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Comprehensive *in vitro* and *in vivo* phenotypic-based screening for the identification of new aza-scorpiand macrocycles agents against *T. cruzi*

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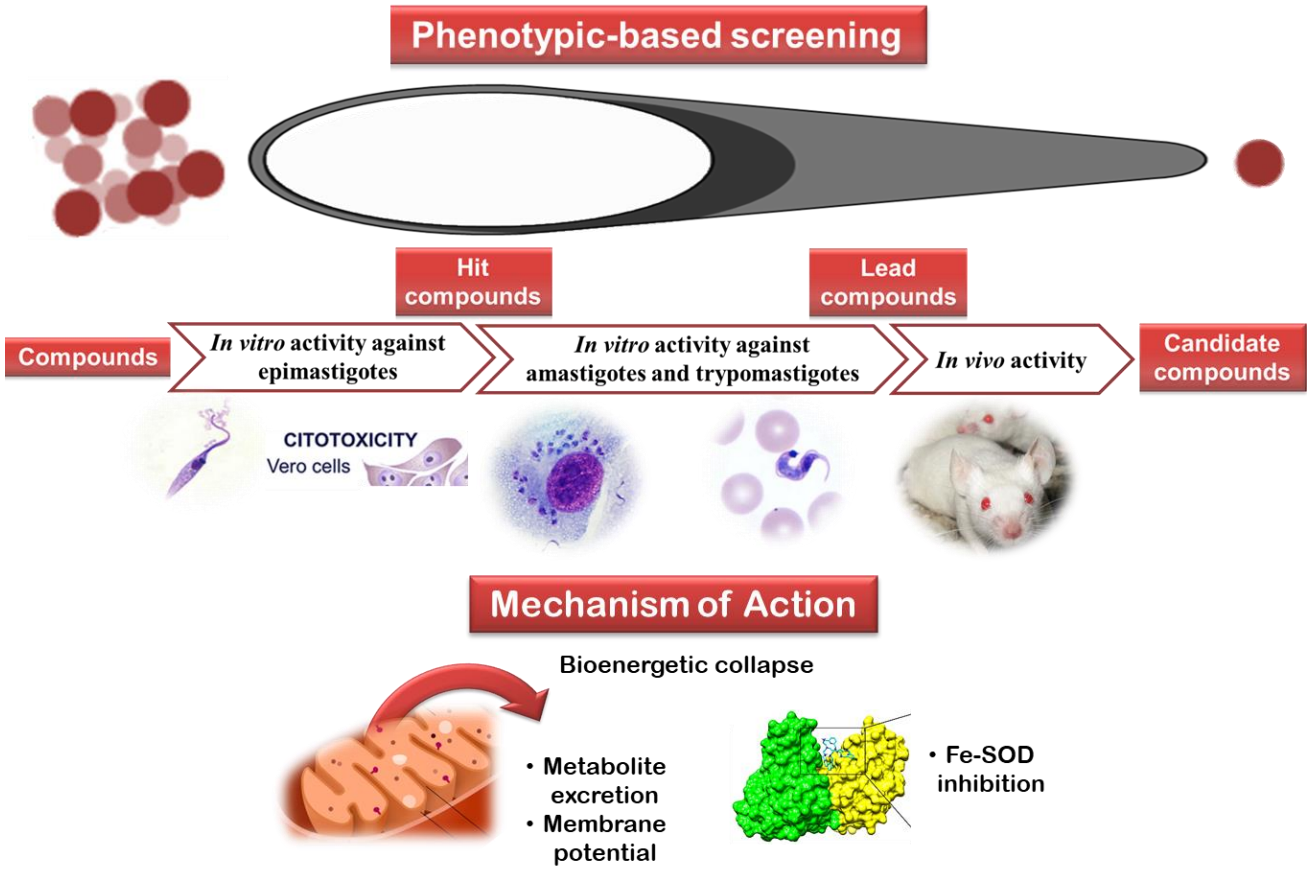
Comprehensive *in vitro* and *in vivo* phenotypic-based screening for the identification of new *aza-scorpiand* macrocycles agents against *T. cruzi*



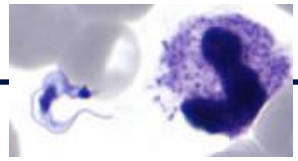
Abstract:

Trypanosoma cruzi, the aetiological agent of Chagas disease, is a genuine parasite with a tremendous genetic diversity and a complex life cycle, causing complicated pathogenesis. The treatment of the disease has been studied by scientists for more than 100 years, but at present Chagas disease is a life-threatening infection and a global public health problem that has no effective treatment and affects 6-8 million people worldwide. Hence, there is an urgent need for effective new drugs to tackle Chagas disease. Here, we describe a comprehensive strategy and a complete *in vitro* and *in vivo* phenotypic-based screening in early drug discovery pipeline for the identification of new effective agents against T. cruzi. In short, 22 *aza-scorpiand* macrocycles were screened in vitro against different *T. cruzi* strains (including a BZN-resistant strain), and lead compounds were evaluated in vivo after oral administration in both the acute and chronic infections in mouse model. The mode of action was also evaluated at the energetic level.

Keywords: Chagas disease; Drug discovery; Neglected tropical diseases; Screening cascade; *Trypanosoma cruzi*

Introduction

Chagas Disease & *Trypanosoma cruzi*



CDC

- Parasitic, systemic, chronic and life-threatening illness.
- Caused by tropical infection with the triatomine-transmitted protozoan parasite ***Trypanosoma cruzi.***

CDC

World Health Organization

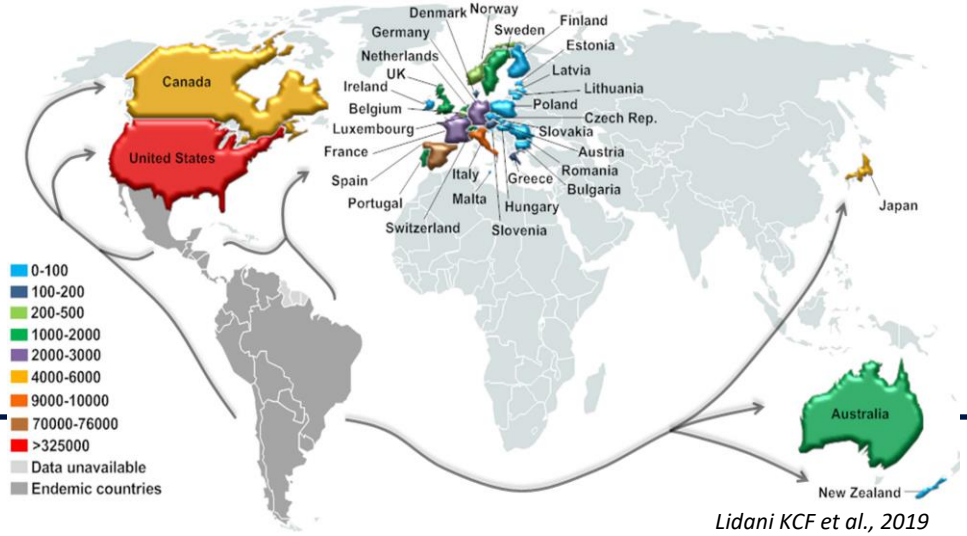
- **Classified as**:
  - A neglected tropical illness.
  - The most important parasitic disease in Latin America.
  - The leading cause of morbimortality in many endemic regions.
  - The most prevalent of the poverty-caused and poverty-promoting illness in Latin America.
  - Fewer than 10 % people are diagnosed and only a few number receive treatment.

Introduction

Chagas Disease & *Trypanosoma cruzi*

- The prevalence of the disease has been reduced in Latin America due to:
  - **Health policies**: compulsory blood-bank screening.
  - **Multinational initiatives**.

- Widespread due to mobility and migration.



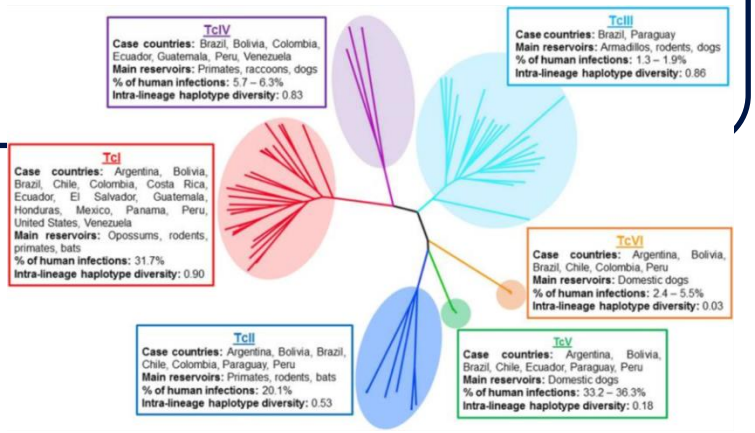
Lidani KCF et al., 2019

- **Global health problem:**
  - 6-8 million infected people.
  - 28 thousand new cases/year.
  - 14-50 thousand deaths/year.
  - 70-100 million people at risk of infection.

Introduction

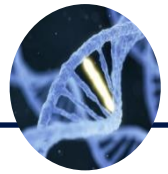
Genetic diversity of *T. cruzi*

- **Pool of strains and isolates** that circulate among vectors and mammalian hosts.
- Extensively studied by biological, biochemical, and molecular methods.
- Difference up to 40 % in DNA content between strains.
- 2009 → 6 genetic lineages or DTUs has been proposed.

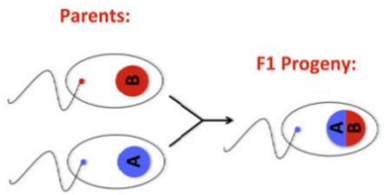


Francisco AF et al., 2017

- **Classical clonal evolution model is challenged**:
  - Binary fission.
  - Discrete mutations.



- **Recombination and genetic exchange** between the dividing amastigote intracellular forms.
- Expected **genetic exchange** in the digestive tract of triatomine vectors.

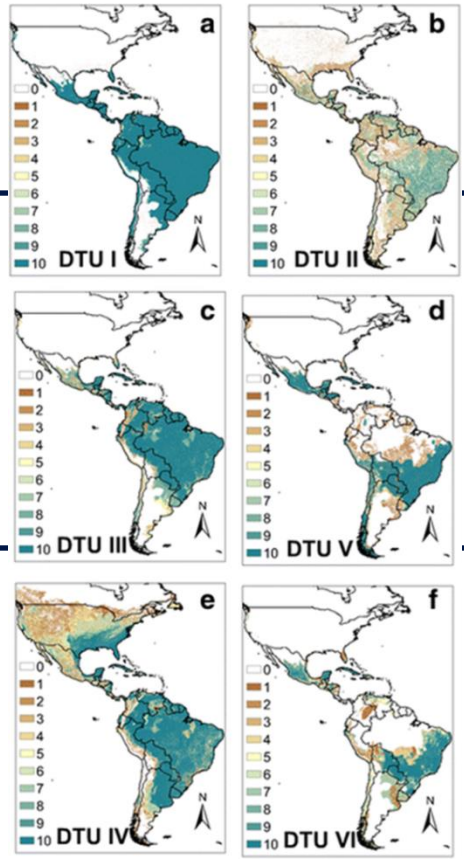


Messenger LA and Miles MA, 2015

Introduction

Genetic diversity of *T. cruzi*

- This heterogeneity could explain the **geographical differences** in:
  - Disease pathology.
  - Morbidity and mortality.
  - The wide divergence in the susceptibility of the current treatments.



Izeta-Alberdi A et al., 2016

However

- **No definitive correlation** between them and parasite lineage has been established.
- TcI isolates, more resistant to the reference drug benznidazole
- There are divergences in susceptibility to current treatments independently of the mitochondrial nitroreductase (TcNTR) sequence.

?

Hence

Additional factors should be studied

To avoid:
- **Drug resistance**
- **Treatment failures**

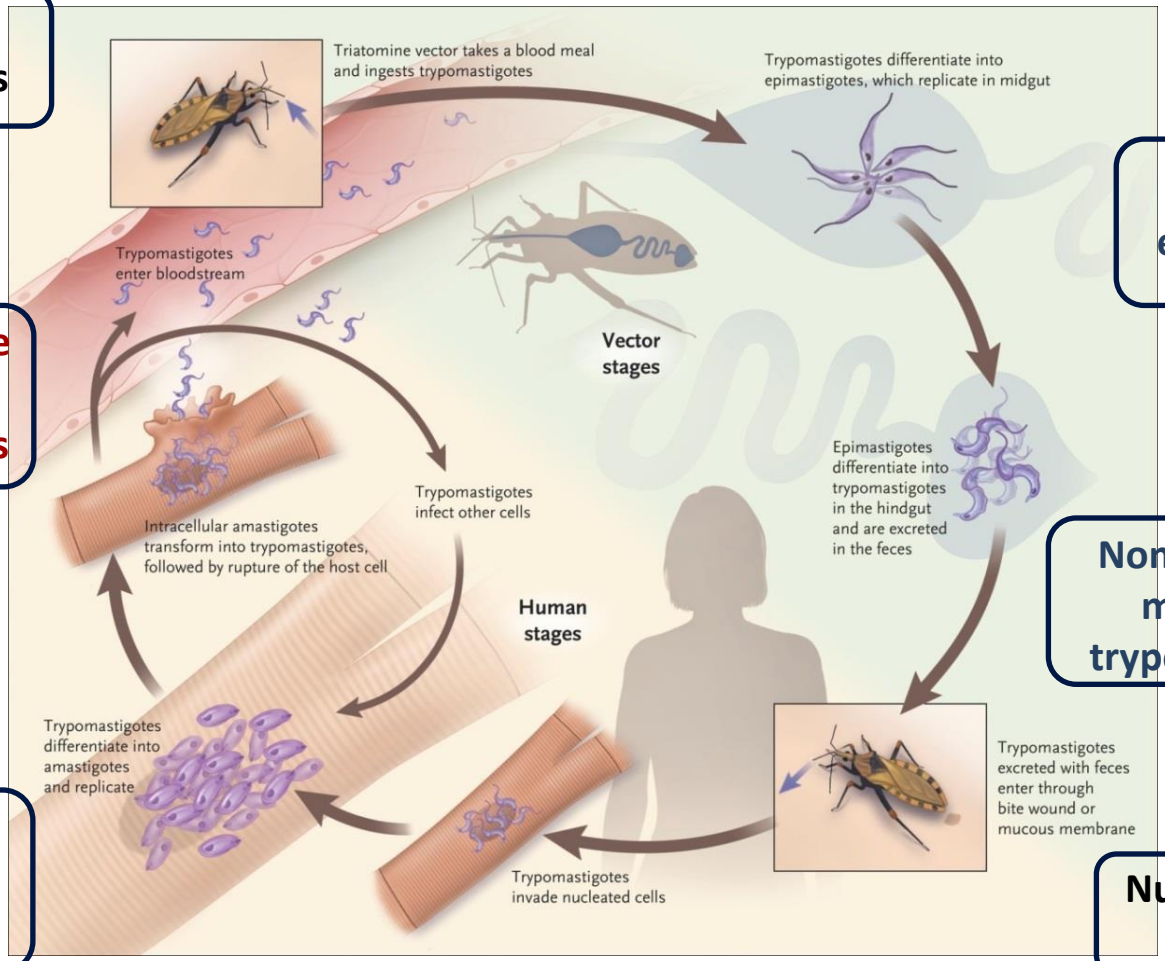
Introduction

Life-cycle of T. cruzi

Heteroxenic protozoan

Pool of different *T. cruzi* strains

Numerous vector species



Replicative epimastigotes

Non-replicative Bloodstream trypomastiotes

Non-replicative metacyclic trypomastigotes

Replicative amastigotes (intracellular)

Numerous mammal host species

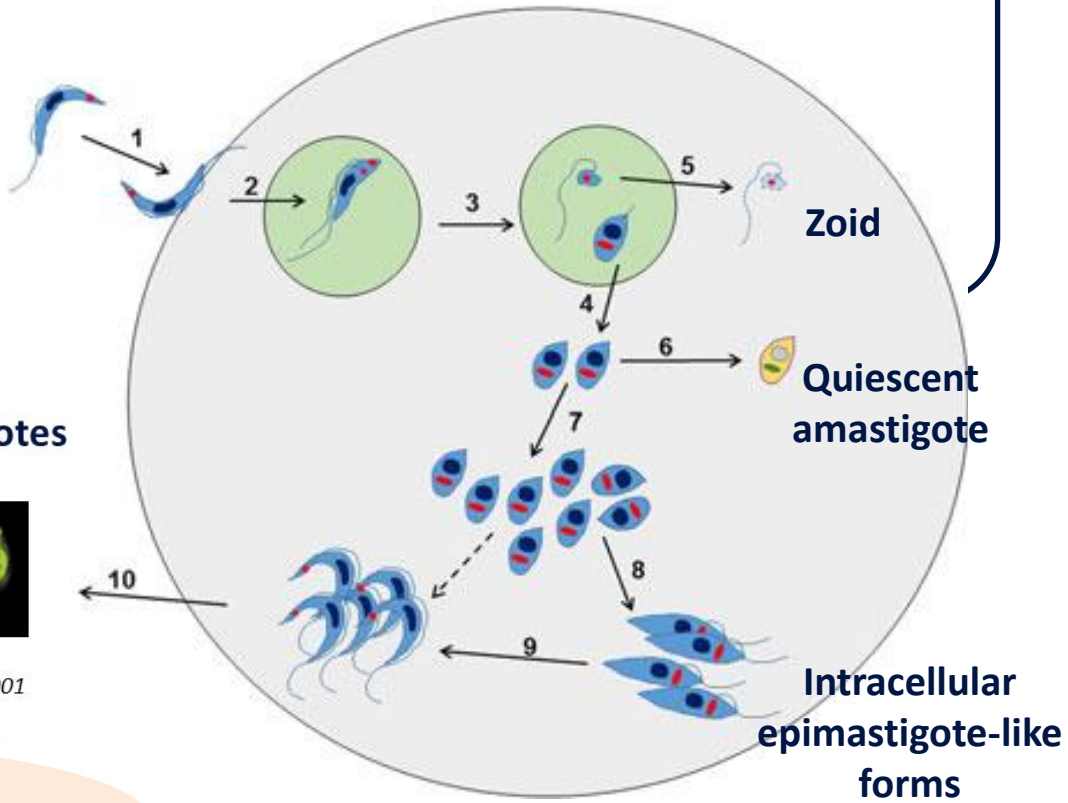
Bern C., 2015

Introduction

Life-cycle of T. cruzi

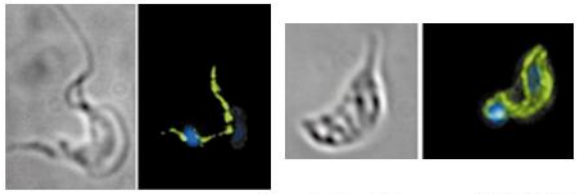
- The process in mammalian host cells is **more complex**.

Intermediate forms are also observed during the T. cruzi life-cycle



Francisco AF et al., 2017

Slender and broad trypomastigotes



Morris K and Engman DM, 2001

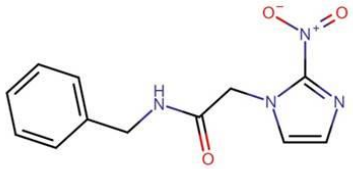
And even an extracellular differentiation to amastigote forms

Introduction

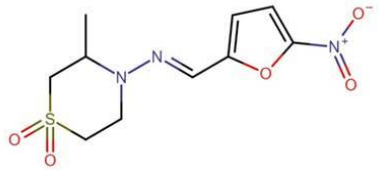
Current treatments

➢ **Limited to two obsolete nitroheterocyclic drugs**:

- Frequently treatment failures.
- Long treatment periods.
- Toxic side-effects.



Benznidazole

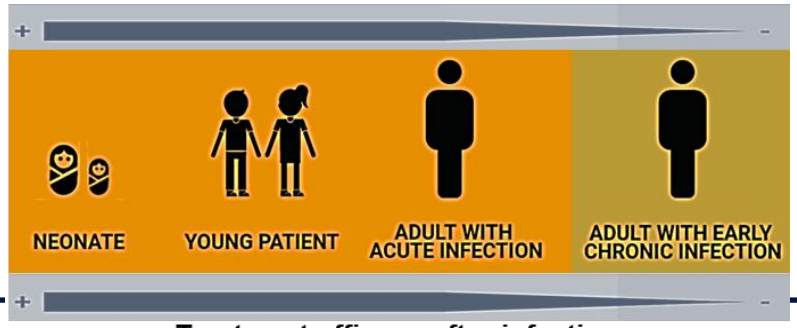


Nifurtimox

➢ **The cure rate depends on several factors**:

- Phase of the disease.
- Associated comorbidities.
- Age and immune response of the patient.
- Susceptibility of the parasite genotype.

➢ **Treatment recommendations:**



Treatment efficacy after infection

ISGlobal

➢ Contraindicated during:

- Pregnancy.
- Kidney or liver insufficiency.
- People with advanced Chagas heart disease.

Introduction

Aims

- **Chagas Clinical Research Platform** (CCRP) (2009):
  - Evaluation and development of **new drugs** for Chagas disease.
  - **Standardization of methodology** to assess drug efficacy.
  - Revision of **alternatives** for using current approved drugs (guidelines, doses, combination).

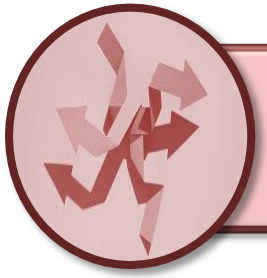
DNDi
Drugs for Neglected Diseases *initiative*

DNDi

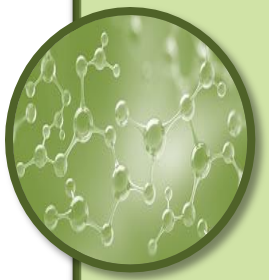
- ✓ **The aim is to find a specific treatment that allows the erradiction of the parasite** and, hence, the elimination of the symptoms of Chagas disease.
- ✓ The development of **new drugs, safer, more effective, that provide a shorter treatment course**, preferably oral, is an important need.

Introduction

Objectives



1. Establish a **comprehensive and complete phenotypic-based screening** in both *in vitro* and *in vivo* models to identify potential compounds against Chagas disease.



2. Develop more effective, safer and affordable compounds since the current therapeutic arsenal to combat Chagas Disease is inadequate and insufficient.

3. Elucidate the mechanism of action of trypanocidal drug candidates.

Introduction

Current target product profile / Objectives

	Acceptable	Ideal
Target population	Chronic	Chronic and Acute
Geographic Distribution	All regions	All regions
Efficacy	Non inferior to benznidazole standard dose* in all regions (parasitological)	Superiority to benznidazole standard dose to different phases of disease (acute and chronic) (parasitological)
Safety	Superiority to benznidazole* in the frequency of definitive treatment discontinuations for medical indication (clinical and laboratory)**	Superiority to benznidazole* in the frequency of definitive treatment discontinuations for medical indication (clinical and laboratory)**
Contraindications	Pregnancy	No contraindications
Precautions	No genotoxicity**; No pro-arrythmic potential	No genotoxicity; No teratogenicity; No pro-arrythmic potential
Interactions	No clinically significant interaction with anti-arrythmic and anticoagulants drugs	No clinically significant interaction
Presentation	Oral/Parenteral (short POC)*** Age-adapted	Oral Age-adapted
Stability	3 years, climatic zone IV	5 years, climatic zone IV
Dosing regimen	Oral – any duration Parenteral – <7 days	<30days
Cost	Current treatments	Lowest possible

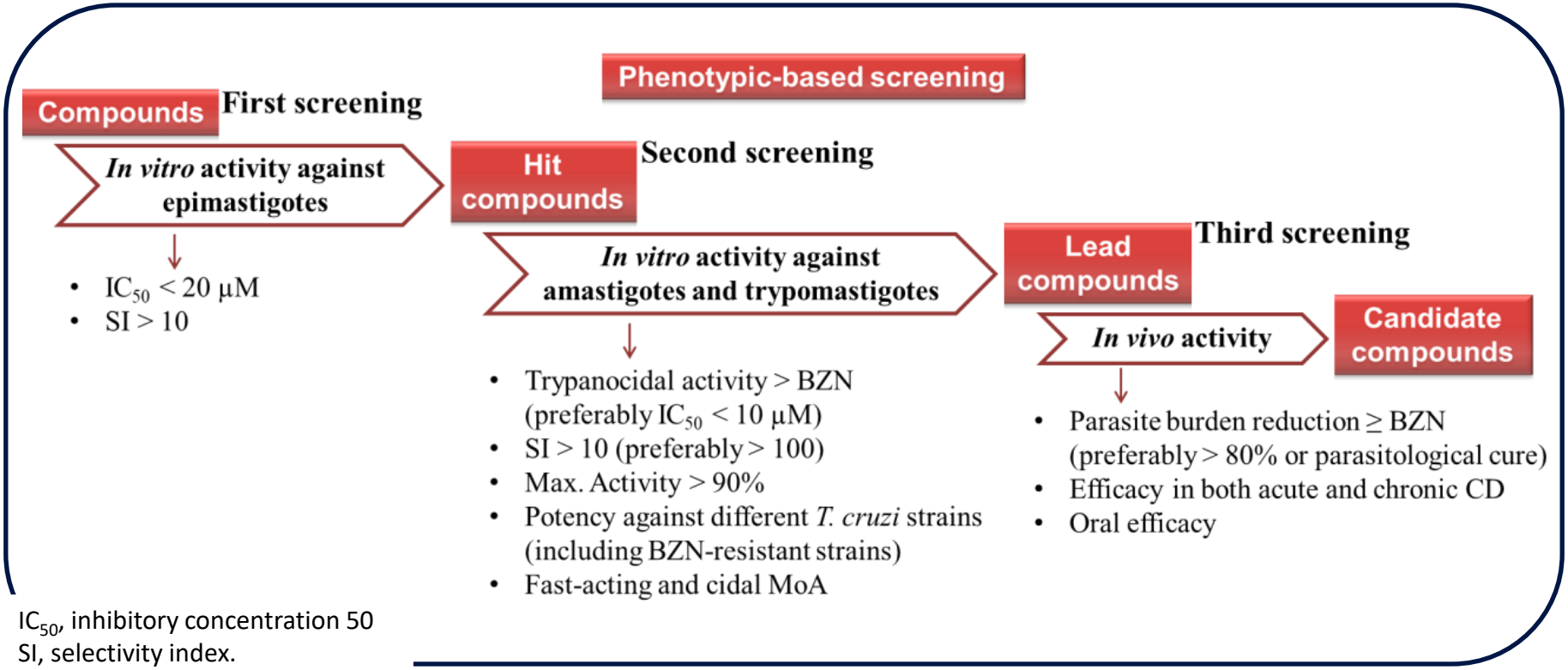
Objectives of this work

* As per WHO recommendation
** No genotoxicity is a condition only for NCEs
*** Need for parenteral treatment for severe disease

DNDi

Introduction

Screening strategy



IC_{50}, inhibitory concentration 50
SI, selectivity index.

Results and discussion

In vitro activity assays

Activity of benznidazole and compounds against the three developmental forms of *Trypanosoma cruzi*, and toxicity on mammalian Vero cells.

Compound	*T. cruzi* Arequipa strain			*T. cruzi* SN3 strain			*T. cruzi* Tulahuen strain			Toxicity
	E	A	T	E	A	T	E	A	T	VERO cells
BZN	16.9 ± 1.8	8.3 ± 0.7	12.4 ± 1.1	36.2 ± 2.4	16.6 ± 1.4	36.1 ± 3.1	19.7 ± 1.7	10.0 ± 0.8	15.1 ± 1.3	80.4± 7.1
2	2.9 ± 0.3	6.2 ± 0.6	4.8 ± 0.5	5.7 ± 0.5	nd	nd	15.8 ± 1.4	nd	nd	38.5 ± 4.1
9	18.1 ± 3.9	nd	nd	36.4 ± 3.4	nd	nd	10.0 ± 1.1	17.0 ± 1.5	12.2 ± 1.2	136.3 ± 14.8
16	9.0 ± 1.0	14.6 ± 1.5	10.3 ± 0.9	15.7 ± 1.2	nd	nd	19.0 ± 1.5	nd	nd	136.4 ± 15.8
19	18.4 ± 1.5	25.9 ± 2.8	27.4 ± 2.4	19.6 ± 2.2	31.5 ± 2.9	25.4 ± 2.7	12.8 ± 1.1	24.1 ± 2.1	22.8 ± 2.2	232.8 ± 27.2
21	6.4 ± 0.6	2.5 ± 0.3	1.6 ± 0.1	16.9 ± 1.4	11.3 ± 0.9	10.6 ± 0.9	11.4 ± 1.1	6.8 ± 0.7	7.8 ± 0.7	654.9 ± 51.9

E, epimastigotes; A, amastigotes; T, trypomastigotes

1-22, new synthesized *aza-scorpiand* macrocycles

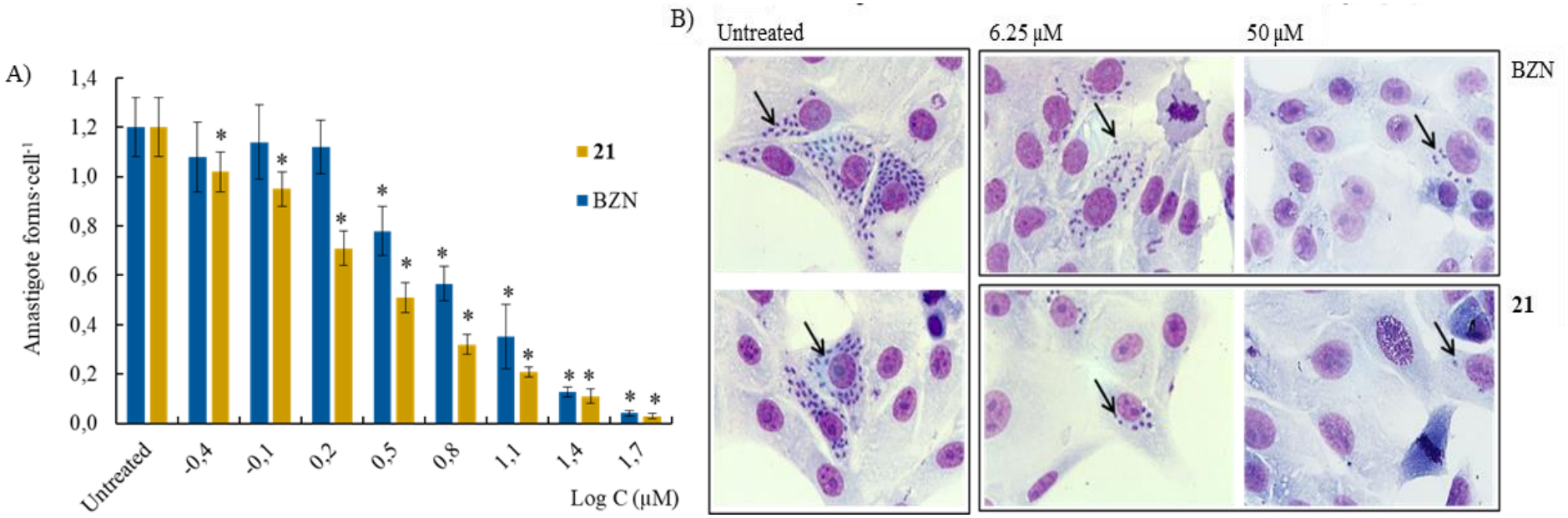
The compounds with the best activity profile are listed in this table.

The value is the mean of three separate determinations ± standard deviation. BZN, benznidazole; nd, not determined.

Activity/toxicity IC_{50} (μM)

Results and discussion

In vitro activity assays

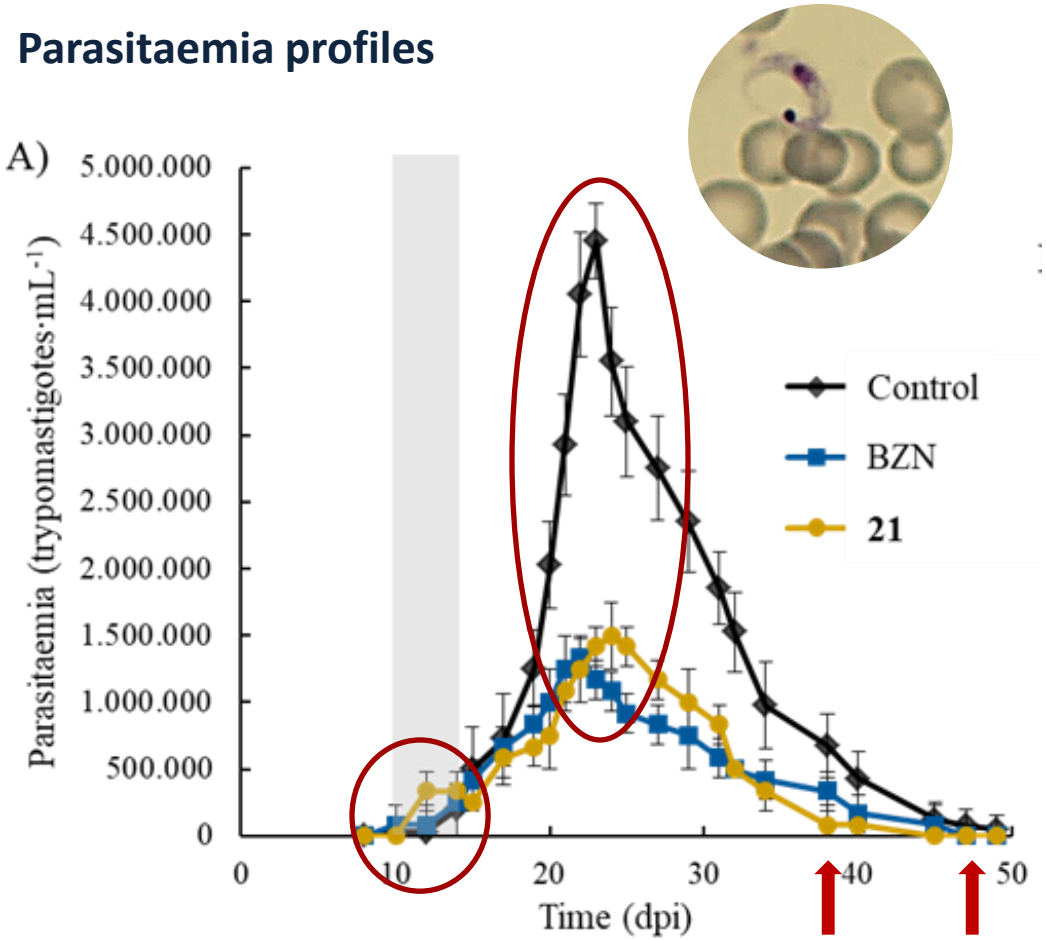


A) Number of amastigotes of *Trypanosoma cruzi* Arequipa strain per Vero cell exposed to benznidazole (BZN) and 21. The value is the mean of three separate determinations ± standard deviation. * Significant differences between control and treated parasites for α = 0.05. B) Representative images of Vero cells infected, treated and Giemsa stained. Arrows point to the amastigotes.

Results and discussion

In vivo activity assays

1/3 Parasitaemia profiles

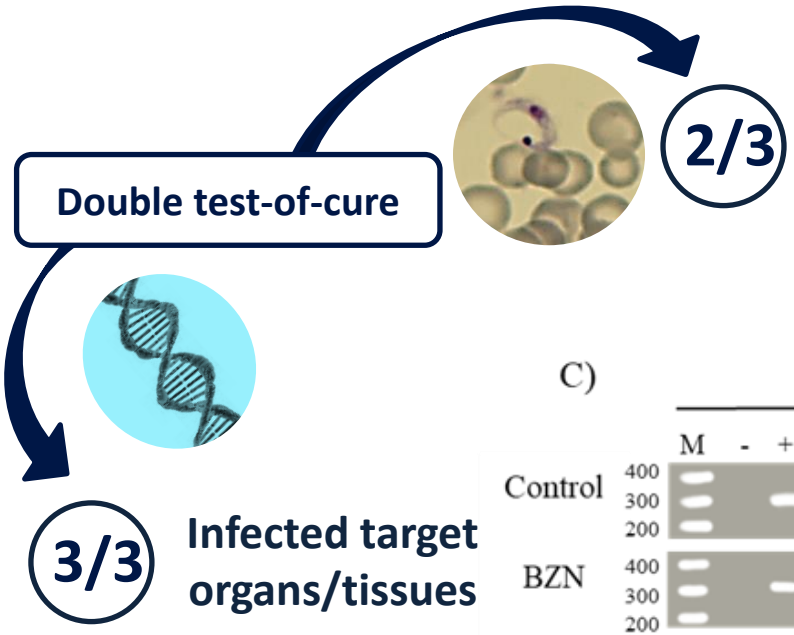


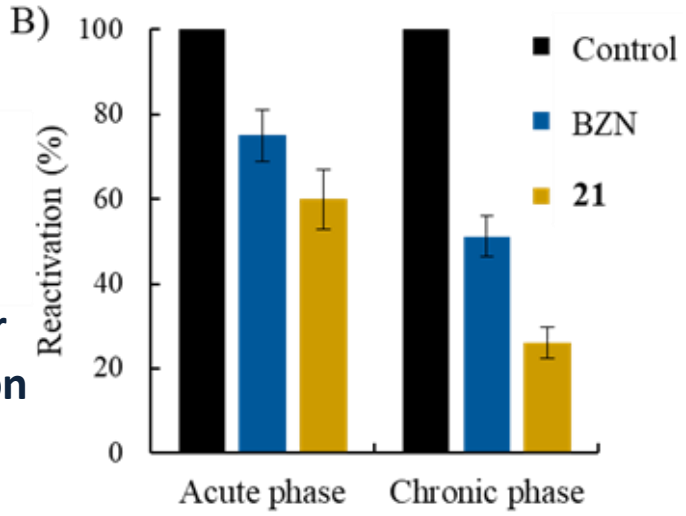
- Treatment period
- Parasitaemia peak
- Last day of parasitaemia

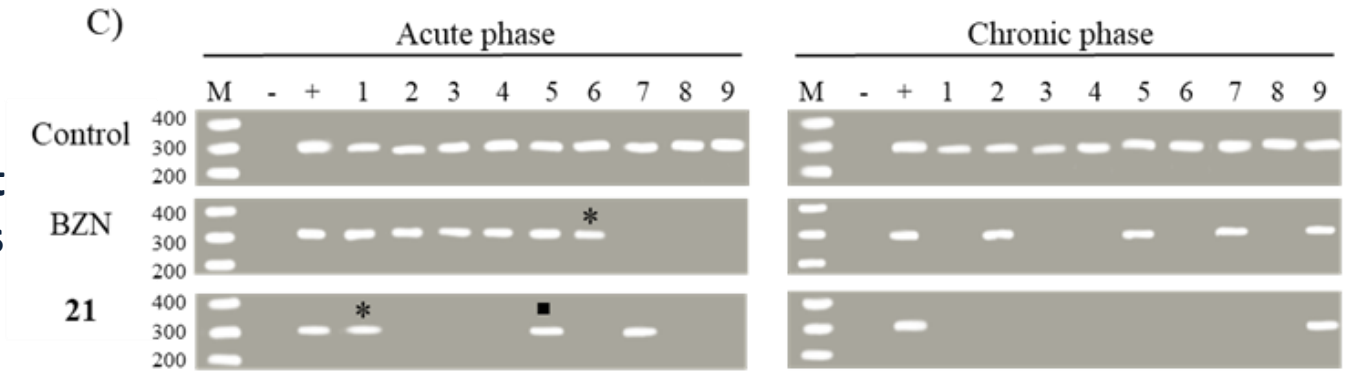
A) Parasitaemia profile during the acute infection. Treatment days are represented in grey. The value is the mean of three mice ± standard deviation. Significant differences between control and treated mice for α = 0.05.

Results and discussion

In vivo activity assays







B) Parasitaemia reactivation after immunosuppression of mice treated during the acute and chronic infection. The value is the mean of three mice ± standard deviation. Significant differences between control and treated mice for α = 0.05. C) PCR analysis of the nine target organs/tissues after treatment of mice during the acute and chronic infection. Lanes: M, base pair marker; -, PCR negative control; +, PCR positive control; 1-9, organs/tissues PCR: 1, adipose; 2, bone marrow; 3, brain; 4, oesophagus; 5, heart; 6, lung; 7, muscle; 8, spleen; 9, stomach. * 1/3 of the mice showed 300 bp PCR product on electrophoresis; ■ 2/3 of the mice showed 300 bp PCR product on electrophoresis. BZN, benznidazole.

Results and discussion

Compound 21 fulfills the *in vitro* requirements stablished for ideal drugs against Chagas disease:

- Higher trypanocidal activity and lower toxicity than BZN.
- Efficacy against a panel of different *T. cruzi* strains.
- Fast time to kill and cidal behaviour.

Compound 21 met many of the *in vivo* criteria stablished for ideal drugs against CD:

- Activity after oral administration.
- Activity in both the acute and chronic phases of Chagas disease.
- Higher efficacy than the reference drug benznidazole.

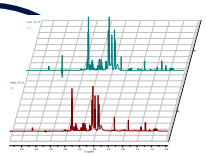
Toxic effects were also analysed by measuring heart, kidney and liver markers. The low toxicity exhibited by compound 21 allows it to be tested at higher doses (partial or total) in order to reach a sterile cure.

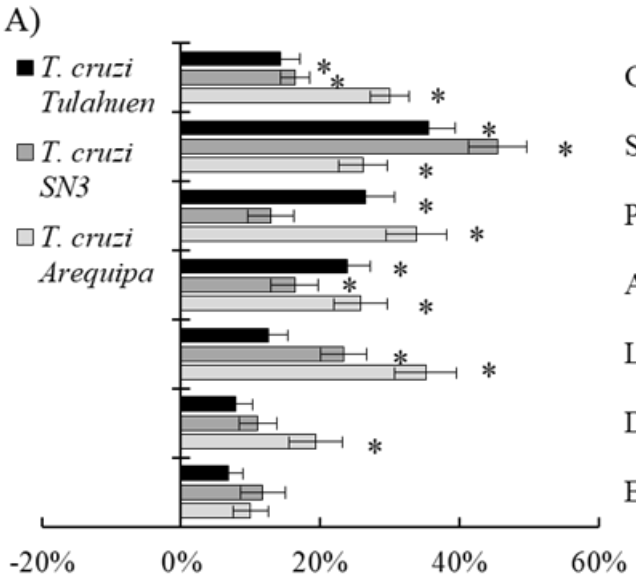
Results and discussion

MoA assays

1/2 Energetic metabolism tests

- **Metabolite excretion (catabolic alterations)**

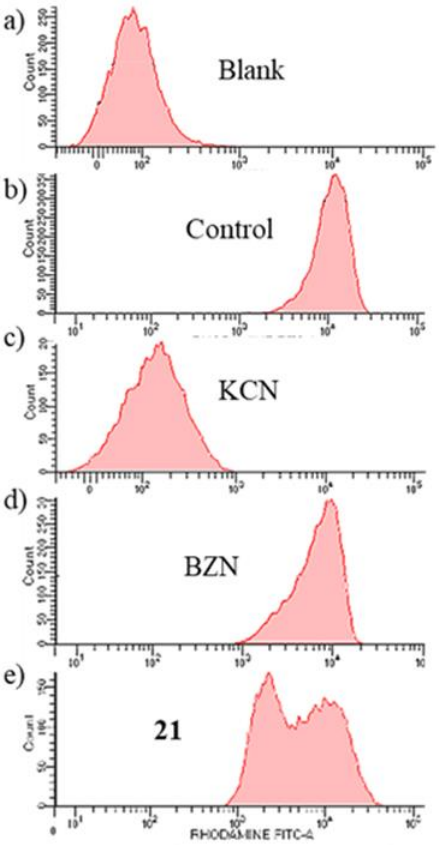




A) Variation of catabolites excreted by epimastigotes of *Trypanosoma cruzi* exposed to 21 at IC_{25} concentrations in comparison to control parasites. The value is the mean of three separate determinations ± standard deviation. * Significant differences between control and treated parasites for α = 0.05. B) Mitochondrial membrane potential from epimastigotes of *Trypanosoma cruzi* Arequipa strain exposed at IC_{25} concentrations: (a) blank, (b) control, (c) potassium cyanide (KCN), (d) BZN, (e) 21, (f) Inhibition on mitochondrial membrane potential with respect to control parasites. The value is the mean of three separate determinations ± standard deviation. Significant differences between control and treated parasites for α = 0.05.

- **Mitochondrial membrane potential (mitochondrial dysfunction)**

B)



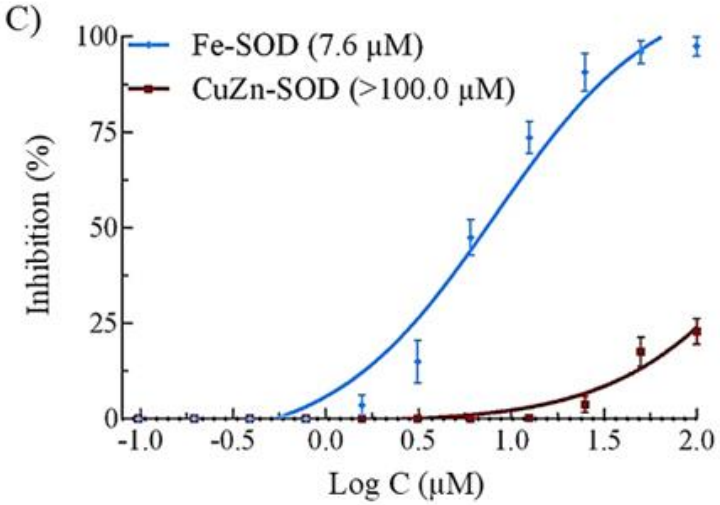
f)

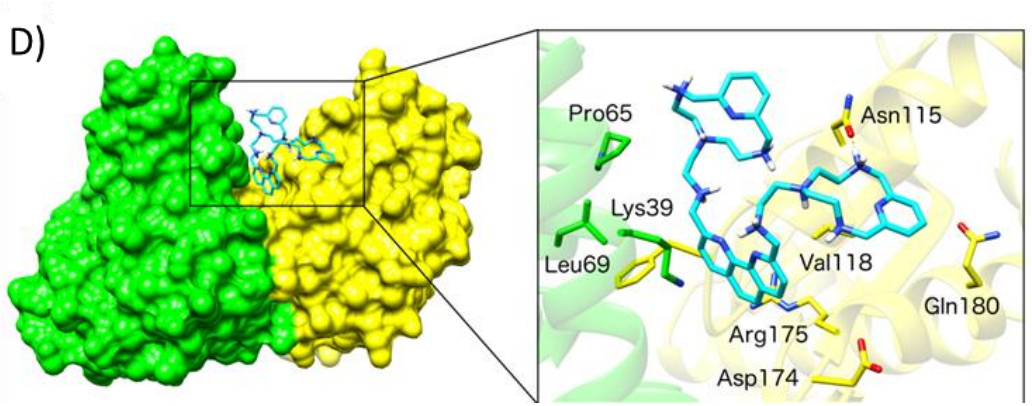
	Inhibition (%)
KCN	95.5 ± 6.1
BZN	35.4 ± 3.2
21	43.1 ± 3.6

Results and discussion

MoA assays

2/2 SOD enzyme inhibition tests



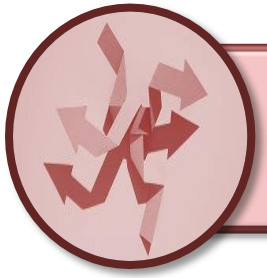


C) Inhibition of Trypanosoma cruzi Fe-SOD – activity 42.0 ± 3.8 U·mg-1 – and human erythrocytes CuZn-SOD – activity 47.3 ± 4.1 U·mg-1 – for 21. The value is the mean of three separate determinations ± standard deviation. In brackets: IC50 value. D) Proposed binding mode of compound 21 to the cytosolic Fe-SOD enzyme (PDB ID 2GPC) (PubMed: 19384994).

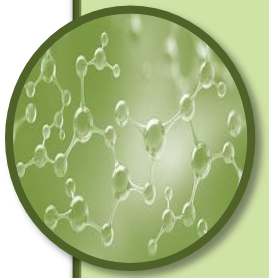
All this lead us to hypothesize that the cidal activity of compound 21 can be attributed to a mitochondria-dependent bioenergetic collapse and redox stress by Fe-SOD inhibition.

The possibility of multitarget activity should however not be rejected.

Conclusions



1. A **comprehensive and complete phenotypic-based strategy** has been developed in both *in vitro* and *in vivo* models to identify potential compounds against Chagas disease.



2. **Compound 21** has been identified as a **potential compound** that meets the most stringent *in vitro* and *in vivo* requirements, whose trypanocidal activity was even higher than that of the reference drug benznidazole.

3. Its **fast-acting and cidal activity profile** could be ascribed to a mitochondria-dependent bioenergetic collapse and redox stress by inhibition of the Fe-SOD enzyme.

4. Given that the ultimate goal is to achieve a **sterile parasitological cure**, new treatment schedules or even a synergistic compound **21**-BZN treatment should be exploited. This combination is likely to improve trypanocidal activity, increase the efficacy, and reduce toxicity.

Acknowledgments

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