In Vitro Imaging of *Trypanosoma cruzi* Host Infection Systems for Evaluation of Compound Activity

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Statement of originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

__________________________
Melissa Sykes
Abstract

Chagas disease, caused by the protozoan parasite, *Trypanosoma cruzi*, is a leading cause of infectious myocarditis in the world and a major cause of morbidity and mortality in endemic areas, in Central and South America. The parasite is not limited to the human host and has a number of mammalian reservoirs. Besides the Triatominae vector, transmission can occur orally from contaminated food, congenital infection or blood and organ donations, meaning that the disease has also spread to non-endemic areas. There are two stages of Chagas disease, the acute and chronic stages. The acute stage may present as a non-specific febrile disease, however during the chronic phase, heart tissue becomes infected, and parasite persistence and autoimmunity results in heart disease, often causing mortality. The drugs used to treat Chagas disease have associated toxicity and in addition, there are doubts about their efficacy in treating the chronic disease. Currently there are few compounds that are clinical candidates for Chagas disease, it is therefore essential that new compounds that are active against *T. cruzi* are identified and evaluated for their potential to progress through the drug discovery pipeline.

In an aim to identify compounds with activity against *T. cruzi*, a phenotypic, high-content, 384-well image-based assay was successfully developed to estimate the effect of compound treatment on *T. cruzi* amastigotes in 3T3 fibroblast cells. In the same well, the effect of compound activity on host cells could also be determined, as 72 hours post infection there was no damage to the host cells observed. During assay development a number of live and fixed-cell fluorescent dye combinations were applied to define *T. cruzi* infected cells, and these will be outlined. A separate, fluorescence based phenotypic live cell assay was developed to establish compound activity against the host cell-free
trypomastigote life cycle form. To develop the assays herein, the life cycle of *T. cruzi* was optimised in 3T3 (mouse embryo) and NHCF-A (normal human heart) fibroblast cell lines. The differentiation of *T. cruzi* epimastigotes, production of trypomastigotes from host cells and the health and age of both host cells and trypanosomes were considered. These assays were reproducible, with Z'-factors of >0.5 and were sensitive to selective and non-selective compounds, demonstrated by the activity of the known drug, nifurtimox and the general protein synthesis inhibitor, puromycin.

The image-based, *T. cruzi* amastigote assay was used to identify active compounds from a library of compounds with either known biological activity, or that have been US FDA approved. Twelve compounds were identified with confirmed activity ranging from 0.0294 µM to 4.5 µM with selectivity indexes of between >622 to >3.5. The azole antifungals ketoconazole, miconazole, clotrimazole and voriconazole; the anti-cancer drug, camptothecin; and the immunosuppressant myconefonolate were identified as active and have previously been reported with inhibitory activity against *T. cruzi*. This supports the use of the amastigote assay to profile further compound libraries against the parasite in the future. The FDA approved drugs ciclopirox olamine (CPX) and clemastine fumarate demonstrated novel activity against *T. cruzi* amastigotes and serve as promising hits for future development. Profiling of these compounds against the extracellular, trypomastigote form of the parasite has identified that CPX has activity against this life cycle stage, with similar activity in comparison to the control drug NFX. This compound was subsequently tested in an acute mouse model of *T. cruzi* infection, and was found to significantly decrease infection. Further *in vitro* profiling of CPX identified that iron chelation is functionally important for the induced cell death of *T. cruzi* amastigotes and trypomastigotes. The action of this compound was estimated over time against trypomastigotes and it was determined that an IC$_{50}$ value of
8.4 µM could be estimated following 12 hours incubation. This relatively short incubation to produce an effect may influence the \textit{in vivo} activity of this compound.

Separately, a library of compounds with previously identified activity against \textit{Plasmodium falciparum}, the Medicines for Malaria Venture (MMV) Malaria Box collection was also profiled against \textit{T. cruzi}. This library afforded 6 probe-like compounds and 4 drug-like compounds with activity against the \textit{T. cruzi} amastigote ranging from 0.0544 µM to 4.64 µM, with selectivity indexes ranging from >169 to >7.9. The suitability of these as hit molecules for Chagas disease research is discussed.
Acknowledgements

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**Oral Presentations**


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Conference Assistant: Keystone conference, Drug Discovery for Protozoan Parasites (J1). Jan 15-20 2012. Santa Fe, New Mexico, USA.

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Best Student Presentation Award from the Australian Society of Parasitology (ASP), at the World Association of Veterinary Parasitology (WAAVP) 24th Annual Meeting Perth, Western Australia, 2013.
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<th>Description</th>
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<tr>
<td>3T3</td>
<td>Swiss mouse embryo cells</td>
</tr>
<tr>
<td>Ax</td>
<td>axoneme</td>
</tr>
<tr>
<td>AZA</td>
<td>22,26-azasterol</td>
</tr>
<tr>
<td>BB</td>
<td>basal body/pro-basal body pair</td>
</tr>
<tr>
<td>BENEFIT</td>
<td>benznidazole evaluation for interrupting trypanosomiasis</td>
</tr>
<tr>
<td>BESM</td>
<td>bovine embryo skeletal muscle</td>
</tr>
<tr>
<td>BZ</td>
<td>benznidazole</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CPX</td>
<td>ciclopirox olamine</td>
</tr>
<tr>
<td>CYP51</td>
<td>sterol 14-alpha demethylase</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DFO</td>
<td>desferrioxamine</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>DNDi</td>
<td>Drugs for Neglected Diseases Initiative</td>
</tr>
<tr>
<td>DOHH</td>
<td>deoxyhypusine hydroxylase</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FC</td>
<td>ferric citrate</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FDA</td>
<td>food and drug administration</td>
</tr>
<tr>
<td>FIC</td>
<td>fractional inhibitory concentration</td>
</tr>
<tr>
<td>FICI</td>
<td>fractional inhibitory concentration index</td>
</tr>
<tr>
<td>FOV</td>
<td>field of view</td>
</tr>
<tr>
<td>FP</td>
<td>flagellar pocket</td>
</tr>
<tr>
<td>FPP</td>
<td>farnesyl diphosphate synthase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GR</td>
<td>glutathione reductase</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>HAT</td>
<td>Human African trypanosomiasis</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HMGCR</td>
<td>HMG-CoA reductase</td>
</tr>
<tr>
<td>HPRT</td>
<td>hypoxanthine phosphoribosyltransferase</td>
</tr>
<tr>
<td>IHA</td>
<td>indirect haemagglutination</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>K777</td>
<td>N-methyl-Pip-F-homoF-vinyl sulfonyl phenyl</td>
</tr>
<tr>
<td>kDNA</td>
<td>kinetoplast DNA</td>
</tr>
<tr>
<td>KP</td>
<td>kinetoplast-posterior distance</td>
</tr>
<tr>
<td>LCMS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LIT</td>
<td>liver infusion tryptone / tryptose</td>
</tr>
<tr>
<td>LNCaP</td>
<td>androgen sensitive human prostate adenocarcinoma</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MMV</td>
<td>Medicines for Malaria Venture</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NFX</td>
<td>nifurtimox</td>
</tr>
<tr>
<td>NHCF-A</td>
<td>normal human atrial cardiac fibroblasts</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>NOAEL</td>
<td>no observable adverse effect level</td>
</tr>
<tr>
<td>NP</td>
<td>nucleus-posterior distance</td>
</tr>
<tr>
<td>NRF2</td>
<td>Nuclear factor erythroid 2</td>
</tr>
<tr>
<td>PAINS</td>
<td>pan assay interference compounds</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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</table>
PFR paraflagellar rod
PFT protein farnesyltransferase
PgP P-glycoprotein
PPP pentose phosphate pathway
PSC3H transformed mouse skin fibroblasts
REOS rapid elimination of swill
RPMI Roswell park memorial institute
SCC saline sodium citrate buffer
SD standard deviation
SI selectivity index
SOD superoxide dismutase
SPSS Statistical Product and Service Solutions
SQS squalene synthase
TAU3AAG artificial triatomie urine supplemented with with L-proline, L-glutamate, L-aspartate and glucose
TcNTR *T. cruzi* mitochondrial nitro-reductase
TcTR *T. cruzi* trypanothione reductase
Td thermal denaturation
TR trypanothione reductase
UDP uridine diphosphate
WHO World Health Organisation
### Glossary of Terms

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
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<tr>
<td>ASKA library</td>
<td>a set of <em>E. coli</em> K-12 open reading frame (ORF) genes</td>
</tr>
<tr>
<td>Asthenia</td>
<td>weakness</td>
</tr>
<tr>
<td>Balb/c</td>
<td>an albino laboratory mouse strain used for basic research and non-clinical drug discovery</td>
</tr>
<tr>
<td>Chagasasin</td>
<td>an endogenous inhibitor of <em>T. cruzi</em> cruzipain</td>
</tr>
<tr>
<td>CL strain</td>
<td>a strain of <em>T. cruzi</em>, isolated from <em>Triatoma infestans</em>, of TcVI lineage</td>
</tr>
<tr>
<td>Cruzipain</td>
<td>a cysteine protease unique to <em>T. cruzi</em></td>
</tr>
<tr>
<td>Dm28c</td>
<td>a clone of <em>T. cruzi</em>, isolated from an opossum</td>
</tr>
<tr>
<td>Dyspnoea</td>
<td>breathing difficulties</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>a membrane lipid found mainly in yeast and fungi</td>
</tr>
<tr>
<td>galE</td>
<td>gene that encodes UDP-galactose 4-epimerase, which epimerizes UDP-galactose and UDP-glucose in the galactose metabolism pathway</td>
</tr>
<tr>
<td>Glucuronidation</td>
<td>enzymatic modification of drugs and other xenobiotics during metabolism</td>
</tr>
<tr>
<td>Hypusine</td>
<td>a eukaryotic amino acid, essential for polyamine incorporation into proteins</td>
</tr>
<tr>
<td>Isobologram</td>
<td>a graph of the fractional inhibitory concentrations of compounds to assess synergy/antagonism</td>
</tr>
<tr>
<td>Kinetoplastid</td>
<td>mitochondrial DNA of kinetoplastid protozoa</td>
</tr>
<tr>
<td>Lipinski’s rule of 5</td>
<td>physiochemical properties of a compound to evaluate drug likeness</td>
</tr>
<tr>
<td>LLC-MK2</td>
<td>normal <em>Macaca mulatta</em> monkey kidney cells</td>
</tr>
<tr>
<td>Loewe additivity theory</td>
<td>a drug does not interact with itself therefore this combination will be additive</td>
</tr>
</tbody>
</table>

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LP10 \(\alpha-[(4\text{-(methylcyclohexyl)}\text{carbonylaminol})-N-4\text{-pyridinyl-1H-indole-3-propanamide}\]

Mevalonate pathway metabolic pathway essential to sterol biosynthesis

Michael acceptor an electron poor organic compound

Myalgia muscle pain

Neuropathy degenerative state of the nervous system

of the TcVI lineage

P-glycoprotein a multi-drug transporter in eukaryotes

Pruritus itching

RAW264.7 mouse leukaemic macrophage cell line

Sterol essential lipid molecules in eukaryotes

Topoisomerase class of enzyme involved in DNA supercoiling

Tulahuen strain a strain of \(T. cruzi\) isolated from \(Tiatomina infestans\),

of TcVI lineage

VERO African green monkey kidney cells

Xenodiagnosis diagnosis of \(T. cruzi\) infection using the Triatomine vector

Y strain TCII reference strain of \(T. cruzi\) isolated from an infected patient

Z'-factor a statistical measurement of assay reproducibility
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Chapter 1: General Introduction

1. General Introduction

American Trypanosomiasis, or Chagas disease, is caused by the protozoan parasite, *Trypanosoma cruzi*. The disease is estimated to cause 14,000 deaths per year [1] and has been recognized by the World Health Organisation (WHO) as one of the world’s 17 most neglected tropical diseases [2]. Chagas disease is transmitted by a number of species of the Triatoma bug and the disease is endemic to areas in which these vectors are prevalent, mainly in Central America, South America and Mexico. There have recently been reports to suggest that oral transmission of the parasite also occurs through consumption of food contaminated with Triatomine faeces, or meat from hunting animals [3]. Housing is an important catalyst for infection, particularly in poor, rural areas, where houses commonly consist of thatched roofs and / or mud walls [4], thus providing an area in which the vector, human and intermediate domestic and sylvatic hosts live in close proximity [5]. Population movement has also meant there have been incidences of the disease in non-endemic countries that do not have the insect vector, including Japan, Australia, and countries within Europe (Figure 1) [6].
There are two stages of Chagas disease, the acute and the chronic phase. The initial stage is usually asymptomatic, or may present as flu-like symptoms [7]. In untreated patients with severe symptoms of Chagas disease, the mortality rate is around 5-10% [8]. The cause of death in acute Chagas disease can be as a result of cardiac failure, meningitis, or encephalitis [9]. Individuals infected with the disease that are asymptomatic can remain so for up to 10 to 30 years [10]. The later and chronic phase results when the cellular response limits parasite proliferation leading to a sustained inflammatory reaction [11], which can ultimately result in digestive damage, with gastrointestinal complications such as constipation, abdominal fullness, acute abdominal pain [12], and enlargement of parts of the digestive tract, or megaesophagus and megacolon [13]. The heart is the most severely affected organ, usually associated with fatal cases of the disease [13],

Figure 1.1. World distribution of Chagas disease in endemic and non-endemic countries. Red shows Chagas endemic areas. Red arrows indicate movement and incidence of disease in non-endemic countries. Reproduced from Coura and Vinas, Nature (2010) [6].
although death can also result from meningitis or encephalitis [14]. Chronic Chagas heart disease can present as heart arrhythmia, thromboembolic events [15] or atypical aneurysms [16]. Sudden cardiac death is linked with left ventricular dysfunction, decreased contractility and increased volume in the heart [17]. In 20-30% of infected individuals, Chagas disease results in cardiomyopathy or megaoesophagus / megacolon [18].

There are 6 discrete sub-typing units of *T. cruzi* based on molecular markers and biological features, classified as *T. cruzi* I to *T. cruzi* VI [19]. A strict separation of human and sylvatic life cycles is not clearly defined, as recent discovery of oral routes of infection mean that the vector is not the only means of infection [3], and genetic sub-types are not restricted to either animal or human hosts. However, in human infections, there has been a predominance of *T. cruzi* I in the USA, Mexico, Central America and countries in the north of South America, whilst TcII, TcV and TcVI are more concentrated in the Southern Cone countries [20]. Due to the limited number of studies undertaken to investigate the genetic basis of *T. cruzi* and related disease outcomes, a defined link between the two has not been well established. However, there has been a link established in Venezuela and Columbia between patients with cardiomyopathy and TcI, and TcII-TcV have been linked to digestive syndromes [21,22]. Other reports show TcI to be predominantly present in the bloodstream, whereas TcII mostly presents in cardiac tissue [22,23]. In a recent study in Argentina, it was shown that TcI isolates induced more skeletal damage and TcVI isolates are associated with inflammation in the heart [24]. However, more extensive studies are required before a definitive genetic link between specific isolates and disease outcomes and severity can be established.
There have been a number of *T. cruzi* strains that have been adapted for the laboratory. TcI reference strains include TCC, Sylvio X10, G and its clone D11, EP, CA-I CL-72 and its clone K98, MIRANDA CL78, 13379 cl7 and SE 9V [25,26], whilst the Y strain is a reference strain for TcII, and the Tulahuen and CL Brener strains have been stated to originate from the TcVI lineage [26]. In the majority of laboratory studies including infection of mammalian cells *in vitro*, the Tulahuen or Y strains of the parasite have been commonly used [27-34]. Since the Tulahuen strain has been associated with the chronic phase of the disease, and inflammation of the heart, it has been employed as the parasite strain in these studies.

1.1 Life cycle of *T. cruzi*

Chagas disease is transmitted to humans by an insect vector, the domestic Triatominae bug, of which there are 140 species that vary geographically [35]. The vector grows to high densities in domestic environments, residing in poor quality housing in endemic areas [36]. Triatomines have hence been targeted for control measures and removal of the vector has been successful in some areas including Chile, Uruguay, Brazil and parts of Argentina and Paraguay [20]. However, most species of *T. cruzi* maintain enzootic life cycles, which complicate potential control measures. The parasite has sylvatic hosts including squirrels, birds, opossums, mice [37], armadillos [38] and domestic hosts including dogs, cats, pigs, goats [39] and caviomorph rodents such as guinea pigs [40]. The sylvatic and domestic infection of *T. cruzi* is important not only in acting as reservoirs for the disease for transmission to the insect vector, but also via direct infection to humans from mammalian hosts. Although the sylvatic/ domestic host to human contact processes that cause transmission are not completely understood [36,41], it is thought to include ingestion of contaminated meat and food contaminated with excrement from infected animals [3]. Many families in endemic areas supplement
their meals by raising guinea pigs in domestic environments as well as armadillos and small mammals, a large number of which can be infected with T. cruzi [20]. Oral infection can also occur from consumption of food contaminated with Triatomine faeces, such as sugar cane [42]. Other modes of transmission of T. cruzi, not involving vectorial transmission, have been reported via blood transfusions in both endemic and non-endemic countries [43], due to either lack of screening of donor samples or poor sensitivity of blood screening methods [44]. As the disease can be asymptomatic, Chagas disease can be passed from mother to child for several generations [45]. Collectively, these factors contribute to the difficulty in the control of Chagas disease and consequently it is thought that the disease will never be eradicated [46].

A feature of all mammalian Chagas disease infections, are the non-replicative bloodstream trypomastigote form, which infect host cells and, once internalised, differentiate into the replicative amastigote form (Figure 1.2). Within the host cell, amastigotes differentiate into trypomastigotes, and are released to form subsequent infections. Trypomastigotes are taken up by a blood meal of the insect vector and differentiate into epimastigotes in the insect mid gut. In the hind gut, epimastigotes transform into infective metacyclic trypomastigotes, which are transmitted to mammalian hosts when the insect has a blood meal and parasite infected faeces enter the bite wound site. It is suggested that during the acute stage of Chagas disease, extracellular and intracellular parasites are main contributors to the disease, whilst in the chronic or late stage, evidence suggests that persistence of intracellular forms may be responsible [47].
Figure 1.2. The life cycle of *T. cruzi*. A blood meal is taken by the Triatomine bug (1), containing trypomastigotes (2). Trypomastigotes differentiate into epimastigotes (3) and multiply in the mid gut (4), differentiate into metacyclic trypomastigotes in the hind gut, and are transmitted to the host bloodstream in a blood meal (6). Trypomastigotes invade cells, form amastigotes (8-11) and differentiate into trypomastigotes (12). Trypomastigotes burst from the host cell (13) to either infect the vector bug (15) or reinfect mammalian host cells. Reproduced from Teixeira et al (2012) [48].

The types of host cells infected depend upon the stage of infection. In the initial acute stage all types of nucleated cells are targets for infection. The main types of cells that have been found to be infected from human histopathology include the mononuclear phagocytes, muscle cells, bone marrow, fibroblasts and those present in the CNS [14,49] and heart [50]. In the CNS, the main cell types infected are glial
cells and astrocytes [14]. In the later chronic phase, it is difficult to demonstrate the presence of parasites in peripheral blood or tissues and identification relies upon serological testing [51]. The mechanism by which tissue damage occurs is still not clear and different hypotheses have been formulated with respect to the pathogenesis of symptomatic chronic Chagas disease. There is evidence that parasite infection can translate into the clinical expression of the disease, however there is also evidence to support autoimmune response damage of target tissue that is free of *T. cruzi* infection [16,52]. There may also be interplay between these various mechanisms. Treatment of the chronic disease is therefore challenging as the biological basis for tissue damage is not well defined. If there is damage caused by an autoimmune mechanism, vaccines could potentially contribute to chronic disease and if parasite persistence is a cause, use of immunosuppressive therapy has the potential to cause re-activation of the disease [53]. If parasites become latent and persist [9], consideration may need to be given to drugs that are able to distribute into deep tissue.

1.2 Chemotherapy of Chagas disease

1.2.1 Side effects of drugs used to treat Chagas disease

The nitro-derivatives, nifurtimox (NFX) and benznidazole (BZ) are the only two drugs currently approved for the treatment of both acute and chronic *T. cruzi* infection. These drugs have associated toxicity and cause side effects including nausea, vomiting, severe weight loss, insomnia, depression, convulsions, vertigo and disorientation [54]. The severity of these side effects can often result in the treatment not being completed or forcibly stopped. A study of BZ tolerance in a cohort of 100 adult patients, with a mean age of 38.7 years, treated in the chronic or intermediate phase of the disease [55] shows treatment was ceased in 9% of
cases due to poor gastrointestinal tolerance, fever or severe urticaria (hives). The most common side effects experienced during treatment were headache (56.2%), urticarial rash (43.4%), paresthesia (“pins and needles” sensation) (27.6%) and gastrointestinal disorders (15%). Recently it has been shown that of a cohort of 54 patients treated with BZ for chronic Chagas, with a mean age 37 years, that 20.4% had to have therapy suspended mainly because of drug related fever and skin disorders [56]. The recommended treatment schedule for BZ is 5-8 mg/kg/day, split in to 2 days for 60 days for both acute and chronic infection [55-57]. Although there is not much known about the pharmacokinetic profile of BZ, recent studies have identified no relationship between serum concentration and adverse side effects [56] and further optimisation of dosing schedules is required in an effort to improve tolerability. A study outlining the treatment of children with BZ found there was a relationship between age and side effects, with a lower incidence and decreased severity of adverse effects in children < 7 years [58]. Therefore age may be a factor to consider in future dosing studies with the use of BZ. Currently, the Benznidazole Evaluation for Interrupting Trypanosomiasis (BENEFIT) trial, to determine the safety and tolerability, as well as the effectiveness of BZ in reducing mortality and cardiovascular outcomes of patients with chronic Chagas disease, is being undertaken [59] and will improve our understanding of the safety of this drug. In this pilot study a group of 600 T. cruzi infected patients, from 18-75 years of age will be treated with BZ and scored for minor effects, significant adverse effects and toxic effects will be monitored through liver and renal function [60].

BZ is believed to be generally better tolerated than NFX, although there are no controlled studies to show this [61]. The use of NFX can depend upon the disease endemic region. Mexico, for instance, uses NFX as a first line treatment for Chagas, as BZ is not a treatment supported within the national guidelines. However the reasoning for this is unknown and there is a lack of data related to
the use of either BZ or NFX to treat Chagas worldwide [61]. The tolerance of NFX has not been as widely reported. A study on the treatment of 81 chronically infected patients treated with NFX, with a mean range of 39, revealed 32% of patients ceased treatment due to either pruritus (itching), asthenia (weakness), neuropathy, dyspnoea (breathing difficulties), headache, myalgia (muscle pain), rash, or a combination of these [62]. The main side effects associated with NFX treatment were headache (73%), anorexia (74.7%), nausea (54.3%) and abdominal pain. The treatment schedule is similar to BZ, with administration of 10 mg/kg/day, divided into 3 doses for 60 days. Simplification of treatment regimes and improvement of tolerability is warranted to improve the safety profile of NFX.

Figure 1.3. The drugs currently used to treat Chagas disease (A) Benznidazole; (B) Nifurtimox.

1.2.2 Diagnosis of Chagas disease

During the acute phase of infection, when levels of parasitaemia are the highest, xenodiagnosis, haemoculture / or buffy coat examination are often undertaken to define infection [63]. Xenodiagnosis is the exposure of the T. cruzi Triatomine vector to blood samples and following the detection and development of parasites [64]. Haemoculture requires samples of blood to be added to media and growth of
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the parasite over a period of time is determined [65]. The buffy coat is a concentrated sample of leukocytes prepared from peripheral blood [66], which, during infection contains trypomastigotes [67]. Both xenodiagnosis and haemoculture could take up to weeks or months to enable multiplication of parasites, require a colony for growing the vector, or require absolute sterility, conditions which are difficult to replicate in the field [68]. These tests are unable to detect low parasite numbers, and although they are sufficient, when applicable, to detect acute *T. cruzi* infection, they are limited in their usefulness in diagnosing chronically infected patients or for monitoring drug efficacy.

Application of the polymerase chain reaction (PCR) to detect *T. cruzi* in blood samples has shown more sensitivity in detecting *T. cruzi* infection, particularly in patients with chronic disease [69]. PCR procedures rely upon amplification of the parasite DNA from blood *in vitro* and although this method affords some increased sensitivity, variability and lack of specificity has been demonstrated [70]. Recently, an international survey that evaluated PCR methods for the detection of *T. cruzi* revealed large variations in accuracy and a lack of quality controls among the 48 PCR studies that were reviewed [70]. The volume and conservation of samples, the parasite sequences used, the primers, reagent and cycling conditions used have been shown to be contributory factors [68,70].

The method recommended by the World Health Organisation (WHO) for identification of *T. cruzi* infection is serological testing [71]. Serological testing detects developed antibodies against the antigenic mixture of *T. cruzi*, and three of the methods are widely used include indirect haemagglutination (IHA), indirect immunofluorescence (IIF) and enzyme linked immunosorbent assays (ELISA) [68]. Assessment of the cure of *T. cruzi* infection by antibody seroconversion usually involves several years of follow-up in adults, as a positive serological test results
could either indicate treatment failure or simply reflect a persistent antibody response [72]. However, in infants that develop acute or congenital Chagas, seroconversion can occur as soon as 3 months of age following treatment [73]. To define a cure in adults, it is recommended by WHO that a 10-20 year serological follow up should be performed [71]. It is also recommended by the Ministry of Health in Brazil that two or more methods are available to confirm a diagnosis of Chagas disease [74]. Further serological markers are desperately needed and are currently being investigated. These have the potential to provide valuable tools in accurately determining infection and evaluating the efficacy of late stage treatment and drug efficacy [75,76].

1.2.3 Success of drug treatment in acute and chronic phase Chagas

Treatment with either BZ or NFX in the acute stage of the disease is reported to produce a cure rate close to 100% in infants, demonstrated by reports of congenital cases of Chagas disease. Of 59 congenitally infected children, from between the ages of 15 days to 10 years, 87.2% of the population were cured, as determined by negative serology, following NFX treatment. This increased to 98% when children were under 3 years old and 100% when under 8 months old [77]. In a separate report, of 32 congenitally infected children, diagnosed at 6 months and treated with either BZ (28 infants) or NFX (3 infants), and monitored to 2 years, there was a cure rate of 93.8% of cases, assessed by negative serology and micro-hematocrit [78]. A more recent study compared 5 mg/kg/day of BZ for 60 days to a reduced treatment schedule of 7.5 mg/kg/day of BZ for 30 days, to treat newborns congenitally infected with Chagas [79]. There were 61 and 59 infants in each group, and an overall cure rate of 99% was observed for both groups. It remains to be seen if reduced treatment schedules are efficacious in adults. This aspect will be to a certain extent looked at in the BENEFIT trial, whereby 60 mg/kg/day of BZ
is adjusted to a maximum daily dose of 300 mg/day, related to a 5 mg/kg/day for 60 day schedule. Therefore a 40 kg individual will take 300 mg/ day for 40 days [60].

It is accepted that BZ and NFX treatment in the acute stage in adults is successful, and many papers that refer to the efficacy of these drugs, state around 80% efficacy in the acute stage and 20% in the chronic stage [80]. However, there are very few publications that show clinical results of treatment of acutely infected adults, and those that do show variable results at the level of efficacy. A study undertaken in 2002 outlines the outcome of 21 acutely infected patients, aged 0.7-60 years for 13 years post-treatment, following administration of 5-10 mg/kg/day of BZ for 32 days [81]. Of these individuals, 76%, showed negative serology, although the time it took to reach negative serology was not reported [81]. A recent publication reports a clinical follow up of 179 patients, aged between 2 and 72 years of age with acute Chagas, treated with BZ for 60-90 days, using antibody titres PCR diagnosis, xenodiagnoses and cardiac evaluation [82]. The clinical follow up was undertaken over a period of 7 years, and it was found that only 26.3% could demonstrate a serological cure, observed at around 4 years for these cases. There were 8.3% of the remaining patients that were positive by PCR, although only 72 randomly chosen patients were assessed by PCR. It would have been beneficial to compare PCR to serology testing for all of these samples. The variability therefore of reported cure rates could be due to factors such as the criteria used to define the cure, the variability of patient ages and how far the acute stage has developed in the patient prior to treatment being intiated [80]. There are no recent reports for the treatment of patients with NFX in the acute stage of the disease.
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Clinical evidence currently available is insufficient to support efficacy of BZ and NFX in chronic Chagas patients [59] and again this is compounded by the fact that the evaluation of the efficacy of these drugs is a controversial issue [83]. A recent study with a group of 21 chronic cases of Chagas, found by PCR analysis performed 13 months post NFX therapy, that there were either 6 cases or 4 cases where treatment failed, dependant on the PCR test employed [83]. The authors note that future determination of cure should involve follow up assessment with both PCR and serology. A separate study, using PCR and serology testing of 37 patients following treatment with NFX 4 years after treatment shows that in 97.3% of patients results could either indicate treatment failure or circulation of antibodies [72], although PCR was negative in all patients. These authors did not use PCR studies before treatment, highlighting poor controls and thus difficulty in assessing results. A more recent study revealed that incomplete treatment with BZ of 81 chronic patients, discontinued because of adverse effects, equivalent to 10 daily doses of 5 mg/kg, resulted in 20% cure by seroconversion [84]. The median time of seroconversion was 9 years, and this highlights the many years of follow up required. It also indicates reduced dosing schedules could be a consideration in the future.

Although the drugs used to treat Chagas have been shown in many cases to reduce or cure disease, a cure is difficult to define and current methods to diagnose the disease are lengthy. Safer and more efficient treatments, together with accurate and early post-treatment tests of cure are important for the successful clinical management of *T. cruzi* infected patients in the future.
1.2.4 Potential drug resistance of *T. cruzi* to current drugs

Drug resistance has been induced with both BZ and NFX drug pressure *in vitro*, and is an important consideration for future research. Independently acquired mutations of *T. cruzi* epimastigotes cultured under NFX drug pressure *in vitro* were identified in the gene encoding mitochondrial nitro-reductase (TcNTR) [85], and distinct drug-resistant clones were formed within a single population. The loss of one copy of TcNTR was not associated with a decrease in infectivity and clones were also cross-resistant to NFX. *In vitro* acquired resistance has also been attributed to the P-glycoprotein (Pgp) efflux pump in *T. cruzi*, demonstrated by overexpression of the genes TcPGP1 and TcPGP2 in epimastigotes, metacyclic trypomastigotes and amastigotes [86]. Strains isolated from human patients, domestic vectors and sylvatic reservoirs or vectors, have been found to exhibit resistance to both NFX and BZ *in vitro* [87, 88] The presence of naturally circulating strains with conferred resistance against BZ *in vitro* has also been documented with strains isolated from infected individuals in Columbia [89]. Similarly, there has been some correlation between *in vitro* results and sensitivity to BZ demonstrated with *in vivo* models of acute and chronic disease in mice [90]. However, determination of drug susceptibility was undertaken with the epimastigote form of the parasite. Moreno and co-workers found that there was no correlation between BZ susceptibility *in vitro* and the clinical outcome in patients, determined with epimastigotes [91]. Cultivation of the infective form of parasites isolated from humans and subsequent correlation of the susceptibility of these parasite forms to BZ/ NFX is thus warranted to gain more of an understanding of the role of resistance in human infections. Although drug resistance has not been definitively demonstrated when correlated with clinical results, demonstration of the ability of parasites to become resistant, albeit *in vitro*, could have implications for future treatment strategies [85]. Taking into consideration that the treatment
duration is often extended, for example NFX therapy is around 90 days [92], and thus fluctuations in drug concentrations due to poor compliance during this period, must be considered a potential contributory factor to resistance [93].

### 1.2.5 Mode of action of antichagasic drugs

The modes of action of both BZ and NFX against *T. cruzi* are not known, however may be due to the nitro moiety present in both drugs that has a non-selective and damaging redox function [94], or alternatively the generation of reactive intermediates by BZ and reactive oxygen species by NFX [54]. Reaction with nucleic acids or production of superoxide radicals causing damage to the parasite has been suggested from studies with the epimastigote form of the parasite for BZ and NFX, respectively [95,96]. Other studies have shown that, at least for NFX, that oxidative stress is not likely to contribute to the action of the drug, at least not against the epimastigote form of the parasite, [94] as redox-cycling was observed at concentrations of greater than 400 μM, two orders of magnitude higher than the concentration required for anti-proliferative activity. Another possibility is that BZ causes an oxidative response in the host, which could contribute to the anti-parasitic activity, and toxicity. It has been found that following treatment of rats with BZ that biomarkers of oxidative stress, such as the activities of catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST) and glutathione reductase (GR), were up-regulated [97]. In *T. brucei* it has been demonstrated that reduction of NFX by a trypanosomal nitro-reductase causes formation of a toxic nitrile derivative, equally toxic to mammalian and parasite cells. The mode of action of this derivative is not known, although the basis for parasite selectivity could be the action of the parasite type 1 nitro-reductase [98]. This would need to be confirmed in an infective culture of *T. cruzi*. It would be beneficial to determine the mode of action of these drugs, as this could aid the
development of new drugs with reduced toxicity that may be more selective toward parasite targets.

1.2.6 Validated *T. cruzi* cellular targets

There is no agreement in the literature about which drug targets are validated versus promising in *T. cruzi* research. Herein, validated targets will be considered those enzymes that have been identified in the intracellular, infective form of the parasite, are important for parasite development and chemical inhibitors that have demonstrated anti-parasitic activity show specific binding to the target of interest. Most importantly, inhibitors of these targets have shown activity *in vivo* [99] by demonstrating clearance of the parasite in at least a murine model of disease.

1.2.6.1 Sterol biosynthesis: Sterol 14-Alpha-Demethylase

There are a few validated targets of *T. cruzi* parasites, of which inhibitors show efficacy both *in vitro* and *in vivo* and inhibitors from some of these classes are currently in clinical trials for treatment of *T. cruzi* infection. Azole antifungals have historically shown activity against *T. cruzi*, including in mouse models for the disease [100-103], and have been demonstrated to target the cytochrome P450 enzyme sterol 14-alpha demethylase (CYP51), required for sterol biosynthesis [104]. CYP51 is also present in different phyla including plants, animals, fungi, lower eukaryotes yeast and bacteria. The enzyme catalyses the removal of the 14-methyl group from sterol precursors and inhibitors of this enzyme prevent binding of the substrate and oxygen activation [105]. Studies have shown that the azole antifungal, ketoconazole, reduces sterol components of *T. cruzi* epimastigotes [106], and through metabolic labelling and spectral studies it has been demonstrated that CYP51 is the likely target of a collection of di-substituted
imidazoles [107]. CYP51 expression is up-regulated in *T. cruzi* epimastigotes treated with sterol biosynthesis inhibitors including terbinafine and ketoconazole [108] and recently the crystal structure of *T. cruzi* recombinant CYP51 was shown to bind to the antifungal drugs posaconazole and fluconazole [109]. Sterol biosynthesis inhibitors have also been shown to reduce the sterol composition in amastigotes [110], and cause extensive ultra-structural alterations [110]. Of the sterol biosynthesis inhibitors, both voriconazole and ketoconazole are not curative in an acute model of *T. cruzi* infection following oral dosing *in vivo*, but have suppressed parasites and improved survival rates [111-113]. Similarly, fluconazole is not curative, however this compound has also displayed less *in vitro* activity than otherazole antifungals with demonstrated activity against *T. cruzi*, having an IC$_{50}$ estimated at 8 µM [114]. In contrast, IC$_{50}$ values of other antifungals with *in vitro* activity against *T. cruzi* include 0.001 µM for itraconazole and ketoconazole, 0.004 µM for voriconazole, 0.02 µM for miconazole and 0.0005 µM for posaconazole as determined utilising β-galactosidase transfected *T. cruzi* parasites to assess activity [115]. It is not clear why fluconazole was used in a mouse model due to the high IC$_{50}$ value in comparison to other azoles, although fluconazole was one of the first azoles identified as active against *T. cruzi*, with the corresponding inhibition of ergosterol synthesis identified *in T. cruzi* epimastigotes *in vitro* [116].

Cure rates have been reported with itraconazole treatment in a murine model of acute *T. cruzi* infection [117], however not in the chronic stage [118]. In the acute model, 120 mg/kg/day of the antifungal was administered orally for 9 to 7 weeks to achieve cure, defined by negative serology, haemocultures and histological sections [117]. In the chronic mouse model of infection, 100 mg/kg/day was administered for three months, with no beneficial results observed. Ravuconazole led to a 70% cure in an acute mouse model of *T. cruzi* infection, when mice were treated twice per day with 10 mg/kg of the compound [119], although using the
same dosing schedule in a chronic model of infection provided no cure, defined by positive serology and parasite detection [117]. Inability of this compound to effect parasites in the chronic phase may be due to a short terminal half-life of 4 hours in mice [103]. As the half-life of ravuconazole in humans is significantly longer at 4-8 days, it may potentially be curative in humans [103]. E1224, developed by the Japanese pharmaceutical company, Eisai to improve the absorption and bioavailability of ravuconazole, was recently evaluated in phase II trials to assess safety and efficacy in the treatment of the chronic phase of the disease in humans [103]. However, first reports of the results of this trial show that although this compound was able to clear infection following treatment, less than one third of the patients continued to maintain parasite clearance [120]. Posaconazole, a triazole derivative, has demonstrated 50-60% efficacy in a chronic mouse model of T. cruzi infection and is currently in clinical trials to determine efficacy in human chronic infection, either as a monotherapy [121], or in combination with BZ [122].

Those compounds that have successfully demonstrated activity in a murine model of Chagas are shown in Figure 1.4. It would be beneficial to assess the failures of some of these compounds observed in the chronic phase. It could be that compounds may not sufficiently penetrate tissues that contain persistent amastigote populations, and therefore do not possess the correct pharmacokinetic properties for this phase of the disease. It has been shown that T. cruzi amastigotes can contain up to 80% (by weight of total sterols) of cholesterol, thought to originate from host cells [123], although susceptibility of amastigotes to CYP51 inhibitors suggests that endogenous sterols are specifically required for survival [124]. The insect stage of T. cruzi contains greater quantities of mRNA of genes involved in sterol biosynthesis, which could suggest availability of sterols from the mammalian host [125]. It has recently been demonstrated that some CYP51
inhibitors have less than 100% efficacy \textit{in vitro} [126], however survival of amastigotes following treatment \textit{in vitro} has not currently been demonstrated. It has been suggested that Leishmania amasigotes can survive with greatly altered sterol profiles when cultured in the presence of low concentrations, although currently this is from a report of unpublished work [127]. The search for more inhibitors of CYP51 still continues, and recent efforts have focussed on the identification of inhibitors of the recombinant enzyme that also demonstrate \textit{in vitro} activity against the intracellular amastigote, by screening a library consisting of a selection of molecular scaffolds against \textit{M. tuberculosis} CYP51 [128]. This has identified the non-azole inhibitor Nalpha-[(4-methylcyclohexyl)carbonyl]-N- pyridin-4-yltryptophanamide (LP10) with a 60% cure rate observed in a murine acute model of \textit{T. cruzi} infection [129] (Figure 1.4).

![Figure 1.4](image.png)

\textbf{Figure 1.4.} Structure of \textit{T. cruzi} sterol 14-\textalpha{}-demethylase inhibitors that have resulted in curative activity in a murine model of acute Chagas disease. (A) Posaconazole, (B) Itraconazole, (C) Ravuconazole, (D) The non-azole inhibitor, LP10.
1.2.6.2 Cruzipain

The cysteine protease cruzipain, belonging to the papain family of enzymes was first discovered in cell free extracts of epimastigotes [130] and has since been suggested to be involved in a number of processes in *T. cruzi*. Chagasin, an endogenous inhibitor of *T. cruzi* cruzipain, has been over expressed in epimastigote cell lines which have demonstrated low rates of differentiation in comparison to wild type parasites [131]. This suggests that cruzipain is a probable requirement for the modulation of differentiation from the epimastigote to the infective metacyclic trypomastigote. Both the inhibitor and the protease have also been identified in the amastigote life cycle stage by labelling with gold-labelled antibodies [132], suggesting that modulation could also occur in relevant clinical life cycle forms. Collectively, these results suggest that cruzipain is involved in *T. cruzi* growth, differentiation and host cell invasion. The cysteine protease inhibitor N-methyl-Pip-F-homoF-vinyl sulfonyl phenyl (N-Pip-F-hF-VSφ; K1177 or K777) has been shown to covalently bind with recombinant cruzipain [133], has demonstrated *in vitro* activity against amastigotes, *in vivo* curative activity in an acute model of infection [134,135], and also has a curative action in *T. cruzi* infected, immuno-compromised mice [136]. In a model of *T. cruzi* infection in dogs, this compound protected the animals from cardiac damage and resulted in a suppression of infection [137]. K777 is currently in late clinical development for the treatment of Chagas disease [136]. To further confirm that K777 is specifically interacting with cruzipain in amastigotes, co-localisation studies would be beneficial. Studies of the localisation of chagasin, using antiserum raised in rabbits, show that it is localised in amastigotes on the cell surface and flagellar pocket and in trypomastigotes in the flagellar pocket and cytoplasmic vesicles [132] (Figure 1.5).
Figure 1.5. Localisation of chagasin in clinically relevant forms of T. cruzi. Areas of localization are circled in green (A) in the amastigote, chagasin is localized in the flagellar pocket and on the cell surface. (B) in the trypomastigote, localization is in the flagellar pocket and in cytoplasmic vesicles. Images are modified from Texeria (2012) [48].

1.2.7 Potential drug targets

1.2.7.1 Sterol biosynthesis: Sterol 24-c-transferase

Enzymes involved into the sterol biosynthetic pathway, other than CYP51, also have the potential to be future targets for T. cruzi chemotherapy (Figure 1.6). Sterol 24-c-methyltransferase (otherwise known as ERG-6), which alkylates sterols at carbon-24, and is vital to the biosynthesis of ergosterol, has been identified in the reservosomes of T. cruzi epimastigotes [138]. The methyl transferase inhibitor C22,26-azasterol (AZA) has been demonstrated to have in vitro activity against amastigotes in African green monkey kidney (VERO) cells with an IC_{100} of 100 nM and improved the survival of mice following in vivo infection, and although it did
not provide a cure, parasitaemia was reduced by 90%. This compound was also shown to potentiate the activity of the azole antifungal ketoconazole *in vitro*, by producing a synergistic effect in combination [139]. A library of synthesised azasterols was tested *in vitro* against *T. cruzi* Tulahuen strain amastigotes in mouse peritoneal macrophages with activities ranging from 6.1 µM to 64.6 µM [140]. A separate collection of azasterols were tested against *T. cruzi* amastigotes, yielding 5 compounds with IC$_{50}$ ranging from 1.71 µM to 3.46 µM with selectivity index (SI) values between 17 and 246 (Table 1.1). The authors note however that these compounds do not inhibit *T. cruzi* sterol 24-c-transferase [141] and that an investigation into the mode of action of these compounds is warranted. Those azasterols with reported activity identified against *T. cruzi* amastigotes *in vitro* are shown in Table 1.1.
### Table 1.1. IC<sub>50</sub> values and selectivity indexes (SI) of azasterols with identified activity in vitro against T. cruzi amastigotes. *Identified as an inhibitor of T. cruzi Sterol 24-c-methyltransferase. NR= not reported.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; value (µM)</th>
<th>Selectivity (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Compound 1" /></td>
<td>0.1 (EC&lt;sub&gt;100&lt;/sub&gt;)*</td>
<td>NR [139]</td>
</tr>
<tr>
<td><img src="image2.png" alt="Compound 2" /></td>
<td>0.3 (EC&lt;sub&gt;100&lt;/sub&gt;)*</td>
<td>NR [139]</td>
</tr>
<tr>
<td><img src="image3.png" alt="Compound 3" /></td>
<td>3.46</td>
<td>172 [142]</td>
</tr>
<tr>
<td><img src="image4.png" alt="Compound 4" /></td>
<td>4.70</td>
<td>&gt;140 [142]</td>
</tr>
<tr>
<td><img src="image5.png" alt="Compound 5" /></td>
<td>2.43</td>
<td>&gt;246 [142]</td>
</tr>
<tr>
<td><img src="image6.png" alt="Compound 6" /></td>
<td>2.54</td>
<td>&gt;229 [142]</td>
</tr>
<tr>
<td><img src="image7.png" alt="Compound 7" /></td>
<td>5.61</td>
<td>16.84 [142]</td>
</tr>
<tr>
<td><img src="image8.png" alt="Compound 8" /></td>
<td>1.71</td>
<td>17 [142]</td>
</tr>
<tr>
<td><img src="image9.png" alt="Compound 9" /></td>
<td>&lt;2.75</td>
<td>&gt;16.38 [142]</td>
</tr>
<tr>
<td><img src="image10.png" alt="Compound 10" /></td>
<td>&lt;2.08</td>
<td>&gt;242 [142]</td>
</tr>
<tr>
<td><img src="image11.png" alt="Compound 11" /></td>
<td>9.52</td>
<td>&gt;52.9 [142]</td>
</tr>
</tbody>
</table>
Figure 1.6. The sterol biosynthesis pathway: in mammals and yeast. The initial steps of sterol biosynthesis are conserved in yeast, mammals and trypanosomatids. Reproduced from Espenshade and Hughes (2007) [143].

1.2.7.2 Sterol biosynthesis: HMG-CoA reductase

HMG-CoA reductase (HMGCR), which catalyses a rate controlling step in the ergosterol biosynthesis pathway (Figure 1.6), has been identified in *T. cruzi* [144]. Lovastatin, an inhibitor of mammalian HMGCR in the cholesterol synthesis pathway, has been tested *in vitro* against *T. cruzi* amastigotes and showed poor activity, with a <30% reduction of infection in comparison to controls following treatment of infected cells with 1 µM of the compound [145]. However, lovastatin potentiated the effect of ketoconazole *in vitro*, having a synergistic effect. When ketoconazole and lovastatin were coadministered to mice infected with *T. cruzi*, in
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an acute model of infection, at 15 mg/kg/day and 20 mg/kg/day respectively for 7 days, parasites were suppressed to undetectable levels. Fluvastatin and cerivastatin have been reported as active against recombinant *T. cruzi* HMGCR [146], although activity against the parasite has not currently been published. Statins have been shown to have cardiovascular protective properties and low doses of simvastatin has been shown to ameliorate cardiac inflammatory remodelling in dogs infected with *T. cruzi*, although circulating parasite levels were not affected. It would be warranted to profile other statins against *T. cruzi* to determine their *in vitro* efficacy, as if it could be achieved, antiparasitic and anti-inflammatory activity would be beneficial particularly in the chronic stage of the disease [147].

1.2.7.3 *Sterol biosynthesis: Squalene synthase*

Squalene synthase (SQS) catalyses the first committed step in ergsterol synthesis in eukaryotes and in cholesterol synthesis in mammals (Figure 1.6), and this membrane bound enzyme has been identified in *T. cruzi* epimastigotes [145]. A specific inhibitor of human SQS, 3-(biphenyl-4-yl)-3-hydroxyquinuclidine, has demonstrated an IC₅₀ value of 3 µM against the amastigote form of the parasite [148]. Two inhibitors developed against human SQS, ER119884 and E5700 (Figure 1.7), inhibited growth of *T. cruzi* in VERO cells with IC₅₀ values of 11 and 8 nM, respectively [149]. Although E5700 reduced parasitaemia, in a murine model of acute *T. cruzi* infection, when dosed at 50 mg/kg/day for 30 days, this compound was unable to clear infection [150]. The requirement of some organs, such as the testes, to maintain elevated endogenous levels of cholesterol could pose a significant limitation to development of both HMGCR and SQS inhibitors as treatment drugs for Chagas disease [102]. Quinuclidine derivatives, which are active against the purified trypanosomal SQS enzyme, show activity against the
amastigote form of the parasite with moderate IC₅₀ values ranging from 4.7 to 16 µM and corresponding SI values of 4.4 and 17 times, respectively [151]. Research into this class of inhibitors is continuing, with an aim to develop compounds with improved activity and selectivity.

![Figure 1.7. Structure of human squalene synthase inhibitors which exhibit activity against *T. cruzi* amastigotes *in vitro*. (A) E5700 and (B) ER11988.]

1.2.7.4 *Mevalonate pathway: Farnesyl diphosphate synthase*

Farnesyl diphosphate synthase (FPP) is an enzyme involved in the mevalonate pathway leading to farnesylated proteins and to the biosynthesis of sterols, such as cholesterol and ergosterol (Figure 1.6). Bisphosphonates target this enzyme and have been used in therapy in humans for the treatment of bone disease, as FPP is also required for the prenylation of signalling proteins that regulate a variety of cell processes important for osteoclast function [152]. Prenylation is also involved in the formation membrane anchors in ras related G-proteins, essential to their function. As these proteins are often upregulated in cancer cells, FPP is also a target for cancer chemotherapy [153]. Bisphosphonates have poor activity against *T. cruzi* amastigotes, ranging from 65 µM for pamidronate [154] to 35 µM for 2-(4-imidazolyl)-1-hydroxyethane-1,1-bisphosphonate [155], and 20-70 µM for a panel of synthesised compounds [156]. Risedronate was identified as active against *T.
cruzi amastigotes at 50 µM, demonstrated following 96-120 treatment of infected VERO cells [157], although the IC$_{50}$ value was not reported. This compound was subsequently tested in a murine model of acute T. cruzi infection. Dosing as low as 1 mg/kg/day for 7 days reduced parasitaemia by 90% [158]. Longer treatment schedules should be undertaken in future studies.

1.2.7.5 Thiol dependant redox metabolism: trypanothione reductase

Trypanothione reductase (TR) is a key enzyme in the antioxidant metabolism of T. cruzi. The role of TR in thiol dependent redox metabolism is distinct from other eukaryotes and prokaryotes, thus providing a potentially selective target. There are reports on target based screening to identify inhibitors against this enzyme, isolated from the epimastigote [159], however there are few inhibitors with activity reported against the T. cruzi parasite. It has been suggested that TR is also present in trypomastigotes, via indirect measurement of NADPH consumption of trypomastigote soluble fractions [160]. From a collection of 2- and 3-substituted 1,4-naphthoquinone derivatives, designed to potentially inhibit T. cruzi TR, three polyamine derivatives exhibited IC$_{50}$ values below 10 µM against T. cruzi amastigotes, with corresponding activity against recombinant T. cruzi TR (TcTR), and no cytotoxicity to human macrophages above 25 µM [161], shown in Table 1.2. Assays were undertaken by Giemsa staining of Y strain infected MDCK (Madin-Darby canine kidney) cells, following exposure of infected cells to compound for 3 days. Further profiling of activity to define the SI would be necessary before considering these as hits. The authors report that the design and synthesis of further napthoquinone derivatives was underway. A recent study has outlined the synthesis and evaluation of napthoquinone derivatives against T. cruzi amastigotes, however these compounds display no selectivity toward the parasite [162].
### Table 1.2. The activity of napthoquinone derivatives against *T. cruzi* amastigotes in MDCK cells, and against recombinant trypanothione reductase (TcTR). Data from Salmon-Chemin et al (2001) [161]. SI = selectivity index of activity of compound toward *T. cruzi* amastigotes over Madin-Darby canine kidney (MDCK) cells.

<table>
<thead>
<tr>
<th>Structure</th>
<th>TcTR IC$_{50}$</th>
<th>IC$_{50}$ value <em>T. cruzi</em></th>
<th>SI (MDCK)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>1.7</td>
<td>4</td>
<td>&gt;6.25</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>1.4</td>
<td>9</td>
<td>&gt;2.78</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>0.45</td>
<td>4.3</td>
<td>&gt;55.5</td>
</tr>
<tr>
<td><img src="image4.png" alt="Structure 4" /></td>
<td>2.0</td>
<td>3.0</td>
<td>&gt;8.33</td>
</tr>
<tr>
<td><img src="image5.png" alt="Structure 5" /></td>
<td>1.7</td>
<td>4.0</td>
<td>&gt;6.25</td>
</tr>
</tbody>
</table>

1.2.7.6 Glyceraldehyde-phosphate-dehydrogenase: glycolysis

Glycolysis has been shown to be an essential process for energy production in *T. cruzi* axenic amastigotes [163], although not yet identified in the intracellular amastigote, because of the interferences caused by host cell metabolism [164]. The structure of the catalytic enzyme involved in glycolysis in *T. cruzi*, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), has been reported [165] and inhibitors against recombinant GAPDH have subsequently been synthesised, and a number
of compounds that are selective against the \textit{T. cruzi} enzyme have been identified [166,167]. Despite this, to date there are few reports of compounds with \textit{T. cruzi} GAPDH activity against the parasite. Bressi and co-workers report a series of adenosine analogues with micromolar activity against \textit{T. cruzi} GADPH, [168]. Only one compound displayed a mean IC$_{50}$ value against the parasite of $<$10 µM, at 7 µM, with a comparatively poor activity against the enzyme, with an IC$_{50}$ of 75 µM. Structure based design of inhibitors of \textit{T. cruzi} GADPH has afforded one compound, synthesized from a 6-iodopurine riboside analogue, with an IC$_{50}$ value of 3 µM against the intracellular amastigote and no activity displayed on 3T3 fibroblasts [164].

1.2.7.7 Glucose-6-phosphate-dehydrogenase: pentose phosphate pathway

Glucose is metabolised through the pentose phosphate pathway (PPP) in \textit{T. cruzi}, and generates NADPH, which in turn enables the reduction of enzymes such as trypanothione reductase [169]. To date 7 enzymes of the PPP have been cloned and expressed from \textit{T. cruzi}, and studies of labelled glucose have suggested that PPP is functional in \textit{T. cruzi} epimastigotes [169]. Expression of one of the enzymes in the pathway, glucose 6- phosphate dehydrogenase, was up-regulated, indicated by a 46 fold increase, in trypomastigotes incubated in the presence of H$_2$O$_2$, suggesting that PPP is important in the regulation of oxidative stress [170]. An assay utilising enzyme specific substrates was used to identify enzyme activity of all enzymes in the PPP in the epimastigote, amastigote, epimastigote and metacyclic trypomastigote [171]. Currently inhibitors of these enzymes have not been identified.
Trypanosomes lack de novo synthesis of purines, essential for DNA and RNA synthesis, and rely on a purine salvage pathway as a source. There has been extensive characterisation of the enzymes in this pathway [172], and this pathway has been suggested by many as a drug target [173-175] although there have been few reports of active inhibitors against either the enzyme or the parasite. 6-methyladenine has been demonstrated to be effective in vitro against two enzymes within this pathway, guanine deaminase and adenosine hydrolase, isolated from the epimastigote [176]. Hypoxanthine phosphoribosyltransferase (HPRT) is a key enzyme in nucleotide synthesis and catalyzes transfer of a phosphoribosyl group from phosphoribosylpyrophosphate to a purine base to form a purine ribonucleotide, essential in DNA synthesis [174]. The crystal structure of this enzyme from the epimastigote has been identified and purine analogues have been shown to inhibit this enzyme [177]. It has been suggested that the purine analogue allopurinol blocks T. cruzi HGPRT [174], however there is no direct evidence to support this in the current literature. The IC$_{50}$ value of allopurinol has been determined to be 3 µM against T. cruzi Tulahuen strain amastigotes, following 7 days incubation [178]. Allopurinol has suppressive, although not curative activity in a murine model of acute T. cruzi infection, and is also not curative following treatment of chronic patients [179]. Structure based synthesis of inhibitors of T. cruzi HPRT, which also inhibit T. cruzi growth has been undertaken, although inhibition of amastigote growth was only observed at <20 µM collectively for these compounds [180]. Trimetrexate, a Food and Drug Administration (FDA) approved drug for the treatment of Pneumocystis carinii infection, has also been found to inhibit T. cruzi dihydrofolate reductase (DHFR), with an IC$_{50}$ of 26 nM against intracellular amastigotes, however also has activity against human HGPRT, and the authors note that this compound could
serve as a template for the rational design of more specific inhibitors of this enzyme [181].

1.2.7.9 Mitochondrial targets

The mitochondria is contained in a complex with the kinetoplast of \textit{T. cruzi} which consists of a structure of mini and maxi circles of DNA [175]. The mitochondria contains an electron transport chain for respiration, proteins for kinetoplast maintenance such as topoisomerases, enzymes involved in DNA supercoiling, and also an essential fatty acid pathway [182]. Topoisomerase II [183] and topoisomerase I [183-186] have been isolated from \textit{T. cruzi} epimastigotes and DNA intercalators have shown activity against recombinant \textit{T. cruzi} topoisomerase I [187]. Inhibitors against topoisomerases in other organisms, have been reported as active against \textit{T.cruzi} epimastigotes [188], and on the amastigote form of the parasite free of host cells [189]. However, Croft and Hogg (1988) note that there was limited activity of a panel of bacterial DNA topoisomerase inhibitors, including nalidixic acid, ciprofloxacin and oxolinic acid against intracellular amastigotes of both \textit{T. cruzi} and \textit{Leishmania donovani} [190]. Improved, selective inhibition would need to consider the homology of the \textit{T. cruzi} enzyme to human topoisomerases, which to date is not published. Moderate sequence identity of this enzyme has been observed between other protozoan species, for instance \textit{L. donovani} and human topoisomerase II have a sequence homology of 47% [191].

There may also be a number of topoisomerases that have not been identified in \textit{T. cruzi}. In \textit{L. donovani}, the atypical topoisomerase 1B is present, in which there are substantial differences to the human enzyme [192]. An inhibitor against this enzyme, with \textit{in vivo} suppression of parasite burden in a mouse model of infection has been identified for \textit{L. donovani} [193].
The respiratory chain of *T. cruzi* is another possible therapeutic target because of potential differences with host enzyme complexes [182]. A cyclopallidated compound (7a), with activity against a panel of tumour cell lines via oxidation of mitochondrial proteins, has been shown to potentially target the mitochondria of *T. cruzi* trypomastigotes. This was estimated by a combination of staining with anti-dihydrolipoamide dehydrogenase, a mitochondrial matrix protein, conjugated with Alexa Fluor 594, and ultrastructural studies, following exposure to the compound [194]. Compound 7a produced an 80% survival rate following IP dosing of *T. cruzi* acutely infected mice, however was not able to clear infection. Further investigation is needed to determine if additional compounds that may target mitochondrial function can demonstrate activity against amastigotes without toxicity to mammalian cells.

Evidence suggests that *T. cruzi* mitochondrial and nuclear DNA potentially serve as targets for diamidine type compounds, exemplified by a dose dependant increase in accumulation in kDNA exhibited by some of these molecules, in the epimastigote form of the parasite [195]. Diamidines, such as DB786 and DB889 have shown activity *in vitro* against amastigotes in the nM range, and DB786 led to a reduction of 98% of parasitaemia in a murine model of acute infection [195]. A recent comparison of trypanocidal activity of 13 structurally related amidines, including 4 diamidines using thermal denaturation (Td) of kDNA as a marker of activity, again with epimastigotes, suggests that kDNA binding may not be the mode of action *per se* [196]. Undertaking these studies using amastigotes, to compare accumulation to the relative Td of these compounds is therefore warranted. DB289, a related diamidine compound was in clinical trials for the treatment of Human African Trypanosomiasis (HAT), however liver toxicity and delayed renal failure meant that this compound was discontinued [197]. Such
potential side effects should be considered for this class of compound, regardless of the potential target.

Identification of the expression of metabolic enzymes in intracellular parasites is often difficult as many enzymes also have homologues in mammalian cells. Although amastigotes from infected cells can be isolated [198,199], it is possible that these cell preparations may still contain host cell artefacts, creating potential problems for genetic profiling/ enzymatic analysis. There has been peptide mapping undertaken for all life cycle stages of T. cruzi, utilising amastigotes from the extracellular differentiation of amastigotes to trypomastigotes (using a low pH) [200]. Although these amastigotes are not derived from intracellular parasites, a difference was observed in expression of proteins between life cycle stages, which could explain some lack of success with target based approaches, based on targets identified from epimastigotes. This highlights the need for a multi-parameter approach to target identification, whereby targets potentially identified against the epimastigote stage can be identified with a degree of certainty in the amastigote stage with a combination of antibody-labelling, proteomic approaches, / reduction or elimination of expression may reduce or eliminate host cell infection and small molecule inhibition. Confirmation of inhibitor activity in the intracellular stage is necessary before some of these targets can be further validated. Due to the suppressive nature against T. cruzi infection in vivo of some of the inhibitors already identified, combinations of compounds, as demonstrated with inhibitors from the sterol biosynthesis pathway, could be a consideration for future investigations.

During the last 3 decades despite numerous in vitro and in vivo studies, and new targets identified (Table 1.3), there have only been a few compounds that have advanced to clinical trials. These facts highlight the ongoing need for the
introduction of new compounds into the drug discovery process for the treatment of Chagas disease, that are non-toxic, and which have potential as the next therapeutic class of drugs with more efficient treatment regimens, and that are active against both the acute and chronic phases of the disease.

<table>
<thead>
<tr>
<th>Function</th>
<th>Target</th>
<th>Life cycle stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterol biosynthesis&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Sterol 1, 4-α-demethylase</td>
<td>E,A [129,144,201,202]</td>
</tr>
<tr>
<td>Growth/ differentiation/ invasion&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cruzipain</td>
<td>E,T,A [132]</td>
</tr>
<tr>
<td>Sterol biosynthesis</td>
<td>Sterol 24-c-transferase</td>
<td>E [202]</td>
</tr>
<tr>
<td>Sterol biosynthesis</td>
<td>HMG-CoA reductase</td>
<td>E [202]</td>
</tr>
<tr>
<td>Sterol biosynthesis</td>
<td>Squalene synthase</td>
<td>E [202]</td>
</tr>
<tr>
<td>Mevalonate pathway</td>
<td>Farnesyl diphosphate synthase</td>
<td>E [203]</td>
</tr>
<tr>
<td>Thiol dependant redox metabolism</td>
<td>Trypanothione reductase</td>
<td>E [204], T [160]</td>
</tr>
<tr>
<td>Glycolysis</td>
<td>Glyceraldehyde-phosphate- dehydrogenase</td>
<td>E [205]</td>
</tr>
<tr>
<td>Pentose phosphate pathway</td>
<td>Glucose-6-phosphate- dehydrogenase</td>
<td>E,T,A [171]</td>
</tr>
<tr>
<td>Purine salvage pathway</td>
<td>Guanine deaminase/ adenosine hydrolase</td>
<td>E [172,206,207]</td>
</tr>
<tr>
<td>Nucleotide synthesis</td>
<td>Hypoxanthine phosphoribosyltransferase</td>
<td>E [208]</td>
</tr>
<tr>
<td>DNA replication</td>
<td>Topoisomerases</td>
<td>E [183,209]</td>
</tr>
</tbody>
</table>

Table 1.3. Enzyme targets identified in *Trypanosoma cruzi*. a) Denotes validated targets. Presence in life cycle stages was identified by enzyme activity/ purification/ whole genome sequencing (*T. cruzi* genome project [210]). b) Sterol analysis of isolated amastigotes suggest the presence of this enzyme.. A= amastigote, E=epimastigote, T=trypomastigote.
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1.3 Identification of compounds with activity against *T. cruzi*

1.3.1 Target based approaches

The knowledge of targets in drug discovery has enabled the structure-based design of chemical inhibitors or selection of inhibitor libraries against *T. cruzi*, as highlighted in section 1.2.4 and further identification of more specific inhibitors of these enzymes may provide future leads for *T. cruzi* drug discovery.

1.3.1.1 Phenotypic approaches

Although knowledge of target structure has aided the development of compounds for *T. cruzi* drug discovery, many compounds that have been identified have been from whole cell based screening of compound collections, otherwise known as phenotypic screening. This enables identification of compound activity against the parasite, with potential follow-up for the determination of the mode of action. If the mode of action of a compound is already known, structure based rational design may be used to improve properties of the active molecules. Phenotypic approaches have the added benefit over target based approaches in that previously unidentified targets can potentially be identified. The success of phenotypic screening is particularly highlighted in HAT drug discovery: with the exception of the drug eflornithine used to treat the disease, the current pipeline of existing and potential new drugs have all resulted from phenotypic screens [211]. Phenotypic screening was also the most successful approach for first-in-class drugs for a variety of disease indications between 1998 and 2008 [212]. Although a number of compounds have been identified in *T. cruzi* drug discovery using knowledge of targets in the case of the inhibition of sterol biosynthesis, there have
been a number of promising compounds identified from compound library screening campaigns, using various assay formats.

1.3.1.2 Maintenance of the T. cruzi life cycle in vitro

An important consideration for T. cruzi phenotypic screening is the in vitro maintenance of the infective life cycle of the parasite. Culture of the T. cruzi life cycle has been described in some detail for HeLa cells [213], however the description of the initiation and ongoing sub-culture of the T. cruzi life cycle in vitro is often limited in the literature. There have been reports of differentiation of the non-infective epimastigote form of the parasite into the metacyclic, infective form using an artificial triatomine bug urine, supplemented with L-proline, L-glutamate, L-aspartate and glucose (TAU3AAG) [214]. An alternative approach is by spontaneous differentiation of epimastigotes during the stationary phase of growth in liquid media [215]. Once metacyclic trypomastigotes infect mammalian tissue, they form amastigotes, differentiate into trypomastigotes, and are released from the host cell. A scheme for the maintenance of the T. cruzi infective life cycle in vitro is shown in Figure 1.8.
Consideration should be given to the age and health of the life cycle stage that is used in an assay to determine compound activity. Also, the effect of the length of time parasites are sub-cultured following differentiation into the infective form should be investigated. Changes in the morphology of the Brazilian strain of *T. cruzi* cultured on murine PSC3H cells have been observed over time, whereby released trypomastigotes more readily form spheromastigotes (rounded forms) than 2-5 week old cultures. This could potentially impact upon antigenicity, infectivity and physiology [216]. Importantly, the life cycle stage used in an assay should be relevant to infection in the human host. There seems to be some confusion in the literature as to what the important clinical target is for *T. cruzi*. Some authors state it is the amastigote stage [123,129], whilst others believe that it is both the trypomastigote and amastigote stage of the disease that are the most
significant [217-220]. Recently, a meeting “Experimental Models in Drug Screening and Development for Chagas Disease”, incorporating a variety of networks and laboratories, was held for discussion of Chagas in vivo and in vitro experimental models [221]. It was agreed from this meeting that there needs to be an in vitro system which allows for monitoring the effects on both amastigotes and trypomastigotes in just one system. This would be beneficial, however separate assays may give more of an understanding of the potential in vivo effects. As it is thought to be important for compounds to remove persistent amastigote populations in the chronic phase of the disease [222], a separate assay to define amastigote activity would be warranted. If compounds were also active on the trypomastigote form, this may potentiate in vivo effects by preventing infection.

1.3.1.3 Phenotypic assays for T. cruzi drug discovery

The methods for determining compound activity against T. cruzi vary considerably in terms of the detection method, the time of compound exposure and the life cycle stages that are incubated with the compound (Table 1). Although there are a number of methods that have been used to determine compound activity on the epimastigote form of the parasite [223,224], this life cycle stage is not clinically relevant to disease. Assays have alternately been developed to estimate compound activity against the trypomastigote, however most are using simple cell counting techniques [219,225], although there has been a report utilising the viability dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [226]. Other authors have assayed compound activity against trypomastigotes for the purpose of sterilising blood, using β-galactosidase transfected parasites, at 4 °C [29]. Using the same transfected parasites, an assay format that has been widely utilised to determine activity of compounds on infective forms of the parasite employs a β-galactosidase reporter gene transfected
parasite [29]. In this assay, trypomastigotes are added to host cells and following 2 hours incubation, compound is added and plates are incubated for 7 days. Although there is no direct evidence to show the time it takes trypomastigotes to infect cells in vivo, infections increase at least over 20 hours for the Y strain of the parasite, when inoculated onto a monolayer of VERO cells [227]. Potentially activity in this assay could therefore indicate activity of a compound against the trypomastigote, the amastigote, and the infection process. A tdTomato reporter gene transfected parasite has been used in an assay to identify compound activity against the amastigote form of the parasite by washing off trypomastigotes following host cell infection, and detection undertaken with a fluorescence-based read out [228]. Recent image-based methods using DNA staining techniques detect the effects of compound on intracellular amastigotes [229]. There is a lack of well established procedures and screening protocols to evaluate compound activity against T. cruzi. From a meeting held to discuss in vivo and in vitro screening protocols in Brazil in 2010, it was recommended that to provide uniformity compound activity against T. cruzi be assessed using an assay format which utilises β-galactosidase transfected parasites and which is currently widely used [29]. It was also recommended that introduction of automation or alternative assay formats would be of benefit [221]. These authors also suggest an assay that allows for the monitoring of the activity of compounds on the trypomastigote and amastigote in one assay system. However, as amastigote persistence is implicated in the chronic phase, and trypomastigotes in the bloodstream are prevalent in the acute stage, it would also be beneficial to have separate assays to identify the action of compound activity on each parasite form separately. It has been suggested that a compound that is active against both life cycle forms may be more effective against the disease [218].
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### Table 1.4: Methods for the identification of compound activity on the life cycle stages of *T. cruzi* parasites.

<table>
<thead>
<tr>
<th>Life cycle stage</th>
<th>Detection System</th>
<th>Drug exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epimastigote [223]</td>
<td>β-galactosidase transfected parasites (fluorescence)</td>
<td>72 hours</td>
</tr>
<tr>
<td>Epimastigote [230]</td>
<td>Resazurin</td>
<td>48 hours</td>
</tr>
<tr>
<td>Trypomastigotes [219,225]</td>
<td>Counting parasites</td>
<td>24 hours, 48 hours</td>
</tr>
<tr>
<td>Trypomastigotes [226]</td>
<td>MTT assay (fluorescence)</td>
<td>24 hours</td>
</tr>
<tr>
<td>Amastigotes [228]</td>
<td>tdTomato transfected parasites (fluorescence)</td>
<td>3-4 days</td>
</tr>
<tr>
<td>Amastigotes [229]</td>
<td>Nuclear staining (image-based)</td>
<td>72 hours</td>
</tr>
<tr>
<td>Amastigotes/trypomastigotes [29]</td>
<td>β-galactosidase transfected parasites (fluorescence)</td>
<td>7 days</td>
</tr>
</tbody>
</table>

### 1.3.1.4 Outcomes of *T. cruzi* phenotypic screening

A β-galactosidase reporter gene-based *T. cruzi* assay has been used in a 96-well format to screen 303,286 molecules, the National Institute of Health (NIH) collection, and active compounds were further profiled in a separate cytotoxicity assay to determine their IC$_{50}$ values against host NIH-3T3 cells [27]. Two of these molecules (Table 1.5) were found to reduce, but not clear, infection in an acute *in vivo* model, utilising recombinant parasites expressing luciferase. Further optimization of these compounds would be required, due to solubility and permeability issues created by the presence of a quaternary ammonium. The β-galactosidase reporter gene assay was used to determine the activity of a collection of agrochemicals against the parasite and identified fenarimol with an IC$_{50}$ value of 350 nM [231]. Analogues of this compound were synthesised as part of a ‘hit to
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lead optimisation programme and one compound was found with low nanomolar activity (Table 1.5) that was able to suppress parasitaemia to undetectable levels in both a murine model of acute infection and an “intermediate” chronic model of infection. However, there was parasite rebound following 3 rounds of immunosuppression, and further work is needed to determine if other analogues are able to provide a cure [232]. As fenarimol inhibits *T. cruzi* recombinant CYP51 as effectively as posaconazole, and has been shown to complex with the enzyme, it appears that this is the target of the compound and this information can be used to design additional compounds to limit off target activity of the CYP mammalian enzyme [233]. This compound is still undergoing lead optimisation [234].

The β-galactosidase assay was also used by Rottman and co-workers (2012) to assess the activity of a collection of agrochemicals, again discovering compounds likely to inhibit CYP51, including clotrimazole [235], however *in vivo* activity of these compounds was not reported. The lead optimisation of the compound tipifarnib is also being guided by the use of the β-galactosidase transfected parasite. Originally this compound was screened as it is an inhibitor of the human protein farnesyltransferase (PFT), thought to be a potential drug target for *T. cruzi*, and found to have an IC\textsubscript{50} value of 4 nM against *T. cruzi* [236]. However, it has since been found that this compound is a *T. cruzi* CYP51 inhibitor and assay guided efforts are underway to reduce human PFT activity and improve *T. cruzi* CYP51 activity [237]. The *in vitro* and *in vivo* efficacy of a lead tipifarnib analog has been evaluated (Table 1.5) and was shown to significantly suppress parasitaemia in an acute mouse model of infection, following oral dosing of 40 mg/kg for 20 days. Unfortunately, this compound did not provide a cure as determined by positive PCR and reactivation of disease in some mice following treatment, as determined microscopically, was reported [238]. Optimisation of the selective
binding of this compound is continuing, based on the published structure of *T. cruzi* CYP51 [109].

A series of novel 2-nitro-1H-imidazole- and 3-nitro-1H-1,2,4-triazole-based aromatic and aliphatic amines have been assessed for activity in the *T. cruzi* β-galactosidase assay, identifying 18 compounds with activity ranging from 40 nM to 1.97 µM, with SI values between 1320 to 45 [239]. Currently, *in vivo* evaluation is being undertaken for these compounds. This assay was also used to profile the activity of over 300,000 compounds from the BROAD Institute of MIT and Harvard (USA) [240]. Fifteen hit-to-lead starting points were identified, and some of these series are still in the process of lead optimisation. Currently, an imidazole-based compound serves as a lead in *T. cruzi* research (Table 1.5) and *in vivo* studies demonstrated that 60% of mice were parasite free following oral treatment in a sub-chronic immunosuppressive mouse model of infection. A cure of infection was not determined by PCR analysis of selected tissue. Mechanism of action studies for this compound are underway and will aid development of further series in the future [240].
<table>
<thead>
<tr>
<th>Compound structure</th>
<th>IC₅₀ (µM) (SI, mammalian cell line)</th>
<th>In vivo activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td>0.07</td>
<td>Suppressed infection in a mouse model of acute infection, using luciferase expressing Y strain parasites to infect BalBc mice [27]</td>
</tr>
<tr>
<td><img src="image2.png" alt="Image" /></td>
<td>0.11</td>
<td>Suppressed infection in a mouse model of acute infection, using luciferase expressing Y strain parasites to infect BalBc mice [27]</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td>0.0015 (3373, L6)*</td>
<td>Clearance of parasites in an acute model of infection using Swiss mice infected with Tulahuen strain parasite. Rebound following 3 cycles of immunosuppression [232]</td>
</tr>
<tr>
<td><img src="image4.png" alt="Image" /></td>
<td>0.0005*</td>
<td>Suppressed infection in an acute model of infection using Swiss mice infected with Y strain parasites [241]</td>
</tr>
<tr>
<td><img src="image5.png" alt="Image" /></td>
<td>0.008 (&gt;12500, L6)*</td>
<td>60% of mice parasite free in an immunosuppressive model of infection using Swiss mice infected with Tulahuen strain parasites [232]</td>
</tr>
</tbody>
</table>
Table 1.5. Compounds identified from *T. cruzi* phenotypic screening campaigns with demonstrated activity *in vivo* in mouse models of *T. cruzi* infection. * denotes lead compound. The selectivity index (SI), if reported, is represented in brackets, with the mammalian cell line used to determine the SI.

Some compound collections have been used for the validation of an assay rather than serving to provide hit compounds. A compound library, comprised of 909 compounds that are mainly FDA approved, was screened in 96-well format using an image-based amastigote specific HTS assay. The hits were used for assay validation, to compare activity to a non-automated assay utilising Giemsa staining of wells [229]. None of the hits from this screen have currently been reported as progressed. In 2012, the Institute Pasteur, Korea screened a Pfizer library of 150,000 compounds against amastigotes using an image-based assay, in a 96-well format, with infected myoblasts [242]. To date the specific methods for the assay and the outcomes of this screening campaign have not been published.

Assays used to estimate compound activity against clinically relevant life cycle forms have enabled the identification of lead compounds for *T. cruzi* drug discovery, and continue to guide optimisation of these compounds. Further development of automated, more sensitive methods for drug discovery can drive identification of new leads against the infective forms of *T. cruzi*. Although the β-galactosidase assay has been a valid method of discovering active compounds for many years, a system that allows visualisation of amastigotes in host cells [229] may be more sensitive. Image-based assays can potentially identify fewer remaining amastigotes in a host cell than whole well fluorescence based methods, and therefore provide a more accurate determination of levels of infection.
1.4 Summary and project aims

BZ and NFX are the only drugs currently used for the treatment of Chagas disease, however, as they commonly present with side effects, variable efficacy and may lead to drug resistance, they are not the most suitable treatments. More selective and safer drugs are needed that are able to efficiently clear *T. cruzi* infection, especially in the chronic phase of the disease.

Few molecular targets for therapy have been identified for the development of new compounds for early drug discovery against *T. cruzi* that are able to demonstrate *in vivo* efficacy. Many targets remain to be validated, and chemical inhibitors with activity against the clinically relevant life cycle forms need to be identified and characterised. The importance of some of these targets in the amastigote stage is hampered by the fact that host cells also often contain the same or similar enzymes or pathways.

Despite a number of compounds entering clinical trials for the treatment of Chagas disease over the last few decades, no compounds have yet demonstrated curative activity. Screening of compound collections in phenotypic, whole cell assays against *T. cruzi* has afforded promising new lead compounds. Further identification of new molecules with activity against the clinically relevant stages of *T. cruzi* is important for early drug discovery against this parasite, and could enable the identification of new target classes. Improvement of existing assay techniques to allow for more sensitive phenotypic screening methods may enhance correlation of *in vitro* to *in vivo* efficacy.
1.4.1 Project aims

1. **Develop a robust method for maintaining* T. cruzi* in the infective stages**
   a. Determine the optimal growth medium for the epimastigote stage of the parasite.
   b. Differentiate the epimastigote to the metacyclic trypomastigote with the most effective production.
   c. Maintain an infective culture of parasite on 3T3 fibroblast host cells, monitor the production of trypomastigotes over time.

2. **Develop sensitive and reproducible assays to estimate the effect of compounds on the clinically relevant forms of* T. cruzi***
   a. Develop an image-based assay to determine compound activity against the intracellular amastigote stage of the parasite. This assay will improve upon existing methods by also staining the cytoplasm of the host cell, allowing for a more accurate determination of compound mediated host cell toxicity.
   b. Develop an assay against the trypomastigote stage of the parasite.
   c. Evaluate the sensitivity of these assays with the reference compounds NFX and puromycin.
   d. Evaluate the reproducibility of these assays by analysing the Z’-factor.

3. **Determine the activity of compounds against* T. cruzi* amastigotes and trypomastigotes**
   a. Determine the activity of a collection of compounds, the Medicines for Malaria Venture (MMV) Malaria Box, demonstrated to be effective against the protozoan parasite, *Plasmodium falciparum* against the life cycle stages of *T. cruzi*, using the above assays. Ascertain the activity of an in-house library of compounds, containing FDA approved drugs and compounds with
known biological activity, against the life cycle stages of *T. cruzi* using the assays described above.

b. *In vivo* activity of identified active, non-toxic compounds with a favourable pharmacological profile

c. *In vivo* testing is to be undertaken using an acute mouse model of *T. cruzi* infection, undertaken at the New York University Anti Infectives Screening Core. This mouse model utilises a transgenic strain of *T. cruzi* expressing firefly luciferase, and the extent of *T. cruzi* infection of mice following compound treatment is estimated by an *in vivo* imaging system, IVIS 100 (Xenogen, Alameda, CA). The methodology is outlined in http://ocs.med.nyu.edu/anti-infectives-screening/protocols.

4. **Combination studies**

   a. To determine the *in vitro* effects of a combination of the compound/s identified from (3) with the currently used drug, NFX.

5. **Compound preliminary mode of action and speed of action studies**

   a. To employ *in vitro* assays to determine the speed of action of the compound/s identified in (3), initially against the trypomastigote form as this life cycle form is non-replicating. This allows for manipulation of phenotypic assay formats to determine the time that it takes for the compound to affect the parasite. This will give an indication as to whether the compound may potentially have time to act on these blood-circulating forms *in vivo*.

   b. To employ *in vitro* assays to ascertain the mode of action of the compound/s, if the mode of action of the compound/s is published against other parasite species/ mammalian cell lines.
Chapter 2: General Materials and Methods

This chapter contains the materials and methods of routine protocols carried out in this thesis. Methods herein represent the optimised protocols and subsequent chapters will outline their development.

Unless stated otherwise chemicals were purchased from Sigma Aldrich (St. Louis, MA, USA) and culture media were from Life Technologies (Carlsbad, CA, USA). The PBS used in these methods was sterile, and supplemented with 100 mg/L MgCl₂ and 100 mg/L CaCl₂.

2 General Materials and Methods

2.1 Cell counting techniques

2.1.1 Formaldehyde fixation

Motile forms of *T. cruzi* (trypomastigotes and epimastigotes) from liquid cultures were diluted in 3.6% formaldehyde and counted on a haemocytometer. The formaldehyde solution was prepared by the addition of 100% formaldehyde in to saline-sodium-citrate buffer (SCC), comprised of 3M NaCl, and 300 mM sodium citrate Na₃C₆H₅O₇ (sodium citrate) at pH 7. Cells were allowed to settle on to the haemocytometer surface for 5 to 10 minutes before counting.

2.1.2 Giemsa staining

A 10% Giemsa solution was prepared by the addition of concentrated stock to PBS, which was prepared freshly before each use. Giemsa staining was used to determine the life cycle stages of mixed populations of epimastigotes and metacyclic trypomastigotes, or the percentage infection rates of amastigote
infected host cells in 384-well assay plates (Collagen1 Coated, CellCarrier, PerkinElmer, Waltham, MA, USA). For mixed motile populations, prepared slides containing smears of parasites were fixed and stained. Samples were prepared for the smear by centrifuging parasite samples for 8 minutes at 3000 g, then the cell pellet was washed twice in PBS. For cultures in-plate, the media volume was removed and wells were washed with PBS twice before staining. Samples were fixed for 5 minutes in methanol (MeOH) and dried for 5 minutes before staining with Giemsa. The stain was then washed from slides with water, or washed off the plate surface by rinsing 4 times with 20 µL PBS.

2.2 Maintenance of mammalian cell lines

All mammalian cell lines were maintained at 37°C, under an atmosphere of 5% CO₂.

2.2.1 3T3 embryonic fibroblast sub-culture

3T3 mouse (Swiss Albino) embryonic fibroblasts (CCL92; ATCC, Manassas, VA, USA) were maintained in Roswell park memorial institute (RPMI) 1640 without phenol red, supplemented with 10% heat inactivated foetal calf serum (FCS). Cells were sub-cultured every 3 days by the addition of 4x10⁵ cells to 25 mL of RPMI supplemented with 10% heat inactivated FCS, in 175 cm² cell-culture flasks (Griener BioOne, Frickenhausen, Germany).

2.2.2 Heart fibroblast sub-culture

Normal human atrial cardiac fibroblasts (NHCF-A; Lonza, BSL, Switzerland) were maintained in fibroblast Clonetics Bullet- Kit™ media (Lonza, Switzerland), in 5
Chapter 2: General Materials and Methods

mL of media in 25 cm² or 25 mL of media in 75 cm² flasks, at 37°C and 5% CO₂. Cells were sub-cultured every 3 days by the addition of 8.75x10⁴ cells to 25 cm² flasks or 2.62x10⁵ cells to 75 cm² flasks, and media was replaced daily. Cells were not cultured for more than 6 passages.

2.2.3 HEK293 Sub-culture

HEK293 cells (CRL1573; ATCC, USA) were maintained in high glucose DMEM, supplemented with 10% FCS. Cells were sub-cultured every 3 days by the addition of 1x10⁶ cells to 175 cm² flasks containing 25 mL of medium.

2.2.4 Long term preservation of mammalian cell lines

For the long-term preservation of mammalian cell lines, these aliquots of cells were frozen down in liquid N₂ at a concentration of 2x10⁵ 3T3 cells/mL, 8.75x10⁴ NHCF-A fibroblasts/mL or 1x10⁸ HEK-293 cells/mL; in 95% FCS and 5% DMSO.

2.3 Culture of T. cruzi life cycle forms

2.3.1 Epimastigote culture

*T. cruzi* Tulahuen strain epimastigotes were provided by Professor Frederick Buckner (Washington University, USA). Y strain parasites were provided by Dr Victor Contreras (Universidad de Carabobo, Bárbara, Valencia, Venezuela). Epimastigotes of both *T. cruzi* strains were cultured in liver infusion tryptone (LIT) medium [215], supplemented with 10% FCS and 100IU/mL penicillin/streptomycin, in 75 cm² or 25 cm² flasks, in 10 mL or 25 mL of LIT respectively, with phenolic, non-vented lids (Corning, NY, USA). Cells were cultured at 28°C, in
Chapter 2: General Materials and Methods

a normal atmosphere. LIT medium contained 68.4 mM NaCl, 5.37 mM KCl, 45.10 mM Na₂HPO₄, 11.10 mM D-glucose, 50g/L liver extract, 50g/L tryptone and 50 mg/L hemin. The medium was adjusted to pH 7.2 before autoclaving at 121°C for 40 minutes.

To enumerate the epimastigote density, the culture was first microscopically assessed. If cells appeared to be clumping (rosetting), the culture was passed slowly through a 26 gauge needle (Becton-Dickinson, San Jose, CA, USA). For the long term storage of epimastigotes, cells were frozen in liquid N₂ at a concentration of 5x10⁶ cells/mL; in 90% FCS and 10% DMSO.

2.3.2 Differentiation of epimastigotes to metacyclic trypomastigotes

Epimastigotes were grown to a density of 2-6x10⁶ cells/mL from frozen stock in 75 cm² flasks. Cells were adjusted to a density of 1x10⁶ cells/mL, and transferred to two flasks. Following incubation for 6 days, when cells were estimated to be at the end of the log phase of growth, flasks were harvested by centrifugation at 3000 g for 8 minutes. Epimastigotes were washed twice in PBS, by centrifugation, and resuspended at a density of 5x10⁸ cells in 15 mL centrifuge tubes (Falcon-Corning, USA) in approximately 2-4 mL of artificial triatomine urine (TAU), supplemented with L-proline, L-glutamate, L-aspartate, glucose and 100 IU/mL penicillin/streptomycin (TAU3AAG, [243], Table 2.1). Cells were incubated for 2 hours at 28°C and subsequently following, were adjusted to 5x10⁶ cells per mL in TAU3AAG. Twenty five mL of the cell suspension was added to 8x 175 cm² flasks, with non-vented caps, and incubated for 3 days at 28°C.
Table 2.1. Composition of the chemically defined medium, artificial Triatomine urine (TAU3AAG), for the differnetiation of *T. cruzi* epimastigotes to metacyclic trypomastigotes.

<table>
<thead>
<tr>
<th>Salt components</th>
<th>mM concentration</th>
</tr>
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<tbody>
<tr>
<td>NaCl</td>
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</tr>
<tr>
<td>KCl</td>
<td>7.5</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.496</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>3.504</td>
</tr>
<tr>
<td>Red Phenol</td>
<td>0.1%</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2</td>
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### Organic Components

<table>
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<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
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<td>50</td>
</tr>
<tr>
<td>Na aspartate</td>
<td>2</td>
</tr>
<tr>
<td>L-proline</td>
<td>10</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
</tr>
</tbody>
</table>

The flask supernatant was harvested, leaving behind cells stuck adhered to the surface of the flask. Medium was removed from the cells by centrifugation at 3000 g for 8 minutes. Cells were washed once in PBS and resuspended in PBS at 5x10⁸ cells/mL. At this concentration a sufficient density of parasites could be seen on a smear, for enabling determination of morphology. In the epimastigote form, the kinetoplast is located between the nucleus and the flagellum, and whereas in the metacyclic trypomastigote, the kinetoplast is sub-terminal [244] (Figure 2.1). Differentiated, metacyclic parasites could be identified from epimastigotes by either Giemsa staining or by fixing and counting on a haemocytometer, at 20x magnification. For Giemsa staining, a smear was prepared by adding 10 µL of the parasite suspension to a glass slide and then slowly moving the liquid across the slide with a 100 µL tip, whilst not touching the glass surface. If parasites were
smeared against the surface, many were flattened and/or lysed. The number of infective forms could also be determined by fixing and viewing a sample of the culture at 20 x magnification on a haemocytometer. Epimastigotes are stumpy, with a short flagella, whereas metacyclic trypomastigotes are thin, often “C” shaped, with a longer flagellum [245].

![Diagram of T. cruzi epimastigote and trypomastigote](image)

Figure 2.1. **Morphological differences between the *T. cruzi* epimastigote and trypomastigote.** (A) Shows each life cycle form, where NP=nucleus-posterior distance KP=kinetoplast-posterior distance (B) Shows the colour key for the nucleus, kinetoplast and flagellum. The basal body/pro-basal body pair (BB), flagellar pocket (FP), Axoneme (Ax) and paraflagellar rod (PFR) are shown in the flagellum structure. In the trypomastigote the KP is smaller and the NP is greater in comparison to the epimastigote, as the kinetoplast is closest to the posterior, and the nucleus is closer to the anterior. Modified from Wheeler and Gull, 2013 [246].

A mixture of metacyclic trypomastigotes and epimastigotes were added to a monolayer of host cells at a multiplicity of 10:1 (trypomastigotes: host cells). To form a monolayer, host cells were added 24 hours prior to infection, at a concentration of 4x10⁵ cells, in 5 mL of RPMI without phenol red with 10% FCS
and 100 IU/mL penicillin/ streptomycin, in a 25 cm² flask. Following 24 hours co-
incubation of host cell and parasites at 37°C and 5% CO₂, non-internalised
trypomastigotes were washed off 3 times with PBS. Medium was replaced and
following approximately 8 days incubation, sufficient trypomastigotes were
released in to the supernatant to initiate subsequent cultures. Trypomastigotes
were collected from the supernatant and used for the continuing subculture of
infective cells.

2.3.3 Subculture of T. cruzi infective forms (amastigotes and trypomastigotes)

2.3.3.1 Subculture of T. cruzi in 3T3 fibroblasts

To form a monolayer of host cells, 4x10⁵ 3T3 cells, in 5 mL of media, were
inoculated in to 25 cm² flasks or 1.2x10⁶ cells, contained in 15 mL of media, were
added to 75 cm² flasks (Griener Bio One, Frickenhausen, Germany). Following 24
hours incubation, trypomastigotes released from 3T3 host cells following infection
with metacyclic trypomastigotes (section 1.3.2) were added to the monolayer of
3T3 cells at a multiplicity of infection (MOI) of 10:1 parasite: host cells. After 24
hours incubation, non-internalised trypomastigotes were washed off three times
with PBS and the media was replaced. Following 2-3 further days incubation,
trypomastigotes were released in to the supernatant from infected cells and were
collected for subsequent sub-culture, or for use in assays, on the third day.
Trypomastigotes were collected by centrifuging the supernatant, and
resuspending the cell pellet in fresh media before counting. T. cruzi co-cultured on
host cells were not maintained for more than 3 months.
2.3.3.2 Subculture of *T. cruzi* in NHCF-A human cardiac fibroblasts

From 2.3.3.1, trypomastigotes released from 3T3 cells were used to infect a monolayer of NHCF-A fibroblasts. One million NHFC-A cells were added per 25 cm² flask, in 5 mL of media (see section 1.2.2), 24 hours prior to infection. Trypomastigotes were added at a MOI of 10:1 parasites: host cell. After 24 hours, trypomastigotes remaining in the supernatant were removed by washing the cell bed 3 times with PBS. After 3 days further incubation, trypomastigotes released into the supernatant were used to infect subsequent cultures, or added to assay plates.

2.4 Assays for the detection of the sensitivity of *T. cruzi* life cycle forms / mammalian cells to compounds

2.4.1 Preparation of NFX

NFX was isolated from Lampit® tablets (Bayer, Berlin, Germany), kindly provided by the Drugs for Neglected Diseases Initiative (DNDi). NFX tablets (Lampit, 4.183 g, 10 tablets) were suspended in dichloromethane (DCM, 100 mL) and water (20 mL) and the layers separated. The DCM layer was further washed with water (2 x 20 mL) then dried (MgSO₄), filtered and the solvent removed under reduced pressure to give the pure compound as an orange powder (1.156 g, > 95 % purity as determined by liquid chromatography-mass spectrometry (Agilent 6120 Quadrupole LCMS).

The identity of NFX was confirmed by ¹H nuclear magnetic resonance (¹H-NMR) (Figure S1) and ¹³C-NMR (Figure S2) (Agilent Unity Inova 500 MHz). ¹H NMR (500 MHz, CDCl₃) d: 7.46 (s, 1H), 7.37 (d, J = 3.5 Hz, 1H), 6.73 (d, J = 4.0 Hz, 1H),
4.24 (dt, J = 15.5 and 4.0 Hz, 1H), 4.07-4.14, (m, 1H), 3.79 (ddd, J = 15.0, 12.0 and 3.0 Hz, 1H), 3.18-3.13 (m, 1H), 3.06 (dt, J = 14.5 and 2.5 Hz, 1H), 2.95 (dd, J = 14.0 Hz, 10.0 Hz, 1H), 2.92-2.87 (m, 1H), 1.55 (d, J = 6.7 Hz, 3H); 13C NMR (125 MHz, CDCl3) δ: 153.9, 125.0, 113.8, 108.9, 57.8, 56.5, 45.5, 43.6, 17.7 (NB one C signal not observed).

2.4.2 Dilution of compounds

Twenty microlitres of water was dispensed by a Multidrop™ microplate dispenser (Thermo Scientific, Newington, NH) in to clear, sterile, 384-well plates (BD Biosciences, Franklin Lanes, NJ, USA). Compounds, dissolved in 100% DMSO, were diluted 1:21 by a Minitrack™ liquid handling system (PerkinElmer, Waltham, MA, USA), by the addition of 1 µL of compound to the 20 µL of water. Five µL of this dilution was then added to each assay plate for testing.

2.4.3 *T. cruzi* amastigote infected 3T3 cells: image-based assay

Additions of parasite and host cells were made with a Multidrop plate dispenser, under sterile conditions. 3T3 host cells were seeded in to 384-well plates (CellCarrier, Collagen1 coated, PerkinElmer, USA) at 1x10^3 cells/well in 50 µL of RPMI, containing no phenol red, supplemented with 10% FCS and 100 IU/mL penicillin/ streptomycin. Cells were allowed to attach to the plate surface at room temperature for 15-20 minutes before incubation for 24 hours at 37°C and 5% CO₂. Trypomastigotes were added at a multiplicity of 5:1, parasite: host cell, in a volume of 10 µL. As it was estimated that host cells approximately double in 24 hours, an MOI of 5:1 parasite: host cells was equivalent to 1x10^4 cells/well of the parasite, at the time of addition. Plates were incubated for 24 hours and trypomastigotes that had not infected host cells were removed from wells by
washing three times with PBS. The first wash was undertaken by manually removing the well volume, in a biological safety cabinet, class PC2. The well volume was emptied from the plate into a reservoir containing a small volume of 10% bleach solution, replaced with 50 µL of PBS and again the well volume was removed in the same manner. Wells were then washed twice on a Bravo liquid handler (Santa Clara, CA, USA) and 50 µL per well of RPMI medium was replaced. Five µL of diluted compound was added with a Minitrak liquid handler (section 1.4.1). Plates were incubated for 48 hours before preparation for imaging. All staining steps were undertaken on a Bravo liquid handler (Agilent Technologies, Foster City, CA, USA) and washes were with sterile PBS. Wells were washed twice before the addition of 20 µL of 4% paraformaldehyde (PFA) and incubated for 15 minutes at room temperature. Twenty µL of 0.01 mg/mL Hoechst 3342 (Life Technologies, USA), diluted in RPMI without FCS, containing 0.1% Triton® X-100 was added and plates were incubated for 30 minutes. Wells were washed twice and 20 µL of 0.05 µg/mL HCS CellMask™ Green (Life Technologies USA) in RPMI without FCS was added, and plates were incubated for 30 minutes. Wells were washed twice and 20 µL of RPMI, without phenol red, supplemented with 100 IU/mL penicillin/streptomycin was added. Wells could be imaged immediately, or stored up to 1-2 weeks at 4°C, light protected, prior to imaging.

Images were taken on an Opera® QEHS High-content imaging system (PerkinElmer, USA), or an Operetta® high-content imaging system (PerkinElmer, USA), at 20 x magnification. On the Opera, images were taken over seven fields in the well, with 2 stacks (differing in a height of 2-4 µm), per field. On the Operetta, 5 fields in the well were imaged, at approximately 2 µm height. If images were taken on one plane using the Opera, some amastigotes appeared to be out of focus. For this reason, multiple heights were taken and then combined to form one image for analysis. For each snap-shot, one image was taken at 405 nm excitation 450/50
nm emission to capture the Hoechst stained nuclei, and another at 488 excitation and 520/35 emission to capture the HCS CellMask Green stained cytoplasm of the host cell. Images captured on the Opera were combined and analysed with Acapella® software (using the Assay Language Interface) and Operetta images were analysed using Harmony® software. Both scripts used in house developed building blocks of in built scripts to analyse images (Figure 2.2 and Table 2.2). Firstly, each script identified the host cell nucleus by analysis of the Hoechst image, by size exclusion of the parasite nuclei. The cytoplasm of the host cell was identified by a cytoplasm detection script. Parasite nuclei within the host cell cytoplasm were detected by spot analysis. Host cells containing ≥5 spots were considered to be infected, which removed the majority of background from the spot detection algorithm. The output on each imaging system was both the number of infected cells (host cells containing ≥5 spots in the cytoplasm) and the number of host cells (objects determined by identifying cells with a nucleus with an area of > 80 µM, and cytoplasm, detected by the find cytoplasm script).

In the assay, both the survival of the host cells and amastigotes could be determined in one well, as during the course of infection there was no observed host cell damage. The positive control used to calculate activity against the host cells was a final concentration of 30 µM of puromycin, a general protein synthesis inhibitor; whilst for the amastigotes 12 µM of the drug used to treat Chagas disease, NFX was used.
Figure 2.2. Method for the detection of infected cells in the amastigote image-based assays on the Opera and Operetta high-content imaging systems, with building blocks in the Assay Language Interface and Harmony. Image was taken from the Opera QEHS image-based system. (A) Input image. (B) Find nuclei. (C) Find cytoplasm. (D) Find spots. (E) Select the population of infected cells.
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<table>
<thead>
<tr>
<th>Find region</th>
<th>Method</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global</td>
<td>Individual threshold</td>
<td>Determines the intensity threshold for each object individually</td>
</tr>
<tr>
<td>Nuclei</td>
<td>Split factor</td>
<td>Parameter influencing whether a large object is split into two or more smaller objects</td>
</tr>
<tr>
<td></td>
<td>Common threshold</td>
<td>The lower level of pixel intensity for the whole image that may belong to nuclei</td>
</tr>
<tr>
<td></td>
<td>Contrast</td>
<td>Parameter setting a lower threshold to the contrast of detected nuclei</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>Common threshold</td>
<td>Parameter determining first guess borders of the cytoplasm</td>
</tr>
<tr>
<td>Spot detection</td>
<td>Radius</td>
<td>Determines how intense a spot must be to be detected</td>
</tr>
<tr>
<td></td>
<td>Contrast</td>
<td>Responsible for split or merge decisions between adjacent spots</td>
</tr>
<tr>
<td></td>
<td>Spot to region intensity</td>
<td>Sets a lower threshold for spot to cell intensity. The ratio of the spot peak intensity to the mean intensity of image.</td>
</tr>
</tbody>
</table>

Table 2.2. Function of the methods within the find region building blocks on the Opera and Operetta imaging systems contained within the amastigote assay script.

2.4.4 *T. cruzi* amastigote infected NHCF-A cells: image-based assay

To identify compound activity on NHCF-A host cells, the same protocol was followed as described above utilising 3T3 cells (section 1.4.2), except that $3 \times 10^3$ host cells were added, and an MOI of 2.5:1 parasite: host cell was used, thus $1.5 \times 10^4$ trypomastigotes / well were added. Also, compounds were diluted by the addition of 1 µL of compound to 80 µL of water, resulting in a final concentration of 0.1% DMSO in the assay.
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2.4.5 *T. cruzi* trypomastigote viability assay

Trypomastigotes were taken from the supernatant of a culture of infected 3T3 cells (section 1.3.3.1), by removing the supernatant and centrifuging for at 3000 g for 8 minutes. The medium was removed and cells were resuspended in RPMI without phenol red, supplemented with 10% FCS and 100 IU/mL penicillin/ streptomycin and adjusted to 2x 10^6 cells/mL. Fifty microlitres of this suspension was added to 384-well, black/ clear sterile plates (BD Biosciences, USA), using a Multidrop dispenser (PerkinElmer, USA). Immediately following addition of the parasites to the plate, 5 µL of compounds (section 1.4.1) were added to wells with a Minitrack liquid handler (PerkinElmer, USA) and plates were incubated for 42 hours at 37°C and 5% CO₂. Ten microlitres of 70% PrestoBlue® (Life Technologies, USA) diluted in RPMI without phenol red, supplemented with 10% FCS and 100IU/mL penicillin/ streptomycin, was added to wells. Plates were further incubated for 6 hours, before reading on an Envision plate reader (PerkinElmer, USA) at an excitation of 530 nm and an emission of 595 nm.

2.4.6 HEK293 cytotoxicity assay

Cells were harvested and diluted in high glucose DMEM supplemented with 10% FCS and 100 IU/mL penicillin/ streptomycin, to a concentration of 7.27×10^4 cells/mL. Under sterile conditions, 55 µL of diluted cells per well were added to a black, clear bottomed 384-well lidded plates (BD Biosciences, Bedford, MA, USA) with a Multidrop liquid handler (PerkinElmer, USA). Cells were incubated for 24 hours before the addition of 5 µL of diluted compounds, with a Minitrak liquid handler, and plates were incubated for 48 hours. Ten microlitres of Alamar Blue® (Life Technologies, USA) was added to wells and plates were incubated for 4 hours at 37°C and 5% CO₂, followed by 20 hours at room
temperature, before reading on an Envision plate reader (PerkinElmer, USA) at an excitation of 530 nm and an emission of 595 nm.

2.4.7 Calculation of compound activity

Percentage inhibition values following exposure of infected cells to compounds in the amastigote assays were calculated by the following formula, where

% cell inhibition = 100 - \[
\frac{(IN_{t}-IN_{r})}{(IN_{c}-IN_{r})}\]

\times 100

IN_{t}= Number of host cells infected following exposure to the test compound
IN_{r}= Number of infected host cells in the presence of the reference compound
IN_{c}= Number of infected host cells without compound added (vehicle control, DMSO)

Similarly, in the trypomastigote assay and HEK293 assays, data for the number of infected cells were substituted with the fluorescence values for each treatment (test compound, reference compound or vehicle control).

The IC_{50} values for compounds were calculated using a sigmoidal dose response equation (variable slope equation) in GraphPad Prism version 5.0, (GraphPad Software, San Diego, CA, USA). Determination of the range of linear response for each assay was estimated by calculating the linear regression of the concentration of cells versus the assay outcome, in each assay format (outcome being either the fluorescence output, number of infected cells, or number of host cells), also calculated in GraphPad Prism. No constraints were placed on the top or bottom of the curve fit generated by GraphPad Prism. The percentage inhibition of the test compound in each assay was calculated relative to the control compound in each assay, rather than an absolute control of no host cells / no parasite. It is therefore it
is possible for a test compound to have greater or less than the activity displayed by the control compound.

2.4.8 Statistical calculations

The reproducibility of each assay was determined by calculating the Z'-factor [247] with the equation:

\[
Z' \text{ factor} = 1 - \frac{3 \cdot SD_A + 3 \cdot SD_B}{\text{mean}_A - \text{mean}_B}
\]

Where: A is the mean end point signal of each assay (number of infected cells/host cells in the amastigote/host cell assays respectively, or fluorescence intensity in the trypomastigote or HEK293 assays); B is the background signal (cells treated with reference compounds). SD refers to the standard deviation. In each assay, a plate containing a third DMSO and one third reference compound was included to calculate the Z'-prime of the assay.
3 Growth and Maintenance of T. cruzi

3.1 Introduction

The optimal maintenance of cell lines is an important and necessary consideration for the development of in vitro, phenotypic-based assays, particularly when both host and parasite cells need to be considered. Reproducibility of the production of the relevant cellular stage and health of these cultures is imperative for ensuring that assay reproducibility and sensitivity are not affected negatively by the culture methods employed.

The life cycle of T. cruzi is complex and information for the initiation and ongoing subculture of T. cruzi in vitro is limited in the literature. Many papers refer to trypomastigotes for phenotypic assays that are simply obtained from the supernatant of infected cells [229,248]. There has been some description of the differentiation of epimastigotes to metacyclic trypomastigotes for the CL strain [228] and recently a more detailed description of cultivation of life cycle forms for the production of GFP-transfected DM28c clone trypomastigotes has been reported [249]. However, rates of infection, days following infection in host-parasite co-culture, which infective cells are harvested, and length of the maintenance of co-cultures are not often outlined, especially for the Tulahuen strain parasite. Potentially, differing T. cruzi cell lines and host cells utilised may affect the culturing conditions, and thus ultimately the assay.

Liver infusion tryptone medium (LIT) is the most commonly used medium to cultivate epimastigotes of various species of T. cruzi, however the constituents of this media vary in the literature. To determine a LIT formulation which best supported the growth of T. cruzi Tulahuen and Y strain epimastigotes, parasites
Chapter 3: Growth and Maintenance of *T. cruzi*

were grown in reported variations of this media [215,250]. Hemin, a source of iron for *T. cruzi* [251] is shown to be required for optimal proliferation [252] and differentiation [253], however excess can cause oxidative stress [254]. In many publications, hemin is added at a concentration of either 50 mg/L or 20 mg/L [251,253], and therefore growth at these concentrations was investigated. The LIT medium used may also influence the differentiation of the parasite. Bourguignon *et al* (2006) [255] found the *T. cruzi* EPM strain produced 72% differentiated metacyclic trypomastigotes in non-autoclaved LIT and no differentiation was observed in autoclaved LIT. Thus the growth in filtered versus autoclaved LIT was also compared as part of this thesis.

Methods for the differentiation of *T. cruzi* from the non-infective epimastigote form of the parasite into the metacyclic, infective form include incubation of epimastigotes in an artificial triatomine bug urine, TAU3AAG [214], and spontaneous differentiation of epimastigotes during the stationary phase of growth [215]. TAU3AAG is thought to cause nutritional stress which initiates differentiation [256] and it is reported that optimal differentiation is obtained from parasites grown to the late log phase of growth [250]. The differentiation of epimastigotes in TAU3AAG, during both the stationary and log phase of growth were compared for the Tulahuen strain of the parasite, following the protocol of Contreras *et al* (1988) [257]. The rates of differentiation were compared to those of parasites grown in LIT medium during the stationary and log phases of growth, to determine the most effective method for differentiating *T. cruzi* Tulahuen strain parasites. The optimised protocols were applied to the Y strain of the parasite to determine if it was also possible to culture this strain under the same conditions.

Metacyclic trypomastigotes generated by incubation of epimastigotes in TAU3AAG were used to initiate infective subcultures of *T. cruzi* in 3T3 host cells.
The 3T3 cell line is a mouse derived, embryonic fibroblast cell line, that has been employed extensively for assays utilising Tulahuen strain parasites, [28,29,258]. The subculture of Tulahuen strain T. cruzi was investigated with this cell line. As many in vivo drug sensitivity models employ mice as the model organism for T. cruzi infection [27], this also supports the use of this cell line in early drug discovery efforts. The infection of a human derived, cardiac fibroblasts (NHCF-A) cells was also investigated as an alternative host system for the parasite. As heart cells have been shown to be infected, particularly in the chronic phase of Chagas disease [259-261], this could be an effective model for the investigation of compound activity. To date there have been no reports of T. cruzi species infecting this specific cell line.

3.2 Materials and Methods

3.2.1 Host cell maintenance

3.2.1.1 Host cell doubling time and obtaining a monolayer of cells

The maintenance and growth media of 3T3 and NHCF-A cells is outlined in section 2.2.1. To determine the doubling time of 3T3 host cells, one hundred cells/well, plated from cells grown to passage #7, were added to 96-well sterile tissue culture plates (BD Biosciences, USA) in 100 µL media. At 24 hour intervals from seeding, 4 wells were harvested, centrifuged at 300 g for 3 minutes, and counted over 6 days. To estimate the doubling time of NHCF-A cells, passage #2 cells were grown in 24-well sterile tissue culture plates (Greiner CellStar, Germany) at an initial seeding density of 6.65x10³ cells/well, in 750 µL media (based on the suppliers recommended seeding density of 3.5x10³ cells/cm² [262]). Four wells were harvested and counted every 24 hours, for 6 days.
The doubling time of cells was determined using the equation:

\[ T_d = \frac{(t_2 - t_1) \times \log(2)}{\log(q_2/q_1)} \]

Where \( t_d \) = doubling time.

Two time points in the log phase of growth were used to calculate the doubling time of cells. The first time point is represented as \( (q_1) \) at time 1 (\( t_1 \)). Following 24 hours, cells were counted \( (q_2) \) at time 2 (\( t_2 \)).

To approximate the number of 3T3 cells at confluence in a 25 cm\(^2\) flask, \( 4 \times 10^5, 2 \times 10^5 \) and \( 1 \times 10^5 \) 3T3 cells were inoculated into duplicate 25 cm\(^2\) flasks, observed until they reached confluence, and the flask contents counted. To approximate the number of NHCF-A cells at confluence, \( 8.75 \times 10^4 \) cells were inoculated into 25 cm\(^2\) flasks, monitored until they appeared to reach confluence, and flasks were harvested and cells enumerated. Confluence was observed by visual examination of the cell monolayer and defined as when all of the cells appeared to be in contact with one another.

3.2.1.2 Host cell passage number

The passage number may affect contact inhibition displayed by some fibroblast or fibroblast-like cell lines [263]. For NHCF-A cells, the manufacturer (Lonza, USA) states that these cells undergo contact inhibition, and thus it is not recommended to passage them beyond 10 population doublings [262]. NHCF-A cells were monitored over 10 passages of growth in 75 cm\(^2\) flasks (Chapter 2.2.2), from passage #1. 3T3 cells, also known to undergo contact inhibition [264] were grown in 175 cm\(^2\) flasks and the cell number was monitored for over 12 passages, from
passage #4, the earliest passage from liquid N\textsubscript{2} stock. The cell layer was observed daily to determine if cells were growing over one another. Mean values were estimated from two flasks for each cell line.

3.2.2 Culture of \textit{T. cruzi} epimastigotes

3.2.2.1 \textit{Tulahuen} strain

Three types of LIT media were compared for their ability to support \textit{T. cruzi} Tulahuen strain epimastigote growth, defined as LIT1, LIT2 and LIT3, and were prepared as outlined:

LIT1 was based upon the medium of De Lima \textit{et al} (2008) [250] containing 5.4 mM KCl, 150 mM NaCl, 24 mM glucose, 10 g/L liver infusion broth, 20 mg/L hemin (porcine), 10 g/L yeast extract, 15 g/L tryptose and 10% FCS. Hemin was dissolved in 1M NaCl before addition, and the pH adjusted to 7.2 before filtering. The mixture was then heated for 60 minutes at 60\textdegree C. FCS and 100IU/mL penicillin/streptomycin were added post filter. Filtration was by passing through a 0.45 \textmu m followed by 0.22 \textmu m cellulose filter system (Corning, USA) via vacuum pump. LIT2 contained the basic LIT1 components similar to Camargo, (1964) [215], comprising 5.4 mM KCl, 68.4 mM NaCl, 24 mM glucose, 50 g/L liver extract, 50 g/L tryptone, 50 mg/L hemin and 10% FCS. All components were autoclaved, excepting the FCS. Hemin was dissolved in 1M NaCl before addition and the pH of the media was adjusted to 7.2 before autoclaving. Following, the solution was filtered through a 0.22 \textmu m cellulose filter system with a vacuum pump (Corning, NY, USA). LIT2 was also prepared as a non-autoclaved solution (filtered only). LIT3 contained the basic components of LIT2, with the addition of hemin following autoclaving, as autoclaving or heat treatment may potentially reduce the
available hemin concentration [265]. Hemin, dissolved in 1M NaOH, was passed through a 0.22 µm filter and added to autoclaved LIT. To buffer the medium with a pH close to 7.2, a final concentration of sterile 10 mM HEPES was added to the media. The pH of this medium was estimated at 7.32.

Two heat inactivated samples from differing suppliers were compared, one sourced from Biowhittaker, (Walkersville, USA), (FCS1); and the other (FCS2) sourced from Invitrogen, (Carlsbad, USA). The FCS was prepared with and without filtration, using a 0.22 µm filter, before addition to the media types. Addition of either 50 mg/L or 20 mg/L of hemin were compared for each media type, as these are the two concentrations most commonly used in LIT formulations in the literature.

Cultures were initiated by growing one vial of 5x10⁶ epimastigotes from liquid N₂ stock in 10 mL of LIT2 media, in a 25 cm² phenolic crew capped flask at 27°C, in a normal atmosphere, to a cell density of 5x10⁶ cells/mL. The culture was spun down at 3000g for 8 minutes and washed twice in PBS, before resuspending at a density of 5.25x10⁴ parasites/mL in 10 mL of each respective LIT, 2 replicate flasks per medium. Cells were counted every 48 hours by fixing in 3.6% formaldehyde (section 2.1.1). Counting was stopped when cells appeared to lose motility or rounded up, which was used as a definition of viability. The doubling time was estimated for the medium which supported the most growth, over the shortest time.
3.2.2.2  Y strain

From 3.1.2.1, the media that supported the greatest density of Tulahuen strain parasites was used to assess the growth of the Y strain parasite, cultured in the same manner and counted every 2 days.

3.2.3  Differentiation of T. cruzi epimastigotes

3.2.3.1  Tulahuen strain

Epimastigotes were inoculated into 10 mL flasks in LIT2, at a density of 1x10^6 epimastigotes/mL, in either media containing 20 or 50 mg/L of hemin. Parasite growth was monitored by counting cells in duplicate flasks every 24 hours, from 96 to 216 hours. Spontaneous differentiation in LIT medium during the approximated log phase of growth was monitored at 120, 144 and 168 hours by preparing slides of parasites, one slide per flask, per time point (slide preparation is outlined in section 2.1.2). Two hundred parasites per slide were counted and the percentage of metacyclic trypomastigotes in cultures was determined. The percentage was calculated as

\[
\% \text{ differentiation} = \frac{\text{metacyclic trypomastigotes counted}}{\text{(epimastigotes + metacyclic trypomastigotes counted)}} \times 100.
\]

The remaining flask volume was used to differentiate parasites in TAU3AAG (section 2.3.2), from cultures grown for 120, 144 and 168 hours, in 10 mL of medium, in duplicate flasks.
For the determination of the rates of differentiation during the approximated stationary phase of growth, cultures of epimastigotes were initiated in 10 mL of media, either containing 50 or 20 mg/L of hemin. Parasites were counted at 168 hours to 216 hours growth, at 24 hour intervals. Differentiation was estimated by spontaneous production in LIT and separately by incubation of epimastigotes in TAU3AAG after growth to these time points.

3.2.3.2 Differentiation of T. cruzi Y strain parasites

Y strain epimastigotes were differentiated in TAU3AAG by following the same protocol for the differentiation of Tulahuen strain parasites (section 2.3.2), except that epimastigotes were grown in LIT containing 20 mg/L hemin, and a smaller scale differentiation was undertaken in 4 x 75 cm² flasks. The aim was to incubate cells with host cells until sufficient trypomastigotes were produced to initiate further subculture.

3.2.4 Maintenance of T. cruzi infective cultures

3.2.4.1 Tulahuen strain

3.2.4.1.1 Co-culture with 3T3 host cells

Two separate populations of Tulahuen strain metacyclic trypomastigotes co-cultured with 3T3 host cells were initiated to investigate the parameters of T. cruzi infection, including the length of time that was required for release of parasites and the optimum host cell concentration under the conditions tested. The primary culture was initiated from metacyclic trypomastigotes inoculated on to a 1500 cells/well of 3T3 host cells in an 8-well plate (Nunc-Lab-Tek Chamber slide™,
Waltham, MA, USA), added 4 hours before infection, at a MOI of 10:1 parasite: host cell. Following 24 hours, parasites remaining in the supernatant were washed off with PBS. Co-cultures were scaled up to sufficient cell densities over successive subcultures to infect 25 cm² flasks. In 25 cm² flasks, host cells were seeded 24 hours prior to infection, at 4x10⁵ cells/mL.

A second culture was initiated on a monolayer of host cells in a 9 cm² flask (Nunc Lab-Tek SlideFlask™) and again scaled up to 25 cm² flasks. Monitoring of these cultures allowed identification of the ability of metacyclic trypomastigotes to infect 3T3 host cells under these conditions, and successive co-cultures of the infective life cycle forms of T. cruzi were based on the time of release of trypomastigotes from 25 cm² flask cultures. Extracellular trypomastigotes and amastigotes were enumerated by fixing cells in 3.6% formaldehyde and counting at 20x magnification on a haemocytometer (section 2.1.1).

3.2.4.1.2 Effect of the long-term maintenance of infective cultures

To monitor the effect of the long term maintenance (age) of cultures on trypomastigote and amastigote production in T. cruzi Tulahuen strain co-culture with 3T3 cells, parasites released in to the supernatant were enumerated and compared over a period of 1 - 8 months subculture. Three separately initiated cultures of differing ages that had been maintained for 3, 12 and 15 months respectively were also compared.

3.2.4.1.3 Co-culture with NHCF-A cells

For the co-culture of T. cruzi Tulahuen trypomastigotes with NHCF-A fibroblasts, trypomastigotes were added to a monolayer of host cells that were inoculated 24
hours prior, at $1\times10^6$ cells/25 cm$^2$ flask, from trypomastigotes released from 3T3 host cells (section 2.3.3). Parasites were released and collected following 4 days co-incubation, and were passaged at least 3 times before use in experiments.

3.2.4.2  Y strain

Differentiated Y strain parasites were added to a monolayer of host 3T3 cells. Following 24 hours incubation, non-infected trypomastigotes/epimastigotes were washed off the cell bed by washing 3 times with PBS, and the culture was monitored for the release of trypomastigotes over time.

3.3  Results

3.3.1  Host cell maintenance

3.3.1.1  Host cell doubling time and monolayer cell density

The doubling time of 3T3 cells was calculated to be $23.37\pm1.00$ hours in the log phase of growth, and the doubling time of NHCF-A cells was calculated to be $22.25\pm1.22$ hours. Both cell lines displayed similar growth patterns over the time frames investigated (Figure 3.1).
Cell numbers at confluence were estimated for 3T3 cells to be approximately $4 \times 10^5$ cells, following 24 hours growth. Therefore the MOI of parasite added to flask monolayers was calculated using $4 \times 10^5$ host cells. However, after leaving the cells for an additional 6 days, there were $8.22 \times 10^5 \pm 1.22 \times 10^4$ cells/flask (Figure 3.2). Further time frames would be needed to determine when this doubling occurred, however this does suggest that there may be contact inhibition, at least during the initial 24 hours of growth. A confluent 25 cm$^2$ flask of NHCF-A cells was at $1.19 \times 10^6 \pm 8.83 \times 10^4$, following 6 days growth. Based on the suppliers instructions, confluence is reached in this cell line following 6-9 days growth [262]. Cells were seeded at $1 \times 10^6$ cells/flask before addition of parasites for co-cultures. Note, when calculating the MOI of parasites: host cells, it was based on this cell number.
3.3.1.2 Host cell passage number

3T3 were cells maintained a constant cell number between passages #4-9, when split every 3 days at 4x10⁵ cells/75 cm² flask. At higher passage numbers, there was an increase in cell number (Figure 3.3), and it could be seen that cells were growing over the top of one another (data not shown). NHCF-A cells also displayed a similar pattern, however at passage #6, the cell number increased (Figure 3.3). As a consequence, 3T3 cells were not grown beyond passage #9 and NHCF-A cells were not grown beyond passage #6.
Figure 3.3. The mean number of 3T3 and NHCF-A fibroblasts over passages. (A) 3T3 growth over 12 passages. (B) NHCF-A growth over 10 passages. Cells were split every 3 days, in 175 cm² flasks for 3T3 cells and 75 cm² flasks for NHCF-A cells. Error bars represent the standard deviation of measurements from duplicate flasks.

3.3.2 Culture of *T. cruzi* epimastigotes

3.3.2.1 Tulahuen strain

Growth of *T. cruzi* parasites in the three separate LIT media formulations are shown for the Tulahuen strain parasite in Figure 3.4. The media that supported the most prolific and healthy epimastigote growth was LIT2, which was comprised of a combination of 50 mg/L hemin, supplementation with non-filtered FCS type 1. Non-filtered FCS supported a slightly higher total growth of parasites for both 20 mg/L and 50 mg/L hemin supplementation in LIT1 variations. LIT3 supported growth at a lower total number of parasites, and LIT1 supported poor growth. By microscopy, cells grown in LIT1 were swollen in appearance, with many having lysed. The doubling time of parasites, calculated in the log phase of growth in LIT2 medium was 23.5±8.2 hours.
Figure 3.4. Growth of T. cruzi epimastigotes in variations of LIT media. (A) LIT 1, (B) LIT2 and (C) LIT3. Differences between the preparation of LIT are described in the methodology. FCS1 and FCS2 were serum samples sourced from different suppliers. NF = Not filtered FCS. F = filtered FCS. Error bars indicate the standard deviation of counts taken from duplicate flasks of epimastigotes.

3.3.2.2 Y strain

The growth of Y strain epimastigotes in LIT2 medium, with either 50 or 20 mg/mL hemin is shown in Figure 3.5. Twenty mg/L of hemin supported a slightly higher density of cells, with a doubling time of $96.26 \pm 10.23$ hours, calculated in the log phase of growth.
Figure 3.5. The mean growth (±SD) of Y strain epimastigotes in LIT2 medium, supplemented with either 20 or 50 mg/L of hemin. Counts are averaged from duplicate flasks of epimastigotes prepared.

3.3.3 Differentiation of *T. cruzi* epimastigotes

3.3.3.1 Tulahuen strain

Differentiation of parasites could be seen clearly by Giemsa staining (Figure 3.6). In the epimastigote form, the kinetoplast is between the nucleus and the flagellum and in the metacyclic trypomastigote, the kinetoplast is sub-terminal [244].
Figure 3.6. Geimsa staining to identify *T. cruzi* life cycle forms. (A) Giemsa stained *T. cruzi* epimastigotes, following incubation in artificial triatomine urine (TAU3AAG), showing a metacyclic trypomastigote within a population of epimastigotes. In the trypomastigote, the nucleus and kinetoplast appear separate, and the kinetoplast is anterior. (B) *T. cruzi* epimastigotes in the log phase of growth. Epimastigotes show a close proximity of nucleus and kinetoplast, and the nucleus is more anterior than the kinetoplast. The image was captured with an inverted stereo microscope (Olympus, IX71) equipped with a digital camera (Olympus, DP72), at 100x magnification.

Following 120 hours incubation of *T. cruzi* epimastigotes in LIT1 supplemented with 20 mg/L of hemin, 1% of the culture underwent spontaneous differentiation. Metacyclic trypomastigotes comprised 1% of the culture incubated for 144 hours in LIT1 medium containing 50 mg/mL hemin (Figure 3.7). When parasites were incubated in TAU3AAG, the highest percentage of metacyclic trypomastigotes (6.0±0.94% of the culture) was observed following 144 hours incubation in LIT,
when the media was removed following 24 hours incubation. However, there were 4 times more cells (parasites) per flask in the samples tested with no removal of the media, thus negating the benefit of the increased percentage of metacyclic trypomastigotes. When media was not removed, the highest rate of differentiation was a 3.15±1.20%, from a culture grown for 168 hours in LIT, estimated by the growth curve to be close to the stationary phase of growth.

![Figure 3.7](image)

**Figure 3.7.** Mean growth and differentiation of *T. cruzi* epimastigotes in LIT supplemented with either 20 or 50 mg/L hemin. (A) Differentiation estimated during the log to late-log phase of growth. (B) Differentiation of *T. cruzi* epimastigotes in TAU3AAG, following 120, 144 and 168 hours growth in LIT medium, supplemented with either 50 or 20 mg/mL of hemin. Error bars represent the standard deviation of duplicate measurements, from one differentiation initiated.

When parasites were determined to be in the stationary phase of growth, spontaneous differentiation occurred in 1% of the culture grown in LIT2 medium containing 50 mg/L of hemin after 168, 192 and 216 hours growth (Figure 3.8). In the LIT2 medium containing 20 mg/L hemin, there was 1% differentiation observed following 216 hours growth. Variable results were seen at 192 hours, with one replicate displaying 5% differentiation and the other 1%. When grown in medium containing 50 mg/L hemin at 168 hours growth, there was a
differentiation rate of 3.62±0.27%, similar to previous results seen at what was estimated to be the beginning of the stationary phase of growth.

Figure 3.8. Mean growth and differentiation of *T. cruzi* epimastigotes in LIT supplemented with either 20 or 50 mg/L hemin, during the stationary phase of growth. (A) Differentiation in LIT culture medium. (B) Differentiation in artificial triatomine urine (TAU3AAG), following growth in LIT for 168, 192 and 216 hours. Error bars indicate the standard deviation of measurements taken from duplicate flasks.

To produce trypomastigotes for further experiments, the conditions used for the differentiation of epimastigotes to metacyclic trypomastigotes was to grow 1x 10⁶ epimastigotes/mL in LIT2 containing 50 mg/L hemin for 168 hours, followed by treatment with TAU3AAG. Two 25 cm² flasks, over two experiments were differentiated using these conditions for the initiation of small scale infections, in 5 mL of media. The percentage of the culture that had differentiated was calculated to be 2.75±1.06%. For the production of metacyclic trypomastigotes sufficient to inoculate a 25 cm² flask, containing 4x10⁵ 3T3 cells, the protocol outlined in section 2.3.2 was applied. The mean percentage differentiation of three replicate, larger scale differentiations was 3.21±1.20% (Figure 3.9), producing a mean of 4.5x10⁶±1.05x10⁶ metacyclic trypomastigotes to infect host cells.
Figure 3.9. The mean differentiation of *T. cruzi* Tulahuen strain epimastigotes into metacyclic trypomastigotes by incubation of parasites in artificial triatomine urine (TAU3AAG). Differentiation was in 75 cm$^2$ flasks, as outlined in section 2.3.2. Error bars represent the standard deviation of one count made from 3 separate experiments.

### 3.3.3.2 Y strain

The percentage of metacyclic forms in culture following differentiation observed for the Y strain parasite was 1.5±0.9%, sufficient for a MOI of 2:1 parasite to host cells, in a 25 cm$^2$ flask.

### 3.3.4 Maintenance of infective cultures of *T. cruzi*

#### 3.3.4.1 *T. cruzi* Tulahuen strain

#### 3.3.4.2 Co-culture with 3T3 host cells

A co-culture of 3T3 cells and *T. cruzi* in an 8-well plate was initiated to determine whether a small scale infection could be achieved. As it was not known how many days it would take for trypomastigotes to be released, host cells were added such that a healthy monolayer would be achieved after 10 days growth, which was determined to be 1400 3T3 cells/mL. At densities above this, host cells appeared
granular, with nuclear blebbing observed (results not shown). Two wells were inoculated with metacyclic trypomastigotes at a MOI of 10:1, produced from 25 cm² flasks (section 1.2.4.1). To ensure that the MOI was accurate, host cells were added to the plate 4 hours before the addition of the parasite. Parasites were washed off following 24 hours incubation and following 5 days further incubation, it could be seen that partial release of trypomastigotes occurred, and after 10 days total incubation, expanded numbers of trypomastigotes were transferred to 25cm² flasks containing $4 \times 10^5$ 3T3 host cells. Infected host cells remaining in the wells were stained with Giemsa and amastigotes were observed in host cells (Figure 3.10).

![Figure 3.10. T. cruzi Tulahuen strain amastigotes within 3T3 host cells, stained with Giemsa. The image was captured with an inverted stereo microscope (Olympus, IX71) equipped with a digital camera (Olympus, DP72), at 100x magnification.](image)

A separate culture was initiated in 9 cm² flasks containing $7 \times 10^4$ host cells, and transferred to 25 cm² flasks, following 10 days incubation. Flasks were inoculated 4 hours prior at $4 \times 10^5$ cells/mL, $2 \times 10^5$ cells/mL and $1 \times 10^5$ 3T3 cells/mL. The
relative populations of trypomastigotes and amastigotes were monitored over 6 days (Figure 3.11), in duplicate flasks. The majority of trypomastigotes were first released following 4 days of incubation, with a mean of $4 \times 10^5$ cells/mL, and the extent of trypomastigote release was related to the host cell density. After 6 days, it was observed that there was a large number of amastigotes in the supernatant and obvious damage to host cells.

Figure 3.11. The mean release of *T. cruzi* trypomastigotes and amastigotes in to the supernatant over 3-6 days following infection of varying concentrations of 3T3 host cells. (A) Trypomastigotes in the supernatant. (B) Amastigotes in the supernatant. Error bars represent the standard deviation of duplicate flasks, with cells prepared from one subculture.

Subsequent subcultures were maintained by addition of parasites to $4 \times 10^5$ 3T3 host cells that were incubated for 24 hours before infection, as it was observed that there was little difference between host cells at 0 and 48 hours at this inoculum (section 3.2.3). Both cultures were compared for the release of trypomastigotes over 3 subcultures in 25 cm$^2$ flasks when prepared in this manner. Although there was a slight difference in the production of trypomastigotes on day 5 in comparison to the initial doses tested, initial release of trypomastigotes also
occurred on day 4 (Figure 3.12). Mean numbers of parasites were estimated from duplicate flasks.

![Graph showing mean release of T. cruzi trypomastigotes (T) and amastigotes (A) in to the supernatant following 3-5 days post-infection of 3T3 cells. Error bars indicate the standard deviation of counts taken from 2 replicate flasks. R1-R3 refer to 3 successive rounds of host cell infection, A=amastigotes in the supernatant, T=trypomastigotes in the supernatant.]

For the initiation of future cultures directly into 25 cm² flasks, 10:1 metacyclic trypomastigotes: 3T3 cells were incubated for 8 days on a bed of 4x10⁵ host cells, and trypomastigotes released in to the supernatant were transferred to new host cells. Released trypomastigotes were collected from the supernatant 4 days after infection (section 2.3.2). Trypomastigote production began to level off following approximately 4 subcultures and these parasites were used for further experiments (Figure 3.13). Passages #1 and #2 were estimated from duplicate counts of the supernatant of one flask; and passages #3-7 were from duplicate flasks.
Figure 3.13. Initiation of an infective culture of *T. cruzi* trypomastigotes on a bed of host 3T3 cells. Passage #1 refers to the production of trypomastigotes following infection of a monolayer of host cells with metacyclic trypomastigotes, which was incubated for 8 days before transferring liberated trypomastigotes. Subsequent cultures were initiated from coculture of parasite and host cells for 4 days. The resulting mean number of released trypomastigotes is shown for each passage on day 4 after infection was initiated. Error bars represent the standard deviation of counts made from duplicate flasks.

3.3.4.3 Effect of long-term maintenance of infective cultures

The prevalence of trypomastigote and amastigote life cycle forms was compared for one infective culture at differing ages. There was a significant increase in the number of amastigotes observed in cultures which were 5 and 8 months old (Figure 3.14).
Figure 3.14. Mean release of trypomastigotes and amastigotes in one infective subculture over time. (A) Enumerated trypomastigotes and (B) enumerated amastigotes. Error bars indicate the standard deviation of counts made from duplicate flasks.

The amastigote and trypomastigotes in the supernatant were compared on days 3 and 4 for cultures maintained for 2, 12 and 15 months (Figure 3.15). It was observed that more trypomastigotes were released earlier in 15 month old subcultures on day 3, and a greater proportion of amastigotes were found in the supernatant on day 4.
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Figure 3.15. The number of trypomastigotes and amastigotes in the supernatant of *T. cruzi* infective cultures, over 3 and 4 days post infection. Cultures were in 25 cm$^2$ flasks (A) Maintained for 2 months, (B) maintained for 1 year and (C) maintained for 15 months. Error bars represent the standard deviation of counts made from duplicate flasks prepared from each subculture.

3.3.4.4 Co-culture with NHCF-A host cells

The release of amastigotes and trypomastigotes into the supernatant when cultured with NHCF-A cells is shown in Figure 3.16. Although there was a slight variation over subcultures, *T. cruzi* Tulahuen strain could be successfully maintained in this cell line.

Figure 3.16. Mean Release of *T. cruzi* trypomastigotes and amastigotes from infected NHCF-A cells over 3 successive subcultures, following infection of NHFC-A cells by trypomastigotes harvested from a co-culture of *T. cruzi* and 3T3 fibroblasts. Counts were made from duplicate flasks per subculture. Error bars represent the standard deviation of measurements.
3.3.4.5  Y strain

Following 2 weeks incubation of Y strain metacyclic trypomastigotes on a monolayer of 3T3 cells in 25 cm² flasks, there were very few trypomastigotes released. As there was a low MOI used (2:1), the whole supernatant from a 25 cm² flask was added to a fresh bed of host cells and again incubated for a further 2 weeks. It appeared that when infected cells burst, the majority of parasites released appeared to have similar morphology to epimastigotes and amastigotes. Cultures were discarded following 1 month. Unfortunately, there was insufficient production of parasites to initiate further experiments.

3.4  Discussion

It was found that LIT2 medium supplemented with 50 mg/L hemin and 10% non-filtered FCS was most effective at supporting the growth of T. cruzi Tulahuen strain epimastigotes, resulting in a doubling time of 23.5±8.2 hours. This doubling time was similar to a previous report on the growth of the Tulahuen strain parasite in LIT containing 20 mg/L hemin resulting in a doubling time of 30 hours [266].

Differentiation of T. cruzi epimastigotes into the metacyclic, infective form in vitro is required for initiation of co-culture with mammalian cells [267]. Differentiation can occur spontaneously during the stationary phase of growth in LIT media, and there have been varying levels of production reported with this method. Camargo (1964) [215] observed 25% metacyclic forms in cultures for the Y strain in the stationary phase of growth, whilst Noguira et al (1975) [268] reported 15% and Manning-Cela (2001) [269] less than 3%. Five percent differentiation of cultures has been reported for the Tulahuen strain, [270] and for the EP strain, 2-5% [271].
Whilst others state 10% of metacyclic forms in culture following differentiation for the strains Chile5, DM28, Tulahuen and Y strains [272]. From the studies undertaken here, it can be demonstrated that when epimastigotes were cultured in a LIT medium containing contained 50 mg hemin/L to a high density, a maximum differentiation of 1% was observed. There was also 1% metacyclic forms differentiated in cultures of epimastigotes in the log phase of growth, following 120 and 144 hours growth in LIT for cultures grown in 50 and 20 mg/L hemin, respectively.

In an effort to increase the production of metacyclic forms for scale up to sufficient number of parasites for infection of host cells, and to minimise the number of epimastigotes, differentiation by a medium mimicking artificial triatomine bug urine, TAU3AAG was explored [250]. TAU3AAG has been used to differentiate a number of different T. cruzi strains [214,250,256,257,273-279]. Removal of the media following 24 hours incubation in TAU3AAG can reduce the levels of non-differentiating parasites [275]. However, although this method increased differentiation to 6.0±0.94%, there was a reduction in the total number of parasites by 25%, therefore negating the benefits of this process. It was observed that with the use of parasites grown in LIT containing either 20 or 50 mg/L of hemin, that the greatest production of trypomastigotes following differentiation in TAU3AAG, without a media change, was at an average of 3.38±0.33%. This was following growth in LIT containing 50 mg/L hemin for 168 hours, with an inoculum density of 1x10^6 cells/mL. Similarly, Y strain parasites were able to be differentiated in this manner, with a differentiation rate of 1.5±0.9%.

An in vitro co-culture system was successfully established for the infection process for the Tulahuen strain of T. cruzi into 3T3 host cells, utilising a MOI of 10:1, which has been applied in previous experiments with T. cruzi [267,280]. It was found that
a confluent monolayer of 3T3 cells, at 4x10^5 cells per 25 cm^2 flask, supported production of the greatest number of trypomastigotes, with the major release of trypomastigotes on day 4 post-infection. Here the infection of NHCF-A fibroblasts by Tulahuen strain *T. cruzi* is also described, which has not previously been reported in the literature. The Y strain parasite was not able to be successfully subcultured in 3T3 cells and highlights the need for optimisation of growth of *T. cruzi* sub-species before assay development can be undertaken. Others have described that the Y strain was unable to be maintained in subculture in VERO cells, whereby the parasite differentiated into amastigote like forms [281], which was also demonstrated here. Although other authors have described Y strain subculture in 3T3 cells, the methodology provided is not detailed. Investigation into the Y strain infectivity of NHCF-A cells for future assay development may be warranted, as it has been well documented that there are differences in drug sensitivity between *T. cruzi* strains [282,283].

During the optimisation of growth of mammalian and parasite cells, there were a number of variables discovered that would be imperative to address to develop a successful phenotypic, image-based assay. Both fibroblast cell lines showed an increase in growth rates beyond passages #10 and #6 for 3T3 cells and NHCF-A cells, respectively, combined with a noticeable loss in contact inhibition. For image-based analysis the absence of a monolayer would make it difficult to determine a single host cell. The doubling time of host cells, both 3T3 and NHCF-A cells, was determined, which is important for consideration of the number of parasites added to host cells, in terms of the MOI. If host cells are added before addition of parasite and grow during this time, the MOI would need to take into account a potential doubling of cells. Also, previously reported doubling times for cells provide an indication of the health of the cultures described herein and thus should also be taken into account. NIH3T3 cells have been reported to have a
doubling time of 24.6 hours [284], similar to that found in this study, namely 23.5±8.2 hours.

The age of the host-parasite co-culture was also identified as an important factor which needed to be taken into consideration, as it impacted upon the morphological forms found in the supernatant of *T. cruzi* Tulahuen strain cultured with 3T3 cells. There was an increase in amastigote, or rounded forms, in cultures maintained over a 15 month period, demonstrated by a comparison of *T. cruzi* infected 3T3 cells subcultured for 2 months and 15 months (Figure 3.15), whereby 4 days after infection of host cells there were 5.87x10^5 and 1.53x10^7 amastigotes released, respectively, a difference of 26 times the number of amastigotes. An increase in the release of trypomastigotes from host cells was also observed on day 3 following infection, which we have previously reported [285]. A culture maintained for 2 months released 2.94x 10^5 trypomastigotes on day 3 following infection, whilst a culture maintained for 15 months released 7.99x10^6 trypomastigotes into the supernatant, a 27 times difference. Previously, there has been a report of similar changes taking place in a culture of Brazil strain *T. cruzi* infected PSC3H fibroblasts, where cultures initiated with freshly isolated trypomastigotes (2-5 weeks old) from infected mice formed less amastigotes in comparison to cultures maintained for > 2 months. This was observed when trypomastigotes were incubated alone, or in the presence of PSC3H host cells [216]. Such morphological changes could correlate with associated genetic changes occurring over time and this could impact on assay techniques and drug sensitivity.

It was determined for the Tulahuen strain of *T. cruzi*, under the conditions tested, that the optimal growth of epimastigotes was in LIT2 medium, supplemented with 50 mg/L hemin (Figure 3.4). Using epimastigotes grown in this medium,
chemical initiation of differentiation in TAU3AAG afforded the greatest percentage of epimastigote conversion to the infective metacyclic trypomastigote life cycle form (2.3-4.8% of the culture, Figure 3.9), used to initiate subcultures of *T. cruzi* on 3T3 fibroblasts. Following incubation of infected cells for 8 days, liberated trypomastigotes were transferred to a new bed of 3T3 host cells and subsequent subcultures were maintained by collecting liberated trypomastigotes 4 days after infection (Figure 3.13). Cultures could be maintained in this way for approximately <5 months, whereby the number of extracellular amastigotes increased 5.7 fold (Figure 3.14). Observation of these characteristics has meant that infective cultures utilised for the research presented here were limited to 3 months. Trypomastigotes liberated from 3T3 fibroblasts in this manner could also be used to initiate subcultures of NHCF-A human heart fibroblasts, and paves the way for optimisation of the growth of this strain in other human heart cells in the future, such as cardiomyocytes. *T. cruzi* has been cultured in rat cardiomyocytes [286] however has not been reported in human cells, and would serve an appropriate model for tissue infected during the chronic phase of Chagas, as it has been shown that myocardial infection occurs in this stage [287].
4 Assay Development

4.1 Introduction

The most widely used assay for the estimation of compound activity against *T. cruzi* parasites utilises β-galactosidase transfected parasites, which are lysed and detected with substrate following the release of expanded numbers of trypomastigotes from infected host cells into media [29]. This assay has been applied successfully in high-throughput format to screen large compound libraries to identify active compounds against *T. cruzi* [231] and also in identifying compounds that are currently in the drug discovery pipeline [238]. However, there are some disadvantages with this method, including the need to lyse the parasite and the necessity for a separate assay to determine compound activity on the host cell. The detection limits of a colourimetric end-point assay may not identify low levels of parasite infection in host cells and compounds could colour quench the signal. Furthermore, compounds could also have direct activity on the expression of the reporter enzyme, which has been demonstrated in a mammalian β-galactosidase transfected tissue culture cell line [288].

Recently, the development of an image-based technique to assess compound activity against the amastigote form of *T. cruzi*, utilising the DNA dye, 4',6-diamidino-2-phenylindole (DAPI), by fixing the parasite and host cells before staining has been described [229]. A script was applied to the acquired images to detect parasites within infected host cells by size exclusion of parasites nuclei in comparison to the host cell nuclei. *T. cruzi* amastigote nuclei are smaller, estimated to be 2-4 µm in diameter [289], in comparison to 3T3 cells, shown to be approximately 12 µm in diameter [290]. Separately, another publication has described the development of a high-content imaging system to define *T. cruzi*
host cell infection rates, utilising DAPI to identify parasite and host cell nuclei, again analysed by size exclusion. However, additionally these authors have included the definition of the host cell cytoplasm through utilization of GFP-transfected 3T3 fibroblasts [291]. This allows for more accurate segmentation of the host cell cytoplasm, for potentially improved definition of *T. cruzi* infected cells and also a more accurate estimation of host cell health by analysis of morphology. Although this assay has not been currently reported for the use of compound screening, such fluorescent markers could improve existing methods.

Initially experiments were undertaken to define if it was possible to use an assay that encompassed two life cycle stages of *T. cruzi* in host cells, namely from amastigote development, release, and reinfection of host cells. A similar approach has been used for Leishmania parasites, however in this format parasites are collected from infected, drug treated cells, and transformed into the infective form [292] before testing. Because of the potential importance of amastigotes in the chronic phase of the disease [261,293-295], the aim was to develop an assay format that would enable determination of whether amastigotes were not completely cleared by a compound, and also whether remaining parasites were able to then go on and re-infect other cells. The life cycle of the parasite, represented by 3T3 cell infection, was studied in a 384-well format over time. The 3T3 cell line was selected as it has been used successfully used in a number of studies described in the literature as a host cell in *T. cruzi* drug discovery assays to identify active compounds *in vitro* [27,29]. A number of compounds identified as active utilising 3T3 cells have also shown *in vivo* efficacy in both acute and chronic models of *T. cruzi* infection, and serve as current leads in drug discovery programs [231,241]. It was found that amastigote contamination did not allow imaging to be employed for incubation periods beyond 3 days post infection. Described herein is the
development of a robust assay based on the incubation of host cells for 3 days post infection, with amastigote infected host cells exposed to compound for 48 hours. Before implementation of in-plate infection, a host cell concentration that would form as close to a confluent monolayer as possible was determined, so that in both estimation of the life cycle of the parasite in a well and in a finalised assay format, there would be minimal doubling of host cells. Growth of host cells would affect the apparent growth of amastigotes, as these intracellular forms are passed from mother to daughter host cells during division [296]. Also, for image-based purposes, host cells growing over one another would make quantification of infected cells difficult. An attempt has been made to decrease host cell division by irradiating host cells [296], however the effect on the host cell/ *T. cruzi* interaction by compromised host cells is unknown. Here, the 3T3 fibroblast cell line has been employed, which is contact inhibited upon the formation of a confluent monolayer. Although this does not mean cessation of growth, this does reduce cell doubling. As heart cells are potentially involved in the chronic phase of the disease, an assay that allowed identification of activity of compounds on the heart fibroblast cell line, NHFC-A was also investigated.

*In vitro* phenotypic assays utilising live cell imaging have the ability to measure accurate cell viability which has recently been demonstrated in malaria research [297,298]. Live-cell imaging methods for *T. cruzi* parasites, have incorporated cadmium telluride quantum dots [299] to assess vector interactions, however currently there are no reported assays that use live cell markers for *T. cruzi* to assess compound activity. Imaging of *T. cruzi* infection of host cells was compared herein with various fluorescent dye combinations for both fixed and live-cell systems, in a 384-well-format. Fluorescent dye combinations to define both the host cell and the parasite were explored. Optimisation of the imaging techniques to be utilised included the effectiveness of dyes in identifying host cell-internalised
amastigotes, the reproducibility of host cell and parasite detection, and the
sensitivity of the assay to known reference drugs / compounds. NFX, currently
used to treat Chagas infections was used as the *T. cruzi* specific compound, along
with the non-specific inhibitor, puromycin. The use of a non-specific inhibitor was
included to enable characterisation of the capability of the assay format to define
activity of compounds with activity against the host cell, an important
consideration for *T. cruzi* drug discovery.

Although there is some debate about the importance of the trypomastigote as a
clinically relevant parasite form for drug discovery (section 1.3.1.3), there is
prevalence of this life cycle form in the acute stage of the disease [300]. Antibodies
with lytic activity against trypomastigotes have been detected in the chronic phase
[301], however as antibodies can remain after infection, their presence is not
necessarily indicative of a current infection [302]. It has proven difficult to identify
trypomastigotes in the chronic stage of Chagas disease, and methods that
currently exist for the detection of parasites, either xenodiagnosis with Triatomine
vector, or haemodiagnosis by culturing blood in LIT medium [303] have low
sensitivity [304]. Repeated haemoculture can identify trypomastigotes in up to
50% of chronic cases [302], however lower numbers of parasites may not be
detectable. PCR has also been applied in the attempt to diagnose chronic Chagas,
however again it is difficult to detect low number of parasites, and there is a high
variability in detection of infection in this method, with both acute and chronic
infections [70]. It has, however, been documented that vertical transmission, and
transmission from blood transfusions during the chronic stage of the disease, can
only occur if there are viable parasites in the blood [305]. Therefore, it is possible
that low levels of trypomastigotes may be circulating in the chronic phase of the
disease. Thus, an assay which has the ability to estimate the activity of compounds
against the trypomastigote form of the disease was also developed, using a
resazurin based dye, PrestoBlue, and is described. As the trypomastigote life cycle form is non-dividing, a single assay format can be used to identify compound activity at a defined end time point, and then at subsequent time points as short as 6 hours incubation.

The aim with the assays developed and described here was to identify compounds with activity against the amastigote form of *T. cruzi* and to also enable determination of compound activity on the trypomastigote life cycle form. As the drugs that are currently used clinically to treat *T. cruzi* possess anti-trypomastigote activity [306], it would be beneficial for new compounds to also exhibit trypomastigote activity, however not entirely essential.

### 4.2 Materials and methods

#### 4.2.1 3T3 host cell growth

#### 4.2.1.1 Plate type and host cell adherence

To determine the micro-titre plate that supported 3T3 fibroblast adherence and maintained this during wash steps, various plate types were compared including 384-well poly-D-lysine coated (CellCarrier, PerkinElmer, USA) and collagen I coated (CellCarrier, PerkinElmer, USA) plates in two separate experiments. Fifty microlitres of 3T3 cells ranging from $4 \times 10^3$ cells/mL to 500 cells/mL were prepared in RPMI medium without phenol red, in a dilution series of 1:2. Fifty microlitres per well were added to two poly-D-lysine plates, 32 wells per concentration (16 wells each for before and after wash analysis), to give final concentrations ranging from 200 cells/well to 25 cells/well. This was repeated for the collagen I coated plates, however as confluence was not reached at the top cell dose from initial
trials, the dose was increased to 400 cells/well. Plates were incubated for 7 days at 37°C and 5% CO₂. Poly-D-lysine plates were washed 5 times with 20 µL PBS, and collagen I coated plates were washed either two or 5 times, using a multichannel pipette (Finn, Thermo Scientific, Waltham, MA, USA). Two washes were compared to 5 as it was estimated that 2-3 washes may be sufficient to wash off parasite, and at least 5 wash steps would be required for host and parasite staining methods. On the last wash step, the well volume was replaced with 50 µL of 10% Alamar Blue, prepared in RPMI supplemented with 10% FCS and plates were incubated for 5 hours before reading on an Envision plate reader (Perkin Elmer, USA).

4.2.1.2 Confluence of the host cell monolayer following 24 and 96 hours growth

To assess monolayer formation, 3T3 fibroblasts were added to 384-well collagen I coated plates at concentrations ranging from 4x10³ to 31 cells per well, in dilution series of 1:2. Wells were assessed for growth following 24 and 96 hours growth by Giemsa stain (section 2.1.1.2). Assessment at 24 hours was undertaken to determine if there may be doubling of host cells following parasite addition, as parasites are added following a 24 hour incubation of host cells. Based upon the results presented in section 3.2.4.2, parasites at an MOI of 10:1 were released following 4 days post infection, therefore as 96 hours incubation of host cells may correspond to the day before parasite release, this was the maximum time that cells were incubated.

4.2.1.3 Alamar Blue redox dye to assess host cell growth over 7 days

As the addition of 1x10³ 3T3 cells/ well appeared to almost form a confluent monolayer following 24 hours incubation, this cell concentration was assessed for
growth and viability over a period of 7 days. One thousand cells/ well were added to a 384-well collagen I coated micro-titre plate. Wells were washed twice in PBS following 24 hours growth and the media was replaced. Following 48, 72, 96 and 192 hours growth, a final concentration of 10% of Alamar Blue in a volume of 10 \( \mu \)L was added to all wells. Plates were incubated for an additional 5 hours at 37\(^\circ\)C, before reading on an Envision plate reader (PerkinElmer, USA) at an excitation of 530 and emission 595 and sixteen wells were analysed per time interval.

4.2.1.4 *PrestoBlue* redox dye to assess host cell growth over 72 hours

As 1x10\(^3\) 3T3 cells/well appeared to almost form a confluent monolayer following 24 hours incubation, this cell concentration was assessed for the ability to double from 24 to 48 hours. If cells were doubling during this incubation time, the MOI calculated when adding parasites to the plate would be affected. Either 4x10\(^3\), 2x10\(^3\) or 1x10\(^3\) cells/well were added to two collagen I coated plates (PerkinElmer, USA) in 50 \( \mu \)L of RPMI media and incubated for 24 hours, 16 wells per cell concentration, per plate. Ten microlitres of 70\% PrestoBlue was added to wells and the plate was read at 20 minute intervals on an Envision plate reader (PerkinElmer, USA) to determine the linear range for each cell concentration. Following 24 hours growth, PrestoBlue was added to wells in the same manner and read following 120 minutes incubation.

4.2.2 Growth of NHCF-A fibroblasts

4.2.2.1 Confluence of the host cell monolayer following 24 and 96 hours growth

To assess the formation of a confluent monolayer, NHCF-A fibroblasts were added to 384-well collagen I coated plates at concentrations ranging from 6x10\(^3\) to
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1.5x10^2 cells per well, in dilution series of 1:2. Wells were assessed for growth following 24 and 96 hours growth by Giemsa stain (section 2.1.1.2). NHCF-A cells were seeded at higher doses than 3T3 cells as it was observed that these cells formed much closer to one another in confluent monolayers in flask cultures. Two wells per cell concentration were analysed.

4.2.3 The life cycle of *T. cruzi* in a 384-well plate

4.2.3.1 Infection of 3T3 fibroblasts over 7 days

3T3 host cells in 50 µL of phenol red free RPMI medium, supplemented with 10% FCS and 100IU/mL penicillin/streptomycin were added to wells in a 384-well collagen I coated plate, at 1x10^3 cells/well. Following 24 hours incubation, *T. cruzi* Tulahuen parasites, in 10 µL of the same RPMI medium, obtained from flask cultures of 3T3/*T. cruzi* (section 3.2.4.2) were added to wells and incubated for 24 hours before washing off the cell bed 3 times with PBS, using a multichannel pipette. Based on the growth of 3T3 cells in-plate (section 3.2.1.1), it was estimated that host cells may double between 0-24 hours and therefore the MOI for the addition of parasite to host cells was calculated accordingly. Parasites were added at MOI values of 10, 5 and 2:1 parasite to host cell. Parasites were enumerated at 3, 4, 5, 6 and 7 days of co-culture to assess the release of trypomastigotes into the supernatant, by taking a sample of the supernatant from two wells and fixing in formaldehyde (section 2.1.1). Infection of host cells was assessed by counting the number of infected cells daily from 3 to 7 days post infection, following staining with Giemsa (section 2.1.2). Two wells were counted to estimate infection, by scoring the number of infected cells per 200 host cells in a well.
As there were a high number of extracellular amastigotes following 7 days incubation with the host cells, this experiment was repeated at host cell concentrations of $1 \times 10^3$, 500, 250, 125, 62 and 31 cells / well, using lower MOI values of 2:1 and 1:1. Duplicate wells were assessed for the presence of trypomastigotes and amastigotes in the supernatant and the number of infected cells were scored.

4.2.3.2  Relationship of MOI and infected cells in 384-well plates

To determine the relationship of the MOI to the number of infected cells, 3T3 host cells were added to collagen I coated plates and incubated for 24 hours, before the addition of parasite, in 10 µL of media. The MOI values were 20:1, 10:1, 5:1 and 2:1, and two wells per MOI were prepared. Plates were incubated for 3 days post infection and Giemsa stained to estimate the number of infected cells. Two hundred host cells were counted to determine the percentage of host cells infected.

4.2.4  Detection of *T. cruzi* infection of host cells with image-based techniques

4.2.4.1  Live-cell imaging of *T. cruzi* infected cells

4.2.4.1.1  Hoechst and C12-resazurin

3T3 fibroblasts were added to collagen I coated plates at $1 \times 10^3$ cells/well and incubated for 24 hours. Trypomastigotes were added at an MOI of 10:1 parasite:host cell and incubated for a further 24 hours. Parasites were washed off three times with PBS and wells were incubated for 3 days. Infected and non-infected cells were stained with either Hoechst 3342 (Life Technologies, USA) alone or Hoechst in combination with the resazurin based dye, C12-resazurin (Life
technologies, USA). Hoechst was added before, after, or together with C₁₂-resazurin. When the dyes were added simultaneously, or C₁₂-resazurin was added alone, wells were incubated for 30 minutes. When Hoechst was used as a single stain, wells were incubated for either 30 minutes, 1 or 2 hours. All incubations were at 37°C and 5% CO₂. Hoechst was added at either 10 µg/mL or 20 µg/mL and C₁₂-resazurin was added at either 5 or 10 µM. Two wells per dye combination/concentration were used. Plates were read on an Opera confocal imaging system (PerkinElmer, USA), at 20x water magnification. To capture Hoechst staining, an image was taken at 405 nm excitation 450/50 nm emission and to capture C₁₂-resazurin staining, an image was taken at 532 nm excitation and 600/40 nm emission. Images were coloured and merged using ImageJ software (http://rsb.info.nih.gov/ij/).

4.2.4.1.2 Hoechst and Triton X-100

To determine if staining of infected cells with Hoechst could be improved with addition of a detergent, Triton X-100 was co-incubated with Hoechst at either 0, 0.1, 0.01 and 0.001% to stain infected and non-infected cells. Instead of staining with Giemsa to determine if cells were infected, wells were fixed for 15 minutes with 4% PFA before staining for 30 minutes with Hoechst. Stains were washed off 2x with PBS and the well volume was replaced with RPMI containing no phenol red, supplemented with 100IU/mL penicillin/streptomycin before imaging. Parasites and host cells were prepared for staining as in section 4.2.4.1.1. Wells were read on the Opera and processed using ImageJ. Three wells were processed per treatment. HCS NuclearMask™ Blue (Life Technologies, USA), prepared as a 1x solution, and DAPI; at either 1 µM or 500 nM, were also compared for their ability to stain parasite cells, following incubation for 1 hour in the presence of these concentrations of detergent. Images were coloured using ImageJ software.
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4.2.4.1.3 Celltracker Green, Hoechst and C₁₂-resazurin

As the nuclear dyes tested were not able to well define infected host cells in a live staining image-based system, a probe stain, Celltracker Green™ (Life Technologies, USA), was used to stain trypomastigotes before addition to the host cells. Host cells were inoculated into wells at a concentration of 1x10⁵ cells/well and following 24 hours incubation, parasites pre-incubated in Celltracker Green were added to wells at a MOI of 10:1 parasite: host cells, in 10 µL of RPMI supplemented with 10% FCS and 100IU/mL penicillin/streptomycin. Before dilution in media, the dye solution was removed from cells by centrifuging for 300g for 8 minutes, and the cell pellet was washed once with PBS. Initially, concentrations of Celltracker Green were varied, from 25, 5 and 1 µM. Parasites were incubated in Celltracker Green, diluted in RPMI with no supplemented FCS for 30 minutes before cells were counted and added to host cells. Wells were incubated for 3 days before addition of Hoechst, at 10 µg/mL followed by C₁₂-resazurin, at 5 µM. Plates were incubated for 30 minutes with each dye before imaging on the Opera at 20x water magnification. Hoechst and C₁₂-resazurin were captured as described in section 4.2.4.1.1 and Celltracker Green was captured at 455/70 excitation and 600/40 emission. Images were coloured and merged using ImageJ software. 12 wells per concentration of CellTracker Green were used. Following initial investigations, the use of a concentration of 5 µM Celltracker Green was investigated further. The order of addition of C₁₂-resazurin and Hoechst was changed, with either Hoechst or C₁₂-resazurin added first, or at the same time.
4.2.4.2 Fixed-cell imaging of T. cruzi infected host cells

4.2.4.2.1 HCS CellMask Red and Hoechst

A combination of HCS CellMask™ Red (Life Technologies, USA) and Hoechst were used to stain non-infected and T. cruzi infected 3T3 host cells, prepared as described in section 1.2.4.1.1. Wells were fixed by removing media, washing 2x in 50 µL of PBS, replaced with 20 µL of 4% PFA and incubated for 15 minutes at room temperature. Hoechst, at a concentration of 10 µg/mL was added to wells in 20 µL of RPMI and wells were incubated for 30 minutes at room temperature. Wells were washed twice with PBS and the volume was replaced with HCS CellMask Red, at either 4 or 200 ng/mL, in 20 µL of RPMI. Samples were incubated for 30 minutes at room temperature before washing twice with PBS and replacing with 20 µL of RPMI, prior to imaging. Images were captured on an Olympus stereo microscope, at 20 x magnification and were coloured and merged using ImageJ.

4.2.4.2.2 Comparison of HCS CellMask Red and HCS CellMask Green

To improve the identification of host cells using a fixed-dye system, the protocol in section 4.2.4.2.1 was repeated, however the detergent Triton x-100, was added at 0.1% into the Hoechst solution before addition to wells containing the cells. Also, the fixatives, glutaraldehyde (25% in H2O), 100% methanol (MeOH) and 4% PFA were compared. Fixation of cells was performed as follows: 20 µL of MeOH was added to cells for 5 minutes and for glutaraldehyde fixation of cells, a 0.5% solution was added to wells and incubated for 15 minutes. For fixation with PFA, 20 µL was added to wells and incubated for 15 minutes. Both glutaraldehyde and PFA were removed before staining, by washing 3 x with 20 µL of PBS. The MeOH
volume was also removed, however wells were not washed, and dried for 10 minutes before staining. HCS CellMask Green and HCS CellMask Red were compared for their ability to stain parasites within host cells. Both dyes were compared at concentrations of 3.75, 2.5, 1.875, and 1.25 ng/mL. Plates were read on the Opera imaging system at 20x water magnification and three wells per treatment were compared. ImageJ was used to colour and combine images.

4.2.5 Assay development to detect compound activity on *T. cruzi* infected fibroblasts using the Operetta and Opera imaging systems

4.2.5.1 3T3 host cells

4.2.5.1.1 Script development and DMSO sensitivity

Infected 3T3 fibroblasts were prepared as in section 4.2.3.1 in duplicate 384-well plates, and half of the plate contained non-infected cells. Varying doses of puromycin were prepared in water in a separate plate, in triplicate, before addition of 5 µL of these dilutions into the assay plate by a multichannel pipette. As the DMSO tolerability of the cells was not known, and puromycin is poorly soluble in DMSO, water was used in the preparation of these dilutions. The final doses of puromycin in the assay ranged from 333 µM to 0.0167 µM. To determine the DMSO tolerability of the assay, a working concentration of 20% DMSO was prepared in water. A two-fold dilution series in water starting from this concentration was prepared. Five microlitres of each dilution were added to the micro-titre plate to give final DMSO concentrations ranging from 1.60% to 0.000203%. Three replicates of each dilution series, for both puromycin and DMSO, were added to both infected and non-infected cells. Images were captured on the Operetta imaging system (PerkinElmer, USA), on the Hoechst 3342 and
Alexa488 channels at 20 x magnification. To determine if either addition of water or media affected the infection of cells or the number of host cells following 48 hours incubation, 5 µL of either water or RPMI medium without phenol red or FCS supplementation were added to 32 wells of both infected and non-infected 3T3 cells.

Wells were fixed with 4% PFA and stained with Hoechst and HCS CellMask Green, following the protocol outlined in 4.2.4.2.2. A script was developed, using the building block commands in the Operetta Imaging system Harmony® Software (PerkinElmer, USA), by applying each step, outlined in section 2.4.2 to the stained cells. It was determined, by eye, which parameters within the script best defined a host cell nucleus, host cell cytoplasm, and spot detection, to define parasites. Initially, 2 and 3 fields per well were read to estimate the reproducibility of the script applied. The Z'-factor using these script parameters was calculated with either the addition of 5 µL of water or medium, using non-infected cells as a positive control. The method for calculating the Z'-factor is outlined in section 2.4.7.

The most reproducible method (5 µL of water or medium, 2 or 3 fields per well imaged) was used to calculate the percentage of infected cells. This estimation was compared to the percentage of cells determined from staining infected wells with Giemsa and counting under a light microscope at 60 x magnification. Two well were stained, following the protocol outlined in section 2.1.2.

4.2.5.1.2 Wash steps with an automated liquid handler

Two wash steps to remove extracellular parasites following the 24 hour incubation time with host cells were undertaken on a Bravo automated liquid handler
(Agilent Technologies, USA), by washing wells with 50 µL of PBS, pre-incubated at 37°C. The addition of PFA, Hoechst and HCS CellMask Green, were also undertaken on the instrument, with two wash steps, by addition of 20 µL of PBS used following each addition. The additions of parasites and host cells were made with a Multidrop (Thermo Scientific, USA), rather than with a multichannel pipette. Two plates were prepared containing 3T3 infected cells. Dose response of the compounds puromycin and NFX were added in triplicate to each plate, in 5 µL of water, by a multichannel pipette. The dilutions were prepared as in section 2.4.1, except that additions were made with a multichannel pipette. Puromycin stocks were prepared in water and NFX was prepared in 100% DMSO.

Wells were analysed as described in section 2.2.5.1, on the Operetta, except that images of Hoechst and HCS CellMask Green staining were taken from 3 fields per well, as outlined in section 4.2.5.1.1. Following the wash steps, 10 µL of media remained in the wells before addition of medium containing RPMI with no phenol red, supplemented with 100IU/mL penicillin/streptomycin, prior to imaging, as outlined in section 2.4.2. Therefore to calculate the activity of reference compounds, a final volume of 65 µL was used.

**4.2.5.1.3 Optimisation of script parameters on the Operetta imaging system using a 10:1 MOI**

To improve the wash steps to remove uninfected trypomastigotes from wells, one wash was undertaken by adding 50 µL of PBS to the plate with a Multidrop liquid handler and removing the volume into a plastic reservoir in a PC2 hood. Two wash steps were then undertaken on the Bravo liquid handler, by addition and removal of 50 µL of PBS. This was performed in an effort to remove as many parasites as possible. A 384-well micro-titre plate was prepared with 3T3 host cells.
in 50 µL of RPMI supplemented with 10% FCS and 100IU/mL penicillin/
streptomycin and infected with *T. cruzi* trypomastigotes, in 10 µL of the same
media, at an MOI of 10:1 parasite: host cell. Following 24 hours, the extracellular
parasites were washed off as outlined above. A 384-well, sterile, clear micro-titre
plate (BD Biosciences USA) containing one third puromycin (8.26 µM) in water, a
third NFX (3.28 mM) in 100% DMSO and a third DMSO (100%) was prepared,
with 128 wells per compound/ DMSO. This plate was diluted and added to the cell
plate as outlined in section 2.4.1. The final concentrations in the assay were 30 µM,
12 µM and 0.36% respectively in the assay, as shown in section 2.4.7, and this plate
was used to calculate the Z’-factor of the assay. Following 3 days post infection,
wells were stained and read on the Operetta imaging system. The script described
in section 2.4.3, taking images from either 3, 4 or 5 fields per well were compared.
The percentage infection and the number of infected cells per well was examined.
The reproducibility for these combinations was compared.

4.2.5.1.4  Relationship of the MOI versus infected cells on the Operetta Imaging system

The linearity of the number of infected cells in relation to the MOI used was
determined on the Operetta. A plate containing MOI values from 40:1 parasite:
host cell down to an MOI of 0.3125:1, with serial dilutions of 2:1 was prepared and
read on the Operetta, using the script outlined in section 2.4.3. There were 32 wells
used to estimate infected and host cells for each MOI value.

4.2.5.1.5  5:1 MOI and 10:1 MOI on the Operetta Imaging System

Two separate experiments were used to compare a MOI of 5:1 and a MOI of 10:1
in terms of the activity of the reference compounds NFX and puromycin, and the
reproducibility of the detection on the Operetta imaging system. Three replicates of a dose response series were used for each MOI, within each experiment.

4.2.5.1.6 Optimisation of the number of fields read on the Opera imaging system using a 5:1 MOI

Using the Assay Language Interface on the Opera confocal imaging system, the same protocol that was used to define infected host cells on the Operetta was applied. The only difference in the imaging of wells on the Opera was 2 stacks (differing in a height of 2-4 µm), were taken per field and the image representative of each field was a composite of these stacks. If images were taken on one plane using the Opera, some amastigotes appeared to be out of focus. For this reason, multiple heights were taken and then combined to form one image for analysis.

For both imagers, the “building block” styles in the analysis software allow for the same criteria to be applied to stained wells on both systems. Two plates were prepared as outlined in section 1.2.5.3, containing a third puromycin, NFX and DMSO. Using the same script as the Opera imaging system, it was determined that imaging 5 fields per well did not result in a Z’- factor greater than 0.5, nominally used as the cut off to define good assay reproducibility [247]. A comparison of data from images taken from 5 and 7 fields per well were compared and the script was applied to determine the number of resulting infected and non-infected cells and the Z’-prime factor for each. To determine if the identification of 5 amastigotes per cell was sufficient to reduce background spots from the spot analysis script, images taken from 5 fields in one infected well were analysed to determine the number of spots per cell by applying either ≥1 spot per cell or ≥5 spots per cell as a cut off for the detection of an infected cell.
4.2.5.1.7 Relationship of the MOI to infected cells on the Opera Imaging system

The linearity of infected host cells in relation to the MOI used was determined using the Opera. One microtitre plate, containing MOI values ranging from 40:1 parasite: host cells, with 16 wells per treatment, to a MOI of 0.078:1 was processed and analysed on the Opera imaging system. Serial dilutions of 2:1 were prepared and read on the system, using the script outlined in section 2.4.3. There were 32 wells for each treatment used to estimate the number of infected host cells.

4.2.5.2 NHCF-A host cells

4.2.5.2.1 Relationship of the MOI versus infected cells on the Opera Imaging system

The same criteria that were applied to detection of infection of T. cruzi infected 3T3 host cells were also applied to T. cruzi infected NHCF-A cells on the Opera confocal imaging system (section 2.4.3), the only difference was that 0.1% of DMSO was used in these experiments. There was a reduction in DMSO as the use of 0.4% DMSO on these cells, resulted in cell death (results not shown). Lower doses of 0.1, 0.05 and 0.001% DMSO were tested against these cells. No obvious effects, on either the host or infected cells, were detected at these concentrations (results not shown).

To determine the relationship of the MOI and the resulting number of infected cells, 3x10^3 host cells/well were added to a collagen I coated 384-well microtitre plate and parasites were added, following 24 hours incubation, at MOI values ranging from 20:1 to 0.039:1. MOI values were based on estimating one doubling of NHCF-A cells over 24 hours from seeding of the host cells.
4.2.5.2.2 Reference compound activity

The activity of puromycin, NFX and amphotericin B against *T. cruzi* infected NHCF-A host cells were estimated for 2 independent experiments, with one dose series in each experiment. The activity of the reference compounds against both host cells and parasites were compared to those estimated for *T. cruzi* infected 3T3 host cells, using the same protocol to estimate infection (section 2.4.3) on the Opera imaging system.

4.2.6 Assay to determine the effects of compounds on trypomastigotes

4.2.6.1 Amastigote formation and cell doses

Fifty microlitres of trypomastigotes were added to 384-well sterile black / clear bottomed plates (BectonDickinson, USA) at cell densities ranging from 8x10^5 to 2.5x10^4 cells/well, in a dilution series of 1:2, in RPMI supplemented with 10% FCS and 100IU/mL penicillin/ streptomycin. Following 24 and 48 hours incubation under standard conditions, 3 wells were counted per cell concentration to determine the number of trypomastigotes remaining, and the number of trypomastigotes that had differentiated into extracellular amastigotes.

4.2.6.2 Relationship of the number of cells to the fluorescent signal

The same cell densities that were prepared in section 1.2.6.1, from 8x10^5 to 2.5x10^4 cells/ well, were used for this experiment to determine the relationship between the number of cells and the fluorescent signal. Micro-titre plates containing the cells were incubated for 42 hours under standard culture conditions before the addition of a final concentration 10% PrestoBlue. Plates were incubated for an
additional 6 hours at 37°C before being read on an Envision plate reader at an excitation of 530 nm and an emission of 595 nm. At this time point, 1x10^5 cells/well gave a signal window of approximately 2 fold. The cell densities which resulted in a linear relationship between the number of cells, and the resulting fluorescent signal, were determined. The reproducibility of the signal in relation to media alone, by estimating the Z’-factor, was determined. The signal window for each cell concentration was calculated, utilising wells containing media alone as the reference point. For each of the cell densities being investigated, there were 32 replicate wells.

4.2.6.3 Activity of reference compounds

Two experiments were performed to determine the activity of the reference compounds, puromycin and NFX, against host cell-free trypomastigotes. Fifty microlitres containing 1x 10^5 trypomastigotes were added to 384-well microtitre plates, using a Multidrop. Five microlitres of puromycin or NFX diluted in water to give a final DMSO concentration of 0.472% were added to the wells and incubated for 42 hours under standard incubation conditions. To calculate the Z’-factor of the assay, wells containing a final concentration of 0.472% DMSO and NFX at a final concentration of 14.2 µM served as the negative and positive controls, respectively. The same stock plates of control compounds / DMSO were used to calculate the Z’-factor and reference compound activity were used for both the trypomastigote and amastigote assays, hence there was a slight change in the final assay concentration of DMSO and reference compounds in the trypomastigote assay (section 4.2.5.1.2). A final concentration of 10% PrestoBlue was added to wells and the plates were incubated for a further 6 hours before determining the fluorescent signal on an Envision plate reader.
4.3 Results

4.3.1 3T3 host cell growth

4.3.1.1 Plate type and host cell adherence

When cells grown in poly-d-lysine coated wells in a 384-well micro-titre plate were washed 5 times with 20 µL of PBS, the Alamar Blue signal was decreased across all cell concentrations tested, when compared with the unwashed control wells (Figure 4.1A). The mean signal retained in comparison to the unwashed control, between 200 and 400 cells/well was 46.15±3.78%, and between 50 to 12 cells/well was 23.43±1.88%. There was a lack of formation of a monolayer particularly evident at 12 cells/well. When cells were plated onto collagen I coated wells, 76.87±6.40% of the signal remained in comparison to the control wells (unwashed), for cell densities ranging from 200 to 400 cells/well (Figure 4.1B). When the cell concentration was between 50 to 12.5 cells/well, 51.25±12.25% of the signal remained following 5 washes. When the wash steps were reduced from five to two washes, the signal was on average 93.39±9.71% in comparison to unwashed controls (100%), for all cell concentrations, except for 12 cells per well, with a reduction to 71% of the signal observed.
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Figure 4.1. Alamar Blue detection of viable cells following wash steps on collagen I or plastic plate surfaces. (A) Poly-D-lysine coated plates. (B) Collagen I coated plates. Error bars represent the standard deviation of the mean fluorescence from 16 wells per cell concentration. The background signal has been removed from measurements.

4.3.1.2  Confluence of the host cell monolayer following 24 and 96 hours growth

An initial cell density of 1x10³ cells/well resulted in a 60-70% confluent host cell monolayer forming following 24 hours incubation (Figure 4.2). At the lower host cell concentrations (from 0.5x 10³ cells/ well to 31 cells/well), a sub-confluent monolayer was observed after the same incubation period. Wells containing initial cell densities of 2x10³ and 4 x10³ cells/well, demonstrated clumping after incubation for the same period of time. In comparison, a confluent monolayer was observed, following 96 hours growth, for cells plated at 1x10³ cells/ well.
4.3.1.3 Alamar Blue redox dye to assess host cell growth over 7 days

An increase of 1.68 times in the Alamar Blue signal was observed between 48 to 72 hours growth whereas minimal changes (0.95 fold) were observed in the signal during the incubation period between 72 and 96 hours, (Figure 4.3). Following 7 days growth, there was an increase in the Alamar Blue signal by 2.28 fold in comparison to the signal observed following 96 hours incubation. It was demonstrated that the maximum detectable signal for Alamar Blue using the Envision was 1x10^6 RFU (results not shown). All reads performed for the above assessments were within these limits.

4.3.1.4 PrestoBlue redox reagent to estimate doubling time of 3T3 cells over 48 hours

To improve the efficiency of host cell detection, specifically the speed, PrestoBlue was used to detect 3T3 cells in wells at 1x10^3, 2x10^3 and 4x10^3 cells/well, following 24 and 48 hours growth. At all cell concentrations tested, a linear relationship was
shown for PrestoBlue incubated with the 3T3 cells for up to 180 minutes at both incubation time points. Following 48 hours growth, the fluorescent signal for each cell concentration was determined after 120 minutes incubation with the dye, within the linear range of detection (Figure 4.3). This signal was compared to cell concentrations incubated for 120 minutes, following 24 hours growth. There was no significant change between the signal determined from 4x10³ cells at 24 hours growth compared to 2x10³ cells following 48 hours growth, suggesting 2x10³ cells had doubled over the 24 hour period. Similarly, the signal was 2.2x10⁵ fluorescence units determined from 2x10³ cells following 24 hours growth and 1x10³ cells grown for 48 hours, suggesting cells at these concentrations may have also doubled over the 24 hour time interval (Figure 4.3B). Although it appears that from 72-96 hours incubation, there was a similar cell number in wells, using an inoculum of 1x10³ cells/well, demonstrated by Alamar Blue staining (section 4.3.1.3).
Figure 4.3. Growth of 3T3 cells in 384-well collagen I coated plates, estimated by resazurin-based viability dyes. (A) Estimation of the growth of $1 \times 10^3$ 3T3 cells in wells over 192 hours, using Alamar Blue. (B) Estimation of the growth of 1 to $4 \times 10^3$ 3T3 cells per well in a collagen coated plate following 24-48 hours, using PrestoBlue. Cells were incubated with PrestoBlue for varying time points, up to 180 minutes. Results were compared for wells incubated for 120 minutes, demonstrated to be within the linear range of detection of the reagent. Error bars represent the standard deviation of measurements, from 16 wells per host cell concentration. The background signal has been subtracted from fluorescent measurements.

4.3.2 Growth of NHCF-A fibroblasts

4.3.2.1 Confluence of the host cell monolayer following 24 and 96 hours growth

With an inoculum of $6 \times 10^3$ NHCF-A fibroblasts/well, it was observed that cells tended to clump during inoculation of wells and following 96 hours incubation, cells could be seen growing over one another. With an inoculum of $1.5 \times 10^3$ cells/well, overgrowth was not observed, however, there was poor confluence following 24 hours incubation. With an addition of $3 \times 10^3$ cells/well, there was approximately 70% confluence observed after 24 hours growth. Some areas could be seen growing over one another following 96 hours growth, however this was minimal. There was a higher density of NHCF-A fibroblasts required than 3T3 cells to obtain a similar cell density following 24 hours incubation and it may be that more NHCF-A cells/cm² are required for confluence. Although this was not determined in-plate, the maximum cell number in 25 cm² flasks was estimated to be greater for NHCF-A cells under the conditions tested (section 3.3.1.1).
4.3.3 The life cycle of *T. cruzi* in a 384- well micro-titre plate

4.3.3.1 Infection of 3T3 fibroblasts over 7 days

Similar to the observations made with cultures grown in flasks, (section 3.2.4.2), parasites cultured in 384-well micro-titre plates were released from host cells 4 days after host cell infection, with expanded numbers of parasites seen on day 5. On day 4 there were $4.15 \times 10^5$, $1.82 \times 10^5$ and $4.25 \times 10^4$ trypomastigotes detected in the medium, from host cells infected with a MOI of 10:1, 5:1 and 2:1, respectively (Figure 4.5). There was a noticeable difference between the number of released parasites, both amastigotes and trypomastigotes, relative to the MOI used (Figure 4.5A and B). There was also an increase in the number of infected cells over time, with the majority of host cells infected on the seventh day after infection (Figure 4.5C).

**Figure 4.4.** Confluence of NHCF-A fibroblasts in collagen I coated 384-well plates, inoculated at $1 \times 10^3$ cells/ well following 24 and 96 hours growth under standard conditions. (A) 24 hours. (B) 96 hours. Scale represents 20 μm.
Figure 4.5. Growth of *T. cruzi* parasites in co-culture with 3T3 fibroblasts in 384-well collagen I coated plates following 3-7 days post infection. (A) trypomastigotes and (B) amastigotes in the supernatant and (C) the percentage of infected 3T3 fibroblasts, from a co-culture of 3T3 and *T. cruzi* in a 384-well plate. Data represents the mean and standard deviation of counts taken from the supernatant of duplicate flasks prepared for each MOI.

Due to the release of parasites following 4 days post infection, the estimation of the number of infected cells was difficult following 7 days post infection, as released / differentiated amastigotes observed from Giemsa staining remained on the surface of the plate (Figure 4.6), which could be seen at all of the MOI values tested. Hence, to define an infected cell, those host cells containing groups of 5 or more parasites, close to one another and within the host cell were estimated to be infected. It is not clear whether these amastigotes on the plate surface and in the supernatant were from burst mammalian host cells, or if they were from released trypomastigotes which had developed into external amastigotes. Figure 4.6 shows...
there was no presence of these amastigotes on the plate surface 3 days post infection with either 2:1 or 10:1 MOI.

Figure 4.6. Giemsa staining of *T. cruzi* infected 3T3 cells following 3 and 7 days post infection. (A) Amastigotes in 3T3 host cells following 3 days infection with a 2:1 MOI parasite: host cell ratio (B) and a 1:10 MOI. Following 7 days infection there were a high number of amastigotes external to the host cell that remained on the surface of the plate, regardless of the MOI. (C) Representative MOI is 10:1 parasite: host cells Red= host cell-external parasites. Scale bars A-D are 20µM. (D) an image at 100 x magnification to show a high population of these are amastigotes. Images were captured on an Olympus stereo microscope, the scale bar represents 100 x magnification.
In a follow up experiment, the life cycle of the parasite was estimated over 7 days by enumerating infected cells and liberated trypomastigotes for MOI values of 1:1 and 2:1, over a range of 3T3 concentrations (Figure 4.7). It was again observed that parasites, both external and internal, increased with an increasing MOI; along with the host cell concentration. At lower concentrations of host cells, particularly at 31 cells/well, host cells dislodged from the edges of the wells, potentially due to the low confluence of cells at these concentrations. Trypomastigotes were only enumerable on day 5, therefore release of cells may have been delayed slightly at a lower MOI, although this did not improve the presence of external amastigotes seen by Giemsa stain on day 7, for any of the cell concentrations.
Figure 4.7. Mean percentage of T. cruzi infected 3T3 cells and release of trypomastigotes from infected cells, in 384-well plates, over 7 days post infection at varying MOIs and host cell concentrations. (A) With a MOI of 1:1 parasite: host cell and (B) 2:1 parasite : host cell. (C) % infected host cells following a 1:1 MOI and (D) with a 2:1 MOI over 7 days post infection. Error bars represent the standard deviation of single counts taken from duplicate flasks, over one experiment.

From these results, the highest number of infected cells, with no interference from host-external parasites was observed with a 10:1 MOI and 1x10^3 3T3 host cells, 3 days post infection. This MOI was therefore used as the basis for further experiments.

4.3.3.2 Relationship of MOI and infected cells in 384-well plates

The number of infected cells was linear in relation to the MOI, up until 20:1 parasite: host cells. However, at a 20:1 MOI, there was a heavy infection load per cell and some fibroblasts were beginning to burst (Figure 4.8).
Figure 4.8. The mean linear relationship of the MOI of *T. cruzi* in relation to the number of infected cells in a 384-well plate. (A) Linear relationship of the MOI to the resulting % of infected cells, $R^2=0.99$. Error bars represent the standard deviation of measurements, taken from two wells, by counting 200 infected cells/well. (B) at an MOI of 20:1, some cells were heavily infected and cells had begun to differentiate in to trypomastigotes, shown by Giemsa stain. The scale bar represents 20 µm.

4.3.4 Detection of *T. cruzi* infection of host cells with image-based techniques

4.3.4.1 Live-cell imaging of *T. cruzi* infected cells

4.3.4.1.1 Hoechst and C12-resazurin

Infected cells were manually determined by Giemsa staining and used for comparison with the automated system being developed. Throughout all of the live-imaging approaches trialed, Hoechst stained the nuclear material of the host cell clearly, however poor staining of the parasite was observed. The incubation time for infected cells with Hoechst was increased from 30 minutes before imaging up to 2 hours, with no improvement in the clarity of the stain. Exposure time during image acquisition was increased from 80 ms to 600 ms, however again poor staining of the parasites was observed. It was demonstrated that C12-resazurin and Hoechst co-staining allowed for identification of host cells, however would not be suitable to apply to an assay for *T. cruzi* amastigote infection (Figure 4.9).
Figure 4.9. Identification of *T. cruzi* infected 3T3 fibroblasts with a combination of Hoechst and C12-resazurin. (A) Hoechst stained the nucleus of the host cell, however poor staining of parasites was observed. (B) C12- resazurin staining, identifying the host cell. (C) an overlay of C12-resazurin and Hoechst images using Image J shows definition of the host cells. Images were taken at 20x water magnification on an Opera imaging system and images were processed with ImageJ [http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/). Scale bars represent 20 µm.

4.3.4.1.2 *Hoechst and Triton X-100*

The effect of Triton X-100 on Hoechst staining of *T. cruzi* infected 3T3 cells is shown in Figure 4.10. In the absence of Triton X-100, poor staining of the infected cells was seen, and high background staining was evident. There was no improvement in the identification of parasite nuclei with the addition of 0.001% of detergent. Increasing the detergent to 0.1% improved detection of parasites, however this caused the release of amastigotes from host cells, which can be seen as spots not associated with host cells. This effect was not observed when cells were fixed with PFA, followed by staining with Hoechst (Figure 4.10). The same effect was observed for both HCS NuclearMask and DAPI staining (results not shown).
Figure 4.10. *T. cruzi* infected 3T3 fibroblasts following infection with a MOI of 10:1 parasite: host cells and stained with Hoechst to identify host and parasite nuclei. (A) Cells stained without the use of Triton x-100. Inset shows poor staining of parasites, with a high background (arrow). (B) Staining with Hoechst and 0.001% Triton X-100. Inset shows some parasite staining, identified as dots adjacent to the host cell nucleus, although clarity is poor, with high background staining (arrow). (C) Following treatment with 0.01% Triton X-100, there were amastigotes detected, however a number had burst from the host cell (inset, arrow). (D) With the use of 0.1% Triton X-100 there was a low density of host cells and poor infection. (E) After fixing with 4% PFA and no addition of detergent, parasites (dots) can clearly be seen (inset, arrow). Scale bars represent 20 µm.
4.3.4.1.3  Celltracker Green, Hoechst and C₁₂-resazurin

When *T. cruzi* Tulahuen strain parasites were pre-treated with 0.5 µM Celltracker Green before infection of host 3T3 cells, it was observed that the stain was retained only in the parasites within infected host cells, whereas with 25 µM Celltracker Green there was also residual staining of host cells (results not shown). However, with the use of 0.5 µM Celltracker Green, the clarity of parasite staining was poor, even with a high exposure time of 1000 ms (results not shown). Although parasites pre-stained with 5 µM using Celltracker Green could clearly be identified within host cells, there was also uneven staining of parasites, some showing dull staining. The order of addition of stains also did not affect the staining. Representative images illustrated in Figure 4.11 were from the addition of C₁₂-resazurin and Hoechst at the same time.
Figure 4.11. 3T3 fibroblasts infected with *T. cruzi* and stained using a combination of Hoechst, C₁₂-resazurin and Celltracker Green, with live host and parasite cells. A-D) staining of infected cells, E-H) non-infected cells. A, E) Hoechst host cell nucleus staining, B, F) C₁₂-resazurin host identified the cell cytoplasm. C, D) and Celltracker Green stained parasites (D, H). Images presented are the resultant merged images for each dye, using ImageJ. Scale bars represent 20 µm.

4.3.4.2 Fixed cell imaging of *T. cruzi* infected cells

4.3.4.2.1 HCS CellMask Red and Hoechst

With a combination of HCS CellMask Red and Hoechst, there was poor staining and irregular distribution of the stain throughout the host cell cytoplasm. Areas within the cytoplasm that did stain showed poor correlation between the CellMask and nuclear stains utilized to identify a host cell, when images were merged (Figure 4.12). The quality of staining was not improved with the use of either MeOH or glutaraldehyde as fixing agents.

Figure 4.12. *T. cruzi* infected 3T3 cells stained with a combination of Hoechst and HCS CellMask Red, following fixing with 4% paraformaldehyde. A) Hoechst staining of parasite and host cells. B) HCS CellMask Red staining of the host cell cytoplasm. C) merge of (A) and (B) with ImageJ.
4.3.4.2.2 Comparison of HCS CellMask Red and HCS CellMask Green

Both CellMask dyes, when used in combination with 0.1% Triton X-100 at any of the concentrations tested, stained the host cell cytoplasm evenly across all samples. It was possible to use 1.25 ng/mL of each dye to easily visualize detection of the cytoplasm at a 40 ms exposure, with a slightly higher intensity of staining seen with the CellMask Green stain (Figure 4.13). Increasing the dye concentration to 3.75 ng/mL resulted in a high fluorescence intensity observed which made delineation of the cytoplasm difficult (results not shown).
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Figure 4.13. Identification of *T. cruzi* amastigotes in 3T3 host cells using nuclear and cytoplasmic markers. A combination of Hoechst (A,D) was used to identify the host and parasite nuclei and either HCS CellMask Red (B) or (E). HCS CellMask Green was used to identify the host cell cytoplasm. (C) and (D) represent merged images of the nuclear and cytoplasmic stains. Scale bars represent 20 µm.

4.3.5 Assay development to detect compound activity on *T. cruzi* infected fibroblasts on the Operetta and Opera imaging systems

4.3.5.1 3T3 host cells

4.3.5.1.1 Script development and DMSO sensitivity

The *T. cruzi* amastigote / 3T3 assay was developed on both the Opera and Operetta imaging-based systems. The software in both systems has the same building block analysis available to build image-based scripts based upon in-built algorithms. Therefore exactly the same script can be built on both machines. The Harmony software was used to initially build the script on the Operetta and the same script was applied to the Assay Language interface on the Opera. Firstly, each of the nuclear analysis building blocks available in the Harmony software interface were applied to the images of the cells and the script that best defined a host cell were determined. This was undertaken by eye. The Harmony Software contains four separate scripts that define the nucleus of a cell, with cut offs that can be applied to the size, splitting factors to split one nucleus from another and contrast to potentially sort identified nuclei based on the fluorescent intensity. The numbers to define criteria used in many of these script building blocks are arbitrary to the system and a process of applying each separately and visualising the defined objects, was applied. In effect the user “teaches” the system to best define the
object of interest. This process was repeated for detection of the cytoplasm. Spot analysis was optimised by comparing infected and non-infected host cells. As the level of the background with spot analysis in the non-infected cells, the script was modified such that ≥5 spots per cell defined an infected cell. This approach reduced the non-specific background detection, and thus was the script utilised for the selection of infected cells. The final script parameters are described in section 2.4.3. The analysis of spots using Harmony software, that are identified as amastigotes within the host cells, are shown in Figure 4.14.

The number of host cells were identified in each well by exporting the number of objects defined from the Harmony script that contained a nucleus and cytoplasm that were co-localised. The number of infected cells was also exported, as those objects that contained ≥5 spots within the cytoplasm.
Figure 4.14. Analysis to determine a *T. cruzi* infected cell on the Operetta imaging system using building blocks in Harmony software. (A) Overlay of Hoechst and HCS CellMask Green fluorescent stains. (B) Detection of the nucleus of host cells. (C) Spot detection to define a parasite (one amastigote=1 spot). (D) Expanded section of (C) to show the effectiveness of spot detection in identifying parasites within the host cell. P=parasite nucleus; H=Host cell nucleus. Scale bars represent 20 µm.

The number of fields imaged per well were compared, along with the effect of the addition of either 5 µL of water or media to cells. The 5 µL addition was made as this was the vehicle for the addition of compounds in the final assay format. When infected cells were enumerated with 2 fields per well, the $Z'$-factor was below 0.5, considered to be cut-off to determine a robust assay [247]. Increasing the number of fields imaged per well to 3 increased the number of cells detected and therefore improved the $Z'$-factor (Figure 4.15). The addition of water or medium did not alter the reproducibility of the detection of either host cells or amastigote infected host cells, exemplified by the $Z'$-factor in each case being >0.5 (Figure 4.15).
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Figure 4.15. The reproducibility, measured by the Z’-factor, of the addition of 5 µL of water or media to wells containing *T. cruzi* infected 3T3 cells. Infected cells, inoculated at a MOI of 10:1 were incubated for 48 hours. Wells were read over 2 or 3 fields applying a script developed on the Operetta image-based system, and the Z’-factor was compared for each treatment. (A) Z’-factor for the detection of *T. cruzi* infected cells. (B) Z’-factor for the detection of 3T3 host cells. The positive control was non-infected 3T3 cells. Error bars represent the standard deviation of 32 wells analysed, over 2 plates, within one experiment.

With an addition of water, the number of infected cells was 184±0.658 and the number of host cells was 582±10.4, identified from infected wells. Therefore a mean of 31.6% cells were detected as infected by the criteria set. Giemsa staining of wells and manual counting of infected cells resulted in an estimated infection rate of 32.4%, confirming the precision of the Operetta script to identify *T. cruzi* infected cells. From an evaluation of wells containing non-infected cells, there were 539±2.73 host cells. As there was no damage or loss of host cells due to infection 72 hours post infection, it was determined that estimation of compound effects against host cells from wells also containing parasite would be an acceptable method of estimating host-mediated cytotoxicity. From an evaluation of non-infected cells, there were 3.92±1.36 infected cells detected using the script, displaying thus a low background from the spot analysis employed (Figure 4.16).
Figure 4.16. The mean number of *T. cruzi* infected 3T3 cells and total 3T3 cells detected in a 384-well plate with the Operetta image-based system following the addition of 5 µL of water to *T. cruzi* infected and non-infected 3T3 cells for 48 hours. (I) Infected wells and (NI) non–infected wells. Data was from duplicate wells, with 32 wells per treatment, over duplicate 384-well plates.

The criteria that were used for further evaluation were images from 3 fields per well, and the use of water to dilute compounds. The IC\(_{50}\) value of puromycin using this method was determined to be 1.75±0.36 µM against the parasite and 1.51±0.36 µM against the host cells, when the host cell response was analysed from infected host cells. The IC\(_{50}\) value of puromycin was demonstrated to be 2.23±0.33 µM when tested against host cells alone, in non-infected wells. As this compound kills host cells, similar activity against both parasite and host cells would be expected.

The potential impact of DMSO on both the host cells and parasites was investigated. A slight effect was observed, namely on the number of host cells and consequently the number of parasites identified, when DMSO concentrations greater than 0.8% were used (Figure 4.17). Activities calculated at this dose,
relative to non-infected cells, were 18.90±4.90% and 25.94±5.84% for detected parasites and host cells, respectively (Figure 4.17). There was similarly an effect on host cells in non-infected wells, with an activity of 21.9±7.2% observed with the addition of 0.8% DMSO.

Figure 4.17. Effect of DMSO concentration on *T. cruzi* amastigotes and 3T3 host cells detected using an Operetta image-based reader and Harmony analysis software. (A) *T. cruzi* amastigotes in 3T3 host cells and (B) total host cells. The percent activity was calculated in reference to wells containing no parasite. Error bars represent the standard deviation of measurements, from triplicate doses over two replicate plates, from one experiment.

4.3.5.1.2 Wash steps with an automated liquid handler

To optimise the assay format such that it was amenable to HTS it was necessary to incorporate automated washing steps. Key was the ability to wash the wells without significant disruption to the cell layer whilst ensuring removal of the unwanted extracellular parasites. By adjusting the Bravo pipette tips to ensure that the tips were as close as possible to the plate surface, the majority of the well volume could be removed from the plate when washing off trypomastigotes, along with parasites contained in the media (Figure 4.18A). From this initial trial, the IC$_{50}$ value of puromycin against *T. cruzi* intracellular parasites was estimated
to be 2.27±0.77 µM, and simultaneously against host cells, in the same well, was estimated to be 3.40±0.80 µM. NFX had an IC$_{50}$ value against *T. cruzi* intracellular parasites of 1.24±0.17 µM, and was 40% active at 150 µM against host cells.

In a follow up experiment, there appeared to be cells removed from the plate surface by tips (Figure 4.18B), which may have been due to either small discrepancies in plate heights or changes in the height settings of the machine over time. By increasing the plate height, all of the trypomastigotes were not removed and when stained, amastigotes could be seen that were retained in the well (Figure 4.18C). For further evaluation of the assay, a manual wash step was included, to compensate for the inability of the automated liquid handler to remove the whole well volume. This method is outlined in 2.2.5.1.3.
Figure 4.18. Staining of *T. cruzi* infected 3T3 cells showing differing wash step effects accumulated during both wash off of non-infected trypomastigotes and during the staining procedures. All wash steps were undertaken using a Bravo liquid handler. (A) No disruption of the host cell monolayer. (B) Damage to the host cell monolayer, with cells lifted from the plate surface (arrow). (C) Not all of the trypomastigotes were removed in wash steps. Inset and arrow shows amastigotes that appear to be extracellular.
4.3.5.1.3 Optimisation of script parameters on the Operetta imaging system using a 10:1 MOI

Increasing the number of fields imaged per well increased the number of captured host cells and therefore parasites. Capturing 3 fields per well resulted in a Z’-factor of 0.49, close to the cut off of 0.5 (Figure 4.19). By increasing the number of fields imaged per well to 5, the output was more statistically relevant and thus the Z’-factor was improved, to 0.55. The Z’-factor for the detection of host cells remained above 0.5, regardless of the number of fields captured. A slight reduction in the Z’-factor was observed when infected cells were calculated as a percentage of the total number of host cells, with a Z- factor of 0.52 when 5 fields were captured, in comparison to when a total number of cells was used as a measure of infection, with a Z’-factor of 0.55.

![Figure 4.19](image)

Figure 4.19. The number of captured images per well of T. cruzi infected 3T3 cells and the resulting parasite and host cell numbers and assay reproducibility on the Operetta. (A) Number of parasites and host cells and (B) the Z’- factor based on the number or percent of infected cells, or total host cells. 384 –well plates were analysed on the Operetta imaging system, at 20x magnification. Error bars represent the standard deviation of measurements, calculated from 128 wells, over one experiment.
For the analysis of infected cells, 5 fields per well were imaged, with an output of the number of infected host cells was used for the Operetta image-based system. A mean infection of $32.26\pm5.46\%$ per well of host cells was determined using this approach, for 2 independent experiments.

4.3.5.1.4 Linearity of MOI versus infected cells on the Operetta imaging system

A linear relationship between the MOI and the number of infected cells detected up to a $5 \times$ MOI was demonstrated (Figure 4.20).

![Graph A](image1.png)  
![Graph B](image2.png)

**Figure 4.20.** The linearity of the relationship between the MOI and the resulting number of *T. cruzi* infected 3T3 fibroblasts per well following 48 hours post infection, determined on the Operetta imaging system. (A) At 20-40:1 MOI, there appears to be a maximum number of host cells infected. (B) A linear relationship was found up to a multiplicity of 5:1 parasite to host cell. Representative data is from one experiment, with 28 wells per MOI. $R^2=0.99$.

Cells containing differentiated trypomastigotes caused some loss of linearity at higher concentrations. This can be seen as the cells are not detected by the script as efficiently, due to trypomastigotes being tightly compact and not as amenable to spot detection. This was particularly noticeable at a 40:1 MOI (Figure 4.21). A
similar number of host cells remained following infection with a 20:1 MOI (95%),
thus the linearity was not due to loss of host cells from the plate surface due to
infection. It is probable that the maximum number of infected cells may also be
achieved at these MOI values (66% at both 20:1 and 40:1 MOI).

Figure 4.21. High MOI values result in heavily infected 3T3 cells that are not well
defined by spot analysis, demonstrated at a MOI of 20:1. (A) Image overlay of HCS
CellMask Green and Hoechst. (B) Spot detection by building blocks in Harmony software.
(C) Definition of infected cells by the developed script in Harmony software, with green
representing infected host cells. Images were taken at 20 x magnification.

4.3.5.1.5 5:1 and 10:1 MOI on the Operetta imaging system

Based on the results from section 4.3.6.3, a 5:1 MOI was used for all subsequent
experiments, as this MOI was defined as within the linear range of the assay.
There was a decrease in the number of infected cells, as expected, detected with a
5:1 MOI, in comparison to a 10:1 MOI, with a ratio of 0.416. However, the number
of actual host cells detected with each MOI did not vary, with a ratio of 1.07. The
Z' factor for the detection of infected cells was estimated to be 0.64 when a 5:1
ratio of parasites to host cells was used (Figure 4.22).
Figure 4.22. Comparison of an MOI of 10:1 and 5:1 *T. cruzi* parasite to host cells and the resulting numbers of infected and host cells identified, and reproducibility, measured by the Z’-factor. Wells were analysed on the Operetta imaging system. (A) The number of amastigote infected 3T3 cells, with and without treatment with nifurtimox. (B) The number of host cells, with and without treatment with puromycin. (C) The % infected cells in the well. (D) The Z’-factor from identification of host and *T. cruzi* infected cells. For the calculation of the Z’-factor and number of infected cells/host cells, 128 wells of *T. cruzi* infected 3T3 cells were analysed, either treated with DMSO or nifurtimox, over two separate experiments. Error bars represent the standard deviation of these measurements.

When compared to a MOI of 5:1, at 10:1 MOI there was negligible difference between the IC$_{50}$ values of NFX observed (Figure 4.23). The IC$_{50}$ value for NFX was 0.689±0.074 as estimated against *T. cruzi* amastigotes using an MOI of 5:1 and when parasites were added at a 10:1 MOI, the IC$_{50}$ value was 0.913±0.122 µM. The activity of NFX against host cells was below 40% for both a 10:1 and 5:1 MOI. At a
5:1 MOI, the general protein synthesis inhibitor, puromycin resulted in IC$_{50}$ values specific for host cells and parasite of 2.65±0.520 µM and 2.12±0.520 µM, respectively. With the addition of a 10:1 parasite: host cell ratio, the IC$_{50}$ value of puromycin for the parasite and host cells were 2.633±0.822 and 4.091±0.795 µM, respectively. The activity of amphotericin B was also tested and activity compared between 10:1 and 5:1 MOI. There was no difference observed in the IC$_{50}$ value obtained between treatments (p<0.05). Based on the lack of variation in activity of these reference compounds under these conditions, it could be possible to use either of these ratios for further evaluation of compound activity. However, to remain within the linearity of the assay detection, a 5:1 MOI was used for further experiments.
Figure 4.23. The effect of a 5:1 and 10:1 MOI on the activity of the reference compounds puromycin, amphotericin B and NFX against T. cruzi amastigotes and 3T3 cells, estimated on the Operetta imaging system. (A) NFX, (B) puromycin and (C) amphotericin B against T. cruzi amastigotes and 3T3 host cells, when parasites were added to plates at either a 5:1 or 10:1 MOI. The reference compound activities were calculated from three replicate dose series of each compound, for each MOI, over two independent experiments. Error bars represent the standard deviation of these measurements.

4.3.5.1.6 Optimisation of the number of fields per well read on the Opera imaging system using a 5:1 MOI

The developed T. cruzi amastigote/host cell assay was also optimized on the Opera QEHS 2.0 Image-based system. The use of the Opera was undertaken to allow a more efficient high-throughput analysis of plates, with the ability to use more than one reader at a time. Also, this may make the protocol optimized herein to be transferrable to more laboratories that have this equipment available. Five or 7-fields within each well were imaged and compared on the Opera, as it was discovered that when images were taken from 5 fields per well, there were 60% less cells identified than previously estimated on the Operetta (section 4.3.6.1.5). The field of view (FOV) is different between these imaging systems, although the camera size is the same. The FOV using a 20 x water magnification on the Opera is 430 x 345 µm² whilst the FOV using 20x magnification on the Operetta is 675 x 509 µm², a difference of 57% between the machines. Therefore the difference in host cell numbers observed was directly related to the difference in the FOV. By increasing the number of fields taken per well, the number of identified and host cells were increased, therefore improving the reproducibility of the results obtained, resulting in a Z’-factor of 0.592±0.0149 for the amastigote assay and 0.814±0.0636 for detection of the 3T3 host cells (Figure 4.24).
Figure 4.24. The effect of using either 5 or 7 fields imaged per well on the Opera imaging system on the number and percentage of *T. cruzi* infected 3T3 cells, the total number of 3T3 cells and the $Z'$-factor of the detection of parasites and host cells. (A) The number of infected cells, with and without puromycin treatment. (B) The number of host cells, with and without puromycin treatment. (C) The $Z'$-factor of the detection of amastigote infected 3T3 cells and host cells. (D) The percentage of host cells infected in a well. To calculate the $Z'$-factor, one experiment was performed, with 42 wells each of *T. cruzi* infected 3T3 cells treated for 48 hours with either nifurtimox or 0.37% DMSO. Error bars represent the standard deviation of measurements.

Based on utilizing 7 fields imaged using the Opera image-based system, the assay and final script is outlined in section 2.4.3 and Figure 4.25 shows the analysis of the infection of cells, using the Assay Language Interface.
Figure 4.25. Identification of *T. cruzi* infected 3T3 cells using the Opera QEHS high-content imaging system. Images are taken at 20x water magnification. (A) Infected host cells. Green represents the host cell cytoplasm detected with HCS CellMask Green. Blue depicts nuclear material from both host cells and parasite stained with Hoechst. (B) Definition of the cytoplasmic region of host cells in which spot detection of parasite nuclei is undertaken. (C) Spot detection to define parasite nuclei. (D) Application of script using the Opera assay language interface to define infected cells. Infected cells are green, red are non-infected. (E-H) follows the same definitions for (A-D), only that these are infected host cells that have been treated with 12 µM NFX for 48 hours.

A criteria of ≥5 spots per host cell cytoplasm was used to identify a *T. cruzi*, amastigote infected cell. By eye it was determined that using this cut-off, most spots identified by the Operetta spot detection script, that did not appear to be stained parasite nuclei or “background” spots were not included in analysis (Figure 4.26C). Also, in most infected cells, there were nests of amastigotes that could be seen, with numerous parasite cells within each (Figure 4.26C). To determine whether the identification of ≥5 amastigotes per well was sufficient to lower or remove these background spots, images from 5 fields in one well were
analysed with a cut-off of either ≥5 spots, or a minimum of ≥1 spot per cell. From these fields, it was possible to detect 416 host cells. Cells containing either 0, 1-5; 6-10; 11-15; or 16-20 spots were binned into independent groups (Figure 4.26). It can be seen in Figure 4.26 that by reducing the detection criteria to ≥1 spot per cell, that the number of infected cells in the bin of 1-5 parasites per well increased from a frequency of 4 to 97 cells. From the image captured, it can be seen that using a criteria of ≥5 spots per cell, those cells containing 1-2 spots that appeared to be background with poor stain were not detected (circled; Figure 4.26). Increasing the spot minimum to cell intensity of selected spots (see section 2.4.3) did not improve background, but rather meant that less infected cells were detected, as fewer spots were detected that were clearly shown to be stained with Hoechst (results not shown).
Figure 4.26. The number of spots per cell (x-axis) used to define an infected cell, defined on the Opera image-based system and the resulting number of infected cells. (A) Using a cut off of ≥1 spots per cell, (B) using a cut off of ≥5 spots per cell and (C) a cut off of ≥5 spots per cell and cells containing what appear to be background detection of spots are not included in the analysis. The frequency refers to the number of infected cells identified using each criteria.

4.3.5.1.7 Relationship of the MOI to the number of infected cells on the Opera imaging system

The linearity of infection was retested and confirmed on the Opera, using the conditions outlined in section 4.3.6.1.4. This confirmed that the assay detection was linear, with respect to the number of infected cells per well, up to a 5:1 MOI (Figure 4.27).
Figure 4.27. The linearity of the relationship between the MOI and the resulting number of *T. cruzi* infected 3T3 fibroblasts per well following 48 hours post infection, determined on the Opera imaging system. (A) At 20-40:1 MOI, there appears to be a maximum number of host cells infected. (B) A linear relationship was found up to a multiplicity of 5:1 parasite to host cell. Representative data is from one experiment, with 28 wells per MOI. R²=0.99.

4.3.5.2  **NHCF-A host cells**

4.3.5.2.1  **Relationship of the MOI to the number of infected cells on the Opera imaging system**

The linearity of the number of infected NHCF-A host cells with a range of MOI values was retested on the Opera, using the conditions outlined in section 1.3.6.5. It was found that the assay performance was linear, with respect to the number of infected cells per well, to a 2.5:1 MOI (Figure 4.28).
Figure 4.28. The linearity of the relationship between the MOI and the resulting number of *T. cruzi* infected NHCF-A fibroblasts per well following 48 hours post infection, determined on the Operetta imaging system. (A) At 20-40:1 MOI, there appears to be a maximum number of host cells infected. (B) A linear relationship was found up to a multiplicity of 2.5:1 parasite to host cell. Representative data is from one experiment, with 9 wells per MOI. R²=0.99.

To detect infected cells in a NHCF-A monolayer, a MOI of 2.5:1 was used, as this was in the linear range following analysis of the relationship of the MOI and the resulting number of cells detected as infected, on the Opera imaging system. The script that was developed for analysing 3T3 cells was also used for the successful identification of *T. cruzi* NHCF-A infected cells. The only difference was that the algorithm, used to define the cytoplasm of the host cell was changed to method C, rather than method B, as it was more readily able to detect the more elongated NHCF-A fibroblast. The Opera cytoplasm detection library contains 6 different algorithms for definition of the cell cytoplasm, given that a nucleus has also been defined. In the “interface” between the Acapella script and the user, known as the Assay Language interface, the defined measurements for each algorithm are not provided. Generated images showing the selected cells from the input image allow the user to apply each detection method and select the most appropriate, by
comparing the applied cellular segmentation to those that appear to be the cytoplasmic borders, by eye (Figure 4.29).

**Figure 4.29. Identification of T. cruzi infected NHCF-A cells using the Opera QEHS high-content imaging system.** Images are taken at 20x water magnification. (A) Infected host cells. Green represents the host cell cytoplasm detected with HCS CellMask Green. Blue depicts nuclear material from both host cells and parasite stained with Hoechst. (B) Definition of the cytoplasmic region of host cells in which spot detection of parasite nuclei is undertaken. (C) Application of the script using the Opera Assay Language Interface to define infected cells. Infected cells are green, red are non-infected. (D-F) follows the same definitions for (A-D), only that these are infected host cells that have been treated with 12 µM NFX for 48 hours.
4.3.5.2.2  Reference compound activity

The IC\textsubscript{50} values of the reference compounds NFX and puromycin, and their ratio to IC\textsubscript{50} values obtained in the assay utilising 3T3 host cells, are shown in Table 4.1. The ratios were calculated as follows, whereby 1 would indicate no variation -

\begin{align*}
\text{Ratio} &= \frac{\text{IC}_{50} \text{ value of the control compound in the 3T3 assay}}{\text{IC}_{50} \text{ value of the control compound in the NHCF-A assay}}
\end{align*}

IC\textsubscript{50} values observed for 3T3 and NHFC-A \textit{T. cruzi} infected cells were similar for NFX, with a ratio of 0.945. Using a two-tailed unpaired t-test, there was no significant difference between the IC\textsubscript{50} value of NFX between both assays (p<0.05). The ratio between the activities for puromycin against 3T3 infected cells: NHCF-A fibroblasts was 0.832 and 3.68 for the parasite and host cells, respectively. Again, there were no significant differences between these values using a two-tailed t-test, however the p-value was 0.07 for the IC\textsubscript{50} values of puromycin against \textit{T. cruzi} amastigote assays in each case, and 0.441 for the IC\textsubscript{50} values of puromycin against the parasite. These results indicate although there was some difference in the parasite sensitivity to puromycin in NHCF-A cells, there was a clear indication that puromycin was cytotoxic to the host cells, with an SI value of 4.6.

There were a greater number of host cells per well found for NHCF-A fibroblast host cells in comparison to 3T3 host cells following 96 hours incubation. The mean number of NHCF-A host cells was estimated at 764±58.95, whereas from section 1.3.6.5, there were 524±28.50 3T3 cells detected. The Z’-factor for the NHCF-A host cells was 0.689±0.00508 and for the \textit{T. cruzi} amastigotes was 0.570±0.00410.
Table 4.1. IC₅₀ values from two independent experiments, of the reference compounds nifurtimox (NFX) and puromycin in the T. cruzi amastigote assays utilizing either the 3T3 or NHCF-A fibroblast cell lines. Data was averaged from one dose series for each compound, from each experiment. SD= standard deviation.

4.3.6 Assay to determine the effect of compounds on trypomastigotes

4.3.6.1 Relationship of the number of trypomastigotes to the fluorescent signal

The detection of trypomastigotes, using the fluorescent viability dye, PrestoBlue, was linear up to 2x10⁵ trypomastigotes/well and the assay remained reproducible with over greater than 5x10⁴ cells/well detected. As trypomastigotes may differentiate into “external amastigotes” in cell free media [307], the number of amastigotes and trypomastigotes in the well were determined following 48 hours incubation. There was an increase in the number of amastigotes following 48 hours incubation, at 2, 4, and 8x10⁵ cells/well containing 22%, 30% and 28% of this life cycle form in wells respectively, in comparison to 1x10⁵ cells/well, with 18% estimated (Figure 4.31).
Figure 4.30. *T. cruzi* life cycle forms present following 24 and 48 hours incubation of trypomastigotes densities ranging from $8 \times 10^5$ to $2.5 \times 10^4$ trypomastigotes/well in a 384-well plate. (A) Percentage of amastigotes in the well medium following 24 and 48 hours incubation. (B) Percentage of the sum of trypomastigotes and amastigotes in relation to the inoculum used. Data is the mean of two replicates wells counted for each trypomastigote/well concentration.

The sum of these two life cycle forms were calculated and compared to the original inoculum following 48 hours incubation (Figure 4.31). At 4 and $8 \times 10^5$ cells/well, there was a reduction in the overall life cycle forms present by 27% at each concentration. Considerable cell clumping was observed at these concentrations, which may be an indication of cell death. For the remaining concentrations tested ($2$-$0.25 \times 10^5$ cells/well), both parasite forms combined to give approximately 100% of the original inoculum density. The trypomastigote concentration that resulted in the least amastigotes with the lowest loss of trypomastigotes, combined with the highest signal window and an acceptable $Z'$-factor of >0.5 at 48 hours incubation, was found to be $1 \times 10^5$ cells/well.
Figure 4.31. Detection of the viability of trypomastigotes at concentrations ranging from from $8 \times 10^5$ to $2.5 \times 10^4$ trypomastigotes/well in a 384-well plate, following 48 hours incubation.

(A) The relationship of the number of cells/well and the fluorescent signal.
(B) The linearity of the number of cells/well and the fluorescence intensity, $R^2=0.99$. (C) The Z'-factor estimated. (D) The signal window estimated. Data is averaged from 24-wells for each trypomastigote well density. Error bars represent the standard deviation of measurements.

4.3.6.2 Activity of reference compounds

Using an inoculum of $1 \times 10^5$ cells/well, the IC$_{50}$ values of the reference compounds puromycin and NFX were estimated, and the Z'-prime of the assay calculated.
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utilizing NFX as the positive control. For N of 2 experiments, the IC$_{50}$ value for NFX was estimated at 1.09±0.433 μM, puromycin was estimated as 0.504±0.0849 μM and amphotericin B was estimated at 0.319±0.0776 μM. The mean Z’-prime was 0.65±0.07, and the signal window was 2±0.05 between cells treated with DMSO alone at a final concentration of 0.43% versus 14.2 μM of NFX.

4.4 Discussion

From the studies undertaken, the successful development of a sensitive, 384-well image-based assay to estimate compound activity on the amastigote form of the Tulahuen strain of *T. cruzi* in 3T3 host cells has been developed and optimised. Furthermore, an assay to determine compound activity on Tulahuen strain parasites in human NHCF-A heart fibroblasts has also been developed. In these assays, the number of host cells and infected host cells were detected following compound exposure for 48 hours. In an effort to understand compound activity on the blood-circulating form of *T. cruzi*, a separate assay using the resazurin-based dye, PrestoBlue, was developed and optimised. The reproducibility of these assays was illustrated by Z’-factors of 0.592 (section 4.3.5.1.6), 0.57 (section 4.3.5.2.2) and 0.65 (section 4.3.6.2) for the amastigote assays utilising 3T3 and NHCF-A host cells, and trypomastigote assays, respectively. For the detection of host cells, the Z’-factor was 0.814 (section 4.3.5.1.6) for 3T3 cells and 0.69 for NHCF-A cells (section 4.3.5.2.2).

Prior to the development of assays, factors that were considered to be important to the reproducibility and reliability of their application included the 384-well plate surface most suitable for host cell adherence, formation of a confluent host cell monolayer before parasite addition, and demonstration of the parasite life cycle, in a 384-well plate. The necessity to incorporate wash steps to remove
trypomastigotes post infection and during fluorescent staining of infected cells, resulted in identifying collagen I coated plates as the best support to retain host cells on the surface of the plate. 3T3 cells were estimated to double between 24-48 hours incubation, and appeared to plateau in number up to 4 days incubation. However, between 4-8 days incubation, there was an increase in cell number, again approximately a doubling of host cell number. This was also observed in a flask culture (section 1.2.1.1). Other authors have found that in high density aggregates 3T3 cells do not double for more than 3 days [308]. In terms of the assay procedure implemented here, it was estimated that host cells may double from the time of addition and up to 48 hours growth, and therefore the MOI utilised in these studies was based on this. Importantly, during the 72 to 96 hours period growth did not appear as a result of a doubling of cells, which is important as growth of parasites is not wholly due to host cell division.

The life cycle of *T. cruzi*, from the infection of 3T3 cells to the release of parasites was demonstrated in a 384-well plate over a 7 day period. However, the re-infection of host cells following a release of trypomastigotes was difficult to gauge, as there were a significant number of amastigotes maintained on the surface of the plate, even following washing out of the media. Therefore, it was determined that a 7 day assay would not be applicable to an image-based system, and alternative assay formats to estimate compound activity on the amastigote form of the parasite were pursued. As parasites were released 4 days post infection, this allowed for an assay to extend to 3 days from the addition of trypomastigotes. Compound exposure following wash off of trypomastigotes was therefore for a total of 48 hours. Other authors have used 72 hours incubation in an image-based format, however have not discussed the release of parasites over time during assay optimisation [229]. Therefore it may be possible that this assay format may have less interference from external parasites, although this was not investigated.
The assays developed within this study were demonstrated to exhibit a linear relationship between the *T. cruzi* trypomastigote inoculum and the resulting image-based detection of parasites. Determination of a linear relationship is an important consideration in assay development, particularly for HTS, to ensure that the assay is within the limits of the detection method [309,310]. In the assays outlined here, other considerations included overall host cell health and the precision of the detection applied with respect to both the parasites and the host cell. In the trypomastigote assay, high concentrations of cells in a well resulted in a plateau of fluorescence detection, which also corresponded with a reduction in the general health of cells. In the amastigote assay, a MOI of >20:1 parasite: host cell resulted in some earlier differentiation of parasites, possibly due to the amount of parasite burden in infected cells. This change in morphology resulted in poor spot detection and therefore the image-based script was less effective in identifying infected cells. The linearity of detection was optimised for each assay, resulting in a Pearson correlation coefficient of determination ($R^2$) of 0.99 for the detection of parasite infected 3T3, NHCF-A cells and trypomastigotes. The DMSO tolerability of the assays was also determined, important for the optimal health of the parasite and host cells during compound incubation to ensure that there was no added effect associated with DMSO. The maximum concentration of DMSO tolerated by *T. cruzi* amastigotes and 3T3 fibroblasts was 0.37% DMSO, and on NHCF-A host cells it was estimated to be lower at 0.1% DMSO, with 0.43% DMSO not affecting the viability of trypomastigotes.

The reference compound NFX was found to be selectively active against *T. cruzi* amastigotes, with a selectivity index (SI) of > 34 for infected NHCF-A host cells and >141 x for 3T3 infected cells (from Table 4.1), at the doses tested. Both assays, using either 3T3 or NHCF-A host cells showed little difference between the activity of NFX, with IC$_{50}$ values of $0.788\pm0.0207$ and $0.949\pm0.0779$ µM respectively
(Table 4.1). Unfortunately, it is difficult to compare the activity of NFX to other published results, as there are varying methods for the detection of compound activity against *T. cruzi*, with differing incubation times, *T. cruzi* strains, host cells and concentration of parasites / host cells used. In addition a variety of reference compounds have been used including amphotericin B, BZ [29] and K1777 [229], and only few report the activity of NFX on mammalian cell lines. NFX used in more manual-based assay methods has exhibited an IC\textsubscript{50} value against intracellular amastigotes of 7 strains of *T. cruzi* parasites, estimated by Giemsa staining, over a range of 3.55-0.47 µM [282]. In another publication, NFX was found to have an IC\textsubscript{50} value against *T. cruzi* MF strain amastigotes and trypomastigotes of 13 µM and 0.6 µM, respectively, based solely on manual microscopy to count live / dead cells [311]. NFX was found to have an IC\textsubscript{50} value of 0.083 µM against the Dm28C strain when the release of trypomastigotes from host cells was counted, following incubation for 4 days and by addition of compound every 24 hours [312]. These authors report that there was no activity observed when NFX was incubated with RAW264.7 (a mouse leukaemic macrophage cell line), although the doses at which the compound was tested were not reported. NFX has exhibited selectivity toward the epimastigote life cycle stage of *T. cruzi*, with a selectivity index of 41[313].

Amphotericin B was found in these studies to have an IC\textsubscript{50} value of 3.59±0.321 µM (Figure 4.23), 0.319±0.0776 µM (section 4.3.6.2) and 3.01±1.34 µM (Figure 4.23) against the amastigote, trypomastigote and 3T3 host cells, respectively. An assay utilising β-galactosidase transfected *T. cruzi* parasites have previously demonstrated an IC\textsubscript{50} value for this compound against *T. cruzi* of 0.2 µM, however this assay incubated infected host cells for 72 hours in the presence of compound, added 2 hours post- infection rather than 24 hours post infection as outlined here [29]. In a separate cytotoxicity assay, Amphotericin B was 50% active against 3T3
cells at 5.4 µM, following 7 days incubation [29]. In the same studies, it was found with trypomastigotes, there was a maximum lysis, at 24 hours, at 1.6 µM. Despite differences in methodology, as NFX and amphotericin B both show activity against trypomastigotes and amastigotes, and NFX is selective to the *T. cruzi* parasite, these results collectively qualify these assays for the identification of compound activity against *T. cruzi* life cycle forms. The 3T3 host-based assay serves as a more efficient screening assay in comparison to NHCF-A cells, as the media required and daily maintenance of these latter host cells is time consuming and expensive. However, the NHCF-A fibroblast assay would be beneficial downstream for the determination of persistence of parasites in heart cell tissue following compound exposure.

This is the first report of live-cell image-based methods to identify *T. cruzi* infected cells for potential use in the identification of compound activity. Further development would be required to determine that the number of cells infected and therefore the ability of *T. cruzi* to infect host cells is not effected by treatment of trypomastigotes with the fluorescent probe Celltracker Green. Although *T. cruzi* parasites could be identified in 3T3 host cells by staining trypomastigotes with Celltracker Green prior to host cell infection, some amastigotes were poorly stained, likely due to the uneven distribution of dye from mother to daughter amastigotes during cell division. This method could be developed further to investigate reduced incubation times, and therefore reduced cell division; as well as the inhibition of the entry of trypomastigotes in to host cells by compounds. The speed of action of a compound could be determined at earlier time points post infection, using the CellTracker dye to identify infected host cells, and may provide less background than Hoechst, as the fluorescent channel differs from that used to detect the host cell nucleus. As C₁₂-resazurin was demonstrated to effectively stain the cytoplasm of live 3T3 cells, it could be possible to use other
markers in the future to define live \textit{T. cruzi} cells, including cyto-based dyes, which have recently been employed successfully in malaria drug discovery [314]. Under the conditions tested herein, the fixed dye system was prioritised for development due to the more accurate definition of amastigotes within infected cells.

The \textit{T. cruzi} amastigote assay was set up on both the Opera and Operetta high-content imaging systems. The Opera utilises a laser-based spinning disk confocal imaging system to capture images, with the option of water immersion to allow high resolution. The Operetta on the other hand uses widefield regular fluorescent microscope optics to capture images, increasing the number of cells obtained in one image, as was found in these studies. The Operetta also has a spinning pinhole disk confocal option, which is built to suppress out of focus light. Using this option to image amastigote infected cells resulted in poor clarity of images and may need further optimisation. With Opera images, the topography of the host cells could be seen with more clarity, meaning that some of the amastigotes were out of focus, therefore two stacks were imaged. As a result, in terms of time, each machine took 1.5 hours per plate to read and analyse images concurrently. The added benefit of the Opera system is that it has a much larger plate reading capability with the use of stackers, whilst the Operetta is a bench unit. Using these two machines allows this assay to be amenable to more facilities that have this instrumentation, and shows the relative ease of optimising the assay from one machine to another. Therefore other laboratories may be able to use this assay on other high-content imaging machines.

This is the first report of the use of a two–dye system to identify both host cells and \textit{T. cruzi} parasites simultaneously within one well. As the assay has been developed in a 384-well format for use on both the Opera and Operetta imaging systems, and utilised automation to support parasite addition, wash steps and
staining procedures, this assay format is suitable for application in high-throughput to assess the activity of compound libraries against the amastigote form of the parasite.
Chapter 5: In vitro Profiling of Compound Collections

5 In vitro Profiling of Compound Collections

5.1 Introduction

Two compound libraries were selected for screening, using both the amastigote and trypomastigote specific T. cruzi phenotypic assays. These libraries, the Medicines for Malaria Venture (MMV) Malaria box and an in-house library of FDA approved compounds and compounds with known biological activity, provided a potential source for the discovery of compounds with novel activity against the T. cruzi parasite. Through the “repurposing” of compounds with known activity against other cell lines and / or targets there is the potential to identify novel targets for T. cruzi. The in-house library contains 741 compounds with known biological activity, some of which are FDA approved including antihistamines, antifungals, antibacterials, anticancer and anti-inflammatory agents. The MMV Malaria Box contains a chemically diverse collection of 400 proprietary compounds with previously determined in vitro activity against P. falciparum asexual form, comprised of 200 ‘probe-like’ and 200 ‘drug-like’ molecules [315]. More recently, these compounds have also been profiled against P. falciparum gametocytes [298,316]. The construction of the MMV Malaria Box library is outlined by Spangenburg et al (2013) [315]. During this process, drug-like compounds were first selected from hits against asexual P. falciparum that demonstrated rule-of-5-compliant physicochemical properties. Subsequently, known toxicophores were removed through application of the substructure filters REOS (Rapid Elimination Of Swill) and PAINS (Pan Assay Interference Compounds) [317,318]. Compounds that did not pass these filters were assigned to the probe-like category. The probe-like category is believed to be more useful as probes for identifying and classifying biological mechanisms, however it is noted that some of the probes may conform to Lipinski’s rules and therefore be
suitable as starting points in drug discovery programs [315]. Information pertaining to each compound can be found in the Table S1.

Potential drug targets of a similar nature have been identified for both *T. cruzi* and *P. falciparum*. The enzyme dihydrofolate reductase (DHFR), that catalyses the reduction of folate is inhibited by trimetrexate, found to be a nano-molar active against *P. falciparum* [319,320], is also similarly active against *T. cruzi* external amastigotes and trypomastigotes, with IC$_{50}$ values of 26 nM and 19 nM, respectively, and no cytotoxicity demonstrated against C12C12 mouse skeletal muscle cells [181] This compound has also been shown to bind to *T. cruzi* recombinant DHFR, although cross-reactivity with human DHFR warrants design of more specific inhibitors against these parasites [321]. Diamidines, minor DNA groove binding ligands, have been demonstrated to have activity both *in vitro* and *in vivo* against *T. cruzi* [322] and *P. falciparum* [322,323]. A diarylthiophene diamidine exhibited an IC$_{50}$ value of 0.62 µM against *T. cruzi* intracellular amastigotes, with a selectivity index (SI) of 36 , and also exhibited mild *in vivo* activity, reducing parasitaemia in a mouse model of acute *T. cruzi* infection by 40%, with 100% survival of mice [322]. The diamidine [2,5-bis(4-amidinophenyl)furan] (DB75) has demonstrated an IC$_{50}$ value of 0.62 µM against *P. falciparum* asexual forms [323] and the active pro-drug of this compound, [2,5-bis(4-amidinophenyl)furan bis-O-methylamidoxime] (DB289) was shown to result in a 96% cure rate in a small clinical trial with patients infected with *P. falciparum* [324]. Synthesised lipophilic bisphosphonates, related to compounds designed as anti-cancer agents to block protein prenylation, exhibited nanomolar *in vitro* activity against *P. falciparum*, with *in vivo* activity against the liver stage of the parasite, protecting mice from sporozoite challenge, and have been suggested as promising leads against malaria [325]. Bisphosphonates have also been shown to have low micromolar activity against intracellular *T. cruzi* amastigotes [326], and found to inhibit recombinant *T. cruzi* farnesyl pyrophosphate synthase [203]. Numerous anti-
cancer agents also possess anti-malarial and anti-trypanosomal activity [327-329]. Collectively, these results suggest that molecules with known anti-malarial activity may represent an excellent starting point for not only identifying new chemical scaffolds possessing T. cruzi activity, but also potentially identifying new drug targets. Screening the MMV Malaria Box against T. cruzi was therefore considered an ideal source for potentially identifying new compounds which can progress into early drug discovery programmes.

Drug repositioning accounts for approximately 30% of new US FDA approved drugs and vaccines in the last ten years [330]. Repositioning of known drugs was recently the target of a NIH campaign, which fostered partnerships between biomedical research institutes and pharmaceutical companies, to discover new therapeutic uses for existing molecules [331]. Repurposing of compounds has recently been undertaken in the area of neglected disease research [332-335] and chemical libraries containing known biologically active and FDA approved compounds, have been profiled in T. cruzi phenotypic assays. For example, a whole cell image-based assay to detect compound activity against T. cruzi amastigotes was used to screen a library of 909 drugs, comprised predominantly of FDA approved compounds [229]. Although this screen was mainly for validation of the screening technology, 17 compounds were identified with at least 5x selectivity between the T. cruzi CA-1/72 strain amastigotes and the bovine embryo skeletal muscle host cells (BESM). A library of agrochemicals has been tested against T. cruzi in a whole cell reporter gene assay, utilising parasites expressing a β-galactosidase reporter gene [235]. Ten compounds, comprising fungicidal compounds and drugs, insecticides, and an antioomycete compound exhibited activity against T. cruzi Tulahuen strain parasites cultured in rat skeletal myoblasts, with IC₅₀ values ranging from 3-55 nM. The cytotoxicity of these compounds against the host cell was not performed and thus would need to be to confirm their suitability for progression, however as these
compounds have previously determined rat LD$_{50}$ values, this paves the way for further studies. One of the key benefits of FDA compounds is that these compounds have a known pharmacological dossier [235], which could potentially save time and money in the drug discovery and development process. Smaller scale studies have identified FDA approved compounds with activity against $T. cruzi$. Amiodarone, the drug most commonly used to treat arrhythmias in late stage Chagas disease, was found to also be active against $T. cruzi$ both $in vitro$, and $in vivo$ [336]. Currently, $in vivo$ tests using Chagas mouse models are being undertaken with dronedarone, an FDA approved drug, developed to treat arrhythmias with reduced thyroid toxicity over amiodarone [337].

The potential for drugs and compounds that have been identified with activity against $T. cruzi$ to be repurposed as lead molecules or drug candidates, has led to the screening of a chemical library containing compounds with already identified biological activity against $T. cruzi$ intracellular amastigotes, and host cell free trypomastigotes. Those compounds, which displayed activity against the amastigote form of $T. cruzi$, and demonstrated selectivity toward the parasite, were also screened against the trypomastigote life cycle stage. The outcomes of the phenotypic screening of $T. cruzi$ against the MMV Malaria Box and an in-house library of compounds with known biological activity will be discussed.

5.2 Materials and methods

5.2.1 MMV malaria box

5.2.1.1 Primary screening
Chapter 5: *In vitro* Profiling of Compound Collections

The MMV Malaria Box is a diverse selection of compounds identified with antimalarial activity *in vitro*, consisting of a set of 400 drug-like and probe-like compounds [315]. The Avery lab received a "pilot" collection of these compounds, consisting of 685 compounds for initial evaluation to determine the compounds selected for the MMV Malaria Box for general distribution. Compounds were stored in 100% DMSO at a concentration of 5 mM, in 384-well plates at -20°C. Following the protocol for identifying the activity of compounds against *T. cruzi* in section 2.4, dilution stocks of these compounds were prepared at 5, 2.5 and 1.25 mM, which resulted in final concentrations of 18.3, 9.2 and 4.6 µM in the assay. The MOI of parasite: host cells was 10:1; and each plate was screened in duplicate. As outlined in section 4.3.6.1.4, the *T. cruzi* amastigote assay was shown to be linear up to an MOI of 5:1. The use of a 10:1 MOI did not seem to effect sensitivity, however to remain as sensitive as possible within the confines of the assay, a MOI of 5:1 was used for follow up retest experiments. Puromycin and NFX were used as positive controls for the host cell and amastigote assays, respectively, and each assay plate contained in-plate controls, with 7 wells each of puromycin, 7 wells of NFX and 16 wells of a final concentration of 0.37% DMSO. Doses for the calculation of the IC$_{50}$ values of NFX and puromycin were on a separate plate, with triplicate doses of each. A plate comprised of a third each of DMSO, puromycin and NFX was used to determine the $Z'$-factor of the assays. Image capture and analysis were undertaken on the Operetta imaging system at a magnification of 20 x.

5.2.1.2 Retest of DMSO samples

Compounds exhibiting activity of >50% in duplicate at a final concentration of 18.3 µM, with associated activity of >50% at 9.15 µM were selected for retest in dose response to determine IC$_{50}$ values. A separate group was also retested, with >50% activity exhibited at 18.3 µM, with single point, or no corresponding activity at 9.15
μM to determine if this group of compounds may re-confirm activity. As many compounds appeared to come out of solution in water at the highest dose of 18.3 µM, the initial dose for retesting compounds was reduced to 9.15 µM. Compounds were cherry-picked from 5 mM stocks in 100% DMSO and diluted in DMSO, to a working concentration of 2.5 mM. Compounds were then diluted into 15 doses in 384-well plates (Axygen, Tewksbury, MA, USA), before dilution into water prior to addition in the assay (section 2.4.1). The final assay concentrations ranged from 9.15 µM to 0.000229 µM. Compounds were retested in two independent experiments. The reference compounds puromycin and NFX were used as positive controls to calculate activity of compounds against the 3T3 host cell and amastigotes respectively, in both in-plate controls as described in section 1.2.1.1. The MOI was 5:1 parasite: host cells. Image capture and analysis was undertaken on the Operetta image-based system at 20x magnification.

Compounds that were considered active had an IC$_{50}$ value of <10 µM, and if the IC$_{50}$ could be determined against 3T3 cells, and a SI >10. If the IC$_{50}$ against 3T3 cells could not be determined, the SI was calculated by dividing the highest dose of 3T3 cells in the assay screened (18.3 µM) by the IC$_{50}$ of the compound against T. cruzi amastigotes. The SI in these cases did not necessarily exceed 10.

5.2.1.3 Activity of re-sourced, solid samples of drug-like compounds

Drug-like compounds were purchased to determine activity in comparison to the library DMSO stocks. MMV666080 was purchased from Asinex (Moscow, Russia), and MMV001230, MMV665909 and MMV665914 were purchased from Ambinter (Orleans, France). Compounds were prepared as 10 mM stocks in 100% DMSO, and diluted into a total of 15 doses in DMSO, ranging from a final concentration of 36.6 µM to 0.000916 µM in the amastigote assay. All samples appeared to be soluble at a
concentration of 10 mM. Compounds were tested for activity in two independent experiments, in the amastigote / host cell assay and in the trypomastigote assay. The procedure for the trypomastigote assay is outlined in section 2.4.4. NFX was used as a positive control to calculate the activity of compounds against the trypomastigote and amastigote forms of *T. cruzi* and puromycin served as a positive control for the 3T3 host cell. Doses of the reference compounds were contained within the same plate that the test compounds were in. Plates were analysed on the Opera imager at 20x water magnification.

Active compounds were defined as described in section 5.2.1.2. Further increases in the compound concentration in the future, and testing against a panel of mammalian cells could aid in determination of a more accurate IC\(_{50}\) value. The reproducibility of activity against the amastigote form of *T. cruzi* from testing compounds prepared fresh from powder stock, compared to DMSO stored samples, was assessed using an unpaired 2-tailed t-test, performed in GraphPad Prism (p<0.05). Two sample sets were compared, one containing IC\(_{50}\) values from two separate experiments from testing the samples stored in DMSO and the other with IC\(_{50}\) values resulting from testing the compounds prepared from solid stock.

### 5.2.1.4 Activity of drug-like and probe-like compounds against HEK293 cells

The activity of the probe-like compounds, from 100% DMSO stocks; and solid re-sourced samples of the drug-like compounds, dissolved in 100% DMSO, were determined against Human embryonic kidney (HEK293) cells, over 2 independant experiments. Puromycin was used as a positive control, and the same samples that were prepared in sections 5.2.1.2 and 5.2.1.3 were used for this testing. The plates were stored at -4°C prior to the testing taking place, for 1 week. The protocol for HEK293 assay is outlined in section 2.4.5.
5.2.2 In-house collection of FDA approved compounds and compounds with known biological activity

5.2.2.1 Primary screening

The compound collection was sourced from a variety of suppliers by in-house expert analysis of known drugs and compounds with biological activity previously described in the literature. The FDA approved compounds contained within the library are used for the treatment of a variety of indications and include antibacterial, antifungal, antipsychotic, anti-HIV and anticancer drugs. Compound stocks in 100% DMSO were stored in the Queensland Compound Library Facility housed within the Eskitis Institute for Drug Discovery. There are 741 compounds in the library, at concentrations between 1.7 and 23.1 mM in 100% DMSO, corresponding to a final assay concentration of 6.4 to 84.8 µM. To identify active compounds from the library, compounds were screened in three doses in the amastigote / host cell assay, in single point at the neat dose, and at 1:10 and 1:100 of the neat dose. For the screening procedure refer to section 2.4, with the exception that there was a 10:1 parasite: host cell ratio employed, as outlined in section 5.2.1.1. Note that no difference was found between the sensitivity of a 5:1 MOI in comparison to a 10:1 MOI (section 4.3.6.1.5). Compounds displaying greater than 80% activity against amastigotes at the highest screening dose, with greater than 50% activity at a 1:10 dilution of this dose were selected to determine the IC\textsubscript{50} value against the amastigote / host cell, and also in the trypomastigote assay. The reference compounds used to assess the reproducibility of the assay and calculate the activity of compounds were puromycin and NFX. Plates were imaged and analysed on the Operetta high-content imaging system at 20x magnification.
Active compounds identified from primary screening were cherry picked from compound library plates and retested in 15 point doses in duplicate, for duplicate experiments, in both the amastigote / host cell assay and the trypomastigote assay. Compounds were diluted from neat stock, ranging from 1.7 to 23.1 mM, to 15 doses in DMSO in 384-well plates (Axygen, USA). For identification of compound activity, images were captured and plates were analysed on an Operetta high-content imaging system at 20x magnification. Compounds that were considered active had an IC\textsubscript{50} value of <10 µM, and if the IC\textsubscript{50} could be determined against 3T3 cells, that the SI was >10 times. If compounds did not exhibit activity at the doses screened against 3T3 cells, the IC\textsubscript{50} was stated as the highest dose screened against 3T3 cells divided by the IC\textsubscript{50} value of the compound against \textit{T. cruzi} amastigotes. Compounds with an SI estimated in this manner were not discarded if the SI value was not greater than 10. Active compounds were investigated, by undertaking a literature search for known activity of the compound in existing \textit{T. cruzi} assays, or an identified mode of action against either \textit{T. cruzi} or other organisms and known safety profiles \textit{in vivo}, if they had been undertaken. Compounds with potentially favourable profiles were re-sourced as solids for IC\textsubscript{50} value determination.

5.2.2.3 Activity of re-sourced solid samples

The IC\textsubscript{50} values of compounds that were re-sourced as solids were determined from 4 independent experiments, against the \textit{T. cruzi} amastigote / host cells and also against the trypomastigote form of the parasite. Clemastine fumarate was purchased from Tocris (Abingdon, OX, UK); voriconazole, ketoconazole clotrimazole and ciclopirox olamine (CPX) were purchased from Sigma-Aldrich (USA) and miconazole nitrate, oxiconazole nitrate and bifonazole were purchased from LKT laboratories (St. Paul,
MN, USA). The reproducibility of activity against the amastigote form of *T. cruzi* from testing compound prepared fresh from powder stock, compared to DMSO stored samples, was assessed using an unpaired 2-tailed t-test, performed in GraphPad Prism (p<0.05). Images were captured and analysed on an Opera high-content imaging system at 20x water magnification. Doses of the reference compounds were contained within the same plate that the test compounds were in. These plates were run over the same 2 experiments with compounds that were identified as active from the MMV Malaria Box compounds, therefore the control compound IC_{50} values were the same for each.

5.2.2.4 Residual spots following compound treatment

It was observed that there were spots, which appeared to be amastigotes, present at high compound doses in host cells, corresponding to the maximal effect, or plateau of the dose response curves generated in GraphPad Prism (section 2.4.6) for all compounds retested in dose that did not produce a cytotoxic effect, in varying degrees, except for the control compound, NFX. The maximum activity or IC_{100} reached in a sigmoidal dose response curve was between 70-95% for these compounds. The IC_{50} value was calculated using this maximum effect, regardless of the % activity displayed, as it was not known if these amastigotes were viable, or potentially apoptotic.

5.2.2.5 Wash off assay

In an effort to understand if residual amastigotes were able to survive following a prolonged incubation after compound removal, an assay was developed to compare the differences between ‘before’ and ‘after’ compound removal, clotrimazole presented as an example. A final concentration of 4 μM of clotrimazole, estimated to
be the IC\textsubscript{100} of the compound, was added to two plates containing infected amastigotes, to 8 wells, in every second column. The remaining 8 wells of the same column contained a final concentration of 30 µM of NFX. One column in each plate also contained 15 doses of clotrimazole, ranging from a final concentration of 36.6 µM to 0.000916 µM. Following addition of the compounds, plates were incubated for 48 hours, before the well volumes were washed off one plate, whilst the other plate was stained and analysed to determine the compound effect as outlined in section 2.1.4. Wells were washed using a multichannel pipette, by removing media containing compound and washing five times with 50 µL of PBS by gently removing and adding the volume. Following further incubation for 3 days, the plate was processed as outlined in section 2.4.3. Images were captured and analysed on an Opera high-content imaging system at 20x water magnification.

5.2.3 Reproducibility of data

The reproducibility in terms of the number of parasite, host cell, percentage infection and Z’-factor were compared, for retest of the compounds from both libraries from 100% DMSO, read on the Operetta imaging system and for the retesting of solid samples, read on the Opera image-based system, as during these screening runs, an MOI of parasite: host cell was 5:1. Separately, these statistics are reported for the primary screening data, undertaken at an MOI of 10:1. The Z’- prime factor for the trypomastigote assay is reported as an average, from primary screening to retest, inclusive. There were 14 plates in total, 3 of which contained a third each puromycin, NFX and DMSO at final assay concentrations of 35.4 µM, 14.2 and 0.43% respectively, the remainder were from in-plate controls, containing one column of 0.43 %DMSO and 7 wells of NFX at a final assay concentration of 14.2 µM. There were 14 plates in total, containing (1) two separate experiments with 4 replicate
plates within each, (2) two separate experiments with 2 replicates within each. The remainder of plates were 2 plates, from 2 separate experiments.

For retest data in relation to testing compound stocks from 100% DMSO, 2 of the plate statistics represented were from Z’-factor plates containing a third each puromycin, NFX and DMSO. The remainder were from in-plate controls, containing one column of a final concentration of 0.37% DMSO and 7 wells of NFX at a final concentration of 12 µM. Plates 1-6 contained 3 plates from 2 independent experiments. Plates 7-10 were duplicate plates from 2 independent experiments.

For retest data in relation to testing compound stocks from solid samples, 4 of the plate statistics represented were from Z’-factor plates containing a third each puromycin, NFX and DMSO. The remainder were from in-plate controls, containing one column of a final concentration of 0.37% DMSO and 7 wells of NFX at a final concentration of 12 µM. Plates 1 and 2 were from 2 separate experiments and plates 2-7 contain 3 replicates within two independent experiments.

5.3 Results

5.3.1 MMV malaria box

5.3.1.1 Primary screening

Of 741 compounds tested, there were 13 compounds which exhibited >50% activity, in duplicate at 18.3 µM, with a corresponding activity of >50% at 9.2 µM in the T. cruzi amastigote assay. From the second group of compounds selected for retest, there were 10 compounds with >50% activity exhibited at 18.3 µM, with <50% activity displayed at 2.5 µM. The IC\textsubscript{50} value of NFX against \textit{T. cruzi} amastigotes was
1.28 µM and 62% activity at 127 µM was exhibited against 3T3 cells. Puromycin demonstrated an IC₅₀ value against *T. cruzi* amastigotes of 2.34 µM and 3.12 µM against host cells.

5.3.1.2 *Restest*

From the group of 10 compounds that were identified as >50% active at 9.2 µM from primary screening of samples, 3 compounds returned activity at >50% at 9.2 µM in the *T. cruzi* amastigote assay, when retested in dose. Three of these compounds could have an IC₅₀ value estimated. Of the 13 compounds that displayed >50% activity at 2.5 µM, 100% displayed >50% activity at 2.5 µM at retest. Ten of these compounds were able to have an IC₅₀ value estimated against the amastigote form of *T. cruzi*. Of the total 13 compounds that could have an IC₅₀ attributed, two could also have an IC₅₀ against the 3T3 host cell determined. These were two batches of the same compound, namely N-(4-methylphenyl)-4-pyridin-2-yl-1,3-thiazol-2-amine. These compounds did not exhibit selectivity toward the parasite, with a SI of 0.779±0.236 for the two batches tested.

Of the remaining 11 compounds with obtainable IC₅₀ values in the amastigote assay, the selectivity toward amastigotes in relation to the host cells was calculated by dividing the highest dose screened, against 3T3 host cells, at 9.2 µM, by the IC₅₀ value estimated in the *T. cruzi* amastigote assay. Five compounds that could have an IC₅₀ value determined were probe-like, and two of these were two batches of the same compound, MMV665994 (Table 5.1). Two compounds did not have a classification attributed to them in the MMV Malaria Box library (see Appendix, Table S1). The identity of these compounds was determined in PubChem (http://pubchem.ncbi.nlm.nih.gov/), by searching for the batch code. These compounds were STK700023, the azole antifungal clotrimazole, and the 9-
aminoacridine, \(N'-(\text{acridin-9-yl})-\text{N,N-dimethylbenzene-1,4-diamine}\), STL088556 (Table 5.1). The IC\(_{50}\) values against the intracellular parasite were 0.0544 \(\mu\)M and 2.37 \(\mu\)M, with SI values of \(>3.88\) to \(>169\) for clotrimazole and STL088556, respectively. Of the remaining compounds, MMV665941 also displayed activity against the trypomastigote, with a mean IC\(_{50}\) value of 0.836 \(\mu\)M. Four compounds were drug-like (Table 5.2). Of these compounds, IC\(_{50}\) values ranged from 0.463 \(\mu\)M to 2.25 \(\mu\)M, with estimated SI values ranging from \(>4.09\) to \(>19.9\). Two of these compounds, MMV666080 and MMV665909 were also active against the trypomastigote life cycle form with IC\(_{50}\) values of 0.898 \(\mu\)M and 1.08 \(\mu\)M, respectively.
<table>
<thead>
<tr>
<th>Compound name</th>
<th>Structure</th>
<th>T. cruzi amastigote IC$_{50}$ (µM±SD)</th>
<th>3T3 % activity highest dose</th>
<th>IC$_{50}$ HEK293 (µM±SD)</th>
<th>IC$_{50}$ T. cruzi Trypomastigote (µM±SD)</th>
<th>SI (3T3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>*STK700023 (clotrimazole)</td>
<td></td>
<td>0.0544±0.0110 NA at 9.2µM</td>
<td>NA at 10.9 µM</td>
<td>NA at 10.9µM</td>
<td>&gt;169</td>
<td></td>
</tr>
<tr>
<td>MMV665941 (crystal / gentian violet)</td>
<td></td>
<td>0.0931±0.0370 88% at 9.2µM</td>
<td>109% at 10.9 µM</td>
<td>0.836±0.13.5</td>
<td>&lt;98.8</td>
<td></td>
</tr>
<tr>
<td>MMV665994</td>
<td></td>
<td>0.657±0.704 (0.949±0.00516$^1$)</td>
<td>NA at 9.2 µM</td>
<td>NA at 10.9 µM</td>
<td>NA at 10.9 µM</td>
<td>&gt;14.0 (&gt;9.69)</td>
</tr>
<tr>
<td>MMV001239</td>
<td></td>
<td>0.864±0.00575 NA at 9.2 µM</td>
<td>NA at 10.9 µM</td>
<td>NA at 10.9 µM</td>
<td>&gt;10.6</td>
<td></td>
</tr>
<tr>
<td>MMV001241</td>
<td></td>
<td>1.34±0.00884 24% at 9.2 µM</td>
<td>NA at 10.9 µM</td>
<td>NA at 10.9 µM</td>
<td>&gt;6.87</td>
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</tr>
<tr>
<td>*STL088556</td>
<td></td>
<td>2.37±1.51 30% at 9.2 µM</td>
<td>98% at 10.9 µM</td>
<td>65% at 10.9 µM</td>
<td>&gt;3.88</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1. The activity of probe-like compounds from the MMV Malaria Box against T. cruzi amastigotes, trypomastigotes and the host cell, 3T3.*Compounds with no classification. The selectivity index (SI) of compounds is the highest dose tested against 3T3 host cells / the IC$_{50}$ value of compounds against the amastigote form of T. cruzi. (1) two batches of the same compound. Data is from two independent experiments. SD=standard deviation. NA= no activity displayed at the highest screening dose (<20% activity).
5.3.1.3 Confirmation of the activity of re-sourced, solid compound samples

Of the four drug-like compounds that were retested from freshly prepared stock, 100% of these re-confirmed activity against both amastigote and (those active against) the trypomastigote life cycle forms (Table 5.2). It was not possible, at the compound doses investigated, to determine the IC$_{50}$ value against the 3T3 host cells, and the SI was based on the top concentration screened of 36.6 µM / the IC$_{50}$ value determined against the intracellular form of *T. cruzi*. The SI for these compounds ranged from >19.6 to >30.5 and the IC$_{50}$ values from 1.87 µM to 1.20 µM. The variability of the activity of fresh versus frozen samples of compounds against the amastigote form of *T. cruzi* was assessed using an unpaired 2-tailed t-test in GraphPad Prism 5.0. There were no significant differences observed for the control compounds or 3 of the 4 drug-like compounds. MMV001230 showed a 2.8 times variation in the IC$_{50}$ values observed, although the solid compound was shown to contain no impurities by LCMS (results not shown).
| Compound name | Structure |  
|---|---|---|---|---|---|---|---|---|
| **T. cruzi** amastigote IC₅₀ | **3T3 % activity highest dose or IC₅₀** | **IC₅₀ HEK293 (µM±SD)** | **IC₅₀ T. cruzi Trypomastigote (µM±SD)** | **SI** |
| MMV001230 | 0.463±0.0945 | NT | NA at 10.8µM | 19.9 |
|  | 1.29±0.707 | NA at 36.6 µM | 50% at 43.3µM | >28.4 |
| MMV666080 | 0.687±0.787 | 40% at 9.2µM | NT | 0.898±0.0636 | >13.4 |
|  | 1.20±0.47 | 45% at 36.6 µM | NT | 0.750±0.000070 | >30.5 |
| MMV665909 | 1.02±0.882 | 35% at 9.2µM | NT | 1.08±0.00849 | >9.02 |
|  | 1.87±0.0849 | 50% at 36.6 µM | 2.82±1.09 | 0.772±0.041 | >19.6 |
| MMV665914 | 2.25±0.308 | 25% at 9.2µM | NT | NA at 10.8 µM | >4.09 |
|  | 1.50±0.177 | 26% at 36.6µM | NA at 43.3µM | >24.4 |
| *Puromycin* | 2.54±0.131 | 3.39±0.284 | NT | 0.505±0.0849 | 1.33 |
|  | 2.69±0.401 | 3.35±0.121 | 0.409±0.0163 | 0.753±0.00445 | 1.25 |
| *NFX* | 0.588±0.0969 | 36% at 127µM | NT | 1.09±0.433 | >216 |
|  | 1.02±0.328 | 36% at 127 µM | 61% at 150µM | 1.18±0.136 | >125 |
Table 5.2. The activity of drug-like compounds from the MMV Malaria Box against *T. cruzi* amastigotes, trypomastigotes and the host cell, 3T3. Bold type represents activity of re-sourced solid samples of compound. All other activities relate to compounds stored in 100% DMSO. Solid samples of the compound were also tested against HEK293 as a secondary measurement of cytotoxicity. The selectivity index (SI) of compounds refers to (the IC$_{50}$ value of compounds against 3T3 fibroblasts, if attainable, or if not the highest dose tested against 3T3 cells) / (the IC$_{50}$ value of compounds against the amastigote form of *T. cruzi*). NA= no activity displayed at the highest screening dose (<20% activity). NT= not tested. All data is from 2 independent experiments. SD= standard deviation. *Control compounds were puromycin and NFX.

5.3.2  In-house collection of FDA approved drugs and compounds with known biological activity

5.3.2.1  Primary screening

Due to the lack of reconfirmation of activity of compounds from the MMV Malaria Box library that were not active at 9.15 µM, the cut-off for activity for the in-house library was increased to >80% at the top dose screened, ranging from a final assay concentration of 6.4 µM to 84.8 µM with >50% at the mid-dose tested at final screening concentrations ranging from 3.2 µM to 42.4 µM, in an effort to enable only compounds that potentially could have an IC$_{50}$ value obtained to be captured. There were 29 compounds that were >50% active at the mid-dose tested. Twenty seven of these compounds displayed >80% activity at the highest dose tested in the assay, corresponding to final concentrations in the assay that ranged from 16.1 to 29.7 µM.
5.3.2.2 Restest

Twenty seven compounds were re-screened in dose response, with 15 dilutions prepared from the neat dose, ranging from a final assay concentrations of 16.1 to 29.7 µM, over N of 2 experimental replicates. 100% of compounds re-confirmed activity at the highest screening concentration, and 56% (15) of these compounds re-confirmed activity at the mid-dose. Of those that did not confirm activity at the mid-dose, three were able to have an IC$_{50}$ value estimated. Of the 15 compounds, salinomycin and mitomycin C did not display a plateau of activity and therefore an IC$_{50}$ value against T. cruzi amastigotes could not be estimated. Two deoxy-5-fluorinidine, did not show a sigmoidal dose response and there was a plateau of activity across host cells of 50-60% across all doses tested. As it was not possible to separate host cell effects from parasite, this compound was de-prioritised. Racecadotril and geldanamycin were cytotoxic against 3T3 cells, with mean IC$_{50}$ values of 1.10 and 0.434 µM respectively, with a corresponding SI for each of <5. Harringtonine also displayed a SI of 5 and was deprioritised.

Nine remaining compounds had an attainable IC$_{50}$ value with an acceptable SI. In total 12 compounds were identified which had IC$_{50}$ values ranging from 5.3 µM to 0.0227 µM, with corresponding SI values of between >3.54 to > 854. The majority of the identified active compounds were azole antifungals (Table 5.3), and the remaining active compounds included antifungal, immunosuppressant, antihistamine and anticancer compounds (Table 5.4). Of all 12 active compounds, there was only one compound that displayed activity against the trypomastigote life cycle stage, which was CPX, with an IC$_{50}$ of 1 µM.
5.3.2.3 Confirmation of the activity of re-sourced, solid compound samples

The antifungal compounds were sourced as solid samples and tested against both *T. cruzi* life cycle forms, except for lanoconazole, which was not available at the time of testing. All of these compounds were able to have an IC₅₀ value estimated (Table 5.3). There were no significant differences observed for the IC₅₀ values of control compounds, in comparison to the original testing undertaken in section 5.3.2.2. The only compounds tested that showed variability from the original DMSO solution stocks to the new solid stock testing were voriconazole and oxiconazole. The mean differences between these data sets were 2.6 and 2.4 times, for voriconazole and oxiconazole nitrate, respectively. In consideration of the two sources of stock compounds, this was considered minimal.
### Chapter 5: *In vitro* Profiling of Compound Collections

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>T. cruzi amastigote IC₅₀ (nM)±SD</th>
<th>3T3 % activity highest dose or IC₅₀ (µM±SD)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lanoconazole</td>
<td><img src="image" alt="Structure" /></td>
<td>67.1±0.155</td>
<td>NA at 20.5µM</td>
<td>&gt;306</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Voriconazole</td>
<td><img src="image" alt="Structure" /></td>
<td>96.5±3.53</td>
<td>NA at 17.8µM</td>
<td>&gt;184</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36.4±2.38</td>
<td>NA at 18.3µM</td>
<td>&gt;503</td>
</tr>
<tr>
<td>Miconazole Nitrate</td>
<td><img src="image" alt="Structure" /></td>
<td>826±267</td>
<td>NA at 16.1µM</td>
<td>&gt;19.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>835±7.36</td>
<td>NA at 18.3µM</td>
<td>&gt;21.9</td>
</tr>
<tr>
<td>Bifonazole</td>
<td><img src="image" alt="Structure" /></td>
<td>204±52.3</td>
<td>NA at 18.7µM</td>
<td>&gt;91.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>159±1.06</td>
<td>NA at 18.3µM</td>
<td>&gt;115</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td><img src="image" alt="Structure" /></td>
<td>187±38.2</td>
<td>NA at 16.3µM</td>
<td>&gt;87.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>166±0.121</td>
<td>NA at 18.3µM</td>
<td>&gt;110</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td><img src="image" alt="Structure" /></td>
<td>22.7±9.07</td>
<td>NA at 19.4µM</td>
<td>&gt;853</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29.4±5.60</td>
<td>NA at 18.3µM</td>
<td>&gt;622</td>
</tr>
<tr>
<td>Oxiconazole Nitrate</td>
<td><img src="image" alt="Structure" /></td>
<td>388±36.1</td>
<td>40% at 17.9µM</td>
<td>&gt;46.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>159±18.8</td>
<td>24% at 18.3µM</td>
<td>&gt;115</td>
</tr>
</tbody>
</table>
Table 5.3. Activity of azole antifungals identified from a library containing compounds with known biological activity and FDA approved compounds against *T. cruzi* amastigotes, trypomastigotes and the host cell 3T3. The selectivity index (SI) of compounds refers to (the IC$_{50}$ value of compounds against 3T3 fibroblasts, if attainable, or if not the highest dose tested against 3T3 cells) / (the IC$_{50}$ value of compounds against the amastigote form of *T. cruzi*). Bold type represents activity of re-sourced solid samples of compound. IC$_{50}$ values are in nM. SD= standard deviation from two independent experiments. IC$_{50}$ values of compounds against *T. cruzi* amastigotes are nanomolar. NA= no activity displayed at the highest screening dose (<20% activity).

Compounds identified as active against *T. cruzi* amastigotes, with various classifications of known activity against other medical conditions, are shown in Table 5.4. Clemastine fumarate was tested against *T. cruzi* in 3T3 host cells in 4 independent experiments and in NHCF-A fibroblasts, in 2 independent experiments. Clemastine fumarate was tested to determine if there was any difference in sensitivity between host cells, and if parasites were able to be cleared from each type of fibroblast cell line. There was no significant difference observed in the IC$_{50}$ values obtained for clemastine fumarate between either host cell line employed (p<0.05). There was also no significant difference noted between IC$_{50}$ values of compounds against *T. cruzi* amastigotes determined for compounds stored in DMSO, or compounds freshly dissolved in DMSO from solid powder (p<0.05), determined using an unpaired 2-tailed t-test in GraphPad Prism.
## Chapter 5: *In vitro* Profiling of Compound Collections

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th><em>T. cruzi</em> amastigote IC₅₀ (µM±SD)</th>
<th>3T3 % activity highest dose or IC₅₀ (µM±SD)</th>
<th>SI</th>
<th><em>T. cruzi</em> Trypomastigote activity (µM)±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antifungal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciclopirox Olamine</td>
<td><img src="image" alt="Structure" /></td>
<td>5.37±0.512</td>
<td>53% at 19.0µM</td>
<td>&gt;3.54</td>
<td>0.904±0.223</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.90±3.00</td>
<td>42% at 136µM</td>
<td>&gt;34.9</td>
<td>1.00±0.492</td>
</tr>
<tr>
<td><strong>Anticancer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daunorubicin HCl</td>
<td><img src="image" alt="Structure" /></td>
<td>4.533±0.147</td>
<td>60% at 16.1 µM</td>
<td>&gt;3.55</td>
<td>63.5% at 19.0µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Camptothecin</td>
<td><img src="image" alt="Structure" /></td>
<td>0.344±0.00</td>
<td>44% at 16.8µM</td>
<td>&gt;48.8</td>
<td>NA at 19.9 µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.0889±0.0275</td>
<td>38% at 5.0µM</td>
<td>&gt;56.2</td>
<td>NA at 5.9µM</td>
</tr>
<tr>
<td><strong>Immunosuppressant</strong></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Mycophenolate Na</td>
<td><img src="image" alt="Structure" /></td>
<td>0.895±0.00353</td>
<td>30% at 17.2µM</td>
<td>&gt;19.2</td>
<td>NA at 20.3µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td><strong>Antihistamine</strong></td>
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</tr>
<tr>
<td>Clemastine fumarate</td>
<td><img src="image" alt="Structure" /></td>
<td>0.759±0.217</td>
<td>NA at 21.5µM</td>
<td>&gt;28.3</td>
<td>80% at 25.4µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.291±0.141*0.355±0.00516</td>
<td>48% at 21.6 µM</td>
<td>&gt;74.2</td>
<td>48% at 25.5µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*NA at 5.4 µM</td>
<td>*NA at 5.4 µM</td>
<td>&gt;15.2</td>
<td></td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Puromycin*</td>
<td><img src="image" alt="Structure" /></td>
<td>2.85±0.237</td>
<td>2.54±0.945</td>
<td>0.891</td>
<td>0.505±0.0849</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.57±1.44</td>
<td>3.27±3.44</td>
<td>2.08</td>
<td>0.625±0.111</td>
</tr>
<tr>
<td>NFX*</td>
<td><img src="image" alt="Structure" /></td>
<td>0.800±0.0668</td>
<td>32% at 127 µM</td>
<td>&gt;159</td>
<td>1.09±0.433</td>
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<tr>
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<td></td>
<td>0.807±0.319</td>
<td>38% at 127 µM</td>
<td>&gt;157</td>
<td>1.24±0.252</td>
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</table>
Table 5.4. Active compounds identified from a library containing compounds with known biological activity / are FDA approved compounds against T. cruzi amastigotes, trypomastigotes and the host cell 3T3. Classifications refer to compounds reported as active against specific diseases or are FDA approved as a treatment. The selectivity index (SI) of compounds refers to (the IC₅₀ value of compounds against 3T3 fibroblasts, if attainable, or if not the highest dose tested against 3T3 cells) / (the IC₅₀ value of compounds against the amastigote form of T. cruzi). Bold type represents activity of re-sourced solid samples of compound, tested in 4 independent experiments. The remainder of the IC₅₀ values are from testing compounds from the compound library, stored in DMSO, over 2 independent experiments. NT= not tested. SD= standard deviation. NA= no activity displayed at the highest screening dose (<20% activity).* Control compounds were puromycin and NFX.

5.3.3 Residual amastigotes

5.3.3.1 MMV malaria box

For all of the MMV Malaria Box drug-like compounds that were identified as active against T. cruzi amastigotes, a low number of amastigotes was observed at the concentration giving the maximum effect of the compound. Representative images are shown in Figure 5.1, taken from treatment of infected 3T3 fibroblasts with 21 µM of compound.
Figure 5.1. Spots remaining following treatment of T. cruzi infected 3T3 cells with 21 µM of the drug-like compounds identified as active against T. cruzi from the MMV Malaria Box. (A) MMV665909, (B) MMV665914, (C) MMV001230 and (D) MMV666080. Images show Hoechst staining of host and parasite cells. The white circle contains what appears to be a small population of amastigotes. Images were captured at a 20x water magnification on an Opera imaging system.

5.3.3.2 FDA approved compounds / compounds with known biological activity

Figure 5.2 shows spots remaining following a 48 hour treatment of infected 3T3 and NHCF-A fibroblasts with clemastine fumarate, at 5 µM for 48 hours. There was no difference observed between the effect in either 3T3 or NHCF-A fibroblast cell lines. Spots were also evident in infected 3T3 cells treated with the azole antifungal compounds identified as active (Table 5.3), following 48 hours exposure. The mean activity at the top 4 doses of freshly prepared samples from powder (18.3, 9.2, 3.7 and
1.8 µM) were compared for the azole antifungals. Miconazole nitrate and bifonazole inhibited 92% of these amastigotes voriconazole and ketoconazole were estimated to remove 88% respectively, whilst clotrimazole demonstrated the poorest clearance, with 78% of spots removed, in comparison to 100% with the control compound NFX. Following treatment with CPX for 48 hours at concentrations of 68 and 34 µM there appeared to be no residual spots, however there were a small amount seen at 13 µM, with 93% of cells estimated to be un-infected.

![Figure 5.2](image.png)

Figure 5.2. Spots remaining in wells following treatment of *T. cruzi* infected 3T3 host cells with 5 µM of clemastine fumarate, in comparison to 12 µM of the control drug, NFX. (A) NHFC-A host cells, inset (arrow) are spots that appear to be amastigotes. (B) Following 48 hours treatment with NFX there are not apparent spots. (C) 3T3 cells after treatment with clemastine fumarate. Inset (arrow) shows spots that appear to be amastigotes. (D) 3T3 cells following treatment with NFX. Images are taken at 20 x magnification with an Opera imaging system.
5.3.3.3 Wash off assay to estimate survival of residual amastigotes

Following an extra 3 days of incubation with 4 µM of clotrimazole, it could be seen that spots were cleared when compared to images taken after 48 hours incubation, and before the compound was washed off.

Figure 5.3. A wash off assay with 4 µM of clotrimazole shows that after 3 days extra incubation of infected *T. cruzi* 3T3 host cells, following wash off of the compound, that there does not appear to be residual populations of *T. cruzi* amastigotes. (A, C) Hoechst staining of the parasite and host cells. A (inset) shows spots that appear to be amastigotes (arrowhead) (B, D) analysis undertaken on the Opera Assay Language interface to estimate the number of infected cells. Images are taken at 20 x magnification. (A, B) 48 hours incubation with the compound, (C, D) 72 hours incubation following wash off of clotrimazole.
Following 48 hours exposure to the compound 12x less cells were identified as infected by spot analysis following treatment with NFX than with clotrimazole, a significantly different number, as observed in section 5.3.3.2. Following wash off of the compounds and 3 further days incubation, there was no observable difference in clotrimazole treatment in comparison to NFX treatment (Figure 5.4), with a ratio of 1.06 between the mean number of infected cells estimated in the Opera Assay Language Interface. There were also no spots detected by either the image-based script, or by eye, down to a concentration of 1.8 µM of clotrimazole.

![Graph showing number of infected cells](image)

Figure 5.4. The number of infected cells determined in the *T. cruzi* amastigote assay following 48 hours treatment with either 4 µM of clotrimazole or 12 µM of NFX (before wash) or following wash off and a further 72 hours incubation with compounds (after wash). Data was averaged from 44 wells of each treatment, error bars represent the standard deviation of measurements.

5.3.4 Reproducibility of data

A difference in the number of infected cells was estimated between the in-house FDA library and MMV Malaria Box primary screening campaigns, with the number of
infected cells estimated to be 153±28 and 380±21 cells, respectively. This was due to variability in the host cell number, with a mean of 590±15 cells from the MMV Malaria Box primary screen, compared to 845±32 cells estimated from screening the in-house FDA library. This was considered to be likely the result of the wash protocol on the Bravo liquid handler, causing a small population of host cells to be dislodged from plates. It was determined that the height of the instrument varied slightly with time, and tips close to the surface of the plate during wash steps caused this to occur. This effect was not seen once the height of the instrument was corrected. However, there was no difference in the sensitivity of these assays, exemplified by the activity of the reference compounds, shown in Table 5.2 and Table 5.4. There was a mean Z’-factor of 0.68±0.06 for the amastigote assay and 0.73±0.04 for the 3T3 host cell assay.

During compound retesting of DMSO-stored solution stocks, undertaken on the Operetta high-content imager, there was a mean parasite number of 175±30, a mean number of host cells of 871±95, and % infection of 19.7±4.6%. The Z’-factor was greater than 0.5 for all of the plates tested. Figure 5.5 shows these values for all of the plates screened during the retest, for both compound libraries. A two tailed t-test, undertaken in Prism 5.0 revealed that there was no significant difference in the IC$_{50}$ values of the reference compounds NFX and puromycin determined for both primary and retest screening campaigns (P<0.5).
Figure 5.5. The reproducibility of the *T. cruzi* amastigote and 3T3 host cell assay during retest of compounds stored in DMSO. The number of (A) infected host cells, (B) host cells (C) % infected cells and (D) the Z’-factor of plates in the *T. cruzi* amastigote assay over plates from the retest of compounds from 100% DMSO stocks over both of the compound libraries. The MOI used in these assays was 5:1 parasite. See the text for explanation of replicates. Error bars represent the standard deviation of measurements. Plates were analysed on the Operetta imaging system at 20x magnification.

Solid sample testing, undertaken on the Opera, also resulted in Z’-factors greater than 0.5 for each assay, with a Z’-factor for the *T. cruzi* amastigote assay of 0.627±0.0491 and the host cell assay of 0.738± 0.087; and there was a mean of 28±2.6% infected cells/well. The mean number of host cells was 871±95 and the mean number of amastigote infected cells was 141±9.27 (Figure 5.6).
Figure 5.6. The reproducibility of the *T. cruzi* amastigote and 3T3 host cell assay during retest of solid compounds. The number of (A) infected host cells, (B) host cells (C) % infected cells and (D) the $Z'$-factor of plates in the *T. cruzi* amastigote assay over plates from the retesting of solid compounds over both of the compound libraries. The MOI used in these assays was 5:1 parasite: host cells and plates were imaged and analysed on the Opera high-content imaging system at 20x water magnification. See the text for explanation of replicates. Error bars represent the standard deviation of measurements.

A mean $Z'$-factor was observed in the trypomastigote assay of 0.75±0.093, with a mean signal window of 2.0±0.340, calculated from all of the plates from the retesting of both compounds stored in DMSO and freshly prepared stocks of compound from powder.
5.4 Discussion

The *T. cruzi* amastigote assay was successfully utilised for identifying active compounds from two independent compound libraries, with follow-up determination of the activity of identified compounds in both the amastigote / host cell imaging assay and the trypomastigote fluorescence based, viability assay. These assays demonstrated $Z'$-factors throughout the screening campaigns exceeding a cut-off of 0.5, illustrating that these assays are reproducible and thus suitable as high throughput screening platforms [247]. The activity of reference compounds also indicated that these assays were robust, with no significant variation exhibited in the IC$_{50}$ values of reference compounds between primary screening and retest campaigns (P<0.5).

A number of antifungal compounds were identified from the in-house FDA and known actives library that were active against *T. cruzi* amastigotes, with no toxicity displayed toward 3T3 host cells. We have previously published the activity of miconazole nitrate, oxiconazole nitrate and lanoconazole against *T. cruzi* amastigotes generated from this screening data [338]. Whilst the activity reported against the *T. cruzi* amastigotes is novel, the activity of miconazole against *T. cruzi* epimastigotes has previously been documented [339]. However, as these are topical antifungal treatments, with no systemic safety or efficacy studies reported, it is likely that modification of these compounds would be necessary before oral dosing could be considered. Also, a potential liability of these compounds is their interaction with human cytochrome P450, of which miconazole has demonstrated activity [340,341]. The remainder of the azole antifungal agents identified serve as a validation of the *T. cruzi* amastigote assay format, as they have previously been reported to have activity against the parasite. Ketoconazole has been identified as active, both *in vivo* and *in vitro* against *T. cruzi*, although does not provide a parasitological cure in mice [342].
An IC$_{50}$ value of 1 nM was shown against *T. cruzi* Y strain amastigotes in mouse peritoneal macrophages [343], 10 nM against Peru strain parasites in VA-13 cells [344], and 1 nM active against *T. cruzi* Tulahuen strain parasites cultured in 3T3 cells, utilising a β-galactosidase transfected parasite [115]. The IC$_{50}$ value observed in these studies was 29 nM, also in the low nanomolar range, (Table 5.3). The IC$_{50}$ values for voriconazole and clotrimazole, also identified in a β-galactosidase-based assays are reported to have IC$_{50}$ values of 4 nM [115] and 14 nM [235] against *T. cruzi*, respectively. From the studies described here, voriconazole displayed an IC$_{50}$ value of 36 nM and clotrimazole inhibited activity against *T. cruzi* amastigotes with an IC$_{50}$ value of 166 nM. It is difficult to compare assays directly, as the *T. cruzi* assays utilising a β-galactosidase transfected parasite incubates parasite with the compounds for 72 hours, and the compound is added before the addition of the parasite [29]. However, all of theseazole antifungals identified in these studies have reported activity in the nanomolar range. These results validate the use of this assay to estimate compound activity against *T. cruzi* parasites.

The assay that has been developed to estimate the effect of compounds against *T. cruzi* amastigotes has demonstrated the presence of a small residual population of spots detected by Hoechst that appear to be amastigotes, following 48 hours treatment with compounds that have been identified as active. This effect includes theazole antifungal compounds, observed at concentrations that correspond to the maximum compound concentration effect when plotted in GraphPad Prism (Figure 5.2). The presence of amastigotes remaining following treatment with the azole antifungal compounds posaconazole and ravuconazole following a 72 hour incubation with GFP-transfected Tulahuen strain parasites in rat cardiomyocytes has been reported previously, however the maximal effect for each of these compound varies; and the presence of residual amastigotes were compared only at one dose for both compounds (7.5 nM) in this publication [345]. A separate study has
demonstrated the incomplete clearance of CAI/72 *T. cruzi* amastigotes from bovine embryo skeletal muscle cells following 72 hours treatment with 123 nM of posaconazole, although the dose where a maximum effect of this compound is seen is not reported [346]. To our knowledge there has not been a report of this effect over multiple compounds and highlights the benefits of employing image-based techniques. Potential persistence of amastigotes is an important consideration during the chronic phase of the disease, whereby parasites reside in tissues that may not be easily accessible by compounds and may be a contributory factor to compounds not demonstrating efficacy in the chronic phase of Chagas disease. However, further work is needed to characterise these effects. From the results observed following wash off of clotrimazole, this suggests that parasites, treated with up to 1.8 µM of the compound may already be apoptotic following 48 hours exposure to the compound. These investigations are also warranted for the other compounds identified in these studies. To further demonstrate this, a separate stain would also be beneficial, such as a GFP-transfected parasite [347]. Another point to consider is that the lipophilic nature of azoles may make wash off of compounds contained within host cells particularly difficult and it may therefore be necessary to also look at increased incubation with compounds as a comparison.

In the consideration that clotrimazole may be able to clear *T. cruzi* amastigotes from 3T3 host cells, this compound serves as a hit for further investigation against *T. cruzi*. Clotrimazole has been identified as active against *P. berghei* in vivo [348] and *P. falciparum* in vitro [349]. Borhade and co-workers have formulated a nano-emulsion of the compound in an effort to improve the aqueous solubility and lipophilicity [350], and compared this to a suspension formulation in vivo by oral gavage of *P. berghei* infected mice. Suppression of parasitaemia was significantly improved with the nano-emulsion in comparison to an oral suspension. There was no toxicity, measured by both physical observations and renal markers of hepatic toxicity, in either the
parent or modified compound at a dose of 10mg/kg/day for 4 days. It would be beneficial to explore formulations that may improve the solubility and bioavailability of this compound.

Non-antifungal compounds identified as active against *T. cruzi* amastigotes from the in-house library, with IC_{50} values of less than 5 µM against *T. cruzi* amastigotes, and SI values of >10 following retest of DMSO samples included daunorubicin HCl, mycophenolate Na, clemastine fumarate and camptothecin. Immunosuppressive therapy with mycophenolate, used for heart transplants for patients with chronic Chagas disease, has been associated with an increase in reactivation the disease therefore leading to a high risk with this compound [351-353]. Mycophenolate Na has was tested against *T. cruzi* in an *in vivo* model of acute infection to determine if there was an increase in the infection rate [354], and was found to result in suppression of infection. However, an acute model of *T. cruzi* infection was used for this study, whereas reactivation would be associated with the chronic form of the disease. Although this supports the effectiveness of the amastigote assay in identifying this compound, the risk for further development is too high and this compound was not tested from stocks prepared from re-sourced solid powder.

Camptothecin and daunorubicin HCl have been identified as mammalian topoisomerase inhibitors [355,356] and both have been shown to cause DNA damage in *T. cruzi* epimastigotes [356,357]. As camptothecin was a more active representative of this class of activity from the compounds reported here, with an IC_{50} value estimated against amastigotes of 344 nM (Table 5.4), daunorubicin HCl was not retested from a fresh powder. Camptothecin was identified as a potential lead against *T. cruzi* some years ago [358], however poor solubility, rapid inactivation and cytotoxicity demonstrated in human trials as an anti-cancer drug candidate have hindered it’s further development [359]. There have been less toxic analogues of
camptothecin identified [360], some recently shown to be active against L. donovani [361,362]. It would be valuable to profile analogues of this compound class against T. cruzi life cycles, and in a sub-confluent, parasite free host cell model to enable estimation of the inhibition of mammalian cell replication.

This is the first report of the FDA approved antihistamine clemastine fumarate and the antifungal drug CPX exhibiting activity against T. cruzi amastigotes. There were a number of other antihistamines, both first and second generation (21 in total, results not shown) also screened in the in-house library and none displayed activity against T. cruzi. It is possible that clemastine fumarate may inhibit T. cruzi CYP enzymes, as this compound has shown affinity toward the human cytochrome P450 enzyme CYP2D6, commonly found for other azole antifungals that exhibit activity against this T. cruzi enzyme, including ketoconazole [363] and miconazole [364]. There may be sufficient homology between the T. cruzi and human enzyme to warrant cross reactivity. Human CYP activity would need to be taken into account with respect to potential side effects, in particular with consideration of the dose of clemastine fumarate that may be needed to clear infection of T. cruzi, along with the side effects of sedation and central nervous system (CNS) depression due to the known dopaminergic and serotonergic off-target activity of this compound [365]. The plasma concentration of clemastine fumarate following a single oral dose of a 2mg/mL syrup based formulation, is reported to be between 4 µM to 21 µM and has a half-life of 21 hours [366], therefore, following determination of clearance of amastigotes from host cells, it may be warranted to pursue this compound in an in vivo model of acute T. cruzi infection.

The non-azole antifungal, CPX, displayed an IC$_{50}$ value of 3.9 µM against T. cruzi amastigotes and 1 µM against trypomastigotes following the retest of solid sample (Table 5.4). This was the only compound from the in-house library that demonstrated
activity against the trypomastigote form of the parasite, at a similar level to the control drug, NFX. Activity against the trypomastigote form of parasite life cycle stage is not commonly reported in drug development. This is based on the fact that the pathogenesis of the chronic stage is not well defined at present [53], the amastigote form is thought to persist in the chronic phase of the disease [9] and trypomastigotes are not often detected from blood work in the chronic phase, although estimated to be around 50% by xenodiagnosis or haemoculture [301]. Also, the time that trypomastigotes remain in the bloodstream is not known. From these studies, it has been observed that there is a seven fold increase in the number of infected cells from 2 to 24 hours exposure with trypomastigotes (results not shown). Therefore, survival at least in vitro, is >2 hours. Another concern may be antibody mediated lysis of trypomastigotes in the bloodstream. However, it has been shown that such antibodies do not kill 100% of trypomastigotes following exposure in vitro [367]. In consideration of these factors, it may be plausible that trypomastigote also play a part in the chronic stage of infection. There has been a recent report that implicates T. cruzi amastigotes and trypomastigotes in myocardial necrosis in vitro, which may be a factor regulating heart damage in the chronic stage of T. cruzi [368]. It is interesting to note that the two drugs used to treat Chagas are active against both of the life cycle forms. Potentially, the efficacy of these compounds in vivo, at least in the acute phase of the disease, may be partially due to the trypomastigote activity observed.

As an oral treatment, CPX has shown good safety profiles in rats, dogs and mice and a single 10 mg/kg dose study in healthy human volunteers did not exhibit any toxicity [369]. Some early pharmacokinetic models have been undertaken in man, utilising ciclopirox [14C]-olamine, suggesting a moderate clearance time of 6 hours [370]. In leukaemia and myeloma cells, it has been shown that CPX induced cell death is caused by chelation of intracellular iron [371], and it is possible that this may
also represent the mode of action in *T. cruzi*. Metal chelating agents have been described as active against *T. cruzi* epimastigotes and trypomastigotes *in vitro* [52] and the iron chelator desferrioxamine (DFO) has been demonstrated to increase survival of *T. cruzi* infected mice [372]. The best means of determining activity of CPX is to assess the effects on *T. cruzi* infection *in vivo*. This will be outlined in detail in subsequent chapters. It should be noted that other compounds without trypomastigote activity, particularly clemastine fumarate, remain for future consideration. However, based on the profile observed, CPX was prioritised at this time.

From the MMV Malaria Box library, clotrimazole and STL088556, which both displayed activity against *T. cruzi* amastigotes, were not classified as drug or probe-like from the information provided with the Malaria Box compounds. Clotrimazole was also identified as active from the in-house library, with an IC\textsubscript{50} value determined as 55 nM in comparison to 166 nM from the sample within the MMV Malaria Box. STL088556, \textit{N'}-(acridin-9-yl)-N,N-dimethylbenzene-1,4-diamine, displayed a moderate IC\textsubscript{50} value of 2.4 µM against *T. cruzi* amastigotes, with 30% activity against the host cell at 9.2 µM, and an activity of 98% was observed at 10.9 µM against HEK293 cells. This compound is a 9-amino-acridine, and compounds within this class have been demonstrated to be toxic and mutagenic [373]. Acridines are known to have mammalian cholinesterase activity [374], and from undertaking a substructure search in SciFinder\textsuperscript{TM} http://www.cas.org/products/scifinder, it was found that 19 publications were related to this activity and a further 36 were related to the DNA quadruplex. The ability of this compound class to bind to mammalian G-quadruplex of DNA strands, is thought to be the mode of action against some cancer cell lines [375]. In consideration of the potential liabilities, this compound was deprioritised.
Probe-like compounds that are within the MMV Malaria Box do not strictly adhere to Lipinski’s rule of 5, however they may be developed as part of an early drug discovery programme, [315] to improve their pharmacokinetics and bioavailability, if there are no other known liabilities in terms of known toxicity. Considering the target for these compounds against T. cruzi is not known, these compounds may serve as investigative tools to identify new targets against T. cruzi parasites. To ascertain the suitability of these compounds for either of these purposes, a substructure search was undertaken in SciFinder. MMV 665941, methylrosaniline, otherwise known as crystal violet, is used for the chemoprophylactic treatment of blood to remove T. cruzi parasites prior to transfusion in countries with endemic Chagas disease [376]. This compound is reported to have an IC$_{50}$ value of 12 µM against T. cruzi trypomastigotes following 24 hours incubation [377]. Crystal violet has associated problems if used orally, including potential mutagenicity [378] and a number of side effects including diarrhea, vomiting, lethargy and anorexia, observed in a number of animal species, including rats and mice [379]. Due to the known toxicity of this compound, it would be unlikely to ever be pursued in T. cruzi research as a potential for oral treatment. As the effect appears to be cytotoxic, potentially targeting the mitochondrion [380] this compound was not considered a tool for further research in this context.

MMV665994, 3-(3,3-Dichloro-2-propen-1-yl)-2-methyl-4-quinolinol, is a quinolone-based compound, with no reported activity against T. cruzi. Quinolone-based compounds have historically been active against malaria, including the drugs used to treat the disease: chloroquine, quinine, mefloquine and primaquine [381], although only primaquine has been reported to display activity against T. cruzi amastigotes in vitro, replication was not eradicated [382]. A substructure search of MMV665994 returned one biological study of a related compound, a hit against a screen to detect inhibitors of bacterial lysosymes [383]. The ligand similarity search engine, electronic
High Throughput Screening Ligand Activity by Surface Similarity Order (SymBioSys LASSO) [384] associates this compound with binding to the human enzyme aldose reductase (Al-R), with a LASSO score of 0.97. LASSO is a virtual tool to estimate the ability of a compound to bind to a known ligand based on the 3D structure of the ligand [385], with 1 being the most similar score. Al-R is an enzyme responsible for detoxification of toxic aldehydes and the gene been identified in *L. donovani* [386] although not currently identified in *T. cruzi*. However, *T. cruzi* does possess a functional glyoxalase system [387]. The enzyme has not yet been shown to be essential in *L. donovani*, however inhibition of Al-R has been shown to result in apoptosis in colon cancer cells [388], and is the target of drugs used to treat symptoms of diabetes in humans, with no side effects [389]. It may be that determination of the activity of this compound could provide a new target for *T. cruzi*. This compound has low molecular weight and a polar surface area of 267 and 29.1 Å², respectively. These are well within the accepted limits of a drug-like compound, as determined by Lipinski’s rule of 5, namely to have a molecular weight of less than 500 and polar surface area of no greater than 140 Å² [78].

MMV001239, or 4-cyano-N-(5-methoxybenzo[d]thiazol-2-yl)-N-(pyridin-3-ylmethyl) benzamide and related substructures do not appear to have been referenced in publications in relation to biological activity in SciFinder. As there was no cytotoxicity detected against either 3T3 or HEK 293 cells, this compound could be investigated further, however in terms of the physiochemical properties, it has a high polar surface area of 107Å², suggesting it may not enter the CNS [390]. As *T. cruzi* has demonstrated trophism for the CNS experimentally [391] during the acute phase of the disease, and been found in the CNS in histopathologic examination of chronically infected patients [392], the polar surface area of this compound or related compounds may be important. Although it is not clear if target product profiles require CNS penetration for antichagasic compounds, this would be a characteristic
that may be taken into consideration when prioritising compounds for progression. MMV001241, 4-Cyano-N-(4-methoxy-7-methyl-1,3-benzothiazol-2-yl)-N-(3-pyridinylmethyl) benzamide also has no related literature from a substructure search in SciFinder. It has a relatively high molecular weight of 414, close to what may be considered a cut off for CNS penetration [390], however could be a candidate for further studies, with an IC$_{50}$ value of 1.3 µM against amastigotes and no toxicity against 3T3 host cells determined up to 9.2 µM. From the doses tested, the SI was estimated to be >6.9 and increasing the concentration of this compound would be needed to determine a more accurate SI.

As the drug-like compounds would potentially have greater success in terms of faster development due to lack of chemical liabilities, these compounds were given priority to retest solid samples. MMV001230, 3-benzothiazol-2-yl)-N-(pyridin-3-ylmethyl) cyclohexanecarboxamide is thiazole-based compound, with no related literature from a substructure search. As thiazole is structurally related to imidazole, there is the possibility that the mode of action may be similar to the known imidazole antifungal, clotrimazole that has been identified from the in-house library in these studies. There have been examples of thiazole containing compounds acting as antifungals, with minimal toxicity [393]. MMV665909, 2-bromo-N-(4-pyridin-2-yl-1,3-thiazol-2-yl) benzamide is another amino-thiazole, however this compound also demonstrated activity against the mammalian cell line HEK293, with an IC$_{50}$ value of 2.82 µM, and against the T. cruzi trypomastigote with an IC$_{50}$ value of 0.772 µM. A series of related 2-aminothiazoles identified in SciFinder have been identified with activity against Mycobacterium tuberculosis [394], however a low therapeutic index was identified against VERO cells and the authors note these compounds may be exerting a cytotoxic effect. As MMV665909 has demonstrated cytotoxicity against HEK293, it would be warranted to screen this compound against a panel of mammalian cell lines to determine if this compound should be pursued further. It
would be valuable to determine what structural differences may cause the variability in cytotoxicity displayed between these two thiazole-containing compounds.

MMV666080, N-[(8-hydroxyquinolin-7-yl)-phenylmethyl] benzamide, is an 8-hydroxyquinolone. There are 24 related sub-structures and 9 related publications identified from a sub-structure search in SciFinder. A publication identifying 8-quinoline structures with activity against a HIV growth factor found that there was associated toxicity against LnCaP (prostate adenocarcinoma) cells with 18 of 42 compounds synthesised [395], however as this is a cancer cell line, this is not the best measure of cytotoxicity. Compounds with anti-cancer activity that have been identified as active against T. cruzi, including Tipifarnib, currently in pre-clinical development for the treatment of Chagas disease [396]. In these studies, MMV666080 was not tested against the non-cancer cell line, HEK293, however did not exhibit cytotoxicity toward the 3T3 host cell. The 8-hydroxyl-quinolines have been identified as iron chelating compounds [397-400]. N-((8-hydroxy-7-quinolinyl) (4-methylphenyl) methyl) benzamide, a related substructure is believed to modulate Wnt/b-catenin signalling via iron chelation, as the compound activity was abrogated using Fe^{2+} [401]. As this compound has a similar activity profile to CPX from the studies outlined here, with activity against both the amastigote and trypomastigote life cycle forms, and this compound is also a known iron chelator, it could be possible that this may be the mode of action of this 8-hydroxyquinoline in the parasite. It would be beneficial to determine if the activity of these compounds can be abrogated by the addition of an exogenous iron source.

MMV665914, 4-chloro-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)-2-(pyridin-3-yl)ethyl) benzene sulfonamide is another novel compound with no related literature or similar substructures. Based on the phenyl piperazine structure embedded within this molecule, it could possess ion channel agonism or antagonism properties
In vitro Profiling of Compound Collections

[402,403]. Alternately, the benzene sulphonamide moiety may be responsible for the activity of this compound. Recently, the anti-malarial activity of a series of compounds containing a benzene sulphonamide fragment have been identified with in vitro activity against *P. falciparum*, with no cytotoxicity observed against neonatal foreskin fibroblasts (NFF). One of these compounds, 4-(3,4-dichlorophenylureido) thioureido-benzene sulfonamide, demonstrated an ED$_{50}$ of 5 mg/kg in a *P. berghei* mouse model of malaria infection, however it is not clear if this compound was able to clear parasitaemia [404]. The novel activity of this compound against *T. cruzi* identified in these studies and lack of cytotoxicity observed supports the further development and investigation of this compound.

There were 21 compounds identified as active, with <10 µM activity that resulted from profiling the activity of two compound libraries against *T. cruzi* amastigotes. Of these, clotrimazole, ketoconazole, voriconazole, miconazole, camptothecin, myconfenolate and MMV665941 (crystal violet) have previously been identified against *T. cruzi*, in vitro or in vivo. This supports the further use of this image-based technology to profile additional compound libraries against the parasite. From the in-house library profiled against *T. cruzi* life cycle forms, the antifungal CPX potentially represents a different mode of activity to the classical azole antifungals identified with activity against *T. cruzi*. As this compound is active against both of the life cycle stages of the parasite, with minimal mammalian toxicity and has an oral no observable adverse effect level (NOAEL) already identified in rats, dogs and rabbits [405], CPX was recommended for in vivo studies in an acute model of *T. cruzi* infection.

Of the MMV Malaria Box collection, there are 7 potential chemical starting points for *T. cruzi* drug discovery. From these studies, we have confirmed the activity of the 4 drug-like compounds from a solid stock of compound. The quinolone containing
compounds, the probe-like MMV665994 and drug-like MMV666080 are recommended for further evaluation. The probe-like thiazole containing compounds MMV001239 and MMV001241 and the drug-like thiazole MMV001230 represent good chemical starting points. MMV665914 is a novel piperazine-containing compound with no sub-structure equivalents, and could represent a novel mode of action against *T. cruzi*. As MMV665909 has displayed activity against HEK293 cells, testing against other mammalian cells would need to be undertaken to confirm the potential toxicity of this compound. Follow up studies would be initiated assays to determine the effectiveness in the removal of amastigote populations post 48 hours incubation with these compounds, followed by profiling of chemical analogues that may be available against *T. cruzi*, and profiling activity of active compounds against mammalian cells. The most active and selective compounds would then be selected for *in vitro* safety testing. Although the MMV Malaria Box represents compounds with a potentially longer route in drug discovery than libraries focussed for repurposing of compounds, the novelty of some of these scaffolds could provide much needed chemical starting points for *T. cruzi* drug discovery.
6 The activity of Ciclopirox Olamine Against T. cruzi

6.1 Introduction

CPX, a hydroxamic acid antifungal, displayed novel activity against both T. cruzi amastigote and trypomastigote life cycle stages in the assays developed as part of this project. CPX is an FDA approved compound used for the treatment of superficial mycoses and the oral pharmacokinetics have been evaluated in animals during development of this drug as an antifungal agent. CPX has demonstrated a good safety profile, with a NOEAL of 30 mg/kg/day for a 3 week treatment in rats and a clearance suggested to be around 6 hours in humans [370]. CPX also has a NOAEL of 10mg/kg/day when doses were given to both dogs and rats daily, over a 3 month period. CPX is undergoing clinical trials for the treatment of myeloid leukaemia, including phase III clinical trials in high risk patients [406], and patients with refractory disease [407]. In consideration of these factors, CPX was considered herein an oral candidate for early drug discovery in Chagas disease.

The in vivo testing of CPX, was undertaken using the service provided by the Anti-Infectives Screening Core at New York University, USA as our institute currently does not have the required animal models established. CPX was initially tested in a mouse model of acute T. cruzi infection (http://ocs.med.nyu.edu/anti-infectives-screening). This short infection model uses the Y strain of T. cruzi, transfected with firefly luciferase (T. cruzi-Luc) to infect mice, and following treatment with compounds, the mice are imaged to estimate the effect of dosing on infection. Compounds with in vitro activity have been previously identified with activity in this model [228]. CPX was found to significantly suppress T. cruzi infection in relation to the control compound, BZ, currently used for the treatment of Chagas disease. In consideration of the suppressive activity of CPX shown in vivo against T. cruzi
infection, further investigations were undertaken in an effort to understand the mode of action of this compound. Differing from the azoles, which have historically been shown to target sterol pathway inhibition in both fungi [408] and T. cruzi [103], the fungal target of CPX is thought to be DNA replication, DNA repair, and cellular transport, shown in genes up-regulated in Saccharomyces cerevisiae [409]. In leukemia and myeloma cells, it has been shown that CPX induced cell death is caused by chelation of intracellular iron [371]. CPX has previously been identified as having activity against L. donovani promastigotes and amastigotes in vitro, and activity against the recombinant Leishmanial enzyme deoxyhypusine hydroxylase (DOHH), involved in hypusine synthesis, may in part describe the in vitro activity of the compound [410]. Hypusine is a unique amino acid found in eukaryotes and during biosynthesis of hypusine, polyamines are incorporated into proteins, a process that is essential for cell division [411]. CPX has also been recently implicated in the abrogation of HIV-1 infection via inhibition of human DOHH, involved in regulation of apoptosis [412]. It is possible, however, as DOHH is a metalloenzyme, containing an inorganic di-iron catalytic centre [413] and CPX is known to be a metal chelator [414], that this compound may be acting via a chelation effect. It is also possible that the effect on DNA in fungi is mediated by ribonuclease reductase, another iron dependant enzyme that is critical for DNA replication [371].

There have been metal chelators reported with activity against T. cruzi [415-417] including DFO, which has demonstrated low in vitro activity against amastigotes [418]. DFO has been reported to affect extracellular iron in other cell lines, including leukaemia, myeloma, actinomycete and rat kidney cells [371,419,420]. In vivo DFO shows no [251], or poor reduction of parasitaemia [421] in mouse models of acute T. cruzi infection. However, as infection rates in vivo are affected by iron levels, it has been noted that modulation of iron availability could be a potential strategy for restricting the development of Chagas disease [422]. To test the iron chelating
capability of this compound, *T. cruzi* amastigotes and trypomastigotes were incubated in the presence of CPX, with varying concentrations of an exogenous iron source. If iron chelation was responsible for the activity of this compound against *T. cruzi*, it was predicted that the activity of CPX would be reduced or abrogated. Reduction of CPX activity against cancer cell lines has been previously demonstrated *in vitro* in the presence of FeSO$_4$ [423] and a decrease in CPX mediated cell death in myeloma cells has been reported, with iron in complex with a transferrin replacement compound, designed to promote intracellular iron uptake in mammalian cells [371]. In these studies presented here ferric citrate (FC) has been used as an iron supplement. As citrate is thought to be important for iron transport, at least into mammalian cells, this form of iron may allow improved transport across the mammalian host cell membrane [424], without the use of exogenous transferrin. It has been reported that FC also prevents the precipitation of free iron salts [425]. From the results presented here, the addition of FC to the culture medium significantly reduced the activity of CPX against both *T. cruzi* trypomastigotes and amastigotes at concentrations that did not affect *T. cruzi* or host cell growth.

In an aim to understand the potential importance of the activity of CPX against the trypomastigote form of *T. cruzi*, the speed of action of CPX was determined against this stage of the life cycle. It is possible that trypomastigotes may remain in the bloodstream for some time, although this has not yet been clearly defined. Action of CPX over a short time frame may indicate the potential success of this compound in having a detrimental effect on trypomastigotes *in vivo*. The possibility also exists to increase the efficacy of CPX, by combination with the known drug currently used to treat Chagas, NFX. Combination therapy can enhance the efficacy of known drugs by utilising compounds which may act upon different cellular targets, potentially reducing toxicity and minimizing the risk of drug resistance. This approach has been successful for known drugs used to treat Chagas disease, *in vitro* and *in vivo*. 

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Buthionine sulfoximine has been shown to increase the effect of NFX in vitro [306] against Trypanosoma cruzi amastigotes, trypomastigotes and epimastigotes [306]. Combinations of intermittent oral dosing of posaconazole and BZ have recently shown promise in vivo in a mouse model of T. cruzi infection, curing 100% of animals, whilst posaconazole alone cures 80% of infections [426]. Combined oral treatment of T. cruzi infected mice with the heterocyclic analogue DB289 and BZ has shown efficacy in a mouse model of T. cruzi infection, reducing infection by 99%, in comparison to 70% and 90% respectively, for the doses tested in these studies [427]. Unfortunately, the targets of NFX and BZ are currently not known [428,429]. However, it has been postulated that type 1 nitro reductases of T. cruzi activate NFX, resulting in the production of an unsaturated open chain nitrile that is active against T. cruzi, which may function as a Michael acceptor and interact non-specifically with a range of cellular components [430]. Michael acceptors are electron poor organic compounds that have toxological potential due to their electrophilic reactivity [431]. As it was found in these studies that the action of CPX is in part due to metal chelation, the activity of these compounds in combination may have a more efficacious effect in vitro against T. cruzi. Reduction of NFX in treatment regimes would be greatly beneficial for the treatment of Chagas disease.

6.2 Materials and methods

6.2.1 Oral administration of CPX in a mouse model of acute T. cruzi infection

Female Balb/c mice, 5 weeks old (20 - 25g) were divided into three groups of five infected mice. Groups of mice were either treated with the control drug BZ, test compound CPX, or vehicle (water). The estimated dose of control or test compound they were receiving was 60 mg/kg/day of either the control drug BZ or or the test compound CPX. As Balb/c mice drink approximately 5.1 mL of water per day [432],

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The compounds were solubilised in water at the appropriate concentrations. Mice were treated with respective compounds or vehicle daily for 10 days. *T. cruzi* used to infect the mice were transgenic *T. cruzi* Y strain, expressing firefly luciferase (*T. cruzi*-Luc). Mice were infected via intraperitoneal (IP) injection with $10^5$ *T. cruzi*-Luc trypomastigotes forms, harvested from the supernatant of infected LLC-Mk2 cells. Three days after infection (day 0) the mice were anaesthetised by inhalation of isofluorane (controlled flow of 1.5% isofluorane in air was administered through a nose cone via a gas anaesthesia system). Mice were injected with 150 mg/kg of D-luciferin potassium-salt (Goldbio, St Louis MO, USA) dissolved in PBS at 20mg/mL. Mice were imaged 5 to 10 minutes after injection of luciferin with an IVIS 100 (Xenogen, Alameda, CA, USA) and the data acquisition and analysis were performed with the software LivingImage (Xenogen). Following imaging, during the same day, treatment with compounds at the desired dose or vehicle control was started. Following 5 days treatment (day 5), mice were imaged again after anaesthesia and injection of luciferin as described above. This was repeated after another 5 days treatment (day 10). Comparison of drug / compound / vehicle treatments was undertaken with a one way ANOVA, using the statistical analysis program, Statistical Product and Service Solutions (SPSS, IBM v20).

6.2.2 The effect of an exogenous iron source on the activity of CPX in *T. cruzi* amastigote and trypomastigote *in vitro* cultures

To determine the effect of the addition of an exogenous iron source on the activity of CPX *in vitro* against the life cycle forms of *T. cruzi*, the shift of the CPX IC$_{50}$ value was determined in the presence of C$_6$H$_5$FeO$_7$; Iron (III) citrate (FC) was determined *in vitro* against both the amastigote and trypomastigote forms of *T. cruzi*. The known iron chelator, deferoxamine mesylate (DFO, Cayman Biochemicals, Ann Arbor, MI, USA) was used as a control, as this compound is thought to chelate iron in *T. cruzi*
infection, and in the presence of an external iron source in vitro (ferric iron, source not reported), activity against amastigotes was reduced [418]. Twelve duplicate dilutions of CPX and DFO in 100% DMSO were prepared on separate 384-well microtitre plates. Each duplicate dose series was prepared separately. These doses were diluted 1:21 before addition, in 5 µL of sterile water, to 384-well microtitre plates containing either infected 3T3 cells, prepared as per section 2.4.3, or host cell free trypomastigotes. Prior to the addition of FC to amastigotes, trypomastigotes were rinsed from wells as in section 2.4.3, and 20 µL of media was added, to which 30 µL of media containing FC was added. For the addition of FC to trypomastigotes, the concentration of trypomastigotes was increased and added in 20 µL in the plate so as to allow for a final concentration of 1x10^5 cells/mL, following the addition of 30 µL of each FC concentration.

To prepare FC, a stock concentration was dissolved in water at 50 mg/mL. To give a final concentration of 100 µM in the assay, a 0.054 mg/mL and a 0.045 mg/mL solution were prepared for addition to the amastigote assay and trypomastigote assays, respectively by diluting the stock into RPMI containing no phenol red, supplemented with 10% FCS and 1% 100IU/mL penicillin/streptomycin. Dilutions of FC were prepared to give final concentrations in each assay ranging from 100 µM to 0.781 µM, in two fold serial dilutions. These concentrations were based upon a 100 µM concentration of ferric ammonium citrate used to load HEK293 cells in a study that measured intracellular chelatable iron, as a guide [423]. Each concentration was added to the duplicate dose series of CPX or DFO. The control for each compound was a dilution series, in the absence of FC, again in duplicate. The control for the effect of FC on T. cruzi parasites was four wells containing each concentration of FC, with no addition of CPX or DFO, with the relevant concentration of DMSO for each assay. Plates were incubated for 48 hours, then processed and analysed as outlined in section for sections 2.4.3 and 2.4.5 for the amastigote and trypomastigote assays,
respectively. The control compounds used for the assay were NFX for the *T. cruzi* amastigote and trypomastigote assays at a final concentration of 12 µM and 14.2 µM respectively, and 30 µM of puromycin to calculate activity against 3T3 host cells.

### 6.2.3 Speed of action of CPX against *T. cruzi* trypomastigotes

To determine the time that it takes for CPX to have a maximal effect on *T. cruzi* trypomastigotes, a dose response series of CPX was added to host cell free parasites, prepared in the same manner as outlined in section 2.4.4. The activity of CPX was determined following 6, 12, 24 and 48 hours total incubation time which included 6 hours of incubation time with PrestoBlue. There were duplicate series of compound doses, at each time point from final concentrations in the assay ranging from 161 µM to 0.008 µM. Positive controls included 14.2 µM NFX and 35.4 µM puromycin, and the negative control was a final concentration of 0.43% DMSO. The IC₅₀ value at each time point was calculated by using a positive control of medium with no addition of parasite, as the speed of action of the control compounds was unknown. The % activity, using either medium, NFX or puromycin as controls at the IC₁₀₀, at each time point, was recorded as an initial measure of the efficacy of CPX in comparison to NFX / puromycin. As NFX is a yellow coloured compound, interference in the assay was estimated by adding a dose response series to media alone and then detecting the activity in the assay. The compound was incubated with PrestoBlue for 6 hours. Doses varied from 150 µM down to 0.04 µM.

### 6.2.4 Activity of CPX and NFX combinations on *T. cruzi* amastigotes

To determine the *in vitro* effect of a combination of CPX and NFX against *T. cruzi* amastigotes a dose series of each compound was prepared, in 100% DMSO, on two separate plates. One 384-well microtitre plate contained dilutions based on the IC₅₀
value of NFX of approximately 1 \( \mu \text{M} \) (section 5.2.1.3). Dilutions ranged from 16x the IC\(_{50}\) value (16 \( \mu \text{M} \)) to 0.5 x the IC\(_{50}\) value (0.5 \( \mu \text{M} \)), over 13 doses, in two-fold dilutions. The other plate also contained two-fold dilutions of CPX, based on an IC\(_{50}\) value of approximately 3 \( \mu \text{M} \) (section 5.2.1.3), ranging from 16 x the IC\(_{50}\) value to 0.5 x the IC\(_{50}\) value, at final assay concentrations of 48 \( \mu \text{M} \) to 1.5 \( \mu \text{M} \). The plates were prepared at working concentrations double that required, and 20 \( \mu \text{L} \) of each were stamped into a stock plate. One microlitre of this was then added to assay plates to give final concentrations in the assay as outlined. As plates were combined, this allowed a variety of combinations of these compounds to be studied, from 16:0.5 to 0.5:16 x the IC\(_{50}\) values of NFX:CPX.

The sum of the fractional inhibitory concentrations (\( \Sigma \text{FIC} \)) for each combination of compounds for which an IC\(_{50}\) could be attained, otherwise known as the FIC Index (FICI), were calculated as follows:

\[
\Sigma \text{FIC (CPX)} = \text{FIC (CPX)} + \text{FIC (NFX)} = (\text{IC}_{50} \text{ for CPX in a combination}/ \text{IC}_{50} \text{ for CPX alone}) + (\text{IC}_{50} \text{ for NFX in a combination}/ \text{IC}_{50} \text{ for NFX alone}).
\]

It is generally accepted that a drug combination is synergistic if the \( \Sigma \text{FIC} \) values are lower than 0.5 and antagonistic if they are greater than 2 \[433\]. A FIC of 0.5-2 is considered to be indifferent; or additive \[434\]. The \( \Sigma \text{FIC} \) is based on the Loewe additivity zero-interaction theory \[435\], which hypothesises that a drug does not interact with itself and this combination will be additive, with an \( \Sigma \text{FIC} \) of 1.

An isobologram was constructed by plotting the \( \Sigma \text{FIC} \) of (CPX) versus the \( \Sigma \text{FIC} \) of (NFX) for each combination. A concave isobologram (FIC <1) is considered more consistent with synergy, whilst convex is consistent with antagonism (FIC>1), and a straight line suggests an additive effect \[436,437\]. The line of additivity on the
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The isobologram is represented by a sum of 1. If dose pairs are below line in an isobologram, this signifies an antagonistic response, whilst below this line represents a synergistic response.

6.3 Results

6.3.1 Oral administration of CPX in a mouse model of acute *T. cruzi* infection

CPX suppressed Y strain-LUC *T. cruzi* infection in mice following both 5 and 10 days oral treatment with the compound (Figure 6.1). For the control drug BZ, there was a significant reduction in the mean luminescent signal between vehicle treated mice and BZ treated mice on day 5 (P<0.01; 22 fold difference) and day 10 (P<0.01; 67 fold difference in mean luminescence). Similarly, when comparing vehicle to CPX treatment, there was a significant difference in mean luminescence on day 5 in comparison to vehicle treated mice (p<0.01; 5.85 times difference) and days 10 (p<0.01; 22 fold difference). In terms of the control mice, there was a significant increase in the mean level of luminescence between days 0 and 5 (p<0.01; 28.62 fold), however between days 5 and 10 there was less of a difference (p=0.07; 1.6 fold). This shows that the *T. cruzi* infection produced close to a maximum luminescence at day 5. There was no significant difference between the mice on day 0 between any of the cages (vehicle to BZ treated; p=0.975; and those mice before CPX treatment to vehicle treated; p=0.983). Figure 6.1A shows three representative mice on day 10 from the vehicle, BZ, and CPX treated groups of 5 mice. Figure 6.1B shows the mean luminescence and standard deviation of each treatment over 10 days, averaged over 5 mice per treatment group.
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![Image of mice infected with firefly transfected T. cruzi and following oral treatment for 10 days with either vehicle (water), 60 mg/kg/day of CPX or 60 mg/kg/day BZ. (A) Images of infected mice. Y strain *T. cruzi* parasites were transfected with firefly luciferase and quantification of the luminescence signal was with an IVIS 100 *in vivo* imaging system. 3 mice are shown as a representative of 5 mice in each group. (B) Quantification of luminescence units, results are expressed as a mean value for each treatment ± standard deviation. Luminescence was averaged over groups of 5 mice for each treatment.]

Figure 6.1. Balb/c mice infected with firefly transfected *T. cruzi* and following oral treatment for 10 days with either vehicle (water), 60 mg/kg/day of CPX or 60 mg/kg/day BZ. (A) Images of infected mice. Y strain *T. cruzi* parasites were transfected with firefly luciferase and quantification of the luminescence signal was with an IVIS 100 *in vivo* imaging system. 3 mice are shown as a representative of 5 mice in each group. (B) Quantification of luminescence units, results are expressed as a mean value for each treatment ± standard deviation. Luminescence was averaged over groups of 5 mice for each treatment.
6.3.2 Activity of CPX in the presence of an exogenous iron source in *T. cruzi* amastigote and trypomastigote *in vitro* cultures

FC affected the growth of host cells and therefore also amastigotes, when added to infected 3T3 host cells at final concentrations of 100, 50 and 25 µM. At these doses, the number of infected cells were reduced to 30%, 50% and 75% of the DMSO control (Figure 6.2). FC affected the viability of trypomastigotes at a concentration of 100 µM, resulting in 81% of the PrestoBlue signal in the assay in comparison to no addition of FC. The IC₅₀ values were only calculated for those dilution series in which FC did not affect the health of the culture. Therefore in the amastigote assay, IC₅₀ values were calculated from 0-12.5 µM of FC, and in the trypomastigote assay, up to 25 µM of FC.

**Figure 6.2.** The effect of varying concentrations of ferric citrate (FC) on *T. cruzi* life cycle forms and 3T3 host cells. (A) The number of *T. cruzi* infected 3T3 cells, (B) total 3T3 cells, estimated in the *T. cruzi* amastigote image-based assay and (C) viability of trypomastigotes estimated by the resazurin-based reagent, PrestoBlue.

The greatest change in the IC₅₀ value of CPX treated, *T. cruzi* 3T3 infected cells was with the addition of 6.25 µM FC, with a reduction in the IC₅₀ value of 9.48 fold. The resulting IC₅₀ value was 29.16±0.926 µM. There was less of a difference observed when cells were treated with 12.5 µM, 3.12 µM, 1.56 µM or 0.781 µM; with a 2.45,
3.20, 2.45 and 1.86 fold reduction in the IC$_{50}$ value in comparison to the control, respectively (Figure 6.3). The IC$_{50}$ value of CPX with no addition of FC was 3.08±0.53 µM.

Figure 6.3. The activity of ciclopirox olamine (CPX) and deferoxamine mesylate (DFO) on *T. cruzi* amastigotes in the presence of varying concentrations of ferric citrate (FC). (A) IC$_{50}$ values of CPX in the presence of doses of FC and (B) IC$_{50}$ values of DFO in the presence of doses of FC. (C) Shows the dose response curves for CPX treatment over varying concentrations of FC and (D) are the representative responses of *T. cruzi* amastigotes to DFO in the presence of concentrations of FC. Error bars represent the standard deviation of the mean of values from duplicate dose series of each compound, at each FC concentration.

FC reduced the IC$_{50}$ value of DFO at concentrations of 12.5 µM and 6.25 µM by 2.10 and 2.90 fold, respectively. There was variability observed in the duplicate values of
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treatment with 3.12 µM of FC with a mean IC$_{50}$ value for DFO of 92.6±60 µM. The mean reduction in the IC$_{50}$ value of DFO in the presence of 3.12 µM of FC was 3.27 fold. It was observed that with treatment of DFO, the IC$_{50}$ value with no addition of FC was 28.29±1.12 µM. Due to this moderate activity, at the doses screened, the activity had just reached what appeared to be the maximum effect (Figure 6.4). This may have caused some variability in the estimation of the IC$_{50}$ values.

When trypomastigotes were treated with DFO and CPX with no addition of FC, the IC$_{50}$ values observed were 13.3±1.12 µM and 0.657±0.130 µM, respectively. The maximum effect was reached with both compounds, defined by the two top concentrations tested were within 10% of one another. These doses were 7.53 µM and 3.77 µM for CPX and 87.4 µM and 43.7 µM for DFO. FC reduced the IC$_{50}$ value of CPX by 11.9 fold in the presence of 3.12 µM of FC, whilst other concentrations varied from a fold difference of 5.64 at 12.5 µM to 2.43 times at 0.781 µM (Figure 6.4A). FC affected the activity of DFO in more of a dose dependant manner and the maximum fold difference was observed 4.76 fold, with the addition of 50 µM of FC (Figure 6.4B).
Figure 6.4. The activity of ciclopirox olamine (CPX) and deferoxamine mesylate (DFO) on *T. cruzi* trypomastigotes in the presence of varying concentrations of ferric citrate (FC) (A) IC$_{50}$ values of CPX in the presence of doses of FC and (B) IC$_{50}$ values of DFO in the presence of doses of FC. (C) Shows the dose response curves for CPX treatment over varying concentrations of FC and (D) are the representative responses of *T. cruzi* amastigotes to DFO in the presence of concentrations of FC. Error bars represent the standard deviation of the mean of values from duplicate dose series of each compound, at each FC concentration.

6.3.3 Speed of action of CPX against *T. cruzi* trypomastigotes

The maximum IC$_{50}$ value of CPX was reached following 48 hours incubation, although an IC$_{50}$ value was able to be determined at both 12 and 24 hour intervals (Table 6.1). The IC$_{100}$ of CPX, following 48 hours incubation was calculated to be 14.8 µM. Using the data at this concentration at each time interval, the % activity was calculated using either medium containing no parasite, NFX, or puromycin. This was
undertaken to give an estimate of the action of CPX in relation to these compounds, at this dose. A mean value of 137±12.4% was displayed using puromycin as the control across all time intervals, indicating that CPX was more active than puromycin. When NFX was used as a positive control, there was 173% and 115% activity in relation to this control shown by CPX at 6 and 12 hours respectively, with a mean value of 108%, over the remaining intervals. The IC₅₀ value for the reference compounds puromycin and NFX following 48 hours exposure to *T. cruzi* trypomastigotes were 1.03±0.197 µM and 2.21±0.0983 µM, respectively. NFX did not affect the assay when co-incubated with the detection reagent, PrestoBlue, for 6 hours in the absence of parasite.

<table>
<thead>
<tr>
<th>Control</th>
<th>6 hours</th>
<th>12 hours</th>
<th>24 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>NA</td>
<td>8.62±0.844</td>
<td>3.41±0.172</td>
<td>0.866±0.092</td>
</tr>
<tr>
<td>µM IC₅₀ of CPX±SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>32%</td>
<td>73%</td>
<td>90%</td>
<td>95%</td>
</tr>
<tr>
<td>% activity of CPX at IC₁₀₀</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Puromycin</td>
<td>136%</td>
<td>154%</td>
<td>136%</td>
<td>124%</td>
</tr>
<tr>
<td>% activity of CPX at IC₁₀₀</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFX</td>
<td>173%</td>
<td>115%</td>
<td>104%</td>
<td>103%</td>
</tr>
<tr>
<td>(% activity of CPX at IC₁₀₀)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.1. The activity of CPX following 6, 12, 24 and 48 hours incubation with host cell free *T. cruzi* trypomastigotes. Percent activity of CPX is shown at the maximum effect IC₁₀₀, in relation to the positive controls NFX and puromycin; and medium without addition of parasite. The IC₅₀ value at each time point was calculated in reference to medium. IC₅₀ values are calculated from triplicate dose series, over one experiment. NA= not active, cannot determine an IC₅₀ value. SD= standard deviation of measurements.
6.3.4 Activity of combinations of CPX and NFX on T. cruzi amastigotes

The ΣFIC was calculated for combinations of CPX and NFX, where the IC$_{50}$ value was able to be estimated (Table 6.2). Less than 50% activity against 3T3 host cells was observed following treatment with these combinations. The ΣFIC values obtained were all within the range of what is considered to be an additive effect.

<table>
<thead>
<tr>
<th>CPX (xIC$_{50}$)</th>
<th>NFX (xIC$_{50}$)</th>
<th>ΣFIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2</td>
<td>0.905±0.0389</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1.22±0.00843</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>0.795±0.0329</td>
</tr>
<tr>
<td>8</td>
<td>0.5</td>
<td>1.09±0.128</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>1.24±0.0956</td>
</tr>
</tbody>
</table>

Table 6.2. Combinations of CPX and NFX, in relation to their IC$_{50}$ values and the ΣFIC values of these combinations.

The FIC of the various CPX and NFX combinations were plotted in GraphPad Prism, in an isobologram format (Figure 6.5). Most points on the isobologram lie in the region indicating additive effects, although 3 combinations, namely 4 x the IC$_{50}$ of CPX in combination with 4 x the IC$_{50}$ of NFX; 4 x the IC$_{50}$ of CPX in combination with 1 x the IC$_{50}$ of NFX; and 8 x the IC$_{50}$ of CPX in combination with 0.5 x the IC$_{50}$ of NFX represent antagonism. A further 2 combinations, namely 4 x the IC$_{50}$ of CPX in combination with 2 x the IC$_{50}$ of NFX; and 8 x the IC$_{50}$ of CPX in combination with 1x the IC$_{50}$ of NFX, display what is often referred to as “mild synergy”[438]. Collectively, these results suggest that the combinations of CPX and NFX tested exert an additive to mildly synergistic action.
6.4 Discussion

In an acute in vivo model of T. cruzi infection, utilising Y strain-LUC parasites, CPX was found to suppress infection, with only 4 fold greater luminescence displayed than the control drug BZ after 5 days treatment, and 3 times greater than BZ after 10 days treatment in comparison to the vehicle treated control mice. After 10 days CPX treatment, this constituted a 22 times reduction in the mean luciferase signal in comparison to the vehicle control. This is the first report of the action of CPX against T. cruzi in vivo, and although this compound did not clear mice of infection, it warrants further investigation for the use of CPX in T. cruzi drug discovery. An increase in the amount of CPX administered in vivo, by the quantity or frequency of dosing, may determine whether CPX is able to clear infection. At 60 mg/kg there were no adverse effects observed in any of the animals used for this study. It would be of benefit to evaluate efficacy using improved methods of delivery, such as oral gavage by dissolution CPX in methylcellulose, propylene glycol or polysorbate 80 [439], or IP route administration. Chemical modification to improve solubility and
bioavailability could be undertaken. Recently there have been a number of patents published for producing pro-drugs of hydroxamic acids to improve aqueous solubility and cellular permeability [440]. The pharmacokinetic properties of other hydroxamic acids, for example vorinostat, a suberoylanilide hydroxamic acid, have been improved by production of micelles [441]. Micelles, formed by block co-polymers in water are solubilising agents for poorly soluble drugs. Polymeric micelle based solid-form tablets have also recently been produced to improve the oral availability of dexibuprofen [442]. If clearance of parasite in an acute and chronic in vivo model could be achieved for CPX, chemical modification would be justified to improve the solubility. To determine whether CPX should be pursued further in Chagas drug discovery, the efficacy of CPX will be determined initially by administering IP in an acute model of T. cruzi infection as a future direction for this project. If CPX is successful in attaining a parasitological cure, testing in chronic models of infection in vivo would be warranted.

Should clearance of T. cruzi in mouse models of infection not be achieved, CPX would be an excellent candidate for combination studies with known drugs or compounds identified herein as active against amastigotes. As CPX has exhibited suppressive activity, synergistic action in combination with known compounds, could results in a parasitological cure. To determine if the in vitro activity of CPX could be improved, combination studies were undertaken with CPX and the drug currently used to treat T. cruzi infection, NFX. These studies have identified, utilising isobologram analysis and the FIC for a range of combinations of NFX and CPX, that there was an additive effect observed against T. cruzi amastigotes in vitro when these two compounds were co-administered. Although isobolgram analysis suggested a slight synergistic action between these compounds, \( \Sigma \text{FIC} \) values ranged from 0.795 to 1.24, within the range of what is generally defined as an additive effect, namely when the \( \Sigma \text{FIC} \) is between 0.5 and 4 [434]. The simultaneous use of an isobologram with
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FIC data is recommended for accurate analysis of the impact of compound combinations [437], as the more visual method of calculating an isobologram is not as accurate, with more user bias, compared with the use of FIC to calculate effects [443]. An additive effect is also termed no interaction [434] or indifferent [444] and does not demonstrate what is expected from individual compound potencies [445]. For this reason, synergistic effects of compound combinations are preferable [446,447]. CPX has been shown to enhance the efficacy of anti-cancer agents, *in vitro* [448-450], for example CPX significantly enhances the ability of the preclinical anti-leukaemia compound, parthenolide, to target acute myeloid leukaemia *in vitro* [449]. It has also been demonstrated *in vitro* that pre-treatment of breast cancer cells with the iron chelator DFO, renders cells more sensitive to treatment with known anti-cancer compounds [451]. Further investigation of CPX in combination with NFX or BZ against *T. cruzi* with pre-treatment using CPX, may improve the combined effect of these compounds. As glucuronidation is the main metabolic pathway of CPX, interactions with drugs metabolized via the cytochrome P450 system are unlikely [452] and therefore the potential for adverse drug-drug interactions is reduced [453]. Thus there is the potential that CPX could be combined with azole antifungals that have already been identified as active against *T. cruzi*, including those identified herein, such as clotrimazole, ketoconazole or voriconazole.

From these studies the addition of an exogenous iron source reduced the activity of both CPX and to a lesser extent, DFO, against *T. cruzi* amastigotes and trypomastigotes, and therefore chelation of intracellular iron is suggested to be functionally important for CPX and DFO-induced cell death of *T. cruzi*. Although the IC$_{50}$ of DFO against amastigotes has not been reported in the literature, it has been found that following exposure of *T. cruzi* Brazil strain infected mouse peritoneal macrophages to 33 µM of DFO for 96 hours, there was a 55% reduction iron content estimated by atomic absorption of cellular digests [418]. The same authors show that
an external iron source reduced the activity of DFO, following 96 hours of incubation of *T. cruzi* amastigote infected cells *in vitro*, although the iron source was not mentioned. The authors have quoted that 22 µg/mL of equimolar concentrations of DFO and Fe reduce the effect of 22 µg/mL DFO (81 µM) to that observed for non-treated amastigotes [454]. Host cell free amastigotes have also shown a response to DFO, although an IC$_{50}$ value was not reported, and a positive control for growth was not used [455]. By the action of DFO against *T. cruzi*, it has been suggested that iron is necessary for growth [455]. CPX has been reported to act at least partially as an iron chelator in numerous cells *in vitro* including cancer cells [371], *Escherichia coli* [456] and *Candida albicans* [457]. When FC was added as an exogenous form of iron in both amastigote and trypomastigote cultures, there was a decrease in the activity of CPX. A maximum 9.5 fold reduction in the activity of CPX was observed in the amastigote assay with the addition of 6.25 µM of FC. In the trypomastigote assay, a 12 fold reduction in the IC$_{50}$ value was observed with the addition of 3.12 µM of FC.

In both assay formats, DFO was less active than CPX against *T. cruzi* life cycle forms, with IC$_{50}$ values estimated at 15.01±2.05 µM and 28.29±1.12 µM in the trypomastigote and amastigote assays, respectively. A maximum 4 fold difference was detected in DFO activity in the presence of 50 µM FC in the amastigote assay and in the trypomastigote assay, a 3.12 µM addition of FC resulted in a 3.3 fold difference in the IC$_{50}$ value. The lower activity of DFO in each assay may be a function of the ability to penetrate cells [371]. DFO has been demonstrated to have low epithelial permeability and coupled with a high molecular weight of 656, resulting in poor bioavailability [458]. CPX, with a decreased molecular weight and lipophilicity, may be a more efficient intracellular iron chelator. CPX may also be a more effective iron chelator *per se* than DFO, as it has been demonstrated from *in vitro* competition studies that CPX has a higher affinity for FeCl$_3$ [459]. DFO reduced parasitemia *in vivo* in a mouse model of *T. cruzi* infection following 6 hours treatment, following treatment with
approximately 1250 mg/kg. However, after 12 hours there was no reduction in the number of parasites. Although the authors comment that this was a trypanostatic effect, it may be that due to moderate activity it was able to remove less of T. cruzi population in comparison to the control, BZ [372]. Treatment of mice for 12 days with approximately 250 mg/kg DFO before T. cruzi infection, followed by a further 14 days post infection resulted in a 4 fold difference to non-infected mice, however there was no control drug used [372]. From the studies reported here, CPX may more efficiently chelate iron, and shows activity in an in vivo model of T. cruzi infection following 5 days treatment, thus additional in vivo and in vitro studies may shed some light on the importance of iron chelation in T. cruzi infection. Monitoring of iron levels following treatment in vivo with CPX treatment would therefore be warranted as a component of future studies.

The regulation of CPX and DFO activity by FC is not dose dependant across all doses of FC. Some dose dependence is indicated up to 3.25 µM concentrations in the amastigote assay when CPX is added to cells in the presence of FC, however beyond concentrations of 12 µM there is a sharp decrease in the effect of FC on the activity of CPX, which does not correlate with a toxicity effect from FC on the number of host cells. A similar effect was seen with doses ≥ 6.25 µM in the trypomastigote assay. It could be that at these higher concentrations, iron may not dissociate as well from the ferric citrate complex, therefore may not be as readily available to CPX. Because of the multinuclear complexes that can form, the composition of ferric citrate solutions is complex [460]. Repeating this experiment with a divalent source of Fe, such as FeCl₂ or FeSO₄, and other metals such as MgCl₂, CuCl₂ and ZnCl₂ and MnCl₂ to determine if these metals have an effect on the activity of CPX is therefore proposed.

Oxidative stress is likely to be an important part of T. cruzi parasitism. Evidence which supports this is that anti-oxidants, including nuclear factor erythroid 2 (NRF2)
activators, implicated in oxidative stress and toxicity in mammals [461] have been shown to reduce macrophage parasite burden in T. cruzi infected mice, whilst the pro-oxidant paraquat increased infection [462]. The mechanism/s that regulate the enhancement of oxidative stress during T. cruzi infection remain/s to be elucidated, although iron mobilization is a distinct possibility [462]. SOD, metalloenzymes upregulated during oxidative stress in eukaryotes catalyse the dismutation of reactive superoxide anion generated in oxidative stress environments [463,464]. Several human SOD isoforms have been described, characterised by different metal cofactors including Mn and Fe [465]. Two cDNA’s coding for SOD have been cloned and characterised in T. cruzi epimastigotes, are expressed in all life cycle stages, and are Fe- dependant [466]. Fe-SOD overexpression has been identified in vitro in epimastigotes with induced resistance to benznidazole, of which the drug action may in part be due to generation of superoxide anion radicals [467]. Chelation of iron by CPX may reduce Fe- dependant SOD in both the host and parasite, therefore creating an increase in the susceptibility of the parasite to oxidative stress, and potentially increase host intracellular superoxide. As CPX exerted activity against the trypomastigote, which was determined to be at least in part due to iron chelation, the effects are not likely to be wholly host cell mediated. From these studies, CPX was active against both the amastigote and trypomastigote life cycle stages, with IC₅₀ values of 0.705±0.139 µM and 3.083±0.532 µM, respectively.

Iron chelation could decrease parasite growth in other ways, including nutritional stress and inhibition of other iron-dependant enzymes. It has been demonstrated that CPX has inhibitory activity against the iron- dependant enzymes ribonuclease reductase in leukaemia cell lines [371] measured by electron paramagnetic resonance, and against recombinant deoxyhypusine hydroxylase (DOHH), both of human [412] and leishmanial origin [468]. Although little is known about iron transport in T. cruzi parasites, amastigotes cultured axenically possess receptors for human transferrin,
detected by a specific, dose dependant and saturable binding of $^{125}$I-transferrin to the amastigote surface. Probing with a monoclonal antibody specific for human transferrin showed cross-reactivity with a 200 kDa $T. cruzi$ surface protein [455]. Although host cell internalised amastigotes are not likely to be exposed to this protein, as it is transported within endosomes in the mammalian cell and it is thought that the low pH of endocytic vesicles facilitate the dissociation of iron from transferrin, and endocytised apotransferrin is then returned to the cell surface [469]. It has been identified that $T. brucei$ trypomastigotes bind transferrin, and modelling has suggested N- glycosylation sites are involved in receptor mediated endocytosis [470]. As $T. brucei$ is extracellular, it has the potential to capture circulating transferrin in the bloodstream and similarly does the $T. cruzi$ trypomastigote. Iron stores, known as acidocalcisomes, have been identified in $T. cruzi$ trypomastigotes, however it has been shown that by co-incubation of parasites with gold-labelled human transferrin, that although there was binding of this complex to the cell surface, no intracellular gold-labelled human transferrin was detected [471]. It would be beneficial to determine which proteins are involved in iron transport in $T. cruzi$, as this could represent a novel target for parasite- specific chemotherapy.

As the activity of CPX is not completely abrogated by FC, particularly against the trypomastigote, there may be other targets for CPX other than iron chelation. CPX has recently been found to effect genes in the galactose salvage pathway in $Escherichia coli$ [472]. These genes were identified from clones of $E. coli$ manufactured to overexpress a number of genes from the $E. coli$ ASKA library (A Complete Set of $E. coli$ K-12 ORF K-12 open reading frame (ORF) archive cloned individual genes) [473], then grown in the presence of CPX. Those isolates with an increased MIC contained the sequenced galE gene that encodes UDP-galactose 4-epimerase, which epimerizes UDP-galactose and UDP-glucose in the galactose metabolism pathway [472]. However, in $E. coli$ there is a polypeptide found upstream from galE that is very
similar to the iron dependant regulatory protein IdeR from *Rhodococcus equi* [474]. This remains to be determined, however could point to iron mediated signalling. If there is success in treating *T. cruzi* infection *in vivo* with CPX, determining the mode of action of CPX against *T. cruzi* is justified. A combined approach using affinity chromatography and metabolomics could aid in narrowing down potential targets. Affinity chromatography has been used to successfully identify the cellular targets of two kinase inhibitors in *T. cruzi* epimastigotes, and could be applied to trypomastigotes and amastigotes isolated from host cells [475] to identify intracellular, parasite mediated targets of CPX. Metabolomic responses in the presence of CPX may support the action of CPX as an iron chelator, or identify other effected metabolic pathways. The metabolome of *Saccharomyces cerevisiae*, has been determined in an iron deficient environment [476]. It may be possible to compare iron deficiency to CPX treatment in the trypomastigote stage of *T. cruzi* using this approach.

It has recently been discovered during pre-clinical testing of CPX to treat hematologic malignancies that the estimated half-life of CPX in human plasma is less than 6 hours, [457]. Considering the *in vivo* efficacy of CPX demonstrated in a mouse model of *T. cruzi* infection in these studies, the time-frames that CPX exhibited an effect against *T. cruzi* trypomastigotes was determined. Although the maximal effect of CPX against trypomastigotes *in vitro* was observed following 48 hours, an IC$_{50}$ value could be estimated following 12 hours exposure of trypomastigotes to concentrations of CPX, at 8.37 µM. The IC$_{100}$ of CPX (14.8 µM) was more efficient at reducing the viability of trypomastigotes than 35.4 µM of puromycin over all time intervals, and 14.2 µM NFX was also less efficient than the IC$_{100}$ of CPX following 6 and 12 hours exposure (Table 6.1). However, these observations would need to be confirmed by determination of the IC$_{50}$ values of these control compounds, over time. The ability of CPX-treated trypomastigotes to infect cells will also determine if a
shorter exposure of this life cycle form may affect the overall life cycle of *T. cruzi*. The speed of action on amastigotes should be determined, by adjusting the image-based assay to look at earlier time points than 48 hours exposure of compound. It may be beneficial to use a CellTracker dye, outlined in section 4.2.4.1.3, to allow separate staining of the host cell nuclear material and the parasite for the definition of an earlier infection. Increasing the magnification used for image capture may also improve analysis of small numbers of host-cell internalised trypomastigotes. The speed of action of CPX on both life cycle forms and infection of host cells will enable an estimation of the regularity of dosing that may be required to attain an *in vivo* cure, if possible. More than one dose per day may be required to maintain an effective plasma concentration of the drug. The current drugs used to treat *T. cruzi* utilize twice daily treatment regimes and many studies to evaluate the efficacy of compounds *in vivo* have used two doses per day [107,345,477,478].
Chapter 7: General Discussion

7 General Discussion

This study was set out to explore methods of fluorescently identifying the *T. cruzi* intracellular life cycle form *in vitro* and applying an optimised protocol to profile compound activity against the parasite. A key to the success of phenotypic assay development is assurance that the cell line can be reproducibly produced and is robust. Reproducible *in vitro* maintenance of the culture is paramount, to ensure confidence that the effect of a compound is not compromised by the health of the cell line, particularly when multiple life cycle forms are to be taken in to consideration. Within this study the parameters for maintaining the *T. cruzi* Tulahuen strain life cycle *in vitro* were optimised and included aspects of both the host cell line and the parasite. Two host cell lines were explored, and it was found that the passage number of the host cell was important for imaging purposes and potentially may also impact the reproducibility of the number of parasites released. As these fibroblast cell lines are contact inhibited, repeat culturing compromised the confluent, planar, monolayer normally formed in routine culture. Therefore the subculture of 3T3 cells and NHFC-A cells were limited to 10 and 5 passages, respectively.

The release of trypomastigotes from both of these cell lines was found to be post infection of host cells, meaning that parasites for sub-culture were collected from culture on day 4, and imaging-based methods were not feasible over greater than 3 days of infection. Leaving cultures for longer than 4 days resulted in trypomastigotes differentiating in to extracellular amastigotes and eventually repeated infections resulted in damage to the host cell bed. It is not clear what role extracellular amastigotes play in the infective process, however minimisation would potentially contribute to more reproducible infections. By comparison of 2 month old and 15 month old subcultures of *T. cruzi* it was discovered that there
were more amastigotes found in the supernatant on 4 days post-infection and more trypomastigotes were released into the supernatant on 3 days after infection. Although it was not determined, such morphological changes would most likely be the result of associated genetic changes and could affect compound sensitivity. A reproducible method for the differentiation of the *T. cruzi* Tulahuen strain parasite, and growth of epimastigotes in LIT is reported. Although there are some reports of the differentiation of Tulahuen parasites in TAU3AAG [228], the percentage of metacyclic forms generated and the reproducibility of differentiation is not reported, and in these studies resulted in an increase the production of metacyclic life cycle forms over the use of log phase parasites by 2-4%. The characterisation of the *in vitro* culturing outlined herein would be of benefit to the broader scientific community when considering assay optimisation for the *T. cruzi* Tulahuen strain parasite. The inability of the *T. cruzi* Y strain parasite to infect 3T3 cells reproducibly highlights the need to optimise the *T. cruzi* life cycle for each strain that is utilised for *in vitro* studies. This brings in to doubt the amenability of one assay to many strains of parasites, as it has been suggested is possible [229].

The optimised *in vitro* culture conditions were utilised to develop a reproducible and sensitive image-based assay to detect compound activity against the amastigote form of *T. cruzi*. Benefits of this assay over existing technologies include increased throughput over current image-based methods, which are currently in 96-well formats. Although a 384-well method has been described, utilising osteoblasts infected with *T. cruzi* amastigotes [242] reports of methodology and compound activity from the application of this assay technology have not currently been published. The image-based assay herein identifies both the parasite and the cytoplasm of the host cell, which potentially allows for an improved definition of an infected cell. Current methods rely on the ratio of the parasite DNA to the host cell nucleus [479] rather than localisation of the parasite
in the host cell. In these studies it was identified that there are varying levels of residual amastigotes in 3T3 host cells that remain after 48 hours treatment of *T. cruzi* infected cells with compounds. An image-based system that relies upon ratios may not be sufficiently sensitive to detect this effect. Further studies are warranted to determine if these amastigotes are removed following >48 hours treatment with compound and could be an important tool in prioritisation of chemical hits.

During development of the *T. cruzi* image-based assay, a number of fluorescent dye combinations were explored to define a *T. cruzi* infected 3T3 host cell. Further investigation of these markers in the future may provide a more accurate determination of host cell viability, utilising the redox-based indicator C12-resazurin, successfully used to identify the cytoplasm of 3T3 fibroblasts in this study. The fluorescent probe CellTracker Green was used to stain *T. cruzi* trypomastigotes and track their development within host cells. However due to amastigote division, the dye was unevenly distributed in parasite cells 72 hours post infection. This dye could be utilised for shorter incubation times, to either identify compound effect on host cell entry of *T. cruzi* or the speed of action of a compound on the amastigote life cycle stage.

The capability of the *T. cruzi* amastigote image-based assay to detect selective and non-selective compounds is demonstrated by the identified selectivity and non-selectivity of the reference compounds NFX and puromycin, respectively. The selectivities of these compounds are expected, as NFX is a drug used to treat Chagas disease, whilst puromycin is an inhibitor of protein synthesis. The 384-well image-based assay has been used in these studies to profile the activity of two compound libraries against the *T. cruzi* parasite, namely the MMV Malaria box and an in-house collection of compounds that are either FDA approved, or have
known biological activity. From these libraries, identification of compounds with previously reported activity against *T. cruzi*, including antifungal, cytochrome P450 inhibitors validates the application of this assay to assess compound activity against the parasite. The assay has also identified compounds with novel activity against the parasite and development of a secondary, whole cell phenotypic assay to determine activity against the trypomastigote form of the parasite describes the potential that some of these compounds may have against this life cycle form to prevent infection. Whether this translates to a more effective *in vivo* effect is not certain, however as the drugs currently used to treat Chagas are active against both life cycle forms, activity against this life cycle form was considered to be beneficial, although not necessary.

From the in-house library the antifungal CPX displayed novel activity with an IC$_{50}$ value $<5$ µM, against both *T. cruzi* amastigotes and trypomastigotes *in vitro*, with a selectivity index to the *T. cruzi* amastigote of $>35$ times over the host cell. Previously published pharmacological profiles in rats, mice and dogs of CPX, observed a NOAEL of 30 mg/kg/day for 4 weeks [370]. In these studies it was demonstrated that CPX suppressed *T. cruzi* infection, reducing estimated infection by 22 fold in comparison to infected control mice, in a mouse model of acute *T. cruzi* infection, when dosed at the NOAEL equivalent in mice, 60 mg/kg [480], daily for 10 days. As no toxicity was observed following these studies, future directions would include increase of dosing, IP dosing and improvement of the dosing vehicle from drinking water, potentially utilising a cellulose-based diluent. If CPX could clear infection in an acute model of *T. cruzi* infection, further testing and chemical modification to improve the physiochemical properties of this compound would be recommended, as well as determination of the mode of action of this compound. From these studies, iron chelation is likely to be a key factor in the activity of CPX, and iron chelation therapy with other identified
Chapter 7: General Discussion

compounds has recently shown suppressive and curative effects against *P. falciparum* and *P. yoelii* mouse models of malaria infection respectively [481]. Iron chelation remains as target for future development in antiparasitic research [482]. Collectively, these factors point toward CPX as a promising, novel, hit compound for early drug discovery in Chagas disease research. The *in vivo* efficacy of this compound also supports the use of the *T. cruzi* assays developed within this study to profile further compound libraries in the future in the search of new chemical leads. The antihistamine clemastine fumarate was also identified as active against the amastigote form of *T. cruzi*, with an IC$_{50}$ value of 0.76 µM and SI of >28, and future studies are aimed at identifying the activity of this compound in a murine model of acute infection.

There were four drug-like compounds and three probe-like compounds identified as active against *T. cruzi* amastigotes from the MMV Malaria box library, considered as hits for further evaluation in *T. cruzi* drug discovery. Two of these compounds, both with a drug-like classification, demonstrated activity against both the *T. cruzi* amastigote and trypomastigote life cycle forms, with SI values between >8 to >28. Further biological evaluation of these compounds in the future is warranted to include *in vitro* time-course studies and mammalian cell line-panel testing before consideration of *in vitro* safety profiling. These compounds are novel chemical hits for *T. cruzi* research, and new chemical leads with differing modes of action are required for the development of new drugs against Chagas disease.


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<td>GNF;GNF;G</td>
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<td>COc1cc(CNCc2ccc(C)c2)cc(OC)c1O</td>
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<td>AAS16/12433214</td>
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## Appendix

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<td>Set</td>
<td>ChEMBL_NTD_ID</td>
<td>Batch</td>
<td>Source</td>
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<td>NplusO Count</td>
<td>Molecular Weight</td>
<td>Num H Donors</td>
<td>ALogP</td>
<td>Smiles</td>
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Table S1. Properties of drug-like and probe-like compounds from the MMV Malaria Box. Drug-like compounds that were identified as active against *T. cruzi* are marked green. Probe-like compounds that were identified as active against *T. cruzi* are marked orange. For the remainder of the compounds in the library collection (366 compounds), no classification was provided. Of these 366 compounds, two were found to be active against *T. cruzi*, and are marked blue. The ChEMBL NTD ID is the code from the neglected tropical disease database (https://www.ebi.ac.uk/chemblntd). The source of the compounds were either the St Jude Research Hospital, USA, GSK (GlaksoSmithKline), UK/ USA or GNF (Genomics Institute of the Novartis Research Foundation), USA. N plus O counts refer to the sum of nitrogen and oxygen atoms. Num H donors= the number of hydrogen donors. Ro5 violation=number of times Lipinskis rule of 5 is violated. AlogP is the calculated partition coefficient. NA= not applicable.
Figure S1. $^1$H NMR of NFX isolated from Lampit® tablets. Experimental conditions are explained in General Materials and Methods.
Figure S2. $^{13}$C NMR of NFX isolated from Lampit® tablets. Experimental conditions are explained in General Materials and Methods.