

# State-of-the-Art in the Drug Discovery Pathway for Chagas Disease: A Framework for Drug Development and Target Validation

Juan Carlos Gabaldón-Figueira <sup>1</sup>, Nieves Martínez-Peinado<sup>1</sup>, Elisa Escabia<sup>1</sup>, Albert Ros-Lucas<sup>1,2</sup>, Eric Chatelain<sup>3</sup>, Ivan Scandale<sup>3</sup>, Joaquim Gascon <sup>1,2</sup>, María-Jesús Pinazo<sup>2,3</sup>, Julio Alonso-Padilla<sup>1,2</sup>

<sup>1</sup>Barcelona Institute for Global Health (ISGlobal), Hospital Clínic—University of Barcelona, Barcelona, Spain; <sup>2</sup>CIBER de Enfermedades Infecciosas, Instituto de Salud Carlos III (CIBERINFEC, ISCIII), Madrid, Spain; <sup>3</sup>Drugs for Neglected Diseases Initiative (DNDi), Geneva, Switzerland

Correspondence: Julio Alonso-Padilla, Barcelona Institute for Global Health (ISGlobal), Hospital Clínic—University of Barcelona. Carrer Rosselló 149, Barcelona, 08036, Spain, Tel +34 932275400 – ext. 4284, Email [julio.a.padilla@isglobal.org](mailto:julio.a.padilla@isglobal.org)

**Abstract:** Chagas disease is the most important protozoan infection in the Americas, and constitutes a significant public health concern throughout the world. Development of new medications against its etiologic agent, *Trypanosoma cruzi*, has been traditionally slow and difficult, lagging in comparison with diseases caused by other kinetoplastid parasites. Among the factors that explain this are the incompletely understood mechanisms of pathogenesis of *T. cruzi* infection and its complex set of interactions with the host in the chronic stage of the disease. These demand the performance of a variety of in vitro and in vivo assays as part of any drug development effort. In this review, we discuss recent breakthroughs in the understanding of the parasite's life cycle and their implications in the search for new chemotherapeutics. For this, we present a framework to guide drug discovery efforts against Chagas disease, considering state-of-the-art preclinical models and recently developed tools for the identification and validation of molecular targets.

**Keywords:** Chagas disease, *Trypanosoma cruzi*, drug development, screenings, target, animal models

## Introduction

Chagas disease, also known as American trypanosomiasis, is the most important neglected tropical disease (NTD) in the Americas. It is caused by the kinetoplastid parasite *Trypanosoma cruzi* and transmitted by reduviid bugs from the *Triatominae* subfamily. The disease affects between 6 and 7 million people, most of them in Latin America, leading to over 9,000 deaths per year and being responsible for the loss of over 280,000 disability-adjusted life years (DALYs).<sup>1</sup> Apart from vector transmission, the infection can be acquired orally from parasite-contaminated food or drinks, by blood or organ transplantation, and vertically, from infected mothers to their offspring. The three latter are of relevance in endemic and non-endemic regions, like North America, Europe, Australia, and Japan.<sup>2,3</sup>

While the disease has an initial, mostly asymptomatic acute stage, the majority of people are diagnosed during the chronic stage, when symptomatology appears, generally decades following infection. Symptomatology is characterized by potentially life-threatening damage to the cardiac and/or digestive tissues and estimated to occur in up to 40% of those chronically infected.<sup>2</sup> Common manifestations include changes in cardiac conductivity (typically, right bundle branch block or left anterior fascicular blocks; and less commonly sinus node dysfunction, or ventricular arrhythmias) and motility (left ventricular hypokinesis, reduction of the ejection fraction, and changes in diastolic function).<sup>4</sup> Digestive symptomatology is typically characterized by enlargement of the colon and esophagus, associated with enteric neuron damage in these tissues.<sup>5</sup> Mixed organ damage can also be observed in some patients developing cardiac and digestive manifestations. Central nervous system involvement is less common and may be secondary to immunosuppression,<sup>5</sup> or stroke associated with cardiac complications such as heart damage, heart failure, or arrhythmias.<sup>6</sup> Diagnosis during the

acute stage can be achieved by parasitological or molecular methods, while it relies mostly on serology during chronic stages.<sup>2,5</sup>

There are two anti-parasitic drugs currently available: benznidazole and nifurtimox. Both are nitroheterocyclic pro-drugs that are metabolized by parasite nitroreductases (TcNTR), which turn them into intermediate products, with anti-parasitic activity producing reactive oxygen species (ROS).<sup>7</sup> While highly effective when administered at the initial stages of the disease, like in the case of vertical infections, their efficacy reduces in the chronic stage. Moreover, both have frequent adverse effects linked to their long administration regimes, which hinder adherence to treatment in around 15–20% of treated patients.<sup>8,9</sup>

In recent years, there have been remarkable advances in drug research for Chagas disease, but progress on the development of new therapies against it is proving slow and difficult. Usually, the process to develop a new drug can take 10–15 years and have an average cost of \$1–2 billion for each newly approved drug.<sup>10–12</sup> As for other NTDs, there are few economic incentives for the research of new drugs for Chagas disease,<sup>13</sup> which has caused modest coordination of research and development efforts, leading to significant knowledge gaps on key aspects of the parasite biology, and ultimately hindering the identification of therapeutic breakthroughs. While the situation is improving, partly due to an increased engagement of pharmaceutical companies and the creation of public–private partnerships such as the Drugs for Neglected Diseases Initiative (DNDi), the drug discovery landscape of Chagas disease remains scarcely populated.<sup>14</sup>

This is further complicated by *T. cruzi*'s antigenic and genetic diversity. The parasite has a diploid genome of between 106 and 110 Mb, organized in a variable number of 19 to 40 chromosomes.<sup>15</sup> It is estimated to carry up to 22,000 coding sequences, including large families or surface proteins like trans-sialidases, mucins, and mucin-associated surface proteins (MASPs). These play a major role in parasite invasion and antigenicity, and are often subject to recombination events.<sup>16,17</sup> Furthermore, *T. cruzi* is genetically classified into seven major lineages known as discrete typing units (DTUs: TcI – TcVI and TcBat), characterized by different geographic distributions, and populated by isolates with diverse virulence and resistance to drugs.<sup>18,19</sup> In addition, the poorly understood mechanisms of pathogenesis at the molecular level and a heterogeneous set of in vitro and in vivo infection models do not contribute to accelerate progress. In this review, we present a comprehensive framework to guide drug development efforts, with a particular emphasis on phenotypic assays, target identification and validation, and available animal models.

## Biological Considerations for the Evaluation of Drugs with Activity Against *T. cruzi*

The life cycle of *T. cruzi* involves arthropod vectors and mammalian hosts. Metacyclic trypomastigotes (metacyclics) develop from replicative epimastigotes during their transit through the vector posterior digestive tract, and are ultimately expelled in their feces upon feeding from a warm-blooded animal. Metacyclics do not replicate, and infect a host by either accessing its bloodstream through superficial wounds or mucosal membranes, or via the consumption of contaminated food or drinks. They quickly invade the cytoplasm of a host cell and differentiate into amastigotes, the intracellular replicative life stage of the parasite in mammals.<sup>2</sup> Upon successive binary replication cycles, amastigotes transform into motile, non-replicative trypomastigotes, which egress from the host cell to the interstice and bloodstream. They can invade cardiac cells and the nervous ganglia below the digestive mucosa, where chronic infection can persist for decades and eventually lead to severe tissue disruptions.<sup>19</sup> Despite its predilection for these sites, *T. cruzi* can invade a wide variety of host cells and tissues, making it necessary for any potential treatment to have a high bioavailability across different anatomic settings.<sup>14</sup> Blood circulating trypomastigotes can be taken up by the vectors, becoming epimastigotes in the initial segments of the digestive tract, completing the infection cycle.<sup>2</sup> Remarkably, each life stage of the parasite is phenotypically singular, presenting different susceptibility to drugs, and highlighting the major relevance of using mammalian infective forms in drug discovery biological assays.

From a metabolic point of view, the behavior of the parasite during the chronic stage of the infection is also diverse, which has direct implications for its susceptibility to drugs. For instance, it has been described that a small subset of metabolically quiescent amastigotes develops spontaneously in both in vitro and in vivo models. These “persister” or “quiescent” parasites replicate very slowly and are less susceptible to nitroheterocyclic drugs.<sup>20,21</sup> Persistent forms can become metabolically active and replicate again in later stages of the infection; a phenomenon that could potentially

explain the failure of existing treatments to permanently clear parasitemia in some patients, and thus the need for long-lasting or intermittent treatment regimens.<sup>22</sup>

## Drug Screening Strategies Used to Identify Potentially Useful Compounds

The development of new compounds for the treatment of Chagas disease is guided by its target product profile (TPP). Different TPPs have been reviewed elsewhere,<sup>23</sup> but they share key characteristics. An acceptable therapy should be able to (i) benefit patients in the chronic state of infection; (ii) target the most widely distributed parasite strains; (iii) allow shorter treatment schemes; and (iv) be safer and at least as effective as currently available drugs. Additionally, it should ideally be soluble enough to allow oral dosing, lack clinically significant interactions with other medications, and be safe during pregnancy.<sup>23,24</sup>

The first step in the development pathway is the identification of hit compounds, whose suitability is further evaluated considering aspects outlined in the TPP. Hit compounds can be identified through either phenotypic or target-based approaches, which ultimately reach a common validation pathway before entering in vivo pre-clinical evaluation (Figure 1).

In target-based screening, molecular biology techniques are used to identify virulence factors likely to be attractive drug targets. For Chagas disease, this approach has been traditionally limited by knowledge gaps that have only recently begun to be addressed, while it has proven useful to identify and de-prioritize targets likely to fail in clinical trials.<sup>25</sup> Despite this, a handful of potentially interesting targets have been identified. These have been reviewed elsewhere,<sup>26</sup> and include parasite enzymes such as trypanothione-reductase,<sup>27,28</sup> enolase,<sup>29</sup> cruzain,<sup>30</sup> ribose-5-phosphate isomerase (RPI),<sup>31</sup> isocitrate dehydrogenase (IDH2),<sup>32</sup> dihydrofolate reductase–thymidylate synthase (DHFR-TS),<sup>33</sup> pteridine reductase,<sup>34</sup> farnesyl diphosphate synthase (FPPS),<sup>35</sup> and the parasite sirtuin family.<sup>36</sup> More recently, compounds that interfere with the parasite proteasome and mRNA processing machinery have been thoroughly evaluated and gained considerable traction as attractive drug candidates.<sup>14</sup> The strategies used to identify and validate these targets are further discussed in the target validation section of this review.

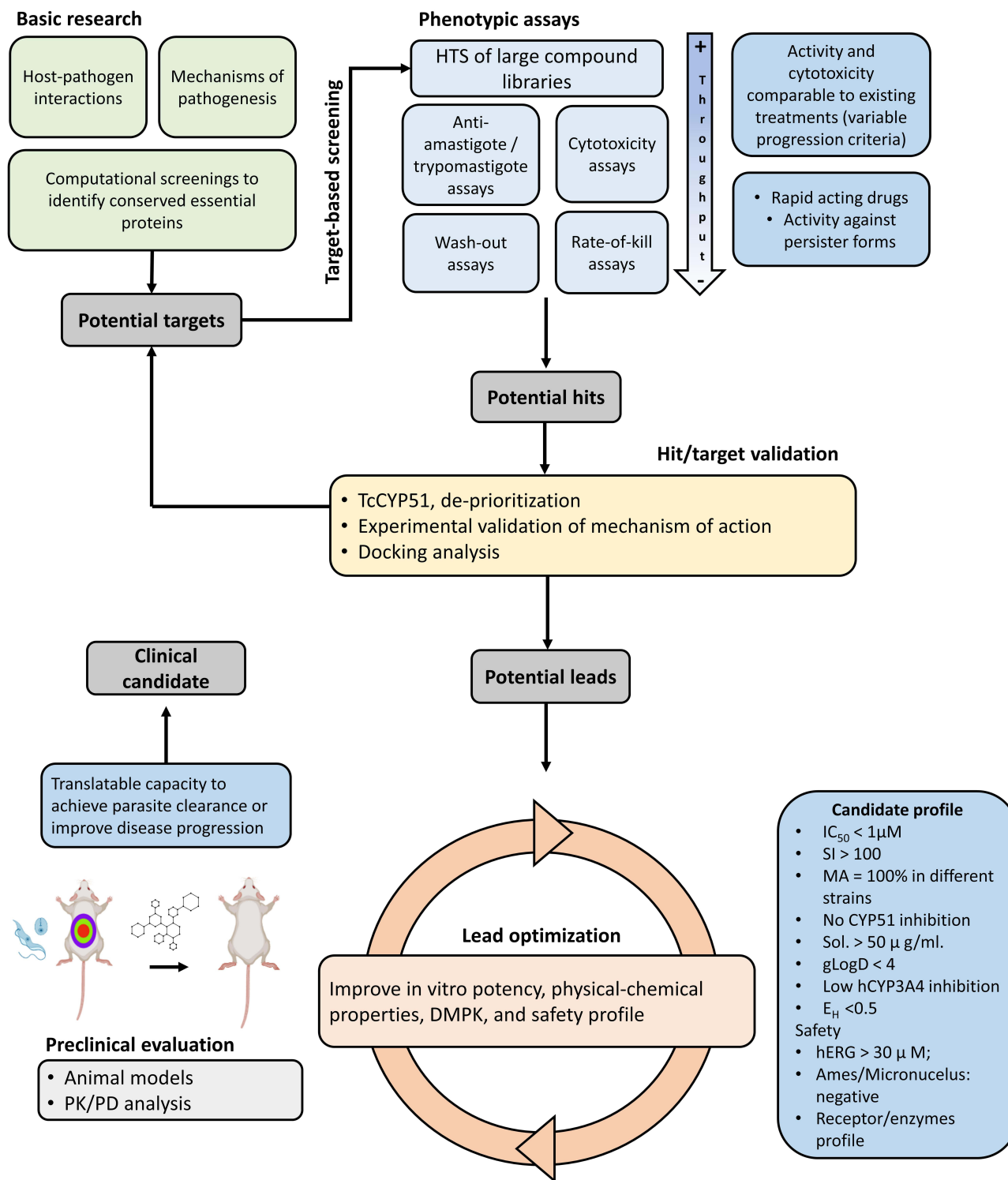
In comparison, the phenotypic screening is a serendipitous approach that uses cell-based assays to quantify the effect of a compound on a desired cell line. Phenotypic screenings are followed by the identification and thorough validation of the targets of hit compounds. Notably, the relative scarcity of well-characterized targets for Chagas disease has made of phenotypic screenings the method of choice for identifying new potential therapies.

## The Phenotypic Screening Pathway

A phenotypic drug development cascade may start with a high-throughput screening (HTS) campaign using a single concentration of the compounds forming part of a large chemical library whose anti-*T. cruzi* activity is evaluated on a primary anti-parasitic assay.<sup>37–40</sup> The initial hits resulting from this screening are studied again with the same assay, using a dose–response design to calculate the half maximal inhibitory concentrations (IC<sub>50</sub>), which then allow comparisons between hits and included reference drugs, ie benznidazole or nifurtimox. Noteworthy, this approach relies on the availability of assays with a certain throughput, so that a high number of compounds can be analyzed simultaneously.

To do so, these assays often rely on genetically engineered parasites, modified to express a reporter's activity that facilitates their identification and quantification using spectrophotometric readouts.<sup>37,41</sup> Due to its genetic stability and trustworthy performance, together with its simple culture conditions and high production rates, several drug discovery anti-*T. cruzi* assays have been based on a Tulahuen parasite strain (TcVI) expressing the *E. coli*  $\beta$ -galactosidase gene.<sup>42</sup> An anti-parasitic assay with these parasites and 3T3 mouse embryo fibroblasts was the basis for the development of subsequent HTS-suited assays using different kinds of mammalian host cells.<sup>37,43–46</sup> Other techniques allowing a medium to high-throughput detection and quantification of parasite growth rely on the detection of ATP production,<sup>47</sup> redox capacity,<sup>48</sup> or the use of fluorescent parasites.<sup>49</sup>

More recently, microscopy-based high-content screening (HCS) approaches have surged as an alternative to obtain quantitative data on the proportion of parasitized cells, or the number of parasites per infected cell. They rely on fluorescent microscopy and image analysis algorithms to identify parasites inside individual cells, allowing the simultaneous processing of several conditions in the host and *T. cruzi* cells, and a medium throughput of compounds.<sup>38,39,50</sup> Remarkably, beyond quantifying parasite growth, these assays can also provide data on the nature and timing of the observed anti-parasitic effects and on the effect of compounds on certain phenotypic characteristics of parasites and cells, including shape, size, presence of vacuoles, or organization of the kinetoplastid



**Figure 1** General framework for the discovery and validation of novel compounds for the treatment of Chagas disease.

**Abbreviations:** CYP51, 14-α demethylase; DMPK, Drug Metabolism and Pharmacokinetics; gLogD, Distribution coefficient; hCYP3A4, Human cytochrome P450 3A4; hERG, Human ether a-go-go gene; HTS, High throughput screening; IC<sub>50</sub>, Half maximal inhibitory concentration; PK/PD, Pharmacokinetics and pharmacodynamics; SI, Selectivity Index; MA, Maximal inhibitory activity, Sol, Solubility at physiological pH.

DNA (kDNA) and cytoskeleton.<sup>51</sup> Additionally, HCS is less likely to be negatively affected by interactions between compounds and reporter enzymes and substrates, or by the effect that colored compounds might have on spectrophotometric readings.<sup>51</sup>

Regardless of the method used to quantify anti-parasitic effects, it is essential to guarantee that these are specific and not derived from induced host cell damage or death. This can be achieved by also evaluating the cytotoxicity of studied compounds on the same host cell line used in antiparasitic assays and calculating their selectivity index (SI), which is defined as the ratio between its half maximal toxic concentration to the host cells ( $TC_{50}$ ) and its  $IC_{50}$ .<sup>45</sup> Importantly, the type of host cell used in anti-parasitic assays must also be taken into consideration when comparing results from different experiments. Parasite infection rates and replication times change from one host cell line to another, modifying the susceptibility observed against certain compounds.<sup>52</sup>

## Specific Complementary Assays

While the evaluation of anti-parasitic effects is easier in epimastigotes and can guide initial drug discovery efforts, the activity of compounds on amastigotes and trypomastigotes should be prioritized, given their central role in the pathogenesis of the disease.

Anti-amastigote assays reflect the potency of compounds on the parasite form typically associated with chronic inflammation and damage and are thus the cornerstone of Chagas disease drug discovery pathways.<sup>37,47,49,53</sup> Standardized anti-trypomastigote assays also exist.<sup>47,54,55</sup> They are important too, given the resistance of this life-stage to certain compounds and the apparent link to their poor-performance in later stages of in vivo evaluation.<sup>47</sup>

The capacity of compounds to kill persister, non-replicating forms should also be studied, given their intrinsic resistance to currently available drugs.<sup>20,22</sup> This can be evaluated, for instance, using wash-out assays, in which infected cultures are incubated for variable periods of time with a compound, which is washed-out after parasites are no longer detectable. Cultures are then maintained for several weeks and monitored daily to record the re-appearance, or not, of trypomastigotes, which would have differentiated from surviving quiescent forms.<sup>14,47</sup> Ideally, host cells should be gamma-irradiated prior to these assays to prevent their overgrowth, which can hamper the appropriate quantification of parasite growth.<sup>20,56</sup>

HCS assays designed to determine whether the parasites simply prevent parasite replication (static effect) or induce a reduction in the number of amastigotes (cidal effect), as well as the time required to produce such effect (rate-of-kill), have also been described and represent increasingly important components of drug discovery cascades.<sup>56–58</sup>

In addition to addressing the compounds toxicity on the host cells supporting parasite replication, other cell toxicity assays are usually included in the drug discovery cascade. A common model uses the human hepatocarcinoma cell line HepG2, since damage to this line suggests liver toxicity.<sup>53,59,60</sup>

Importantly, given the wide geographic-phenotypic diversity of *T. cruzi*, and the fact that any developed drug would be expected to work throughout all endemic regions, complementary in vitro assays should also include evaluating the compounds' activity against different strains and DTUs.<sup>61,62</sup> This seems to be particularly important if potentially slow-acting compounds are being evaluated, as inter-strain differences in susceptibility appear to be more evident following 72 hr of compound exposure.<sup>63</sup>

Similarly, the de-prioritization of drugs that act on the parasite 14- $\alpha$ -demethylase (TcCYP51), an enzyme involved in the synthesis of sterols for the parasite membrane,<sup>56</sup> is also becoming increasingly common. The latter is justified on the failure of azoles posaconazole, ravuconazole, and fosravuconazole in clinical trials.<sup>54,64–66</sup> All of these compounds target TcCYP51 and their disappointing clinical results make the case for the importance of understanding parasite biology in detail. Although TcCYP51 inhibitors are commonly identified as promising candidates in screenings, it is now known that endogenous sterol depletion is necessary before cell death occurs, making the activity of these compounds slow and dependent on parasite replication, explaining their lack of anti-parasitic activity on non-replicative trypomastigotes and slow-dividing parasite strains.<sup>47,56</sup> These findings have prompted the research community to discard any compounds that act by inhibiting that pathway, regardless of their performance in vitro,<sup>56,67</sup> prioritizing fast-acting compounds instead.

Recently, the incorporation of 3D culture systems in drug discovery pathways has gained traction, as these mimic gene transcription and expression more closely in in vivo environments, increasing their translatability. Although 3D cultures have been used to study *T. cruzi* host–parasite interactions and mechanisms of pathogenesis,<sup>68,69</sup> its use in drug discovery remains scarce and mostly restricted to the use of Vero cells and murine primary cardiomyocytes.<sup>70,71</sup>

It is worth to highlight that as the complexity of assays increases, their throughput decreases, so fewer compounds can move forward in the cascade. While there are no universally accepted pathway progression criteria, an  $IC_{50} < 1\mu\text{M}$ , or at least within  $<10X$  that observed for benznidazole, a  $SI > 10$  (ideally  $>100$ ), and a  $TC_{50} > 25\mu\text{M}$  in HepG2 cells have been used in the past.<sup>23,37,40,53,61,72,73</sup> A summary of other commonly used criteria is presented in Table 1.

## Computer-Assisted Selection of Proteins for Target-Based Screening

An ideal drug target should be essential in pathologically relevant parasite life stages; sufficiently different from related mammalian proteins to be selective; and druggable, ie presenting well-defined domains expected to bind certain types of compounds.<sup>74</sup> Large-scale evaluation of these characteristics can greatly reduce the number of potential candidates,<sup>75,76</sup> prioritizing proteins that are conserved across different DTUs, but not in mammals,<sup>77</sup> and stably expressed in amastigote and trypomastigote stages. Drug targets that are evolutionarily conserved among different parasite strains but differ from the human proteome are expected to have wider clinical efficacy, while minimizing off-target effects. Indeed, some of the most-studied targets in the development of treatments for Chagas disease, such as TcNTR, TcCYP51, or trypanothione-reductase, all lack orthologues in humans.<sup>78,79</sup>

Other metabolic pathways that differ from the host, such as the purine salvage and folate synthesis pathways, also represent potentially attractive targets.<sup>78–80</sup> However, a target should not be discarded solely based on the presence of human orthologues, because highly selective inhibitors of the parasite protein could still be synthesized. Such is the case with compounds inhibiting the cleavage and polyadenylation specificity factor subunit 3 (CPSF3), a protein present in humans but sufficiently different in kinetoplastids so as to be considered a promising target for the development of new treatments against these pathogens.<sup>14,81,82</sup> The presence of orthologues in other trypanosomatids can also be incorporated into search strategies. For example, using phenotypic studies against *Trypanosoma brucei* or *Leishmania spp.*, to identify potential targets in *T. cruzi*.

While computational target selection still has significant caveats, identifying proteins that comply with the aforementioned criteria is expected to greatly reduce development times and facilitate the design of target-based screenings.<sup>83</sup> Furthermore, most of these analyses can be performed quickly using freely available bioinformatic tools, such as those hosted at TriTrypDB (<https://tritrypdb.org/tritrypdb/app>), part of the VeuPathDB suite, which includes extensive genomic, proteomic, and metabolomic data, as well as information on specific gene expression levels and their involvement in

**Table 1** Commonly Used Criteria to Determine the Progression of a Compound in Phenotypic Development Pathways

	Chatelain, Ioset 2017 <sup>61</sup>	Martin-Escolano, 2020 <sup>23</sup>	Kratz et al, 2022 <sup>72</sup>	Neitz 2015 <sup>73</sup>	Germain 2011 <sup>40</sup>
$IC_{50}$	$< 5\mu\text{M}$	$< 5\mu\text{M}$	$< 5\mu\text{M}$ ideally $< 1\mu\text{M}$ )	$< 1\mu\text{M}$	$< 1\mu\text{M}$
Max. activity	$> 90\%$	$> 90\%$	$> 95\%$	-	$>55\%$
SI	$> 10$ (3T3 cells)	$> 10$ (ideally $>100$ )	$> 10$ ( $> 50$ )	$>25$	$>100$
MoA	Fast-acting	Fast-acting, cidal	Fast-acting, cidal	-	-
TcCYP51 activity	$> 10\mu\text{M}$	-	Removal of CYP51 inhibitors	$>5\mu\text{M}$	-
Solubility	$> 50\mu\text{g/mL}$			$>50\mu\text{M}^a$	$>100\mu\text{M}$
CYP 3A4	$> 10\mu\text{M}$			$>25\mu\text{M}$	-
Suggested parasite genotypes to be targeted	All (priority to TcI, TcII, TcV, TcVI)	All	Stable activity among strains	Stable activity among strains (evaluated in TcI, TcII, and TcVI)	Only evaluated in DTU VI

**Notes:** Data from Chatelain, Ioset 2017,<sup>61</sup> Martín-Escolano, 2020,<sup>23</sup> Kratz et al 2022,<sup>72</sup> Neitz 2015,<sup>73</sup> and Germain 2011.<sup>40</sup> <sup>a</sup>Saturation in aqueous solution at pH 6.8.

**Abbreviations:**  $IC_{50}$ : Half maximal inhibitory concentration, MoA: Mechanism of action, SI: Selectivity Index, CYP51: 14- $\alpha$  demethylase.

different signaling pathways.<sup>84,85</sup> Apart from being essential in refining the number of targets of potential interest, bioinformatic methods are also necessary to study the targets' interactions with ligands of therapeutic interest. This is a key first step in understanding a drug's mechanism of action, and highly advisable before progressing any compound into in vivo pre-clinical evaluation.

## Evaluation of the Tridimensional Structure of Targets for in silico Experiments

The morphologic study of potential targets in *T. cruzi*, and therefore, the full pharmacodynamic characterization of compounds likely to bind them has been hindered by the lack of detailed protein models in this species. The first genome sequence of *T. cruzi*, produced almost 20 years ago, was obtained from the CL Brener strain, a hybrid, polymorphic strain belonging to TcVI that remains the reference for the entire species.<sup>86</sup> At present, there are at least 30 genomes from different *T. cruzi* strains available online, although most remain incomplete and poorly annotated. Only recently, another, less polymorphic TcI strain (Sylvio X10) has been sequenced and described in detail.<sup>17,87</sup>

The lack of properly annotated sequences, along with the high cost and complexity of crystallographic methods, still limits the number of protein structures available for in silico evaluation. When available, these models are mostly based on CL Brener genes, reducing their generalizability to other DTUs. A partial solution to this issue is the use of computer-generated protein structures. In this regard, AlphaFold, an artificial intelligence (AI) system developed by DeepMind (<https://www.deepmind.com/research/highlighted-research/alphafold>) represents a valuable tool.<sup>88–90</sup> Using deep learning algorithms, the system is capable of predicting accurate protein structures based entirely on their amino acid sequences.<sup>91</sup> Moreover, over 200 million predicted structures are already available in the AlphaFold Protein Structure Database (<https://alphafold.ebi.ac.uk/>), a virtual repository created in a joint partnership between DeepMind and the European Molecular Biology Laboratory Bioinformatics Institute (EMBL-EBI).<sup>89</sup> AlphaFold models of targets of interest can be easily incorporated into drug discovery pipelines by using them, for instance, in in silico docking experiments, where the binding affinity between small molecules and potential targets is predicted, or in inverse virtual screening experiments to determine the mode of action of prospective drugs.<sup>75</sup>

Nevertheless, before using these models, it is important to evaluate their quality. One option is evaluating the model's per-residue predicted local distance difference test (pLDDT) score, which scales from 0 to 100. Regions with a pLDDT > 90 are comparable to experimentally generated crystal structures, with amino acid side chains usually set in the correct orientation. This is crucial when modeling active sites in which the orientation of residues is of paramount importance. In addition, the relative positions of a protein's domains are measured by their predicted aligned error (PAE). A high PAE between two residues indicates that their relative positions are not certain, and their inclusion in docking experiments should be avoided. In particular, considering that a single side chain oriented in the wrong direction can disrupt the results of docking simulations.<sup>89</sup> This is a major limitation for the evaluation of targets based on their computationally predicted structures.<sup>92</sup>

Since AlphaFold relies on multiple sequence alignments, the accuracy of the predictions is also hampered by alignments with low depth. In the context of NTDs, and in particular of Chagas disease, the limited extent of sequence annotation across different *T. cruzi* strains, and the fact that trypanosomatids are evolutionarily distant from other better-studied organisms entails that many *T. cruzi* entries in the AlphaFold DB show a comparatively low prediction score.<sup>93</sup> In addition, the current release of AlphaFold DB does not contain multimeric structures, greatly hindering the incorporation of some targets into computer-assisted drug discovery.

Even though new iterations of AlphaFold are expected to address some of these issues,<sup>89,94,95</sup> the experimental validation of any computationally selected target is still essential before its progression down the development pathway.

## Novel Strategies for Experimental Target Validation

Independently of the type of screening strategy used, the appropriate validation of drug targets requires experimental evidence of their predicted role in the parasite. Until recently, the lack of a simple tool for genetic edition in *T. cruzi* severely limited the capacity to do this. Unlike other trypanosomatid parasites, *T. cruzi* and some *Leishmania* species lack the molecular apparatus needed to carry out RNA interference (RNAi) gene knock-out (KO) experiments, widely used to validate the role of potential targets in *T. brucei*.<sup>96</sup> The recent development of different CRISPR-cas9-based editing

systems in *T. cruzi* now offers a quick, relatively simple, and highly versatile way to experimentally validate the role of certain proteins as drug targets in the parasite.<sup>97–100</sup> This approach has been used to validate AGC Kinase 1 (AEK1), previously identified as essential in other kinetoplastid parasites using RNAi.<sup>101,102</sup> Using a CRISPR-cas9 system, it was demonstrated that the single KO of one of the two copies of the AEK1 gene present in *T. cruzi* Y strain (TcII) caused an abnormal development of the kinetoplast and the flagella of transfected parasites and that its blockage impaired cytokinesis and cell cycle progression, confirming the role of AEK1 as an attractive drug target.<sup>103</sup>

A similar approach was used to confirm the role of CPSF3, a protein involved in messenger RNA processing, as the target of AN15368, a new benzoxaborole analog with anti-parasitic activity and apparent low toxicity, and to identify its nature as a prodrug metabolized by a parasite serine carboxypeptidase.<sup>82</sup> The same method was used to identify the  $\beta$ 4/ $\beta$ 5 interface in the parasite's proteasome as the target of a new arylsulfonamide previously shown to present in vitro anti-parasitic activity,<sup>37,104</sup> confirming results from previous studies that suggested that inhibition of proteasome activity is an exploitable pathway in the treatment of kinetoplastid infections.<sup>105,106</sup> At least five compounds targeting CPSF3 or the kinetoplastid proteasome are currently in development pipelines for the identification of new treatments against kinetoplastid parasites, and three of these are potentially useful in the treatment of Chagas disease (see corresponding section below).<sup>14,107</sup>

Apart from revealing potentially useful pharmacodynamic (PD) information and possible resistance mechanisms, validating the targets of new compounds is also essential to identify those unlikely to perform well in clinical trials, and discard them early in the development process, as is now regularly done with TcCPY51 inhibitors.<sup>14</sup>

## Lead Generation and Optimization

Hit compounds that show potent specific anti-parasitic properties and certain desired metabolic characteristics are considered leads.<sup>24</sup> Lead compounds must have a good PD profile, characterized by a high in vitro activity (killing 100% of parasites), high potency (ideally in the nanomolar range), a high selectivity (SI > 100), a fast mechanism of action, and activity against different *T. cruzi* strains. Additionally, leads must achieve sterile cure in animal models of chronic infection, while presenting an adequate pharmacokinetic (PK) and safety profile.<sup>24</sup>

Notably, some of the most pharmacologically relevant physical-chemical characteristics of compounds can be preliminarily evaluated using chemoinformatic servers and in vitro metabolism models, thus providing a rapid assessment of a drug's absorption, distribution, metabolism, and excretion (ADME) profile.<sup>108</sup> Lead candidates should present a high kinetic solubility at physiologically relevant pH (>50  $\mu$ g/mL), not be unduly hydrophobic at neutral pH ( $\text{logD}_{7.4} < 4$ ), show a low inhibition of human cytochrome P450 (CYP3A4) ( $\text{IC}_{50} > 10 \mu\text{M}$ ), and a low hepatic extraction ratio ( $E_H < 0.5$ ).<sup>24</sup>

Furthermore, the potential for off-target effects should be addressed through standard toxicology screening assays to study interactions with key enzymes and receptors, such as the human ether a-go-go related gene ion channel (hERG or KCNH2), which predicts cardiac toxicity. Similarly, candidate drugs should not be mutagenic (as determined by the Ames or micronucleus assays) and should not cause any significant adverse effects on preliminary animal experiments.<sup>14,24</sup> Analogs of lead compounds can then be synthesized, including minor modifications expected to improve their efficacy, or minimize the probability of off-target effects, a process known as hit-to-lead optimization, generally the last step prior to a proof-of-concept evaluation in an animal model of the disease.<sup>14,24</sup>

## Animal Models for in vivo Evaluation

The complex biology of *T. cruzi* has also hindered the evaluation of new drugs in animal models. The fact that these models had been mostly used to determine the pathogenesis of the different manifestations of chronic disease led to significant diversity, as the parasite strains, animal species, and infection routes used in each one depended on the specific aspects of the disease to be studied.<sup>109–111</sup> Such experimental and physiological diversity makes the selection of cure criteria, and the interpretation of results, challenging. Furthermore, a better understanding of the dynamics of Chagas disease has forced the research community to use new endpoints accounting for tissue-specific parasite loads to define therapeutic cure, adding extra complexity to the development and selection of adequate models.<sup>112</sup> A variety of animal



models of *T. cruzi* infection have been used to evaluate the performance of promising compounds.<sup>109,112</sup> Among those, murine, canine, and primate models are furtherly addressed here, given their importance.

## Murine Models

As it happens for other diseases, murine models are the most extensively used in the study of Chagas disease. Until recently, the evaluation of drugs with trypanocide activity had primarily relied on acute models of infection, given their short duration and the high parasitemia observed in this stage, which provides a greater window to detect any trypanocide effect.<sup>113</sup> In these models, mice are treated with the test drug during the peak of parasitemia, which usually lasts for 2–4 weeks, depending on the *T. cruzi* strain used. Traditionally, death (or mortality prevention following drug administration) was the main endpoint assessed; although this has changed recently, due to ethical reasons and the development of new experimental methods, as described in this section.

Chronic models, on the other hand, mimic the indeterminate stage of the disease, when parasitemia is low. In this case, mice are treated and monitored over a variable period, typically from 1 week to 2 months. The main efficacy outcome in these studies is the capacity of the compound to achieve sterile cure (parasite clearance in all tissues). Animals are then immunosuppressed to induce the relapse of any remaining parasites and observe a possible reactivation of the infection.<sup>114</sup> Distinct combinations of mouse and parasite strains can be used to reflect different aspects of the pathogenesis of human infection. BALB/c, CH3, or C57BL6 mice infected with different parasite strains, including Sylvio X10/4 (TcI), CL Brener (TcVI), Tulahuén (TcVI), Y (TcII), and Brazil (TcI), have been shown to develop cardiac disease with the presence of lymphoid infiltration, fibrosis, and electrocardiographic alterations, similar to those observed in humans.<sup>115–118</sup> These findings were reported following both intraperitoneal infection using blood-derived trypomastigotes and oral ingestion of metacyclic trypomastigotes.<sup>108</sup>

The incorporation of parasites genetically modified to express bioluminescent and fluorescent markers has allowed a detailed monitoring of the dynamics of infection, identifying the persistence of *T. cruzi* in immune-privileged tissues or following-up the elimination of parasites in other anatomical settings.<sup>97,119</sup> These bioluminescence imaging (BLI) models are based on the incorporation of a reporter luciferase gene in the parasites, thereby detecting the light emitted after being exposed to luciferin, the enzyme's substrate.<sup>97,119</sup> BLI models also allow longitudinal, non-invasive evaluation of the drugs' efficacy to reduce or clear the parasite burden. This, in turn, involves the use of fewer animals, complying with current ethical recommendations for animal testing.<sup>120</sup> Bioluminescence and fluorescence can also be observed *ex vivo*, following necropsy, facilitating the identification of residual parasite nests.

BLI models are currently considered the gold standard for the *in vivo* evaluation of drugs with trypanocide action, in addition, they can provide valuable insight on the mechanism of action of compounds.<sup>121</sup> For instance, BLI in models of chronic infection demonstrated that posaconazole, a TcCYP51 inhibitor, fails to clear parasites from certain infected tissues, leading to increases in parasitemia after immune-suppression, matching results from *in vitro* studies that explained its lack of clinical benefits.<sup>114</sup> These results contrast those obtained with earlier infection models, in which posaconazole was interpreted to be an effective therapy.<sup>122</sup> Similar studies have identified serologic profiles associated with parasite clearance, potentially useful for the longitudinal monitoring of treated patients.<sup>123</sup>

Additionally, murine models of Chagas disease have been developed to study specific characteristics of human infection: Swiss Webster and C57/BL6 mice, respectively, infected with *T. cruzi* Y and Brazil strains developed significant increases in intestinal transit times and intestinal dilation, which are common manifestations of chronic human digestive Chagas disease.<sup>124,125</sup> Although the appearance of those symptoms during the acute infection stage limited their applicability. More recently, the use of BLI/fluorescent models with CH3 mice and parasites from the JR strain (TcI), mimicked human digestive disease more closely, with the delayed transit phenotype persisting and worsening in chronic stages of infection, despite significant reductions in systemic parasitemia.<sup>126</sup>

Other models for the study of congenital transmission and immune control of infection also exist.<sup>127</sup> The latter are particularly relevant as differences in the immune response to infection with *T. cruzi* of humans and mice can limit the generalizability of studies evaluating the performance of drugs in these animals.<sup>14,112</sup> Recently, a genetically modified murine model of Chagas disease that, like humans, lacks the  $\alpha$ -1,3-galactosyltransferase ( $\alpha$ -GalT) gene has been developed.<sup>128</sup> Considering that lytic anti- $\alpha$ -Gal antibodies are an important component of IgG responses in patients

with Chagas disease,<sup>129,130</sup> such model would resemble the human humoral response against the parasite more closely than other mice that express the enzyme and are therefore tolerant to  $\alpha$ -Gal epitopes.<sup>14</sup> The use of humanized mice expressing human tissues also translates into more clinically translatable results, but remain prohibitively costly.<sup>112</sup>

Mice are also used in tolerability studies that precede the evaluation of in vivo efficacy and have the objective of studying the safety and potential adverse effects of experimental drugs, as well as determining their maximum tolerated dose (MTD). This is an important parameter that helps in defining the appropriate dose for subsequent efficacy and safety studies in larger models and in humans.

## Canine Models

Dogs are natural hosts of *T. cruzi* and have been traditionally considered adequate models of Chagas disease, with an electrocardiographic and myocardial pathology that reflects human disease.<sup>109,131–133</sup> In particular, dogs can develop a form of the disease that resembles the chronic phase of Chagas disease more closely, with changes in response outcomes following treatment with benznidazole in these animals also matching those observed in human clinical trials.<sup>109,133,134</sup> Nonetheless, differences in the half-life of certain drugs in dogs and humans can limit the translational potential of results obtained in this species.<sup>112</sup> Furthermore, ethical issues and the slow progression of infection in these animals entail high costs and significant logistical challenges that limit their applicability.

## Non-Human Primates

Non-human primates (NHPs) are the most physiologically similar model to human infection and were traditionally considered an important part of the drug development pathway. However, the cost and logistical difficulties of managing these animals have made their use relatively rare.<sup>135,136</sup>

Most of the studies assessing compounds in NHP models of Chagas disease have involved colonies of naturally infected animals.<sup>137,138</sup> A particularly good example of this approach is the recent use of rhesus monkeys (*Macaca mulatta*) naturally exposed to *T. cruzi* in the southern United States to evaluate the performance of benzoxaborole AN15368.<sup>82</sup>

In general, the performance of drugs in NHP models provides valuable clues about their expected efficacy in humans. For instance, the use of posaconazole in primates at plasma levels equivalent to those observed in humans failed to clear parasitaemia, in agreement with results of clinical trials evaluating the drug.<sup>139</sup> Nonetheless, infection of NHP with different strains of *T. cruzi* can lead to varying degrees of disease that do not always correlate with findings observed in human infection.<sup>140</sup> The ethical and economic considerations of handling primates in captivity, together with the availability of novel, translatable, and robust murine models, makes the latter a more suitable alternative.<sup>112</sup>

Discussions on the relevance and translational value of one species over another also tend to miss the fact that current outcomes used in these models are not directly translatable to clinical trials. For instance, the determination of parasitological sterilizing cure, understood as the elimination of parasites from both the blood and tissues, while feasible in animal models, is not possible in human patients. Further improvement on the development of animal models for the disease should, therefore, include the identification of new biomarkers or outcomes that are translatable to a clinical context.

## Targeting the Pathogenesis of Chronic Damage as a Complementary Strategy for Drug Development

Work based on murine models suggests that the scarce yet constant presence of parasites in immune-privileged tissues, like in the digestive tract of infected individuals, is necessary for the development of the chronic manifestations of the disease, as these sites are likely to allow the episodic seeding of parasites to other, less permissive organs, like the heart.<sup>19</sup> Therefore, a treatment with sterilizing capacity in these anatomical settings remains the main aim of any drug discovery strategy. However, the use of compounds able to target some of the key pathways involved in the development of chronic disease, without necessarily killing the parasites might as well be a promising host-focused complementary strategy.

Similar approaches are already used for the treatment of other protozoan infections, such as malaria or leishmaniasis.<sup>141,142</sup>

Symptomatology of chronic infection with *T. cruzi* is mostly associated with the development of chronic chagasic cardiomyopathy (CCC), and/or diverse gastrointestinal problems characterized by the dilation of segments of the digestive tract. The clinical and pathological characteristics of CCC are well defined, but the mechanisms through which the condition evolves remain poorly understood, limiting the development of new drugs that prevent it.<sup>143</sup>

It is accepted that cardiac damage in *T. cruzi* infection is multifactorial, involving a direct lytic effect on parasitized cells, autonomic dysfunction, and probably the occurrence of immune dysregulation, all ultimately triggered by the presence of parasites in the cardiac parenchyma.<sup>143,144</sup> Myocardial fibrosis is also a staple pathological finding,<sup>143,145</sup> and might in turn be explained by changes in the coronary microcirculation and the appearance of ischemic areas.<sup>144</sup> Thrombotic events, caused both by a direct cytolytic effect of parasitism of endothelial cells, and by the synthesis and secretion of pro-inflammatory and pro-aggregative products by parasites and infected cells, most likely play a role in this.

A lipid inflammatory mediator produced in *T. cruzi* infection, lysophosphatidylcholine (LPC) C18:1 has been associated with that process.<sup>146</sup> LPCs are known to have chemotactic effects on phagocytic cells, while favoring parasite survival upon invasion, making them important virulence factors for several protozoan parasites.<sup>147</sup> In addition to this, C18:1-LPC is also a structural analog of the mammal platelet activating factor (PAF), sharing its pro-aggregating effect on platelets.<sup>146,148</sup> Inhibiting the synthesis of this and other parasite mediators, or their interaction with their corresponding receptors, could therefore limit the development of organ damage associated with thrombotic effects in the cardiac and systemic microvasculatures. A structural analysis of this molecule suggests that it is synthesized by an enzyme with phospholipase A2 activity (PLA2), although it remains unclear whether this derives from the parasite, its host cell, or both.<sup>146</sup> While PLA2-putative genes exist in the genome of *T. cruzi*,<sup>149</sup> their expression and activity on mammalian life stages remains to be confirmed, making further research on this metabolic pathway necessary to determine its role as a potential drug target.

Another route of potential therapeutic interest is that of tumoral growth factor beta (TGF- $\beta$ ), an immune-regulatory cytokine involved in parasite development and progression to chronicity.<sup>150,151</sup> TGF- $\beta$  plays a key role in proliferation and differentiation of tissues, as well as in the remodeling of their extracellular matrix.<sup>152</sup> It has also been described to be involved in preventing the development of uncontrolled, potentially life-threatening, inflammatory responses during the acute phases of infection.<sup>19</sup> TGF- $\beta$  polymorphisms have been associated with increased risk of developing CCC and its expression is greatly increased in the early and chronic stages of *T. cruzi* infection.<sup>151,152</sup> Similarly, TGF- $\beta$ -producing cells are also abundant in the heart of animals with chronic infection, which is also more responsive to the cytokine, showing over-expression of its receptors and mediators, like the connective tissue growth factor (CTGF) and fibronectin, directly associated with collagen deposition and the development of fibrosis.<sup>153</sup>

Furthermore, treatment with inhibitors of the TGF- $\beta$  receptor kinase activity reversed collagen deposition and fibrosis in mice infected with the highly virulent Colombiana strain (TcI) of *T. cruzi*, without reducing the heart parasite load. Such treatment also improved electro and echocardiographic parameters and atrioventricular conduction, suggesting that blocking the TGF- $\beta$  pathway might lead to improved cardiovascular function in chronic *T. cruzi* infection, regardless of whether parasite clearance is achieved or not.<sup>154</sup>

Preventing the development of arrhythmias in infected patients might also be an approach to reduce the burden of the disease. In this context, amiodarone, a widely used class III anti-arrhythmic agent, has also been proposed as a potential complementary treatment for Chagas disease. Apart from its anti-arrhythmic effect, amiodarone also induces the release of Ca<sup>+2</sup> from the parasite mitochondrion and acidocalcisomes and kinetoplast, which has been suggested to give it additional anti-parasitic effects.<sup>155</sup> Its combination with benznidazole in mouse models of infection also improved electrocardiographic markers of cardiac damage and reduced levels of pro-inflammatory markers and collagen deposition.<sup>156</sup> Its use in naturally infected dogs led to improved survival.<sup>157</sup> However, a recent meta-analysis of patients with chronic Chagas disease who received amiodarone confirmed its antiarrhythmic benefits, but did not find any evidence of reductions in mortality or hospitalizations.<sup>158</sup>

In terms of digestive symptomatology, extracts from *Lycopodium clavatum* have been described to reduce the progression of gastrointestinal disease in rodent models of Chagas disease, probably through modulation of IL-10

production and an enhanced CD4/CD8 T cell response.<sup>159,160</sup> Other host-directed therapies can also mediate anti-microbial effects, as it has been demonstrated with the anti-leishmanial effect of ibrutinib, a monoclonal antibody against Bruton's tyrosine-kinase (BTK), a key modulator of B-cell maturation.<sup>142</sup>

In any case, it is important to bear in mind that the early clearance of parasites in infected patients remains the only intervention proven to prevent the development of chronic symptomatology, and targeting its pathogenesis without simultaneously killing the parasites is risky and might have potentially dangerous consequences, particularly if it involves the use of immune-modulatory therapies.

## Compounds Under Clinical and Preclinical Evaluation

Despite significant progress in the development of new techniques to understand the complex biology of *T. cruzi*, the number of compounds reaching clinical stage remains low (Table 2).

### Fexinidazole

Fexinidazole is a nitroimidazole recently approved for its usage in the treatment of human African trypanosomiasis,<sup>164</sup> with proven in vivo activity against *T. cruzi*.<sup>165</sup> Fexinidazole is activated by a parasite nitroreductase to a reactive intermediate, which then likely acts against multiple targets within the parasite.<sup>166</sup> In a phase II study in Bolivia, the drug revealed a quick and sustained anti-parasitic effect in patients with chronic infection, but the trial was stopped following the development of neutropenia in 20% of the treated patients (clinicaltrials.gov id: NCT02498782). Other alterations, including neuropsychiatric disorders and hepatic toxicity were also observed, and linked to a higher exposure to the drug.<sup>161</sup> More recently, data from a multi-center study carried out in Spain (NCT03587766) did not show efficacy with shorter, low-dose regimes of fexinidazole after 12 months of follow-up.<sup>167</sup>

Other drugs undergoing preclinical evaluation and likely to progress down the clinical pathway include the following:

### Proteasome Inhibitors

A kinetoplastid proteasome inhibitor, GNF6702 has demonstrated remarkable in vitro and in vivo activity against *T. cruzi*, *T. brucei*, *Leishmania donovani*, and *Leishmania major*.<sup>44</sup> A new derived molecule with improved solubility, LXE408,<sup>162</sup> is currently undergoing phase I clinical trials for its use in visceral leishmaniasis (NCT05593666) but has not been tested against *T. cruzi*.

### Oxaboroles

A benzoxaborole targeting the kinetoplastid mRNA processing factor CPSF3, named AN15368, has demonstrated substantial sterilizing in vitro and in vivo activity against different strains of *T. cruzi*. The compound also cleared the parasitemia in 19 treated, previously PCR-positive NHPs naturally infected with *T. cruzi*, as well as its presence in the

**Table 2** Compounds Under Evaluation for Their Use in Chagas Disease

Compound	Class	Stage of Development	Comments
Fexinidazole	Nitroimidazole	Phase II clinical trial	Significant haematological and neuropsychiatric alterations following standard treatment regimes. Lack of anti-parasitic efficacy in short-term regimes. <sup>161</sup>
LXE408	Proteasome inhibitor	Phase I clinical trial <sup>a</sup>	Derived from compound GNF6702, which demonstrated activity against different <i>Leishmania</i> species and <i>T. cruzi</i> . <sup>44,162</sup>
AN15368	Oxaborole	Pre-clinical evaluation	Remarkable in vitro and in vivo anti-parasitic activity, including in naturally infected NHPs. <sup>82</sup>
DNDi-6148	Oxaborole	Phase I clinical trial <sup>a</sup>	Demonstrated anti-kinetoplastid activity in vivo. Safe in phase I trial. <sup>163</sup>

**Notes:** <sup>a</sup>Currently evaluated for use in leishmaniasis but suggested as candidate drugs for treatment of Chagas disease.

tissues of euthanized animals. Importantly, the remaining animals showed declining anti-*T. cruzi* antibody titers in the next 42 months of follow-up, meeting current criteria used to assess treatment efficacy in humans.<sup>82</sup>

DNDI-6148, another benzoxaborole, is currently in development for the treatment of visceral leishmaniasis.<sup>165</sup> It has been recently nominated as a clinical candidate for the treatment of *T. cruzi* infection.<sup>163</sup> While its mechanism of action in *T. cruzi* has not been described, it is likely to involve, like AN15368, CPSF3 inhibition. An initial study with a single oral ascending dose in healthy volunteers has been completed (EudraCT: 2018–004023-37), and a phase I multiple ascending dose trial is expected to begin in the coming months.

## Conclusions

In the last few years, drug discovery efforts focused on kinetoplastid parasites have produced significant advances in the treatment of human African trypanosomiasis and visceral leishmaniasis. In comparison, the drug development landscape for Chagas disease remains scarcely populated and mostly unchanged. The silent clinical progression of the disease, the complex biology of *T. cruzi*, the poorly understood host–parasite interactions of the long-lasting chronic phase of the infection, as well as the parasite’s remarkable genetic and antigenic diversity pose a challenge to change that reality.

Despite this, the availability of an increasingly large volume of data derived from host-parasite *omics* studies, together with novel bioinformatic tools, gene editing strategies, and improved animal models better reflecting the human chronic infection phase, have all led to the optimization of existing drug development pathways, and the incorporation of further steps that increase the translatability of *in vitro* and *in vivo* experiments to pre-clinical and clinical settings.

This is already leading to the identification and validation of previously unexplored molecular pathways as attractive targets for the development of new treatments. Pending further clinical evaluation of promising compounds, the future treatment of Chagas disease will require the development of drugs with an improved anti-parasitic efficacy, and of complementary therapies that prevent or reverse the organ damage associated with chronic infection.

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## Disclosure

The authors report no conflicts of interest in this work.

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