Identification of Inhibitors of *T.cruzi* Intracellular Amastigote Replication by *in vitro* Imaging Assays

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INTRODUCTION

Chagas disease, (CD) caused by *Trypanosoma cruzi*, is endemic to south America and results in greater than 15 million deaths per year[1] CD presents in an initial acute stage, followed buy a chronic stage, in which 10-30% of infections result in cardiomyopathy and gastrointestinal disease[2] Life cycle stages of the parasite that exist in the mammalian host are the trypomastigote (Fig 1A) and the intracellular amastigote (Figure 1B). Trypomastigotes are found in blood samples in the acute phase, but rarely in the chronic phase[3] New compounds are needed in drug discovery for CD, especially for potential treatment the chronic phase, as drugs currently available have limited effectivity. We aim to identify compounds with activity against the intracellular amastigote, thought to be most prevalent in the chronic phase. Follow up assays will be used to determine activity against the trypomastigote form. Although not essential, trypomastgote activity would potentially be more beneficial for a compound to treat both stages of the disease.

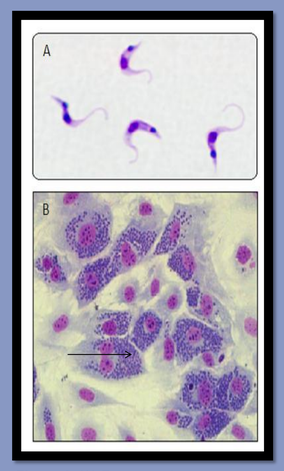

Figure 1: (A) *T. cruzi* motile trypomastigote form ;(B) 3T3 rat fibroblasts infected with *T. cruzi* amastigotes, seen in the cytoplasm (arrowhead) .

METHOD: AMASTIGOTE ASSAY

Primary assay: $1x10^3$ 3T3 fibroblasts/ well in RPMI +10% FCS were added to 384 well plates (PerkinElmer Cell Carrier; Collagen1). After 24 hours, 10µL of $1x10^4$ trypomastigotes were added. Following 24 hours, wells were washed with PBS, once by hand, flicking PBS out of the wells, then 2x on a Bravo liquid handling platform (Agilent) and 50µL of media added. Compounds in 10µL of water were added with a Minitrak™ (PerkinElmer; PE) liquid handler. After 48 hours, wells were fixed with PFA and stained with Hoechst and HCS CellMask Green™ (Life Technologies). Analysis was on an Operetta® high content imager (PE).

Human host cell line: To estimate infection in a primary culture of NHCF human heart fibroblasts (Lonza), $3x10^6$ cells were added and the protocol was followed as above. Analysis was with an Opera® 2.0 high content imager (PE).

METHOD: TRYPOMASTIGOTE ASSAY

$1x10^5$ *T. cruzi* trypomastigotes were added to black/ clear 384 well plates (Beckton Dickinson) in 50µL of RPMI. 10µL compounds were added with a Minitrak liquid handler (PerkinElmer). Plates were incubated for 48 hours before addition of 10µL of a final of 10% Presto Blue (Life Technologies). Plates were then incubated for 6 hours before reading fluorescence at Ex 355, Em 595. Linearity was determined over concentrations of trypomastigotes. Amastigote conversion was 20% of the population at $1x10^5$ cells/ well.

TRYPOMASTIGOTE ASSAY SIGNAL

The assay displayed a signal plateau with greater than $4x10^5$ trypomastigotes per well (Fig 2). This may be due to the cell concentration, as there was a reduction in viability and increase in amastigote conversion at these doses. The assay signal was linear to $2x10^5$ cells/well (Fig 2).

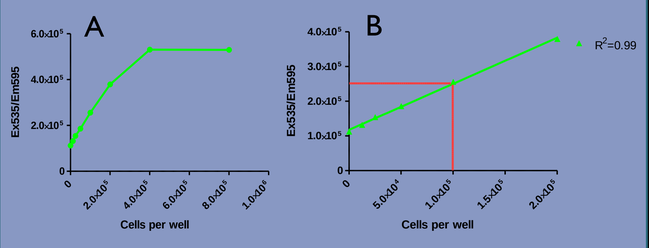

Figure 2: (A) Limits of detection in the trypomastigote assay; (B) Linearity of detection. Red= concentraton of cells per well used in the assay format.

AMASTIGOTE ASSAY ANALYSIS

Hoechst and CellMask images were combined to form a composite image for infected 3T3 or heart cells. Algorithms were written in Harmony®/ Acapella® to identify parasites as spots (Fig 3). For heart cells, an infected cell was defined as >2 spots per host cell cytoplasm.

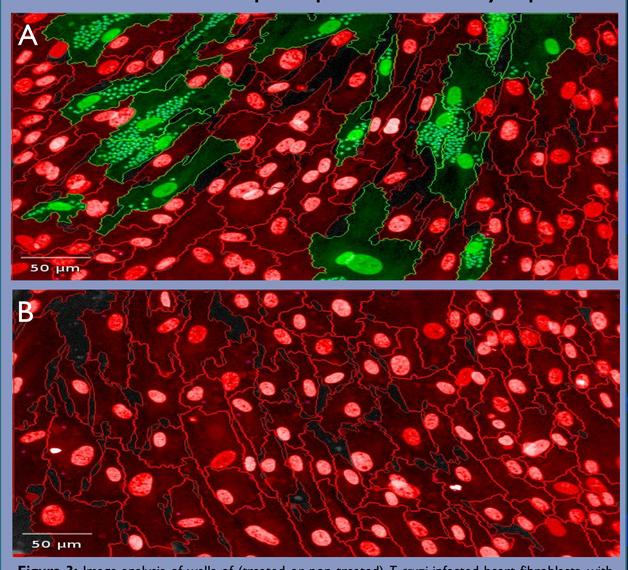

Figure 3: Image analysis of wells of (treated or non treated) *T. cruzi* infected heart fibroblasts, with Opera® 2.0 Acapella® software (using the Assay Language Interface). 20x mag. Red= non-infected cells and green=infected cells. (A) DMSO control; (B) 8µM nifurtimox. For the 3T3 infected cell assay >5 spots was used as there was a higher background associated with these cells/ assay.

COMPOUND PROFILE 1: Activity

From a library of 741 FDA approved and known biologicals, a compound (compound 1) was identified that had previously unreported activity against *T. cruzi* amastigotes, with a potentially novel mode of action. Poor trypomastigote activity was detected (Fig 4).

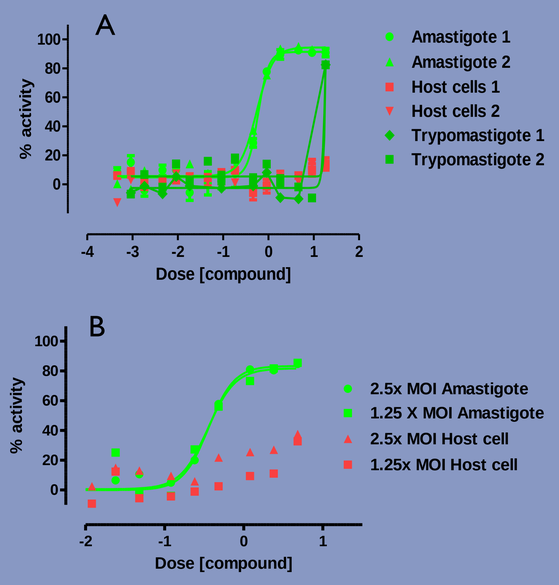

Figure 4: Activity of compound 1 against (A) *T. cruzi* amastigotes in 3T3 fibroblasts over N of 2 experiments, with an IC_{50} of 0.570±0.028µM . An IC_{50} value against trypomastigotes could not be estimated; (B) The compound was active against *T. cruzi* amastigotes in NHCF human heart fibroblasts with an IC_{50} of 0.378±0.029µM, with an infectivity of 2.5 and 1.25x multiplicity of infection (MOI). The reference compound nifurtimox has an IC_{50} of 0.949±0.078µM.

COMPOUND PROFILE 1: Images

2.5µM of compound 1, after 48 hours exposure, shows clearance of the majority of the parasite population with no heart cell effect (Fig 5). To determine if these spots, circled, are parasites that can replicate.

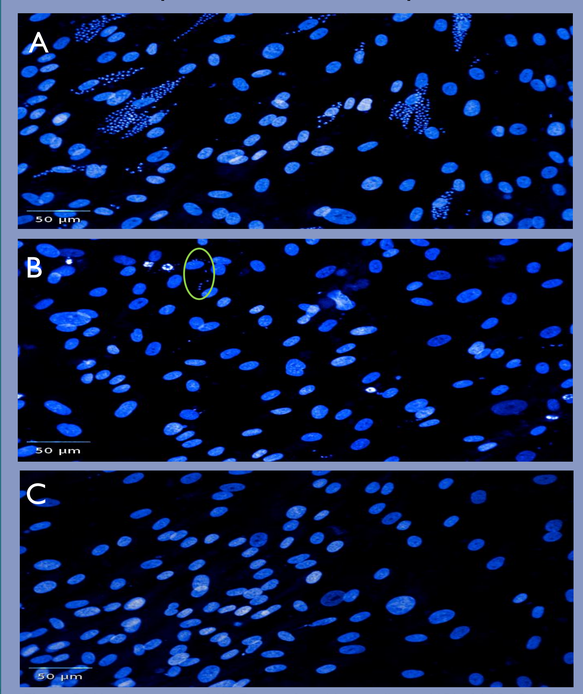

Figure 5: Hoechst staining of (A) *T. cruzi* infected heart cells at a MOI of 1.25x. Parasites are small spots, large nuclei are host cells; (B) *T. cruzi* infected heart cells treated with 2.5µM of compound 1. Green circle=are these viable parasites remaining following treatment; (C) Infected heart cells treated with 8µM nifurtimox. Taken with an Opera® imager. 20x mag.

COMPOUND PROFILE 2: Activity

From a library of 685 compounds with activity previously identified against *P. falciparum*, two compounds (compounds 2 and 3) were identified with activity against both *T. cruzi* amastigotes in 3T3 cells, and trypomastigotes, with <50% host cell activity at 9µM. These compounds have not been re-tested in the heart cell assay (Fig 6).

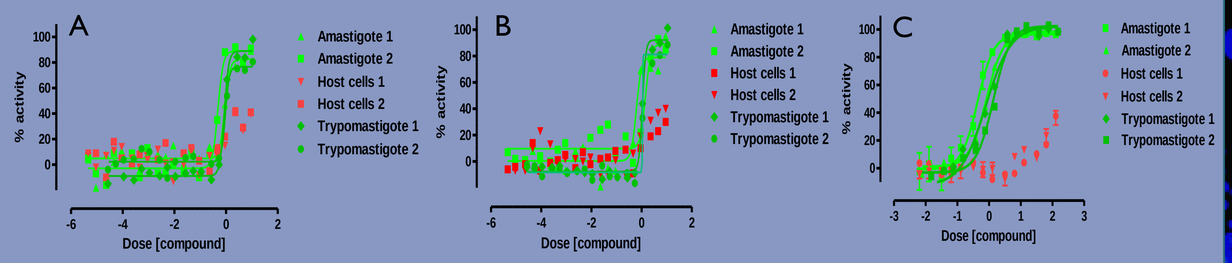

Figure 6: Compounds 2 (A) and 3 (B) with activity against amastigotes in 3T3 fibroblasts, and host cell free trypomastigotes: (A) Amastigote IC_{50} =0.685±0.283µM; trypomastigote IC_{50}=0.898 ±0.063µM; (B) Activity against amastigotes= 1.609±0.255µM and trypomastigotes=1.079±0.008 µM. (C)The reference compound nifurtimox , currently used to treat CD, has an IC_{50} of 0.632±0.277µM against amastigotes in 3T3 cells and1.086±0.421µM against host cell free trypomastigotes.

CONCLUSIONS

The amastigote active compound 1 will be further tested for activity. To repeat the assay and remove compound following 48 hours exposure, to determine if parasite cells can be detected following longer incubation. To add compound before addition of trypomastigotes to host cells. Can this compound prevent infection? Compounds 2 and 3 showed comparable activity to the reference compound and are being sourced to test in the heart cell assay. Both of these types of compounds could be considered hits for early drug discovery for CD, although compounds 2 and 3 would potentially be more beneficial candidate compounds for both stages of the disease.

REFERENCES AND AKNOWLEDGEMENTS

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