Chagas Disease Drug Discovery: Toward a New Era

Eric Chatelain

Abstract
American trypanosomiasis, or Chagas disease, is the result of infection by the Trypanosoma cruzi parasite. Endemic in Latin America where it is the major cause of death from cardiomyopathy, the impact of the disease is reaching global proportions through migrating populations. New drugs that are safe, efficacious, low cost, and adapted to the field are critically needed. Over the past five years, there has been increased interest in the disease and a surge in activities within various organizations. However, recent clinical trials with azoles, specifically posaconazole and the ravuconazole prodrug E1224, were disappointing, with treatment failure in Chagas patients reaching 70% to 90%, as opposed to 6% to 30% failure for benznidazole-treated patients. The lack of translation from in vitro and in vivo models to the clinic observed for the azoles raises several questions. There is a scientific requirement to review and challenge whether we are indeed using the right tools and decision-making processes to progress compounds forward for the treatment of this disease. New developments in the Chagas field, including new technologies and tools now available, will be discussed, and a redesign of the current screening strategy during the discovery process is proposed.

Keywords
Trypanosoma cruzi, drug discovery, secondary assays, translation, compound progression criteria

Introduction
Chagas disease (CD), or American trypanosomiasis, is the result of human infection by the protozoan parasite Trypanosoma cruzi (T. cruzi). More than a century after its first description by Carlos Chagas, the disease is endemic in 21 Latin American countries, with Bolivia currently experiencing the highest disease burden. It remains an important public health issue in Latin America; however, cases of CD are being increasingly detected in the United States, Canada, many European countries, and some Western Pacific countries, owing to a rise in population movements between Latin America and other continents. According to World Health Organization estimates, the number of infected people globally amounts to 7 to 8 million, and more than 10,000 deaths are thought to occur annually. However, exact morbidity and mortality figures are hard to determine accurately. Recent reports state that CD is the leading cause of death in the elderly population in Brazil and a significant contributor to the global burden of cardiovascular disease.

CD primarily affects the poor. The economic burden due to reduced worker productivity, premature disability, and death amounts to 667,000 disability-adjusted life-years lost, with an annual estimated cost of more than 7 billion U.S. dollars in wages and industrial productivity globally related to CD.

The disease is usually transmitted by the so-called “kissing bug,” a blood-sucking reduviid bug of the subfamily Triatominae. It can also be transmitted to man by nonvectorial mechanisms, such as by blood transfusion, organ transplantation, from mother to child (congenital transmission), and orally through the consumption of contaminated food or drink. The majority of patients remain undiagnosed until later stages.

Clinically, human CD has two phases: the acute and the chronic phase (see Fig. 1). The acute phase is most often asymptomatic, but in untreated acute cases, it may present fatality rates ranging from 2% to 8%. During this phase, the parasite load is at its highest and can be detected by direct examination of fresh blood using either microscopy or PCR; hemoculture and xenodiagnoses are also diagnostic methods used at that stage. Following infection, the immune system kicks in and induces a reduction of the parasite load with subsequent control of the balance host/parasite.

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Following the acute infection phase, which lasts between 1 and 2 mo, the patient has evidence of immunity but remains infected. The effective immune responses control infection but do not prevent development of the disease. With the immunological balance between the host and the parasite population, the reduction in the number of circulating parasites is the method of choice for diagnosis of CD; techniques used include indirect immunofluorescent or hemagglutination assays, as well as enzyme-linked immunosorbent assay (ELISA), and the Pan American Health Organization recommends the use of two different tests in parallel to avoid inconclusive results. These tests can remain positive for decades after treatment, making the assessment of treatment efficacy very difficult. If untreated, the chronic phase continues for the duration of a person’s life. About 60% to 70% of patients will never develop disease with clinical manifestations. This indeterminate form of chronic CD is characterized by the presence of antibodies against T. cruzi and a lack of target organ involvement. The remaining 30% to 40% of patients will evolve to chronic disease with cardiac, digestive (megaeosophagus and megacolon), or cardiodigestive involvement, usually 10 to 30 years after the initial infection.

Clinical presentation varies widely according to disease duration and the extent and location of cardiac lesions; digestive forms of CD result from chronic inflammation and destruction of parasympathetic neurons, leading to progressive enlargement of the esophagus or the rectosigmoid colon. CD can also be reactivated if patients in the chronic phase are immune compromised or in the case of co-infection with HIV. Progressive heart failure (70%) and sudden death (30%) remain the most common causes of death in patients with CD.

The complicated evolution and pathology of CD is due to T. cruzi, a parasite that has adapted to changes in the host through an equally complex life cycle. The human stage of T. cruzi (see Fig. 2) is composed of two main forms with different biological functions and morphology (in addition to intermediate forms): one circulating infective but not replicative form, known as the trypomastigote form, and a replicative and intracellular form, known as the amastigote form. In short, the Triatomine bug defecates while taking a blood meal, and metacyclic trypomastigotes present in the feces enter mucosa and cells at the site of infection following scratching of the bite wound. The trypomastigotes then differentiate into amastigotes that undergo several rounds of replication inside cells before a dedifferentiation back to trypomastigotes and bursting of the infected cell occur. The released trypomastigotes infect other surrounding cells or enter the blood stream to infect cells at other body sites, and this cycle of transmission continues until circulating trypomastigotes are taken up by the vector during a blood meal.

T. cruzi has been called the “cruzi complex” because of the considerable genetic polymorphisms observed between parasite populations. The T. cruzi species is quite diverse, with multiple genotypes and phenotypes. Over recent years, with improved and standardized techniques for genotyping T. cruzi, new information is being generated about the epidemiology/distribution of CD and improved understanding of the population genetics of the parasite. In 2009, a new consensus was reached on the nomenclature of T. cruzi. T. cruzi lineages are now referred to by six discrete typing units, from TcI to TcVI. Most recently, further subdivision has been proposed following the demonstration of considerable genetic variability within T. cruzi I. The presence of multiple strains in the same geographical area and the possibility of mixed infection with strains of different tropism or virulence in the host are additional challenges for the already complex pathology.

Current treatment options for CD are limited to only two old nitro-heterocyclic drugs: benznidazole (Rochagan/LAFEPE and Abarax/ELEA) and nifurtimox (LAMPIT/Bayer). The side effects of these drugs, including allergic dermatitis, pruritus, fever, and gastrointestinal intolerance,
among others, are well documented and have limited their broader use, particularly in adult populations. Access to currently available treatments, including a clearer process to obtain them, has also been an issue, and the actual number of patients treated remains very low.

As a consequence, there is an urgent need for safe and efficacious new drug treatments for both acute and chronic phases of the disease. However, the task of designing and developing new compounds for CD is hindered in many different ways besides the already complicated drug discovery and development process. Indeed, the host-parasite interactions and relevance of the various T. cruzi strains in the etiology of the disease are not fully understood. The tools available for testing both in vitro and in vivo experimental animal models so far have a low predictive value, which obviously affects our understanding of the pharmacokinetic/pharmacodynamic (PK/PD) relationships for CD, during the drug discovery process and beyond.

CD Research and Development Landscape

Ten years ago, efforts to deliver new drug candidates for CD were very limited, and the number of investigators involved in drug discovery were few. Indeed, at the time, the activities ongoing worldwide were generally with low critical mass and working on only a few compounds. Moreover, the quality of the compounds that were available and assessed was clearly poor and very often associated with potential or demonstrated toxicity or druggability issues: these include naphtoquinones, diamidines, nitroimidazoles and related compounds, and ruthenium complexes. Both target-based and phenotypic approaches for screening were used, with the former concentrating on putative targets and pathways of potential importance for T. cruzi such as cysteine protease, trypanothione reductase, and ergosterol synthesis. Phenotypic assays were usually available in low-throughput format, allowing the testing of only a few compounds at a time. These approaches had clear limitations, as the number of well-validated targets was scarce and very often could not be translated into phenotypic assays.

Evolution during the past 10 years has been threefold: (1) the appearance of new investigators on the scene, (2) technological developments that allowed testing of new strategies for Chagas research and development (R&D), and (3) the efficacy assessment of two new chemical entities for the treatment of CD patients in the indeterminate stage of the disease, in the first clinical trials for the disease in 40 years.

Indeed, besides the Drugs for Neglected Diseases initiative (DNDi) discovery and Lead Optimization (LO) program for CD, new institutions have joined the effort. The Chagas Drug Discovery Consortium, whose goal is to discover and evaluate new candidate drugs for the disease, was created in 2010 following funding from the U.S. National Institutes of Health (NIH) and included several laboratories, mainly from the United States. A significant number of new consortia for Neglected Tropical Diseases sponsored by the European Union FP7 framework (KINDRED,
NMTrypl, PDE4NPD, A-PARADISE) started drug discovery activities at the end of 2013–early 2014 in the field of neglected tropical diseases (NTDs), including CD.52 Pharmaceutical companies have started to be active in the area. GSK with the Drug Discovery Unit at Dundee University received funding from the Wellcome Trust for NTDs research, which includes a discovery program for CD.53 The Genomics Institute of the Novartis Research Foundation (GNF) has been screening its own libraries to identify interesting compounds and has built strong relationships with academic groups involved in the field of neglected diseases such as the UCSF/Sandler Institute and the University of Washington.54 In late 2013, the Broad Institute, partnering with Eisai, was granted funds from the Global Health Innovative Technology Fund (GHIT) for the optimization of diversity-oriented synthesis–derived small molecules.55 An initiative of GSK-DDW at Tres Cantos, the Open Lab Foundation, provides opportunities to researchers to identify and optimize compounds with the support of GSK assets and expertise.56 Together with the arrival of these new investigators, processes and strategies to identify and move forward compounds for CD have evolved. In particular, there has been a switch from target-based screening toward phenotypic screens; indeed, most of the identified targets were not well validated, and as is often the case in the anti-infective field, the translation from target to infectious agent is poor.57 The possibility of screening compounds in high-throughput format (384-well plate format) using good and robust assays has opened up new possibilities; various strategies for identifying new chemical starting points for further optimization or moving forward to proof-of-concept (PoC) studies are available.

In the early days, given the low throughput of assays available to identify compounds with anti–T. cruzi activity, the so-called “low-hanging fruit approach” was very attractive as compound data were available and potentially few studies needed to be performed before PoC. Repurposing from one indication to another is one such an approach. Fexinidazole, a nongenotoxic 5-nitroimidazole, is a typical example of this approach, as it was rediscovered through a compound-mining exercise developed for sleeping sickness.58 Fexinidazole will enter PoC for CD as it was found to be efficacious in curing mice in in vivo experimental CD models.59,60 For the same reason as above, the screening of small focused sets of compounds, specific chemical series or inhibitors of a specific target for which structure-activity relationships (SAR) are available, was also very attractive. A typical example of this exercise is the oxaborole series, which has shown potential for CD.61,62 These approaches have the advantage that one can quickly move to PoC without using too many resources because annotations for these compounds, for example, solubility, metabolism data, and plasma exposure, are available.

Another approach is more a classical one in which, following identification of a hit, all of the data and SAR have to be generated through a standard LO program; a typical example is the delivery of potential candidates issued from one single hit, fenarimol.63

With the increased capacity to assess thousands of compounds quickly, one has seen a trend of accessing big diversity libraries and data, mostly from pharmaceutical companies, but also from the public domain. Such an example is the Broad screen initiative, which resulted in the high-throughput screening (HTS) of 300,000 compounds from the NIH library, the results of which were put in the public domain, thus allowing various groups to start chemistry programs.64,65

In addition, attempts at standardization of the assays used both in vitro and in vivo started to appear.66 Quality control plates containing a range of selected active and inactive compounds have been put together by various institutions and sent to different laboratories for testing. This allows the assessment of the reproducibility of results between different organizations and comparison of results. The organization of meetings in which data, assays, and progress are shared is also a way or attempt to move the CD research community in a common direction.

Recent Clinical Trials for CD

Significant changes have occurred in the clinical space as well in the past 10 years. Before that period, the poor quality and inconsistency of the data generated required caution for the interpretation, as stated in the Cochrane reports of 2014.67 Treatment duration and dose used for both standard of care (SoC) drugs, benznidazole and nifurtimox, were determined empirically rather than based on data. Moreover, these drugs have a bad reputation both in terms of safety and efficacy; it is well known and described that treatment with these drugs is associated with side effects, and it is difficult to assess their efficacy given the different methods used and the lack of test-of-cure or treatment efficacy.

The BENEFIT clinical trial is a long-term phase 3 clinical trial that started in 2005, looking at the potential clinical benefit of benznidazole treatment in CD cardiomyopathic patients, mainly for prevention of cardiac disease progression and death. This trial is still ongoing and is planned to finish by 2015.68,69

In the meantime, two controlled PoC trials to assess the potential of azoles as anti-chagasic agents have been concluded. The first one, CHAGASAZOL, compared benznidazole (SoC) with posaconazole delivered at two doses for 60 days. The outcome of this trial showed the failure of posaconazole to maintain a sustained response during follow-up after end of treatment as determined by PCR.70 Conversely, all but one patient treated with benznidazole showed sustained response (i.e., no relapse).
The E1224 trial was designed to assess the efficacy of another azole, ravuconazole (E1224 being the prodrug form of the active moiety), in comparison to placebo and benznidazole. Although E1224 showed good safety and was effective in clearing the parasite, it had little to no sustained efficacy 1 year after the end of treatment, compared with benznidazole with 80% sustained clearance of parasite as determined by PCR (DND/Eisai E1224 phase 2 trial, site presentation at the ASTMH Symposium November 14, 2013, Washington, DC). The STOPCHAGAS trial, sponsored by Merck, comparing posaconazole to placebo and including a posaconazole/benznidazole arm, is still ongoing.

In conclusion, the two finalized PoC trials examining the anti-chagasic efficacy of azoles in indeterminate CD patients show that (1) azoles are not efficacious as monotherapy for the treatment of CD patients in the indeterminate phase of the disease and (2) benznidazole has been shown to be an efficacious drug to maintain sustained clearance of parasite even 1 year later.

Considering that the number of PCR-positive CD patients is very variable (in the best cases, up to 60%) and that we do not really know if sustained parasite clearance (in blood) can be associated with clinical outcome (i.e., stop the progression of the disease), benznidazole will remain the SoC for CD treatment for the years to come. However, it is clearly not the ultimate answer, and there is a need for an efficacious drug with fewer side effects as well as a test of cure or treatment efficacy.

But more importantly, these recent data from clinical trials point to another problem of importance for those involved in the discovery and development of new drugs for CD: the lack of translation/correlation between preclinical data and clinical outcomes for a given compound (i.e., the difficulty in predicting efficacy in the clinic based on results from nonclinical studies in in vitro and in vivo models).

### Why Didn’t We Predict These Clinical Outcomes?

The fact that preclinical data did not predict the failure of posaconazole and the prodrug of ravuconazole, E1224, raises several questions regarding the current understanding of the host/parasite interactions. For example, are the right questions being asked in the experimental models of the disease used? Are the models valid? What characteristics should be looked for in such candidate compounds? At the same time, it is a great opportunity to try to understand why azoles failed in clinical trials whereas benznidazole did not; it will help rethink the strategy and process when moving compounds forward into PoC studies for CD. Answering these questions with evidence-based data might help researchers to resolve some of the outstanding questions still present in the field of CD.

A point of prime importance because it has critical impact on the types of assays used and the data that should be generated for future candidate drugs is to define what we are aiming for during the treatment of CD patients.

If parasite persistence is considered the main cause of the disease, then one should aim at eradication of the parasite and so develop compounds that kill the parasite or lead to its irreversible growth arrest. There are reports suggesting that the reduction of parasite load is already good enough; the only issue is that during the chronic phase of CD, the parasite load, at least based on what is measurable in blood, is already very low. One could therefore question the benefit of reducing it further if at all. Furthermore, there is no evidence so far that parasitemia correlates with what would be found in tissues, nor is there evidence that parasite load reduction, or a lower parasite load, is associated with slower disease progression. Even if there has been a lot of progress in PCR methods and in the collection process of blood samples, it is a technical challenge to quantify such low levels of parasites in blood of these patients, and not all patients are PCR positive. Only the future will tell if eradication of the parasite has a clinical benefit and stops progression of the disease. But because there is no practical way to assess this apart from a 10- to 20-year follow-up of treated patients, it is best to aim at killing the *T. cruzi* parasite.

This point is also related to the definition of clinical cure for CD patients. The only test of cure so far is based on conventional serology methods, but it can take up to 20 years to observe seronegative conversion in treated adult patients. There is therefore a clear need for surrogate biomarkers to assess treatment efficacy, as PCR, the current standard in phase 2 clinical trials, is very useful but in practice gives only an indication of treatment success or failure, with no proof of efficacy for a given drug.

When the first data from the PoC CHAGASAZOL trial were presented at the end of 2012, one hypothesis to explain the failure of posaconazole was that the population included in the clinical trial might not be representative of infection with all *T. cruzi* strains or perhaps represented only a subset of those. Genotyping of isolated strains from the patients would be needed to give a clearer answer. Common sense would argue, however, that the drug treatment should be effective against *T. cruzi* parasites belonging to all of the groups defined so far. It is actually one of the criteria of the target product profile (TPP; see Table 1).

Another argument put forward to try to explain the failure of posaconazole was the fact that the systemic exposure of posaconazole observed in patients during this trial, although corresponding to that observed previously, was 5 to 10 times lower than that found in mice following administration of the corresponding dose of 20 mg/kg, which cures the disease in these animals. There is indeed a disconnect between average concentration (\(C_{av}\)) in mice...
and humans; however, the concentration of the drug found in patients treated (between 1.3 and 2.4 µM, corresponding to the dose of 100 mg twice daily and 400 mg twice daily, respectively) was still about 1000 times higher than the commonly reported IC50 for that compound (1 nM or lower). In addition, given the excellent distribution of posaconazole in tissues, one would expect that its failure would not be due to its PK properties.

Lastly, the shorter treatment duration of 60 days has been proposed as the reason for the failure of posaconazole. Indeed, it has been reported that in a patient with chronic CD and systemic Lupus erythematosus, 90 days of posaconazole at 400 mg given twice daily gave negative PCR results 12 months after the end of treatment. It should, however, be mentioned that this was an isolated case and that this patient was concomitantly receiving immunosuppressive treatment. One cannot exclude the fact that longer treatment with this drug could lead to a better outcome. But this would still need to be proven under controlled conditions in clinical trials, and one can question whether longer treatment duration is desirable and would fit with the TPP. This raises the issue of the necessary treatment duration needed to cure CD patients and the determination of this duration based on nonclinical data. It also highlights the importance of being able to predict or distinguish between slow- and fast-acting compounds as well.

As described above, the CHAGASAZOL and E1224 results showed that compounds belonging to the class of azoles that are T. cruzi C14α-demethylase inhibitors (better known as TcCYP51 inhibitors) are not adequate as monotherapy for the treatment of CD patients in the chronic phase. This raises the issue of target validation and the understanding of target/pathway essentiality for the parasite. Ergosterol, the end product in a pathway involving the TcCYP51 enzyme, might be essential for highly replicating parasites but not for non- to slow-replicating ones. This may explain why sustained parasite clearance was not observed with the azoles.

The existence of dormant or nonreplicating amastigote parasites is still debatable and needs to be proven. One could argue that differences in the metabolism of parasites in the chronic stage of the disease might lead to differential susceptibility to azoles. Moreover, and even if it is subject to controversy, one might ask if a candidate compound should also be active against the nondividing trypomastigote stage of the parasite as well as to the intracellular amastigote stage. In malaria, for example, one of the criteria for compounds or combinations thereof is that one should be able to target all of the stages of the parasite to avoid any relapse. It is indeed interesting to note that benznidazole and nifurtimox are active at similar concentration in vitro against both stages of the parasites (data not shown; Melissa Sykes, personal communication), whereas azoles, as expected given their mode of action (MoA), are active only against the intracellular amastigote stage of T. cruzi, the trypomastigote being the nonreplicating form of the parasite (data not shown; Melissa Sykes, personal communication). Indeed, an analysis of the literature on azoles showed that their activity in in vivo models was related to a suppressive rather than a curative effect.

### Table 1. Chagas disease target product profile.

<table>
<thead>
<tr>
<th></th>
<th>Acceptable</th>
<th>Ideal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Target population</strong></td>
<td>Chronic</td>
<td>Chronic and acute (reactivation)</td>
</tr>
<tr>
<td><strong>Strains</strong></td>
<td>TcI, TcII, TcV, and TcVID</td>
<td>All</td>
</tr>
<tr>
<td><strong>Distribution</strong></td>
<td>All areas</td>
<td>All areas</td>
</tr>
<tr>
<td><strong>Adult/children</strong></td>
<td>Adult</td>
<td>All</td>
</tr>
<tr>
<td><strong>Clinical efficacy</strong></td>
<td>Noninferior to benznidazole in all regions</td>
<td>Superiority to benznidazole in different phases of the disease—acute and chronic (parasitological)</td>
</tr>
<tr>
<td></td>
<td>(parasitological)</td>
<td>Superiority to benznidazole or nifurtimox</td>
</tr>
<tr>
<td><strong>Safety</strong></td>
<td>Superiority to benznidazole</td>
<td>No CE or LE or ECG needed during treatment</td>
</tr>
<tr>
<td></td>
<td>3 CE plus 2 standard LE or ECG during treatment</td>
<td>Active against nitrofuran- and nitroimidazole-resistant T. cruzi strains</td>
</tr>
<tr>
<td><strong>Activity against resistant strains</strong></td>
<td>Not necessary</td>
<td></td>
</tr>
<tr>
<td><strong>Contraindications</strong></td>
<td>Pregnancy/lactation</td>
<td>None</td>
</tr>
<tr>
<td><strong>Precautions</strong></td>
<td>No genotoxicity; no proarrhythmic potential</td>
<td>No genotoxicity; no teratogenicity; no negative inotropic effect; no proarrhythmic potential</td>
</tr>
<tr>
<td><strong>Drug-drug interactions</strong></td>
<td>No clinically significant interaction with antihypertensive, antiarrhythmic, anticoagulant drugs</td>
<td>None</td>
</tr>
<tr>
<td><strong>Presentation</strong></td>
<td>Oral</td>
<td>Oral</td>
</tr>
<tr>
<td><strong>Stability</strong></td>
<td>3 years, climatic zone IV</td>
<td>5 years, climatic zone IV</td>
</tr>
<tr>
<td><strong>Dosing regimen</strong></td>
<td>Comparable to systemic antifungal treatments</td>
<td>Once daily/30 days</td>
</tr>
</tbody>
</table>

aClassification according to Zingales et al. 200943; requires further research for definition of target strains for evaluation.
bCE, clinical evaluation; LE, laboratory evaluation; ECG, electrocardiogram.
What about the experimental models used in CD and their predictability? The variability of the animal models used for CD, together with the different readouts used to define cure (parasitemia, PCR in blood, PCR in blood and in tissues), has led to very variable results for both benznidazole and posaconazole as well as ravuconazole. This makes it very difficult to obtain a clear comparison, not to say a good prediction of potential outcome for these compounds. There has always been the belief that posaconazole was better than benznidazole, although with similarly variability in results.\(^{84-86}\) For example, it has been shown that 20 mg/kg treatment with posaconazole starting at day 8 postinfection using the Tulahuen strain of *T. cruzi* led on average to a 40% to 60% cure rate and was always superior or equal to benznidazole tested under the same conditions (cure assessed by PCR in tissues and blood after three rounds of immunosuppression). However, the subsequent trials results showed that no translation was possible with clinical data using this model.\(^{62,63}\)

Bearing in mind these considerations, one could question if the right chemical starting points are being worked on and if the right assays are in place to profile compounds. Indeed, a lot of different groups working on CD have been focusing on CYP51 inhibitors, exemplified by the azole class of compounds and other scaffolds.\(^{87-90}\) Three years ago, to look for more diversity, DND was already intending to filter out CYP51 inhibitor compounds as starting points because of the already substantial number of such inhibitors in the field.

All of these questions highlight a major translational challenge for CD R&D and the need for better or new assays and tools and further testing/generation of data to confirm or refute hypotheses. Better experimental design is clearly needed to answer specific questions and to translate research data to assays compatible with the drug discovery and development process.\(^{91}\)

**Recent Advances in the Field**

Fortunately, the outlook for the future is bright, and the past few years have seen a surge in new technologies and developments that will be very useful to answer the questions described earlier and the need for new drugs.\(^{92,93}\)

**In Vitro Tools**

**HTS and dose-response curves.** Since the advent of the “classical” screening assay for *T. cruzi* based on absorbance readout,\(^{84}\) developed by Fred Buckner and collaborators and used by numerous laboratories on a small scale, advances have been made in terms of HTS format. The Broad Institute developed this assay in 384-well plate format that allows the screening of 300,000 compounds.\(^{64}\) In addition, new transgenic parasites expressing tdTomato or luciferase genes\(^{95}\) and the application of imaging software that allows the identification and quantification of parasites within cells (as well as giving the first indications of the potential cytotoxicity of compounds tested) have also been implemented.\(^{96-98}\)

Using imaging software in a standardized way is very helpful to determine not only the number of infected cells but also the number of parasites remaining following exposure to compounds. Analysis of the effect versus concentration allows selection of compounds that can indeed produce 100% killing of *T. cruzi* parasites inside cells. It is a way to discriminate between activity versus potency of compounds, and if the aim is to eradicate parasites, a 100% response should be sought. Using these new techniques, Moraes and collaborators\(^{99}\) were able to distinguish the activities of benznidazole versus posaconazole and ravuconazole in vitro with respect to the ability of these compounds to induce 100% killing of the parasite. It was indeed striking to see that benznidazole was able to induce 100% killing of all the strains of *T. cruzi* tested, whereas this was not the case for the TcCYP51 inhibitors assessed including posaconazole, ravuconazole, and fenarimols.\(^{99}\) The greater activity of nitro-derived compounds correlated with a low variability between strains. So-called benznidazole-resistant strains such as the Colombiana strain were not tested in this particular study, but it should be noted that data from other laboratories showed similar activity and potency (about 4.8 μM) for benznidazole with this strain as compared with the range of values obtained for the other strains.\(^{93}\) These data suggest that *T. cruzi* susceptibility to benznidazole varies between strains but remains within a factor of 10 for the potency, and in all cases, 100% killing of the parasite is obtained in vitro.

**Parasite stage-specific assay.** *T. cruzi* parasite exists in the human host in several forms according to its life cycle, including trypomastigotes, amastigotes, and intermediate forms thereof.\(^{100}\) Analogous to malaria,\(^{83}\) it would make sense for a compound to target all stages of the parasite occurring in humans (i.e., nonreplicating, circulating, and infective trypomastigotes and replicating intracellular amastigotes). Cell culture assays using trypomastigotes and blood trypomastigotes are available in low-throughput format and might be useful to profile identified hit compounds. Data have to be generated with compounds being profiled in these different stage-specific assays to assess the importance, or not, of identifying compounds with these characteristics.

**Time-kill and washout/reversibility assays.** Time-kill assays give an idea of the rate of kill for a compound (i.e., to identify whether a compound is fast or slow acting). These types
of assays have been pretty well defined for other parasitic diseases such as human African trypanosomiasis and malaria, but not so extensively described for CD. This assay coupled with studies in vivo can be helpful in predicting the speed of parasite killing in humans to get a first indication of the treatment duration needed. A fast-acting and long-lasting compound would be optimal. Benznidazole is surely a fast-acting compound, and it is questionable whether a slow-acting compound is desirable.

There is very often confusion between the time-kill assay and the so-called washout/reversibility assay. The latter is the ultimate test to assess if there is any regrowth of the parasites once one stops drug pressure (i.e., if a compound is static or cidal or causing reversible versus irreversible growth arrest or apoptosis commitment). It should be noted that the activity assay based on imaging parasites can already partially give this information if no parasites can be identified following drug treatment at a given concentration. These assays can help to predict the exposure needed to achieve eradication of the parasite in vivo in the human host.

**Other specific assays/technologies.** Additional assays have recently been developed that allow further profiling of compounds and the selection of the right chemical starting point. In particular, a functional *T. cruzi* CYP51 assay has been developed by the Drug Discovery Unit at the University of Dundee to filter out CYP51 inhibitor compounds (Kevin Read, personal communication).

Whole genome sequencing of generated *T. cruzi* strains/clones resistant to azoles, for example, and clinical isolates obtained from patients who did not respond to treatment with ravuconazole might help to identify potential mutations in specific genes or identify the copy number of genes for specific targets of interest. Target deconvolution using omics, bioinformatics and genetic technologies, applied to *T. cruzi* could lead to target-based strategies to identify new chemical starting points; they could also give important information related to potential safety issues or identify specific markers to look at during further development.

**In Vivo Experimental Animal Models**

Although a consensus for in vivo animal models was published in 2010, the lack of predictability of these models, as observed for the azoles that went into clinical trials, indicate a need to review these models. There is considerable variation depending on animal species, parasite strain, and readouts used, to cite only a few issues to challenge their adequacy. One main common goal should be to design testing hypotheses to improve their predictability and translation to human disease and also to look at new technologies and models to test these hypotheses. A better understanding of these models and answers to the questions we are asking will be achieved only through the generation of systematic data while remaining compatible with the drug discovery process.

Recently, new technologies involving the use of transgenic *T. cruzi* parasites and live in vivo imaging of infected mice have made it possible to follow the course of an infection with this parasite; it also enabled the same animal to be followed for a determined period of time using bioluminescence, thereby reducing the number of animals needed for a given study. **Bioluminescent imaging might also shed further light on what happens during the course of *T. cruzi* infection, such as the dynamics of the parasite during infection and the target organs.** Recent data indeed show that there is still a lot to learn about the disease evolution and that cumulative damage with time could be responsible for cardiomyopathy, as the heart is not necessarily a residing organ for the parasite (at least with *T. cruzi* Tulahuen strain in this model). Moreover, with posaconazole/ravuconazole and benznidazole, research tools are now available that will be very helpful in translating back clinical data to the in vivo model of choice to adjust them to improve their predictability.

In addition, assessing potential biomarkers identified in sera of treated patients in these models could lead to further refinement of the models and better predictability.

These recent developments and new tools, together with the information they generate, have led DNDi to rethink the screening strategy and the way to select new chemical series for LO for CD. Indeed, a panel of assays is now available that can help during the early hit selection/prioritization and potentially increase the chance of success when moving forward. A new screening strategy as depicted in **Figure 3** is proposed.

The screening strategy takes into account all of the points discussed above and considers the assays needed, that is, those that should be on the critical path and those that would be nice to have (increasing our confidence in the decision-making process to move compounds forward but not essential at that stage, leaving room for optimization).

For example, data obtained from screening assays using imaging software to identify parasites in cells can be used to identify hit compounds that kill at least 90% to 95% of parasites at the first most efficacious concentration. This cutoff is based on the fact that azoles such as posaconazole as well as fenarimols, for example, give activity of 70% to 80% in this assay as compared with 95% to 100% activity with benznidazole or nifurtimox. This, together with the potency data (measured as IC$_{50}$—concentration producing 50% reduction of infected cells) and a selectivity index (relative activity of a compound or IC$_{50}$ compared with its cytotoxicity to host cells) higher than 10 for a hit and 100 for a lead, should be considered. Assays such as the trypomastigote assay, *T. cruzi* strain susceptibility testing, time-kill, and reversibility are other important assays to further assess...
compound properties; these would be considered as “nice to have” in the first place for a hit but would be essential once a compound moves forward into LO. The TcCYP51 functional assay was not put on the critical path for the simple reason that we do not want to filter out compounds that are positive in this assay but could still lead to 95% activity, possibly suggesting an off-target mechanism. Given that the choice of experimental in vivo models to be used that are most predictive of potential clinical outcome is unclear, a lead is defined as a compound that has reasonable exposure and achieves parasitemia reduction/eradication in infected mice following 5 days of treatment during the acute stage (besides more classical in vitro primary absorption-distribution-metabolism-excretion characterization). This can be considered as a first proof of principle for the series under investigation before moving into LO (Fig. 3A). For the optimal candidate issued from LO, one should aim at the following properties (see Fig. 3B): good drug metabolism and PK properties and safety profile as assessed using standard assays in vitro and in vivo, good in vitro activity (cidal,
killing 100% of the parasites), high potency (ideally in the nM range—the more potent, the larger the therapeutic index), a fast MoA, and efficacy against a panel of strains representative of the different T. cruzi groups. In addition, such a compound should eradicate T. cruzi parasites in mice chronically infected (treatment with the compound during the chronic stage). Preliminary data suggest that this scheme is currently the most predictive of clinical outcome, at least for the reference compounds tested so far.

It is obvious that this strategy will be subject to discussion and that some assays or criteria might be found to be irrelevant in the future. But it is only through testing and generation of data that hypotheses will be confirmed or invalidated.

This new proposed screening cascade could form the basis and be a first step toward a consolidated target candidate profile acknowledged by investigators involved in CD drug discovery, taking as an example the recent description of the various target candidate profiles for malaria.83

Perspectives

CD drug discovery is entering a new and exciting era, and we are much better off today than a few years ago. One can look to the future with more confidence, even if there is still a long way to go in understanding the complexity of this disease and the host-parasite interactions.

Indeed, even though the recent PoC clinical trials with the two new drug candidates from the azole class failed, one has to acknowledge that the clinical data generated will be very useful, both to the Chagas research community in the short term and to the patients in the longer term. Besides challenging the community and scientific dogma, these clinical data, together with the overall improvements of assays and the recent development of new tools, should help improve our understanding of the characteristics needed for a compound to be progressed toward preclinical development. Taken together, this information will give us more confidence that the candidates moving forward will be efficacious in the clinic. However, this will be possible only if new chemical starting points/series are identified and sustained funding remains available.

Translational challenges are being tackled, but there is still a need for research to better understand the pathology, the host-parasite interactions, and the issue of the relevance of T. cruzi strains, among others. It is critical to understand the reason for the failure of azoles; the combination of in vitro and in vivo experimental data generated with these compounds using these new assays (back-translation of clinical data) will be helpful to confirm current or identify new preclinical models relevant to move compounds forward. It is only through addressing the right questions in these assays and models and through the generation of systematic data to test hypotheses that we will be able to assess which assays or models are critical and most predictive of the clinical outcome. Ultimately, this should lead to a better understanding of the PK/PD relationships for these compounds.

The integration of the MoA when available, or the generation of MoA data through omics and other techniques that are starting to be applied to NTDs, will be helpful not only for the efficacy studies but also to predict potential safety issues and monitor specific parameters during preclinical and clinical development (toxicology studies and clinical trials). Moreover, these techniques could lead to new approaches to identify new chemical starting points through target-based screens.

Although there is still, without doubt, a knowledge gap, we are moving toward the definition of a consensus for a target candidate profile for new compounds for PoC for CD. Moreover, the new data generated and knowledge acquired should encourage a revision of the Chagas target product profile.

The major change of the Chagas drug discovery landscape due to the appearance of new investigators and initiatives from the pharmaceutical industry, academic groups, and consortia is also very encouraging and will surely promote further research and development in the area. The need for open collaboration and data sharing should, however, be strongly emphasized in order to use resources efficiently and also, more critically, to avoid duplication or replication of work given the still scarce funding available for CD drug discovery.

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