresponding ester prodrugs were effective in killing parasites in vitro. Bioluminescent *T. cruzi* assays in mice showed that JR1531, a TcPRAC inhibitor prodrug, can kill parasites in living animals, with boosted action when combined with low doses of benznidazole.

Conclusion: : This approach, based on TcPRAC inhibitor prodrugs in association with low doses of benznidazole, may lead to more effective, specific and non-toxic therapies against Chagas disease.

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1. Introduction

Chagas disease, caused by the parasitic protozoan *Trypanosoma cruzi*, is one of the most important causes of death from infectious diseases in South and Central America. Nearly 7 million people are infected and another 25 million are at risk. With human migration, the disease has extended to northern countries and has become a global concern [1,2]. Moreover, transmission of *T. cruzi* without an insect vector, by the oral route, has been reported in endemic regions [3], and *T. cruzi* is now one of the most prominent emerging foodborne parasitic protozoans [4]. There is no approved vaccine against Chagas disease, and currently only two nitroaromatic

drugs (benznidazole and nifurtimox) are available for treatment of Chagas disease [5]. Although these drugs can be effective in the acute phase, their efficacy against chronic forms of the disease is questionable. Altogether, with high toxicity impairing the benefit–risk ratio, poor criteria for cure or treatment monitoring [5–7] and a lack of innovative molecules [2], novel effective treatments are needed and their development is considered a priority.

We have previously identified *T. cruzi* proline racemase (TcPRAC) as a promising target to fight Chagas disease [8,9]. This enzyme, present in all stages of the parasite's life cycle, contributes to immune escape and persistence [8]. Indeed, *T. cruzi* loses viability with TcPRAC knockdown and gains virulence with TcPRAC over-expression [10]. The crystal structure of TcPRAC complexed with a transition analogue, 2-pyrrolicarboxylic acid, revealed tightly closed and highly specific catalytic sites (PDB 1W61). This left almost no space for the modulation of inhibitor candidates but, interestingly, in the hemisaturated TcPRAC structure (PDB 1W62) the ligand-free catalytic site was opened, showing an accessible surface area of the inhibitor-free monomer. This suggested that the ligand promotes a closure movement of the monomer and, consequently, an opening/closing functional mechanism of the enzyme affording the possibility to model plausible intermediate conformations better suited to efficiently anchor an inhibitor [11,12]. This strategy allowed us to enlarge the chemical search space in virtual screening and led us to identify two irreversible inhibitors: (E)-4-oxopent-2-enoic acid and its derivative (E)-5-bromo-4-oxopent-2-enoic acid. Optimisation of these molecules by the addition of groups and cyclisation led to a potent TcPRAC inhibitor, NG-P27 [13], that exhibited good trypanocidal activity against *T. cruzi*.

Exploiting these findings, this study aimed: (i) to evaluate the inhibitory activity and specificity for TcPRAC of three NG-P27-derived aryl ketones (JR1530, PU-24 and NG-P62), which further exploit the modelled catalytic site expansion; (ii) to assess the in vitro efficacy and toxicity of these compounds and corresponding prodrugs (JR1531, PU-24CO₂Me and NG-P65) against epimastigotes and intracellular amastigotes of *T. cruzi*; and (iii) to investigate the in vivo activity of the best-characterised prodrug (JR1531), alone or in combination with benznidazole, using bioluminescent virulent parasites and a rapid live-imaging screening method. We found that JR1531 is effective in killing parasites in living animals and that it acts in synergy with benznidazole. Therefore, the association of TcPRAC inhibitor prodrugs with low doses of benznidazole may lead to specific, non-toxic and thus more effective therapeutic approaches against Chagas disease.

2. Methods

2.1. Compound synthesis and analysis

Syntheses of TcPRAC inhibitors and prodrugs are described in the Supplementary material. Stock solutions of TcPRAC inhibitors and prodrugs (1 M) and benznidazole (50 mg/mL) were prepared in dimethyl sulfoxide (DMSO).

Recombinant *T. cruzi* proline racemase (EC 5.1.1.4) was produced in *Escherichia coli* BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) and was purified on nickel columns as described previously [8]. TcPRAC proline racemisation was assayed as described elsewhere [9].

2.2. Parasite cultures

Trypanosoma cruzi epimastigotes (strain CL Brener, clone F11-F5) were maintained by weekly passage in liver infusion tryptose (LIT) medium at 27°C. In vitro amastigote/trypomastigote forms of *T. cruzi* were maintained by weekly passage in Vero cells in RPMI 1640 medium (R0883; Sigma-Aldrich) supplemented with 4 mM glutamine, 0.17 mM β-mercaptoethanol, 100 U/mL penicillin,

100 μg/mL streptomycin and 5% (v/v) fetal calf serum (29-101-54; MP Biomedicals, Santa Ana, CA, USA) at 37°C and 5% CO₂. *Trypanosoma cruzi* blood trypomastigotes were maintained in 6-week-old male C3H/HeNRj mice (Janvier Laboratories, Le Genest-Saint-Isle, France). All parasite forms express the firefly luciferase gene constitutively [14].

2.3. Specificity of TcPRAC inhibitors

The specificity of TcPRAC inhibitors was assessed using enzymes bearing catalytic cysteines, namely papain (P4762; Sigma-Aldrich) and bromelain (B4882; Sigma-Aldrich). Briefly, 10 U of each enzyme, diluted in a 1 mL final volume of 10 mM ethylene diamine tetra-acetic acid (EDTA), 60 mM β-mercaptoethanol and 50 mM cysteine, was activated for 20 min at room temperature. Inhibitor:enzyme molar ratios of 1:1, 10:1 and 100:1 were tested. E-64 protease inhibitor (E3132; Sigma-Aldrich) was used at 0.1 mM as a positive inhibitory control. The reaction was revealed with 1% (w/v) azocasein (A2765; Sigma-Aldrich) and measured by absorbance at 440 nm.

2.4. Cytotoxicity of TcPRAC inhibitor prodrugs in non-infected cells

Cytotoxicity and cell viability were assessed using a CellTox™ Green Cytotoxicity Assay (G8741; Promega) and CellTiter-Glo® Luminescent Cell Viability Assay (G7570; Promega) on non-infected Vero cells (5 × 10³ cells/well) in 96-well plates incubated with different concentrations of freshly diluted prodrugs at 37°C with 5% CO₂ for 72 h. Cell morphology changes were evaluated on an Opera® high-content screening system (PerkinElmer) using the same cell, prodrug, temperature, time and CO₂ conditions with 100 nM MitoTracker™ Red CMXRos (M7512; Molecular Probes) and 1 μg/mL Hoechst 33342 staining.

2.5. In vitro trypanocidal/trypanostatic activity of TcPRAC inhibitors and prodrugs

Epimastigotes were incubated in 96-well white plates in LIT medium (1 × 10⁵ parasites/well) with different concentrations of freshly diluted compounds at 28°C for 72 h. Live parasite luminescence emitted upon addition of 0.3 mg/mL D-luciferin (122799; PerkinElmer) was quantified using a TECAN luminometer. For intracellular amastigotes, non-infected Vero cells were seeded in a 96-well white plate in supplemented RPMI 1640 medium (5 × 10³ cells/well) and were infected with 5 × 10⁴ trypomastigotes/well at 37°C for 24 h. Cells were washed and were incubated with different concentrations of freshly diluted compounds at 37°C under 5% CO₂ for 72 h. The presence of live parasites was measured using a Luciferase Reporter Gene Assay (11814036001; Roche).

2.6. In vivo toxicity of TcPRAC inhibitor prodrugs in zebrafish embryos

AB wild-type zebrafish (*Danio rerio*) embryos were raised at 28°C in embryo water composed of mineral water supplemented with 280 μg/mL methylene blue (M-4159; Sigma-Aldrich) and 30 μg/mL 1-phenyl-2-thiourea (P-7629; Sigma-Aldrich) and were handled according to institutionally approved guidelines. At 72 h post-fertilisation, embryos were distributed in 24-well plates (10 embryos/well) in 500 μL of embryo water containing freshly diluted drugs and were incubated at 28°C for 72 h in the dark. Quantification of live embryos and possible malformations was performed using a stereomicroscope.

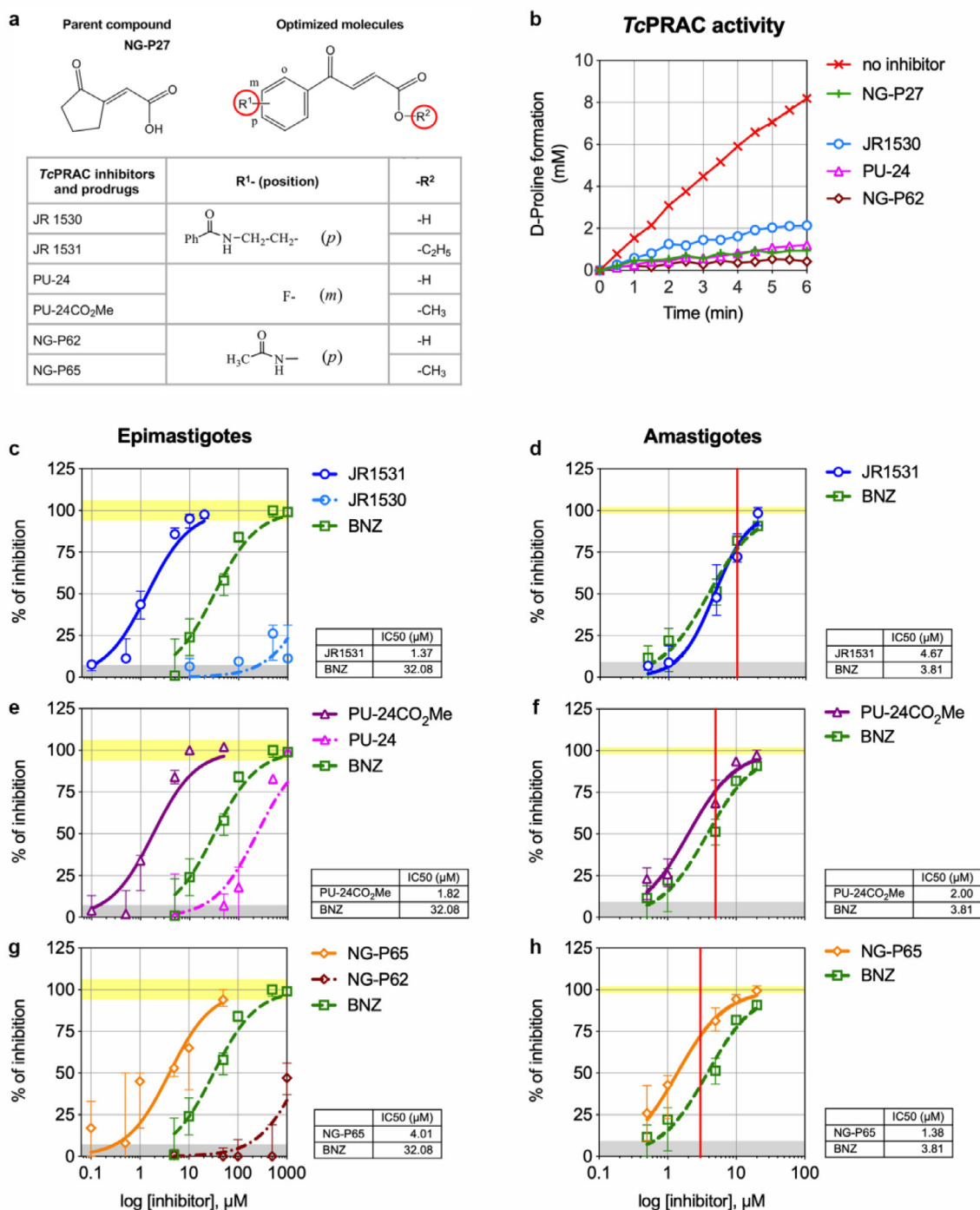


Fig. 1. Structure and activity of *Trypanosoma cruzi* proline racemase (*TcPRAC*) inhibitors and prodrugs. (b) Kinetics of 40 mM L-proline racemisation by *TcPRAC* alone or in the presence of 5 µM compound, either NG-P27, JR1530, PU-24 or NG-P65. (c–h) Dose–response curves of *TcPRAC* inhibitors and their corresponding ester prodrug activity on epimastigotes (c,e,g) and *TcPRAC* inhibitor prodrugs on intracellular amastigotes (d,f,h) of *T. cruzi* (strain CL Brener) in comparison with benznidazole (BNZ): (c,d) JR1530 and prodrug JR1531; (e,f) PU-24 and prodrug PU-24CO₂Me; and (g,h) NG-P62 and prodrug NG-P65. The grey shaded areas correspond to parasites incubated with dimethyl sulfoxide (DMSO) and the yellow shaded areas correspond to the absence of parasites (medium only). Vertical red lines indicate the estimated NOAEL (no observed adverse effect level) for the *TcPRAC* inhibitor prodrugs. Data are expressed as the median and interquartile range. IC₅₀ (µM) values of the *TcPRAC* inhibitor prodrugs are shown on the graphs.

2.7. In vivo rapid mouse model to test the trypanocidal/trypanostatic activity of *TcPRAC* inhibitor prodrugs

Six- to eight-week-old male RjOrl:SWISS mice (purchased from Janvier Laboratories) were handled according to the institutional guidelines of the Central Animal Facility at Institut Pasteur. Mice

were shaved on the dorsal skin and were injected subcutaneously with 1×10^4 blood trypomastigotes in a final volume of 50 µL. Infected mice were treated using five different formulas: (i) vehicle (DMSO); (ii) 50 mg/kg benznidazole (reference treatment); (iii) 5 mg/kg benznidazole; (iv) 50 mg/kg JR1531; and (v) 50 mg/kg JR1531 + 5 mg/kg benznidazole. Five days of treatment, consist-

Table 1

Cytotoxicity (CC₅₀), NOAEL (no observed adverse effect level), trypanocidal/trypanostatic activity (IC₅₀), selectivity index (SI) and median lethal dose (LC₅₀) in zebrafish embryos of the *Trypanosoma cruzi* proline racemase (TcPRAC) inhibitor prodrugs

TcPRAC prodrug	CC ₅₀ (μM)	NOAEL (μM)	Amastigotes IC ₅₀ (μM)	SI	Zebrafish LC ₅₀ (μM)
JR1531	20.01	10	4.67	4.28	24.22
PU-24CO ₂ Me	8.63	5	2.00	4.32	11.00
NG-P65	7.50	3	1.38	5.43	8.76

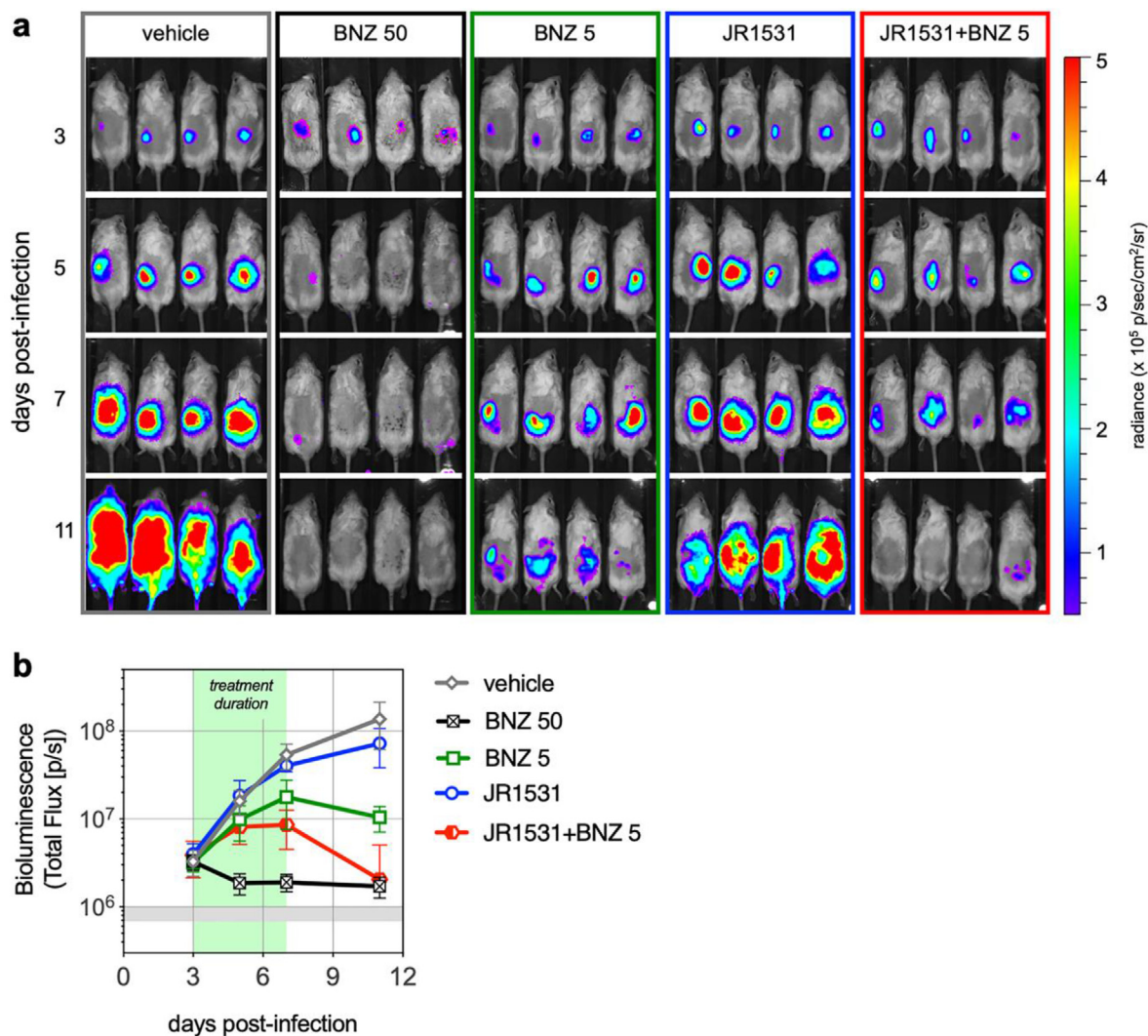


Fig. 2. In vivo trypanocidal/trypanostatic activity of *Trypanosoma cruzi* proline racemase (TcPRAC) inhibitor prodrugs in a rapid mouse model. (a) Representative images illustrating the two-dimensional bioluminescence signals related to *T. cruzi* presence in the dorsal region of mice from the different treatment groups at 3, 5, 7 and 11 days post-infection. (b) Efficacy of the 5-day in situ treatment expressed as bioluminescent signals (total flux in photons/s). Lines represent the median (and interquartile range) of the dorsal bioluminescent signals from mice treated with vehicle (DMSO), 50 mg/kg benznidazole (BNZ 50), 5 mg/kg benznidazole (BNZ 5), 50 mg/kg TcPRAC inhibitor prodrug (JR1531) or the combination of 50 mg/kg TcPRAC inhibitor prodrug and 5 mg/kg benznidazole (JR1531+BNZ 5). The green shaded area corresponds to the period of treatment and the grey shaded area corresponds to background signals.

ing of a daily subcutaneous injection of 50 μL of each formula at the site of the infection, started 3 days after infection. At Days 3, 5, 7 and 11 post-infection, the luciferase substrate D-luciferin was injected intraperitoneally at 12.5 mg/kg; animals were anaesthetised intraperitoneally with 75 mg/kg ketamine (Imalgène 1000; Merial) and 15 mg/kg xylazine (Rompum; Bayer), placed in a confinement box and imaged using an IVIS® Spectrum In Vivo Imaging System (PerkinElmer). Two-dimensional bioluminescence images were recorded, and photon emission was quantified (photons/s) in a region of interest defined using Living Image software (PerkinElmer).

2.8. Ethical statement

Animals were housed in the Institut Pasteur animal facilities accredited by the French Ministry of Agriculture for performing experiments on live rodents. Animal experiments were performed in compliance with French and European regulations on the care and protection of laboratory animals (EC Directive 2010/63, French Law 2013-118, 6 February 2013). All experiments were approved by the Ethics Committee #89 and were registered under reference #2013-0047.

3. Results and discussion

3.1. Synthesis and inhibitory activity of (E)-4-aryl-4-oxobut-2-enoic acids

Chagas disease remains one of the most neglected diseases and the development of effective treatments is considered a priority [2]. Expanding the structure of NG-P27, a potent TcPRAC inhibitor acting both in vitro and in vivo [13], we produced (E)-4-aryl-4-oxobut-2-enoic acids either by the Friedel–Crafts reaction or by crotonic condensation involving aryl methyl ketones. Corresponding esters were obtained by reaction with diethyl sulfate or methyl iodide. All compounds were purified and fully characterised (Fig. 1a; Supplementary Table S1). These compounds, named JR1530, PU-24 and NG-P62, irreversibly inhibited recombinant TcPRAC (Fig. 1b). These compounds displayed no inhibition on other tested cysteine enzymes, suggesting that they were specific for TcPRAC (Supplementary Fig. S1) [13].

3.2. Efficacy against *Trypanosoma cruzi* and cell viability shown with ester prodrugs

Due to their acidic nature, the TcPRAC inhibitors were poorly permeable and not effective in killing parasites in vitro (Fig. 1c–h). To overcome this issue, we synthesised corresponding ester prodrugs following described examples for esterase-activated prodrugs against parasites such as *Plasmodium falciparum* [15] and *Trypanosoma brucei rhodesiense* [16].

The TcPRAC inhibitor prodrugs JR1531, PU-24CO₂Me and NG-P65 presented a marked dose-dependent effect on *T. cruzi* viability, requiring 23-fold lower concentrations than benznidazole to kill epimastigotes. The efficacy against intracellular amastigotes was moderate (Table 1), with doses still similar to that of benznidazole (Fig. 1c–h). The dose-dependent cytotoxicity and NOEL (no observed adverse effect level) of the prodrugs indicated a moderate selectivity index (Table 1). In vivo toxicity tested on zebrafish embryos [17] showed that JR1531 was the prodrug with the lowest dose-dependent toxicity (Supplementary Fig. S2; Table 1) and, consequently, it was selected to be tested in the mouse model.

3.3. Noticeably favourable synergy of JR1531 with benznidazole in mice

Our rapid mouse model with bioluminescent *T. cruzi* trypomastigotes allowed us to detect the effect of prodrug JR1531 treatment as soon as 5 days after infection, which is useful for in vivo drug screening (Fig. 2a).

Prolonged chemotherapy with benznidazole and nifurtimox produces several adverse effects in almost all treated patients, which correlates with poor quality of life and frequent treatment discontinuation [6]. Hence, as JR1531 showed stronger efficacy than benznidazole on live parasites despite a lower efficacy in vivo (Fig. 2b), we tested whether it could have synergistic effects with benznidazole, possibly allowing us to reduce its dose and thus the risk of adverse side effects [18–20]. Remarkably, co-administration of JR1531 with suboptimal doses of benznidazole (5 mg/kg benznidazole) was effective in inducing complete clearance of local parasites within 11 days post-infection (Fig. 2). Hence, the TcPRAC inhibitor prodrug JR1531 was effective in killing parasites in living animals in synergy with benznidazole.

In the absence of an efficient test for cure, especially in the chronic phase [1,7], the quest for better Chagas disease therapies remains a challenge. None the less, the association of TcPRAC inhibitor prodrugs with low doses of benznidazole may lead to a more effective, specific and non-toxic treatment against Chagas disease.

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Competing Interests

None declared.

Ethical approval

Animals were housed in the Institut Pasteur animal facilities accredited by the French Ministry of Agriculture for performing experiments on live rodents. Animal experiments were performed in compliance with French and European regulations on the care and protection of laboratory animals (EC Directive 2010/63, French Law 2013-118, 6 February 2013). All experiments were approved by the Ethics Committee #89 and were registered under reference #2013-0047.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2021.10.030.

References

- [1] Bern C, Messenger LA, Whitman JD, Maguire JH. Chagas disease in the United States: a public health approach. *Clin Microbiol Rev* 2019;33 e00023-19. doi:10.1128/cmr.00023-19.
- [2] Santos SS, de Araújo RV, Giarolla J, Seoud OE, Ferreira EI. Searching for drugs for Chagas disease, leishmaniasis and schistosomiasis: a review. *Int J Antimicrob Agents* 2020;55:105906. doi:10.1016/j.ijantimicag.2020.105906.
- [3] Santana RAG, Guerra M, Sousa DR, Couceiro K, Ortiz JV, Oliveira M, et al. Oral transmission of *Trypanosoma cruzi*, Brazilian Amazon. *Emerg Infect Dis* 2019;25:132–5. doi:10.3201/eid2501.180646.
- [4] Yoshida N, Tyler KM, Llewellyn MS. Invasion mechanisms among emerging food-borne protozoan parasites. *Trends Parasitol* 2011;27:459–66. doi:10.1016/j.pt.2011.06.006.
- [5] World Health Organization (WHO) *Research priorities for Chagas disease, human African trypanosomiasis and leishmaniasis*. Technical report series n°975. Geneva, Switzerland: WHO; 2012.
- [6] Jackson Y, Wyssa B, Chappuis F. Tolerance to nifurtimox and benznidazole in adult patients with chronic Chagas' disease. *J Antimicrob Chemother* 2020;75:690–6. doi:10.1093/jac/dkz473.
- [7] Lana M, Martins-Filho OA. Revisiting the posttherapeutic cure criterion in Chagas disease: time for new methods, more questions, doubts, and polemics or time to change old concepts? *Biomed Res Int* 2015;2015:652985. doi:10.1155/2015/652985.
- [8] Reina-San-Martin B, Degraeve W, Rougeot C, Cosson A, Chamond N, Cordeiro-Da-Silva A, et al. A B-cell mitogen from a pathogenic trypanosome is a eukaryotic proline racemase. *Nat Med* 2000;6:890–7. doi:10.1038/78651.
- [9] Chamond N, Gregoire C, Coatnoan N, Rougeot C, Freitas-Junior LH, da Silveira JF, et al. Biochemical characterization of proline racemases from the human protozoan parasite *Trypanosoma cruzi* and definition of putative protein signatures. *J Biol Chem* 2003;278:15484–94. doi:10.1074/jbc.m210830200.
- [10] Chamond N, Goytia M, Coatnoan N, Barale JC, Cosson A, Degraeve WM, et al. *Trypanosoma cruzi* proline racemases are involved in parasite differentiation and infectivity. *Mol Microbiol* 2005;58:46–60. doi:10.1111/j.1365-2958.2005.04808.x.

- [11] Buschiazzi A, Goytia M, Schaeffer F, Degrave W, Shepard W, Gregoire C, et al. Crystal structure, catalytic mechanism, and mitogenic properties of *Trypanosoma cruzi* proline racemase. *Proc Natl Acad Sci U S A* 2006;103:1705–10. doi:10.1073/pnas.0509010103.
- [12] Berneman A, Montout L, Goyard S, Chamond N, Cosson A, d'Archivio S, et al. Combined approaches for drug design points the way to novel proline racemase inhibitor candidates to fight Chagas' disease. *PLoS One* 2013;8:e60955. doi:10.1371/journal.pone.0060955.
- [13] Amaral PA, Autheman D, de Melo GD, Gouault N, Cupif JF, Goyard S, et al. Designed mono- and di-covalent inhibitors trap modeled functional motions for *Trypanosoma cruzi* proline racemase in crystallography. *PLoS Negl Trop Dis* 2018;12:e0006853. doi:10.1371/journal.pntd.0006853.
- [14] Goyard S, Dutra PL, Deolindo P, Autheman D, D'Archivio S, Minoprio P. In vivo imaging of trypanosomes for a better assessment of host–parasite relationships and drug efficacy. *Parasitol Int* 2014;63:260–8. doi:10.1016/j.parint.2013.07.011.
- [15] Istvan ES, Mallari JP, Corey VC, Dharia NV, Marshall GR, Winzeler EA, et al. Esterase mutation is a mechanism of resistance to antimalarial compounds. *Nat Commun* 2017;8:14240. doi:10.1038/ncomms14240.
- [16] Klein P, Johe P, Wagner A, Jung S, Kühnborn J, Barthels F, et al. New cysteine protease inhibitors: electrophilic (het)arenes and unexpected prodrug identification for the *Trypanosoma* protease rhodesain. *Molecules* 2020;25:1451. doi:10.3390/molecules25061451.
- [17] Zon LI, Peterson RT. In vivo drug discovery in the zebrafish. *Nat Rev Drug Discov* 2005;4:35–44. doi:10.1038/nrd1606.
- [18] Diniz LF, Mazzeti AL, Caldas IS, Ribeiro I, Bahia MT. Outcome of E1224–benznidazole combination treatment for infection with a multidrug-resistant *Trypanosoma cruzi* strain in mice. *Antimicrob Agents Chemother* 2018;62:e00401-18. doi:10.1128/aac.00401-18.
- [19] García MC, Ponce NE, Sanmarco LM, Manzo RH, Jimenez-Kairuz AF, Aoki MP. Clomipramine and benznidazole act synergistically and ameliorate the outcome of experimental Chagas disease. *Antimicrob Agents Chemother* 2016;60:3700–8. doi:10.1128/aac.00404-16.
- [20] Aguilera E, Varela J, Serna E, Torres S, Yaluff G, Bilbao NV, et al. Looking for combination of benznidazole and *Trypanosoma cruzi*-triosephosphate isomerase inhibitors for Chagas disease treatment. *Mem Inst Oswaldo Cruz* 2018;113:153–60. doi:10.1590/0074-02760170267.