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Methodological advances in drug discovery for Chagas disease

Juan M. Bustamante[1] and **Rick L. Tarleton**[1,2,*]

[1] Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, 500 D.W Brooks Dr. S310 Coverdell Center. GA, 30602, USA

[2] Department of Cellular Biology, University of Georgia, Athens, 500 D.W Brooks Dr. S310 Coverdell Center. GA, 30602, USA

Abstract

Introduction—Chagas disease is the highest impact human infectious disease in Latin America, and the leading worldwide cause of myocarditis. Despite the availability of several compounds that have demonstrated efficacy in limiting the effects of *T. cruzi*, these compounds are rarely used due to their variable efficacy, substantial side effects and the lack of methodologies for confirming their effectiveness. Furthermore, the development of more efficacious compounds is challenged by limitations of systems for assessing drug efficacy in vitro and in vivo.

Areas covered—Herein, the authors review the development of Chagas disease drug discovery methodology, focusing on recent developments in high throughput screening, in vivo testing methods and assessments of efficacy in humans. Particularly, this review documents the significant progress that has taken place over the last 5 years that have paved the way for both target-focused and high-throughput screens of compound libraries.

Expert opinion—The tools for in vitro and in vivo screening of anti-*T. cruzi* compounds have improved dramatically in the last few years and there are now a number of excellent in vivo testing models available; this somewhat alleviates the bottleneck issue of quickly and definitively demonstrating in vivo efficacy in a relevant host animal system. These advances emphasize the potential for additional progress resulting in new treatments for Chagas disease in the coming years. That being said, national and international agencies must improve the coordination of research and development efforts in addition to cultivating the funding sources for the development of these new treatments.

Keywords

Chagas disease; high-throughput screening; reporter genes; surrogate markers; *Trypanosoma cruzi*

1. Introduction

Chagas disease, an endemic infection caused by the protozoan parasite *Trypanosoma cruzi* is a major public health problem in Latin America. Estimates of the prevalence of Chagas vary widely but it is likely that between 10 and 20 million people are infected and between 25,000 and 50,000 die per year as a result. Transmitted to humans primarily by reduviid

*Corresponding author. Mailing address: Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, 500 D.W Brooks Dr. S310 Coverdell Center. GA 30602, USA. Phone: 706-542-3378. Fax: 706-542-3582. tarleton@cb.uga.edu.

Declaration of interest

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insects, the infection generally has modest acute impact (swelling near the infection site, fever, fatigue, and enlarged lymphatic organs) and is well-controlled by the host immune response but appears to rarely be completely eliminated. Thus, individuals often do not know they are infected until decades later, and in many cases, not until clinical symptoms (primarily cardiac insufficiency or failure and/or gut dysfunction) are evident [1–2].

The *T. cruzi* life-cycle includes four distinct stages: the non-replicative bloodstream trypomastigotes and the replicative intracellular amastigotes in mammalian hosts and epimastigotes and mammalian-infective metacyclic trypomastigotes in the triatomine vector. The two drugs available for the treatment of *T. cruzi* infection, benznidazole (BZ, *N*-benzyl-2- nitroimidazolylacetamide) and Nifurtimox (NFX, (*R*,*S*)-3-methyl-N(italic)-[(1*E*)-(5-nitro-2- furyl)methylene]thiomorpholin-4-amine-1,1-dioxide) have substantial potential side effects and variable efficacy that is also difficult to measure [3–5]. These attributes have combined to limit the use of these drugs, particularly in treating those with chronic infections. Thus, there is a crucial need to identify new and more effective compounds to treat *T. cruzi* infection. However, one important factor in the search for better therapies is the scarcity of efficient *in vitro* and *in vivo* systems to determine compound efficacy. Recently, there have been several improvements in this regard that are reviewed here.

2. In vitro screening for anti-*T. cruzi* compounds

Manual microscopic counting of parasite growth has long been a important tool for the measurement of the inhibitory effects of compounds on *T. cruzi* [6–8]. This approach is suitable for assaying a small number of compounds but is clearly unacceptable for screening large compound libraries. Both colorimetric [9–13] and fluorometric [14–15] methods have been employed to increase throughput in these assays. Although these latter approaches are more rapid and have greater objectivity than manual counting by microscopy, they are limited in that they are only useful for monitoring inhibitory effects on epimastigotes but not for measuring the growth of intracellular amastigotes, the forms that are the replicating stage of *T. cruzi* in the mammalian hosts. Automated, high content microscopy has recently been applied to this challenge. By combining high resolution microscopy with image analysis and often times, robotic sample handling, this methodology allows the analysis of images of cells for drug discovery [16]. Engle and co-workers used an imaged cell-based high throughput screening (HTS) assay to simultaneously measure the anti-*T.cruzi* efficacy and host cell toxicity of >900 compounds, identifying 55 new hits [17]. A larger, high content HTS assay of a 4,000 compound library against *T. cruzi* Y and Dm28c strains in host cells was also recently reported by Freitas- Junior and colleagues (Freitas-Junior et al 2010 Int. Congress of Parasitol. Melbourne, Australia). An advantage of the high content imaging approach is that potentially any of the hundreds of *T. cruzi* lines currently available in laboratories (or freshly isolated from infected hosts) can be used in HTS assays without the need of incorporating a reporter molecule.

More than a decade ago, Buckner, et al developed transgenic lines of the *T. cruzi* , CL and Tulahuen strain, that express the reporter enzyme β-galactosidase (β-gal), from *Escherichia coli*, also known as LacZ [18]. These strains allow detection of parasite growth by measuring the β-gal activity, thus facilitating the facile enumeration of both extracellular and intracellular parasite growth. Parasites expressing β-gal have also been used for screening of compounds for activity against other parasitic pathogens, including *Toxoplasma gondii*, *Leishmania mexicana* and *Trypanosoma brucei* [18–20]. Colorometric substrates (chlorophenol-red- β-D-galactopyranoside (CPRG) and o-nitrophenyl-β-D-galactoside (ONPG)) have classically been used for quantifying β-gal activity but the increased sensitivity needed for scale-up to 384 well and higher density plates is provided by chemoluminescent substrates based on 1,2-dioxetanes [21]. *T. cruzi*-β-gal parasites were

successfully used to screen compounds for activity against epimastigotes and in a HTS against *T. cruzi* amastigotes [22]. Using this approach, investigators at NYU and the Broad Institute recently completed a HTS of the ~300,000 compounds in the NIH compound collection, providing nearly 4,000 conclusive hits and approximately 1,600 of these confirmed hits showed an IC_{50}<1.2 μM and a 100 fold selectivity [23].

T. cruzi lines expressing other reporters such as firefly luciferase have also been used to screen for trypanocidal compounds [24]. This system uses the substrate luciferin that is oxidized by the luciferase yielding light [25]. One disadvantage of both high content microscopy screening 6 methods and screens employing β-gal or luciferase is the need for a processing step at the end of each assay – the fixation and staining with a DNA stain and/or antibodies in the case of high content microscopy and substrate addition in the case of β-gal or luciferase. This end-point processing step also means that only a single time point for the measurement of parasite growth is possible in these assays. This is not a problem in the case of true HTS assays, where only a single point for assessing growth is needed, but is an issue when an evaluation of the kinetics of growth (and thus insights into the mechanisms of action of active compounds) is desired.

For the latter purpose, parasite lines expressing reporter molecules that do not require substrates for their detection are useful. A wide range of naturally fluorescent proteins are now available and a number have been expressed in *T. cruzi* for vector-biology as well as for *in vitro* and *in vivo* infectivity studies, including analysis of tissue tropism, mechanisms of cell invasion, and genetic exchange among parasites [26–29]. We have recently used *T. cruzi* lines constitutively expressing the tandem tomato fluorescence protein (tdTomato) for the screening of potential anti-*T. cruzi* compounds (Figure 1; [30]. Among the advantages of these particular parasite lines for drug screening, in addition to the elimination of the need for fixation or cell permeabilization, is that the fluorescence signal is sufficiently bright to be able to quantified with a fluorimeter (measuring the entire fluorescence of individual wells) in addition to high content imaging systems (that can quantify parasite growth at the individual host cell level but with much increased time required). In addition, the use of these parasites allows for the continuous measurement of parasite replication or growth inhibition by drugs over time with a high intra- and inter-assay consistency that could be easily scaled up to a 384 well format, allowing for the development of HTS. Multiple parasite strains differing in susceptibility to existing anti-*T. cruzi* compounds have also been produced (Figure 2; [30].

3. In vivo drug screening and testing

T. cruzi naturally infects a large number of mammals in addition to humans, and mice have been the model of choice for the *in vivo* testing of anti-*T. cruzi* compounds. The standard measures that have been used to assess *in vivo* drug efficacy in this model are the suppression of parasitemia early in the acute infection and/or the quantification of survival rates following a normally lethal infection [31–33]. Although both of these measures are indicative of anti-*T. cruzi* activity, neither inform on the ability of compounds to bring about parasitological cure. Many compounds suppress parasitemia and prevent or delay death following an otherwise lethal infection but fail to completely clear the infection [30]. Additionally, since *T. cruzi* is very challenging to detect in hosts during the chronic phase of the infection – even with the use of amplification techniques such as PCR - drugs are virtually never tested for efficacy in this stage. So neither existing drugs nor newly discovered ones are tested for efficacy in the task that is most needed of a drug for use in *T. cruzi* infection – the ability to completely eradicate the chronic infection.

With this problem in mind, we recently used immune suppression as a tool to determine if BZ could achieve parasitological cure in mice. This approach is based upon the premise that the loss of immune control will reveal infection that is otherwise not easily or consistently detectable, particularly in a well-established infection. Immunosuppression has been shown to intensify infection in experimental models of *T. cruzi* infection [30, 34] and as well in human infections as illustrated with HIV-infected subjects who exhibit exacerbated parasitemia and tissue parasite load [35–37]. In the initial study in mice, a 40 day course of BZ treatment during either the acute or chronic phases of the infection was found to completely eliminate *T. cruzi* infection as indicated by the inability of cyclophosphamide-induced immunosuppression to reveal *T. cruzi* or *T. cruzi* DNA using multiple highly sensitive methods [34]. In contrast, non-treated mice with infections of varying lengths (up to 460 days) showed rapid reoccurrence of parasitemia after cyclophosphamide treatment. The conclusion that mice were parasite-free following BZ treatment was further supported by the conversion in parasite-specific T cells to a central memory phenotype, indicative of the absence of continuous antigenic stimulation. We went on to show that other compounds that could quickly suppress parasitemia within a few days of the initiation of treatment were rarely effective in attaining complete parasitological cure [30], further establishing that suppression of parasitemia is a poor indicator of *in vivo* efficacy. These results create a new standard for assessing the anti-*T. cruzi* activity of compounds *in vivo*. The assay for cure requires no specific parasite lines or reagents (other than a method of immunosuppression) nor special equipment. Also, BZ can be used as a standard of efficacy against which other compounds can be compared.

Although this treatment/suppression system is a powerful and sensitive method to address *in vivo* drug efficacy in *T. cruzi* infection, the limitation of this protocol is the long course of the experiment, requiring approximately 80 days for the specific treatment and the subsequent immunosuppression. Library screens such as those reported by the Broad/NYU group are identifying 1000s of compounds that are active *in vitro* and that need to be tested *in vivo.* For 9 this purpose, a more rapid *in vivo* screening assay would be very useful. To address this issue, we have developed an assay that measures the ability of compounds to alter *in vivo* parasite growth using whole animal imaging (Figure 3; [30]. Previous investigators have used *T. cruzi* lines expressing luciferase [38] or betagalactosidase [39] to track parasite growth and persistence in *T. cruzi* infection. To determine if reporter gene-expressing parasites could be used to more quickly evaluate treatment efficacy, *T. cruzi* lines expressing luciferase or tdTomato protein were injected into the footpads of mice, and parasite growth was monitored in this site with or without treatment. In the original assay, mice were submitted to treatment between day 6 and 11 post infection, and the change in fluorescence or bioluminescence intensity before, during and after completion of treatment was used as a surrogate of parasite load [30]. Since the measurement is non-invasive, the kinetics of parasite growth can be determined by periodic (daily if desired) measurements in the same animals. And as parasite replication only occurs intracellularly in mammals, by division of amastigote, the assay measures the ability of compounds to reach parasites inside of host cells – where they spend the majority of their time in this phase of the life cycle.

More recent modifications of the assay have reduced the length to less than 1 week – parasites are injected on day 0 and the first imaging and first drug treatment is given at day 2 (Figure 3). Only 1 treatment dose is needed for highly effective compounds such as BZ, but our standard protocol is to treat twice (days 2 and 3). A second imaging is done at day 6 and the relative change in parasite growth is determined by comparing pre- (day 2) and post- (day 6) treatment images. Both luminescent and fluorescent parasite lines work well for these assays Figure 3 and [30]; fluorescent parasites have the added advantage of not requiring injection of substrate in order to image.

A particularly important aspect of this short term assay is that compounds that are effective at achieving cure in the long-term treatment followed by immunosuppression protocol (e.g. BZ and posaconazole (POS)) are also highly effective at controlling parasite growth in the short-term assay [30]. Alternatively, those compounds that fail to cure mice in the long-term, including ones that strongly suppress epimastigote and amastigote growth *in vitro*, and suppress the level of parasites in the blood of acutely infected mice, also fail to suppress parasite replication in the tissues in the short-term assay. Thus this short-term assay appears to faithfully evaluate the characteristics that make an anti-*T. cruzi* compound highly effective and capable to achieving parasitological cure. Using this system we have screened more than 150 compounds in a few months and have identified some promising leads which are under a more extensive *in vivo* screening.

In addition to assessing compound effectiveness versus *T. cruzi*, the short-term assay yields at the same time data on compound toxicity *in vivo* (a number of compounds show toxicity (death) in mice in as few as 1–2 doses). It should also be noted that this rapid assay is a screening tool and will miss compounds which, by virtue of their structure, have poor bioavailability or *in vivo* stability. Thus, negative results in this assay should not be used to rule-out otherwise promising compounds (which might be improved by other modifications). However positive results indicate not only good anti-*T. cruzi* activity but also relatively low *in vivo* toxicity and good distribution.

Both the long and short-term assays are also providing insights into optimization for treatment protocols using existing drugs. The toxicity of benznidazole and nifurtimox, is thought to be cumulative [5] so any regiment that reduces the total drug dose should reduce toxicity. Preliminary results using the long-term treatment protocol suggest that parasitological cure in mice can be achieved using a much reduced frequency of doses (Bustamante, in preparation). Also being explored in the long-term treatment model are regimens that combine promising new drugs having good safety profiles with doses of BZ or NFX that are below toxicity thresholds.

4. Surrogate markers for treatment efficacy in Chagas disease

Monitoring treatment efficacy in human *T. cruzi* infection remains not only a major challenge but also a huge impediment to the development of new therapies. The improved methods for drug discovery noted above are useless if these drugs cannot be tested and proven effective in humans. The problem here is actually a direct result of the efficiency of immune responses in controlling *T. cruzi* infection to the point of making infection detection very difficult. This is especially problematic in those with chronic infection – the vast majority of cases requiring treatment. Detection of *T. cruzi* in chronically infected subjects always requires an amplification step – either of the parasite themselves using blood cultures or the insect vectors themselves as a “culture vessel” – a practice known as xenodiagnosis – or of parasite DNA by polymerase chain reaction (PCR). And even with amplification, direct confirmation of *T. cruzi* infection is possible in only a fraction of individuals – sometimes as few as 10% but rarely greater than 50%. Thus, using even the best technology, failure to detect *T. cruzi* is more the norm than the exception. This conclusion also means detection of *T. cruzi* after drug treatment is indicative of treatment failure but the lack of detection is largely meaningless, even in those who were positive prior to treatment.

Two surrogates of parasitological cure have been used to assess treatment effectiveness in humans – negativization of conventional diagnostic tests [40–41] and the prevention of progression of symptomatic disease [42–44] Unfortunately, changes in both of these parameters requires years if not decades of follow-up in those treated many years after the initial infection [45], making the methods unacceptable for the evaluation of new treatments.

Recent developments in serological tests may offer some improvements in this regard. By measuring changes in antibody titers to individual recombinant proteins among a set of ~15 used in a multiplex format, it is possible to detect consistent deceases in antibody titers as early 12 months post-treatment [46–47]. Similar changes are more difficult, or impossible to detect over the same time period using conventional serological tests, which mostly use whole parasite preparations, or pools or fusions of a few proteins. Thus, the ability to monitor the responses to individual parasite proteins seems to make a significant difference when using serology as a marker for treatment efficacy.

In mice – the only model in which definitive cure following drug treatment has been demonstrated [34] – immunosuppression is used to determine if treatment has been effective (see above). Although this protocol cannot be routinely used humans, in a single chronic human Chagas disease case where immunosuppression was required to treat systemic lupus 13 erythematosus, experimental use of POS was found to resolve the infection when BZ treatment had failed [48]. Mice cured of chronic *T. cruzi* infection via BZ treatment exhibit a lower frequency of *T. cruzi*-specific CD8+ T cells and an increase in the number of a subpopulation of memory T cells known as central memory T cells (T_{CM}), as compared to their untreated counterparts [34]. T_{CM} cells, in contrast to T effector memory cells (T_{EM}), express the L-selectin receptor (CD62L) as well as IL-7Rα (CD127), molecules involved in T cells entry into lymph nodes and Peyer's patches and homeostatic maintenance of memory T cells, respectively [49–50]. A shift in the *T. cruzi*-specific CD8+ T cells from the predominantly T_{EM} phenotype (CD62Llo, CD127lo) characteristic of the persistent infection to a majority T_{CM} cell population (CD62Lhi, CD127hi) is also seen when cure is obtained using POS or the nitrotriazole derivative NTLA-1 (Bustamante, et al., in preparation). Thus, T cell phenotypic markers have potential as surrogates for the assessment of drug efficacy and cure.

In human infections, the tracking and phenotyping *T. cruzi* –specific T cells is complicated by their low frequency and largely unknown antigen specificity [51–52]. Nevertheless, alterations in *T. cruzi*–specific T cell responses can be detected in a substantial proportion of BZ-treated subjects and these changes correlate with changes in antibody titers detected by multiplex tests [47]. Similar alterations in T cell responses were not evident either prior to treatment in the same individuals or in untreated subjects over the same observation time. These are technically more challenging tests to perform, as compared to serology, and so are not likely to be easily implemented in non-research laboratories. Nevertheless, they may be useful as an adjunct, especially in this situation where other quality benchmarks are limiting. 14

5. Conclusions

The tools for in vitro and in vivo screening of anti-*T. cruzi* compounds have improved dramatically in the last few years. Very high throughput (millions of compounds) in vitro screening assays are now possible. There is also substantial interest in the screening of these large compound libraries by entities with access to them (e.g. big Pharma and public-private partnerships like Drugs for Neglected Diseases initiative (DNDi)). So expectations are high that a substantial number of hits and leads will emerge from these screens in the coming years. The in vivo follow-up screening assays recently developed are among the fastest and most definitive in the infectious diseases area, leaving few impediments to quickly determining the in vivo efficacy of compounds. Useful surrogate markers for treatment efficacy in humans are also emerging although additional investigation is needed to validate these.

6. Expert Opinion

Drug development for Chagas disease has been crippled by two exasperating situations: 1) the persistent view that the disease is not treatable as an infectious disease because of a putative autoimmune etiology, and 2) the failure to appreciate the benefits of treatment irrespective of the stage of infection (e.g. acute or chronic). In short, most infected patients are untreated because the data on the cause of the disease and the benefits of treatment are limited or misinterpreted. Added to this is the conundrum that proving treatment efficacy in a persistent, low-level infection such as *T. cruzi* is very challenging and the fact that research in Chagas disease is grossly underfunded, even compared to other neglected diseases [53]. The end result is a situation where there is little impetus for the development of new drugs – because they cannot be easily tested to validate their efficacy and will not be widely used even if proven efficacious. Fortunately, this situation is changing. It is now widely appreciated that Chagas disease is, in fact, a persistent parasitic infection that requires treatment and parasitological cure in order to prevent progressive clinical disease. Data on the efficacy of current drugs in achieving cure and reducing disease severity is growing, despite the fact that these compounds are not optimal and are under-utilized. The combination of these advances with the scientific innovations reviewed above provides the opportunity for rapid progress in drug discovery for Chagas disease. Several additional high-throughput screens for in vitro-active compounds are likely to be completed in the coming years and the tools for more informative in vivo evaluation of hits and leads from these screens are now in place. Barriers to the realization of efficient clinical trials for promising compounds remain: In the absence of a method to reliably detect persisting infection, surrogates of cure will have to be used. Just as we rely on the detection of immune responses to certify the presence of infection, we will very likely have to depend on the immune system to indicate when treatment has worked. Several promising options for surrogate markers exist and the proposed clinical trials for the licensed anti-fungal posaconazole by Merck and the ergosterol biosynthesis inhibitor ravuconazole pro-drug E1224 by DNDi and Eisai in the near future will provide opportunities for more fully evaluating these and other biomarkers of cure in *T. cruzi* infection and Chagas disease. As importantly, national and international agencies have to do a much better job of coordinating research and development efforts and cultivating the funding sources for all of these efforts with the potential for significant advancement in drug development for Chagas disease.

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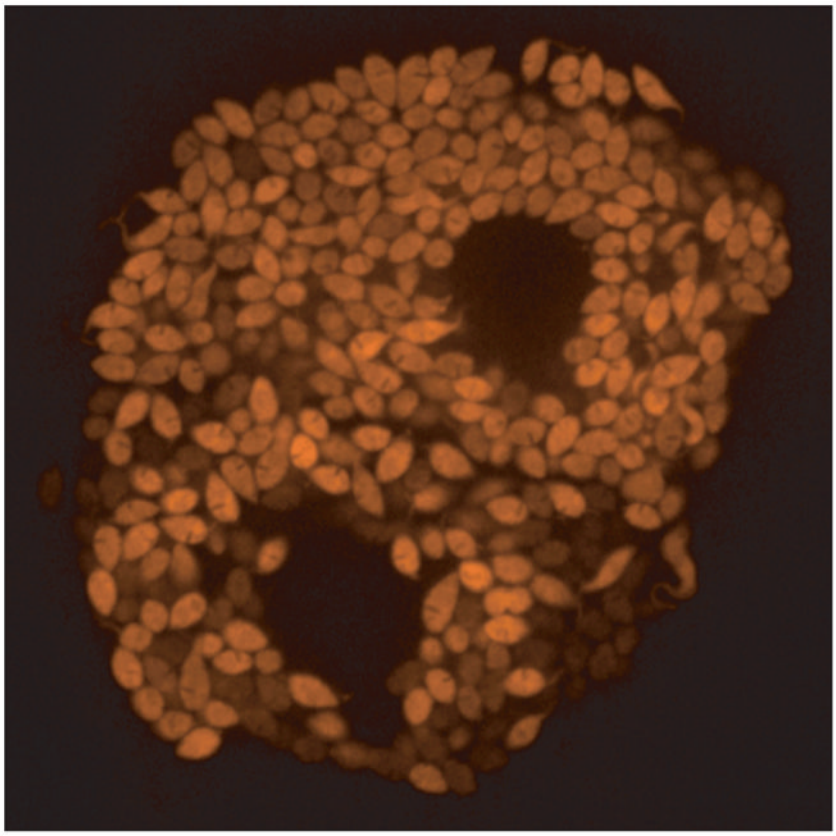

Figure 1.
Image of tdTomato-expressing amastigotes of *T. cruzi* replicating in vitro within host cells.

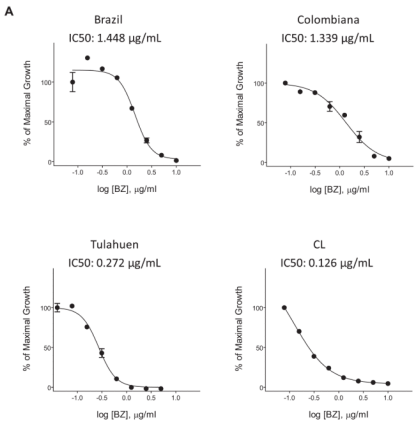

Figure 2.

Monitoring effect of benznidazole on growth of the indicated strains of *T. cruzi* amastigotes in Vero cells by the expression of the tdTomato protein. IC50 were calculated at 3 days of treatment.

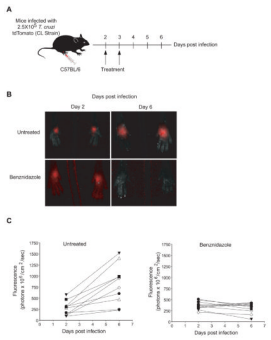

Figure 3.

(A) Schematic of the short *in vivo* assay used to screen anti-*T. cruzi* compounds in 1 week. Mice are infected in the hind foot pads with 2.5×10^5 *T. cruzi*-tdTomato trypomastigotes of the CL strain, and the specific treatment (Benznidazole) was given at days 2 and 3 postinfection. B) Parasites are imaged at day 2 and 6 post-infection. C) Quantification of the fluorescent signal from the individual mice at day 2 and 6 post infection. The efficiency factor (EF) was calculated using the following formula: [Fluorescence of Treated mice day 6 - Fluorescence of Treated mice day 2)/(Fluorescence of Untreated mice day 6 - Fluorescence of Untreated mice day 2)] X 100.