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To cite this version:
Guilherme Dias de Melo, Nicolas Coatnoan, Nicolas Gouault, Jean-François Cupif, Jacques Renault, et al.. Prodrugs as new therapies against Chagas disease: In vivo synergy between Trypanosoma cruzi proline racemase inhibitors and benznidazole. Journal of Global Antimicrobial Resistance, 2022, 28, pp.84-89. 10.1016/j.jgar.2021.10.030. hal-03504248

HAL Id: hal-03504248
https://hal.science/hal-03504248
Submitted on 30 May 2022

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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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A R T I C L E    I N F O

Article history:
Received 9 April 2021
Revised 4 October 2021
Accepted 21 October 2021
Available online 17 December 2021

Editor: edited by Prof Fabrizio Bruschi

Keywords:
Antiparasitic agents
Chagas disease
Drug development
Enzyme inhibitor
Medicinal chemistry

A B S T R A C T

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 open 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 (benzimidazole 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 overexpression [10]. The crystal structure of TcPRAC complexed with a transition analogue, 2-pyrrolocarboxylic 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 allowing the possibility to model plausible intermediate conformations better suited to efficiently anchor an inhibitor [11,12]. This strategy allowed us to enlarge the chemical 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/HeNJ 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 cysteine(s), 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. E64 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 CellToxTM 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 MitoTrackerTM Red CMXRos (M7512; Molecular Probes) and 1 μg/mL Hoechst 33342 staining.

2.5. In vitro trypanocidal/trypansomatic 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.
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 $1 \times 10^4$ 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

<table>
<thead>
<tr>
<th>TcPRAC prodrug</th>
<th>CC₅₀ (μM)</th>
<th>NOAEL (μM)</th>
<th>Amastigotes IC₅₀ (μM)</th>
<th>SI</th>
<th>Zebrafish LC₅₀ (μM)</th>
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</thead>
<tbody>
<tr>
<td>JR1531</td>
<td>20.01</td>
<td>10</td>
<td>4.67</td>
<td>4.28</td>
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<tr>
<td>PU-24CO₂Me</td>
<td>8.63</td>
<td>5</td>
<td>2.00</td>
<td>4.32</td>
<td>11.00</td>
</tr>
<tr>
<td>NG-P65</td>
<td>7.50</td>
<td>3</td>
<td>1.38</td>
<td>5.43</td>
<td>8.76</td>
</tr>
</tbody>
</table>

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 bioluminescent 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.

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 NOAEL (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 trypanostigmates 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.

Funding

This research project received financial support from the Agence Nationale pour la Recherche [ANR-14-CE16-0001-01] and the recurring budget of the Institut Pasteur to the Laboratoire des Processus Infectieux à Trypanosomatidés. GDM was supported by ANR and Institut Carnot–Pasteur Microbes et Santé (Pasteur M&S) fellowships. Part of this work was performed at the UtechS Photonic BioImaging (PBI) platform, a member of the France Life Imaging network [grant ANR-11-INBS-0006].

Competing interests

None declared.

Ethical approval

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Acknowledgments

The authors would like to thank the Imagopole-Citech, especially Marie-Anne Nicola for help and support with the in vivo imaging analyses, Nathalie Aulner for help with the high-throughput screening analyses, and Emi Murayama and Catherine Vivier for assistance with the zebrafish embryo assays.

Supplementary materials


References


