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Preliminary Results Indicate the Anti-Proliferative Effect of HIV-1 Protease Inhibitors on *Trypanosoma brucei* Cells can be due to the Non-Specific Targeting of Metallo-and Cysteine-Proteases

Netsanet Worku[1*] **and Gerd Birkenmeier**[2]

[1]*Institute of Public Health, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia*

[2]*Institute of Biochemistry, University of Leipzig, Leipzig, Germany*

[*]**Corresponding author:** Netsanet Worku, Institute of Public Health, College of Medicine and Health Sciences, University of Gondar, P.O. Box 198, Gondar, Ethiopia, Tel: +251911941866; E-mail: netsanet32000@yahoo.com

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Abstract

Background: Initial studies have confirmed the efficacy of protease inhibitors in the treatment of *Trypanosoma cruzi*, *Plasmodium falciparum,* and *Leishmania major*. However, studies on efficacy and specific protease inhibition of HIV-1 protease inhibitors on *Trypanosoma brucei* cells remained untouched. The objective of the current study was to determine the efficacy of two HIV-1 protease inhibitors, ritonavir, and saquinavir, in *Trypanosoma brucei* proliferation and to determine if these HIV-1 protease inhibitors target the activity of the *Trypanosoma brucei* major proteases.

Methods: Time dependency test at variable increasing concentrations, motile cell counts, alamarBlue® cell proliferation/viability assay and zymography were among the methods applied.

Results: Both ritonavir (IC_{50}=12.23 ± 0.33 μM) and saquinavir (IC_{50}=11.49 ± 0.31 μM) effectively inhibited *Trypanosoma brucei* cells proliferation. The major proteases identified in these cells were the cysteine (~29 kDa Mr) and metallo- (~66 kDa Mr) proteases. Protein band densitometry results showed a statistically significant (P-value<0.05) inhibition in metallo-and cysteine-proteases' activity in *Trypanosoma brucei* cells.

Conclusion: The results suggest that RTV and SQV showed an anti-proliferative effect in *Trypanosoma brucei* cells possibly due to the non-specific targeting of the cysteine- and metallo-protease activities of the parasite.

Keywords: HIV-1 protease inhibitors; Ritonavir/saquinavir; Cysteine-proteases; Metallo-proteases; *Trypanosoma brucei*

List of Abbreviations BBB: Blood-Brain Barrier; BMECs: Brain Microvascular Endothelial Cells; ELISA: Enzyme Linked Immuno Sorbent Assay; HAT: Human African Trypanosomiasis; HIV-1: Human Immunodeficiency Virus-1; MSP: Major Surface Protease; NECT: Nifurtimox-Eflornithine Combination Therapy; NTD: Neglected Tropical Disease; RTV: Ritonavir; SQV: Saquinavir; VSG-Variant Surface Glycoprotein

Introduction

Human African Trypanosomiasis (HAT), also known as sleeping sickness, is a life-threatening neglected tropical parasitic disease affecting the blood, lymphatics and the central nervous system. The disease is caused by two sub-species of the *Kinetoplastid* protozoan parasite, *Trypanosoma brucei: T. b. gambiense* and *T. b. rhodensiense*. A third sub-species, *T. b. brucei*, is only infectious to animals and is commonly used as an experimental model for the first two parasite sub-species [1,2]. The two human infective subspecies of the parasite have their own distinct distribution within the African continent. *T. b. gambiense* is distributed in the west and central parts of Africa causing the chronic form of the disease and *T. b. rhodesiense* is distributed in the east and southern parts of Africa resulting in the acute form of the disease [3,4]. Sixty million people in 36 sub-Sahara African countries, mainly living in poverty-stricken, remote rural areas, are at risk of the disease. Annually the disease causes an estimate of 50,000-70,000 cases, 48,000 deaths and 1,525,000 Disability Adjusted Life Years (DALYs) [3,5]. In 2012, however, the annual number of cases reported had substantially declined to just below 10,000 and the current estimate is 20,000 [6,7]. The progress towards disease control has continued to effectively reduce the number of reported cases to 3,796 with less than 15,000 estimated cases in 2014. The recent WHO Fact sheet report also showed the number of new cases has continued to decline to 2,804, the estimate being lower than 10,000 [8,9]. Compared to other tropical diseases, these figures might seem relatively small and it can also consider that disease control or eradication is no more a far-reaching possibility. However, besides its fatal nature, HAT, without intervention, has the capacity to result in epidemics and is, therefore, a major public health problem.

Since the development of preventive vaccines remains a challenge due to the high degree of antigenic variability expressed by the parasite surface coat glycoprotein, chemotherapy and vector control became the only viable solutions to control HAT. In addition, almost all of the registered drugs in the list were developed over 60 years ago. During treatment, all of these drugs have a number of shortcomings. This includes parasite resistance, allergic reactions, undesirable effects in the urinary tract and reactive encephalopathy (with 3-10% fatality due to

melarsoprol specific therapy). Some regimens also need highly trained personnel and hospitalization of patients for drug administration [3,10]. Searching for new, safe and effective drugs for both the heamolymphatic and cerebral stages of the disease is, therefore, in an urgent need.

In response to this research gap, identification of specific parasite molecules that are essential to the parasite's life cycle or the pathogenesis of the disease they produce is an ideal strategy for successful drug development. Accordingly, many studies and reviews are done on the classification, biological function, and chemotherapeutic role of proteases in different life forms [11-16]. Hence it is known that proteases are ubiquitous in all forms of life and catalyze the enzymatic degradation of proteins. In trypanosomatids, the study of proteases attracted considerable attention because these enzymes are known to get involved in various activities fostering disease pathogenesis including modulation of the host immune system, invasion, and destruction of host cells or tissues, and parasite migration. They also support cell growth, development, and proliferation by taking part in the acquisition of essential nutrients for survival. Besides, they are considered to be parasite virulence factors [17,18]. Therefore, the parasite-derived proteases are also promising targets for the design of antimicrobials against trypanosomatids [17].

Accordingly, initial *in vitro* and *in vivo* studies have confirmed the efficacy of protease inhibitors against the proliferation of protozoan parasites such as *Trypanosoma cruzi, Plasmodium falciparum, Cryptosporidium parvum* and *Leishmania major* [19-23]. Recently, it was also demonstrated that HIV-1 protease inhibitors like nelfinavir, lopinavir, indinavir, and saquinavir have a direct effect on the causative agents of leishmaniasis including *Leishmania amazonensis*, *Leishmania infantum,* and *Leishmania major* promastigotes *in vitro* [24,25]. The effectiveness of these HIV-1 protease inhibitors in inhibiting parasitic multiplication may probably be associated with their capacity to modulate or block the parasite-specific proteases [26,27]. For instance, it has been indicated that the antiretroviral drugs like saquinavir, ritonavir, and lopinavir were observed to target the plasmepsins II and IV in *P. falciparum* protozoa [28] even though it was not possible to prove this result by Parikh et al. [29]. Similarly, the HIV protease inhibitors are also known to target the Ddi1 protein, which is an ortholog of the yeast Ddi1 protein and the only known member of aspartate protease in *L. major* parasites [30]. However, according to our current knowledge, there is no previous work done in the application of these antiretroviral protease inhibitors in *T. brucei*. Hence we hypothesise that they may also exhibit anti-proliferative effect against trypanosomes by inhibiting the activity of one or a group of major proteases essential for the survival of these parasites. The objective of this study project was, therefore, to determine the effect of HIV-1 protease inhibitors Ritonavir (RTV) and Saquinavir (SQV), in *T. brucei* cell lines and to identify if these protease inhibitors also inhibit the activity of the major proteases expressed in *T. brucei* cell extracts.

Materials and Methods

In vitro cultures of *T. brucei* cells

Laboratory-adapted *T. b. brucei* cells (strain TC-221) were obtained from Prof. A. Stich, Würzburg, Germany [31]. Cells were cultured in complete Baltz medium which was composed of 82% Baltz basic solution, 0.8% ß-mercaptoethanol, 0.8% penicillin/streptomycin (10,000 U/ml), and 16.4% heat-inactivated Fetal Calf Serum (FCS). Cells were cultured using 50 ml sterile cell culture flasks (Greiner Bio-One, Frickenhausen, Germany) at 37°C and 5% CO_2 in 100% humidified environment. The medium was changed every 2-3 days.

Cell proliferation/viability assay and its principle

The AlamarBlue® cell proliferation assay was employed to measure the metabolically active cells. The principle behind this assay relates to the fact that metabolically active cells create a reducing environment that promotes the conversion of resazurin (non-fluorescent) to resorufin (highly fluorescent). The absorbance was measured using an ELISA reader/96-well multi-scanner (Tecan, Crailsheim, Germany) at ex550/em630 nm.

Cell extract preparation and determination of protein concentration

To prepare cell extracts the cell pellet was first re-suspended at 1:4 ratio in a lysis buffer containing (25 mM Tris, 2 mM PMSF, 10% Glycerol, 1% Tritonx100, 2 mM EDTA and freshly added 0.3% protease inhibitor cocktail) at pH 8.03. The suspension was repeatedly vortexed every 1-2 minutes within 15 min duration. The cell sediment was always kept on ice. Then the mix was centrifuged for 15 min at x13000 g in 5°C. The supernatant was carefully pipetted into a new Eppendorf tube and the sediment was discarded. The protein content of samples was determined by means of the Bradford method [32].

Concentration-Time-dependency of the effect of drugs on *T. brucei* cell count

Concentration-Time-dependency of effect of drugs on cell count was done in 24-well plates where 1 ml of cell suspension (2×10^5 cells/well) plus 100 µl of ritonavir (Cat.4622; Lot. 110018) or saquinavir (Cat. 4658; Lot. 01989) at increasing concentrations (5 to 100 µM range) or Pentamidine (Pentacarinat® 300, Sanofi Aventis, Gouda, Netherlands) (12.8 to 128 nM) or 100 µl of fresh medium containing 1% DMSO (control). The mixtures were incubated at 37°C in a humidified environment containing 5% CO_2 for 24 hrs. Prior to cell counting the plate was slowly shaken in a slow circular motion to homogenize cell distribution within the growing medium in each well. After a certain time (1, 3, 6 and 24 hrs) of incubation, a volume of 10 µl of the cell suspension was taken for microscopic counting of actively motile cells using a Neubauer hemacytometer (Marienfeld, Germany). All tests were done in triplicate and the mean values were calculated.

Concentration-response test on *T. brucie* cells proliferation

The cell proliferation/viability assay was performed in 96-well cell culture plates to which 100 µl of cell suspension (2×10^4 cells) in medium containing 1% DMSO, 100 µl of RTV or SQV at variable concentrations ranging from 5 µM to 100 µM and 20 µl of AlamarBlue® was added. As a control 100 µl of Pentamidine-isethionate (Pentacarinat® 300, Sanofi Aventis, Gouda, Netherlands) at variable concentrations ranging from 0.5 nM to 128 nM has been employed. In all cases, control wells contained 200 µl of an equal number of cells in fresh medium containing 1% DMSO without drugs. The change in absorbance was recorded at the 48th hr of incubation. Tests were done in triplicate and the IC50 and standard deviation were calculated using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA).

Zymography

A non-reduced 12% SDS polyacrylamide gel copolymerized with 1% gelatin substrate (SDS PAGE) was used for detection of protease activity in *T. brucei* cell extracts. Prior to running the SDS PAGE, the cell extract was mixed with an equal volume of SDS sample buffer in a non-reducing condition. Then 40 µl (18.3 µg protein) of cell extract-sample buffer mix was added to each lane. Protein marker (each 6 µg per lane) was used as a molecular standard. The electrophoresis was run at 125 V for 1.5 hrs. The gel was then slowly removed from the gel chamber and incubated at 22°C for 30 min with an aqueous solution of 2.5% Triton X-100 for renaturation and removal of SDS from the gel. The gel was then incubated again at 22°C in developing buffer containing 8 mM $CaCl_2$ in 50 mM Tris, pH 6.8 for another 30 min. The developing buffer was changed and the zymogram was incubated overnight with fresh developing buffer at 37°C. The gel was then stained with 0.05% Coomassie Brilliant Blue R250 for 3 hrs. Distaining was achieved by incubation with a mixture of water/methanol/acetic acid (50:40:10 v/v) for 30 min. The presence of active proteases was indicated by white bands when contrasted with the blue background of the stained gel.

Application of zymography for identification of proteases in *T. brucei* cells

To detect different species of proteases in the cell extract by zymography, the gels were separately incubated with either 20 mM EDTA (pH 8.0), 5 mM PMSF, 5 mM iodoacetamide or 1 mM Pepstatin A for inhibition of metalloproteinases, serine proteases, cysteine or aspartyl proteases, respectively. Finally, the gels were scanned and the protein bands showing active substrate digestion in the control gels were white colored but those inhibited by the respective specific protease inhibitors were blue colored bands.

Reverse zymography using ritonavir and saquinavir

The same protocol as the zymography was applied except that 100 µM RTV and 100 µM SQV are applied. Briefly, the respective sample gels were incubated in 100 µM RTV and 100 µM SQV in a 50 ml fresh developing buffer (50 mM Tris, pH 8.0 containing 8 mM $CaCl_2$) overnight at 37°C. The control gel was incubated in a developing buffer containing 1% DMSO without either of the HIV-1 protease inhibitors. Finally, the gels were scanned in a computer scanner.

Statistical data analysis

The graphs were drawn using GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, USA). Statistical comparison of the difference between means has been done using two-tailed t-test in SPSS version 15.0. Results were considered statistically significant when the p-value was less than 0.05. The log value of the inhibitor has been applied to calculate SD and IC50 values using nonlinear regression analysis in GraphPad prism. ImageJ software was also applied to analyze the protein band density measurements [33] to determine the anti-Metallo-and anti-cysteine-protease activity of RTV and SQV. All the experiments were done in triplicates unless otherwise indicated.

Results

Effect of HIV-1 protease inhibitors on the proliferation of *T. brucei* cells

Our results demonstrated that RTV at concentrations above or equal to 50 µM was most effective, killing more than 70% of the cells in 24 hrs of exposure (Figure 1A). SQV resulted in similar growth inhibition starting from the 20 µM concentration (Figure 1B and 1C).

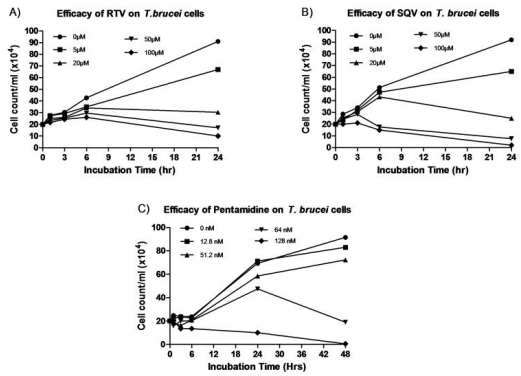

Figure 1: Response of *T. brucei* cells exposed to (A) ritonavir (B) saquinavir and (C) pentamidine. 1 ml of 2×10^5 cells per well in a 24-well plate is applied at the beginning of the experiment. Cell were exposed t o drugs at variable increasing concentrations and cell was done at variable time points within 24 hrs (for test drug) or 48 hrs (for pentamidine) of incubation time. The experiments were done in the triplex and the mean values were taken.

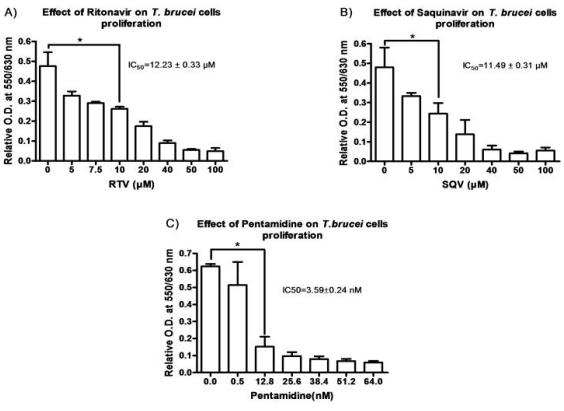

Figure 2: The anti-proliferative effect of (A) ritonavir (B) saquinavir and (C) pentamidine in *T. brucei* cells. 100 µl of 2×10^4 cells per well in a 96-well plate were incubated with 100 µl of each inhibitor at increasing variable concentrations within 5 to 100 µM range. The control wells contained cells in 200 µl medium containing 1% DMSO without drugs. AlamarBlue® proliferation assay was employed and absorbance was measured at ex550/em620 nm wavelength. The experiments were done in a triplex and the mean and standard deviation values were calculated.

In the AlamarBlue®cell proliferation assay, the concentration-dependent effect of these two drugs was observed using 96-well plates. Both RTV and SQV resulted in statistically significant inhibition ($p<0.05$) at 10 μM concentration (Figure 2A and 2B). SQV acts a little better than RTV resulting equivalent effects with a lower IC50 (Figure 2C). Figures 1 and 2, therefore, confirmed that both RTV (IC_{50}=12.23 ± 0.33 μM) and SQV (IC_{50}=11.49 ± 0.31 μM) can effectively inhibit *T. brucei* cells proliferation.

Identification of the major proteases in *T. brucei* cell extracts

To determine targets of these two drugs it was first important to identify the major proteases available in *T. brucei* cells. As indicated in Figure 3, we were able to identify the activity of two major proteases that can clearly be seen as white bands in the lanes of the control gel (n=3) with molecular masses of ~66 kDa and ~29 kDa. The next question was to identify the type or class of these proteases using Metallo-, serine-, cysteine- and aspartyl-specific protease inhibitors. The second gel (n=3) in Figure 3 indicates activity inhibition of proteases by Ethylenediaminetetraacetic Acid (EDTA) at the ~66 kDa position confirming it is a metalloprotease. The third gel indicated inhibition of the ~29 kDa protease activity by the iodoacetamide, showing that it is a cysteine protease. Supporting this result PMSF also inhibited the ~29 kDa protease, only partially, affirming it is a papain family cysteine protease type. Pepstatin A, however, showed no activity inhibition of the two identified protease bands indicating either of these proteases may not be from aspartate protease family members. Those bands at the ~29 kDa are thicker than the band size of the ~66 kDa proteases showing the cysteine proteases are also proportionally more abundant or showed better gelatinolytic activity than the metalloproteases in *T. brucei* cells. This zymographic data, therefore, indicates that the two major proteases, that are clearly expressed using gelatin as a substrate, are papain-like-cysteine proteases and metalloproteases (Figure 3).

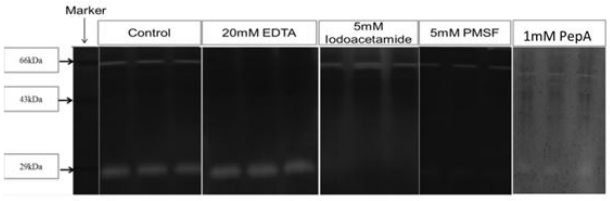

Figure 3: Identification of proteases in *T. brucei* cell extracts. *T. brucei* cell extract was first mixed with equal volume of sample buffer without a reducing agent and 40 μl of a sample containing 18.3 μg of protein concentration was loaded in each lane in an SDS-substate gel before running the electrophoresis. A Roti-protein marker was used as a standard. Control lanes (n=3) showed the proteases expressed as white bands at the ~66 kDa and ~29 kDa level. Application of 20 mM EDTA (n=3 lanes) showed inhibition of the white band's expression at the ~66 kDa level indicating that this is a metalloprotease. Application of 5 mM iodoacetamide (n=3 lanes) on the other hand inhibits expression of the ~29 kDa protease indicating that this is a cysteine protease. The gel (n=3 lanes) where 5 mM PMSF was applied as an inhibitor showed partial inhibition of the ~29 kDa protease telling that this is a papain-like-cysteine protease.

The last column gel (n=3) to the right of the figure demonstrates the application of 1 mM Pepstatin A showed no effect on the activity of the two visible bands of proteases confirming both may not be Aspartyl type of proteases. Effect of HIV-1 protease inhibitors on the proteolytic activity of the major proteases in *T. brucei*

As can be seen in Figure 4A, the effect of HIV-1 protease inhibitors, RTV and SQV, on the activity of these two major proteases has been tested. The results demonstrate that there is a statistically significant ($p<0.05$) reduction in the activity of these proteases by RTV and SQV (Figure 4A and 4B). According to the figures calculated, 72% and 80% of metalloprotease activity were inhibited due to RTV and SQV, respectively. Similarly, 24% and 50% of the cysteine protease activity was inhibited due to RTV and SQV, respectively. Corresponding to the observed results in the anti-proliferative experiments, SQV has also shown a better anti-Metallo and anti-cysteine protease effect when compared to RTV (Figure 4B).

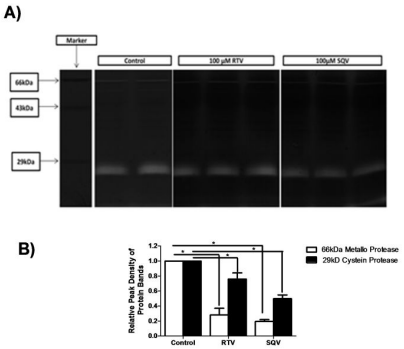

Figure 4: A) Inhibition of the identified proteases using ritonavir and saquinavir: Roti-protein marker (6 μg) is used in the standard lane. Control lanes (n=2) show the metalloproteases expressed as white bands at the ~66 kDa and papain-like cysteine proteases at the ~29 kDa position. Exposure of the proteases with 100 μM RTV and 100 μM SQV at the last two gels (each n=3 lanes) indicated that there is a partial inhibition on the expression of both the metallo- and cysteine-proteases of *T. brucei* cells. B) Image J Densitometry: The relative peak density of the ~66 kDa metalloprotease band (unshaded bars) and the ~29 kDa papain-like cysteine protease band (shaded bars). The graph shows a statistically significant inhibition ($p<0.05$) of Metallo-and cysteine-protease activity due to RTV and SQV.

Discussion

As a current solution on drug discovery of NTDs, new product development partnerships such as Medicines Sans Frontiers and the Geneva-based Drugs for Neglected Diseases Initiative (DNDi) are established by various research and collaborative institutes including the institutes like the WHO special programme on Tropical Diseases Research (WHO/TDR) [34]. As a cost- and time-effective mechanism

of new drugs development against HAT, these initiatives mostly advise the advantage of applying those drugs (including eflornithine and nifurtimox, and the most recently approved fexinidazole) [35], which were under clinical use or trial for other chronic illnesses like cancer and Chagas disease.

We similarly tried to share this experience/strategy through the application of HIV-1 protease inhibitors against *T. brucei in vitro*. As a matter of fact, there is ample evidence on the anti-leishmanial and anti-malarial effects of HIV protease inhibitors both *in vitro* and in clinical isolates [24-30]. However, the effect of these peptidase inhibitors in human African trypanosomes is not yet reported. Since trypanosome and mammalian proteases differ in terms of their substrate specificity [36,] specific and non-toxic protease inhibitors are the rational choice for further anti-trypanosomal drug development [37,38].

Thus, in our project, the question for an apparent effect of HIV-1 aspartate protease inhibitors, RTV and SQV, in *T. brucei* cells in vitro was addressed. Depending on the microscopic count of motile (actively moving) cells, our findings showed that SQV and RTV, respectively, induced up to 85% and 75% cell death at 50 µM concentration. At 20 µM both drugs inhibited survival of more than 50% of cells after 24 hrs of incubation (Figure 1). The same result was obtained using the AlamarBlue® cell proliferation assay which indicated a dose-dependent inhibition of proliferation with IC_{50s} of 12.23 ± 0.33 µM for RTV and 11.49 ± 0.31 µM for SQV (Figure 2). These results, when compared with the standard drug pentamidine, however, showed a significant difference. Pentamidine is more effective at a nanomolar level (IC_{50}=3.59 ± 0.24 nM), unlike RTV and SQV which act in the micromolar level. During observation of the efficacy of drugs using cell count, pentamidine took 48 hours to kill all the cells at 128 nM. SQV, however, brought a comparable result in 24 hrs at 50 and 100 µM concentrations. This may indicate the fast-acting property of SQV compared to pentamidine.

A study was done by Santos and colleagues [25], for instance, indicated a concentration-dependent growth inhibition of *Leishmania* cells by HIV-1 protease inhibitors like lopinavir, nelfinavir, amprenavir, and indinavir. In their findings, lopinavir and nelfinavir resulted in a statistically significant growth inhibition at 50 µM. Our findings, on the other hand, showed statistically significant inhibition of *T. brucei* cells at 10 µM concentration of RTV and SQV. This indicates *T. brucei* are more susceptible to HIV PIs when compared with *Leishmania* parasites (*L. amazonensis*) [39].

Our findings also showed the cysteine- and metalloproteases were the major proteases identified in *T. brucei* cells which corresponds to the study done by [17] who indicated these two proteases together comprise more than 95% of all the proteases identified. An earlier study done by [40] using fibrinogen as a substrate in SDS-PAGE demonstrates proteases with Mr of 28 kDa, 42 kDa, 60 kDa, 90 kDa and 105 kDa in *T. brucei* cells. Even though not well expressed as in the fibrinogen co-polymerized gels, they also identified proteases at the 28 kDa, 31 kDa, 93 kDa and 105 kDa from the same cell extract when using collagen as a substrate. Therefore, our results concur with the previous findings at the ~29 kDa and at the ~66 kDa protein zones. In all studies the 27/28/29 kDa Mr protease was confirmed to be a cysteine protease which is in agreement with our findings. It should be noted that more bands may be expressed depending on the substrate specificity of the various proteases available within the cells. Lonsdale and Mpimbaza also mentioned that the variation in the relative amount of higher Mr bands from one preparation to another appears to be related to the number of steps used in the purification of trypanosomes, whereby excessive handling results in increased amounts of lower-Mr enzyme forms with a concomitant reduction in the higher-Mr forms. The parasite clone differences may also be another possibility for this difference. The author also mentioned the variation in the expression of the different protease bands depends on the freshness of the cell extracts loaded in the gels, whereby fresh extracts express more bands.

Our findings, for the first time, revealed the two major proteases, metallo, and papain-family- cysteine-proteases, of *T. brucei* were found to be the target for RTV and SQV. It can be suggested that the anti-proliferative effect of SQV and RTV might also be caused by incidental cell death, necrosis or apoptosis [41]. According to the genome of the *T. brucei* [42], a hypothetical Caspase protein is identified. This protein is a cysteine-dependent aspartate-directed protease that mediates apoptosis in *T. brucei* cells. Besides to the direct consequences of inhibition of the major proteases by RTV and SQV, the scientific justification to our current findings can also be explained in such a way that RTV and SQV might be peptidomimetic to *T. brucei* aspartate, activating the aspartate-directed hypothetical Caspase protease. Inhibition of the cysteine proteases (increased intracellular cysteine concentration) can then support the induction or mediation of apoptosis. To confirm this, further experiments including morphological analysis using electron microscopy, cell cycle progression, sub-cellular alterations, measuring ROS concentration, determination of DNA degradation, loss of mitochondrial membrane potential, and phosphatidylserine exposure using FACS analyses and cell death [43,44] should be done.

In comparison, SQV presented a better inhibition of both proteases than RTV. This is also in agreement with the concentration-dependent results and IC50 values in the anti-proliferation results. In nature, aspartic peptidase genes in trypanosomatids were poorly characterized. According to Santos et al. (2013) [39], aspartate proteases constituted only 2% when compared with the major proteases (serine-, Metallo- and cysteine-ptoteases). This might probably be why we failed to detect aspartyl proteases in *T. brucei*. Otherwise, the enzyme-substrate specificity or the high pH applied during our zymography experiment can be considered as a factor.

As confirmed by Santos et al. [25] on *Leishmania amazonensis*, lopinavir (a drug co-administered with sub-therapeutic doses of RTV in HIV patients) and nelfinavir effectively inhibited the aspartic proteolytic hydrolysis of the HIV-1 peptidase substrate by the cells. Amprenavir, however, had no inhibitory effect at the highest applied concentration (10 µM). Another study done on *Plasmodium falciparum* cells to determine inhibition of activity of plasmepsin II, an acidic food vacuole aspartate protease that appears to play a role in the initial hydrolysis of haemoglobin by intra-erythrocytic malaria parasites, indicated that both lopinavir and ritonavir inhibited its activity with IC_{50} of 2.7 µM and 3.1 µM, respectively [26]. These two findings indirectly indicated that the different HIV-1 protease inhibitors may target different classes or types of proteases in parasites.

Even though it may not be easy to infer *in vitro* experimental results into clinical applications, RTV can be clinically relevant as its IC_{50} is below the C*max* (14.8 µM) of the serum concentration achievable during the standard dosing of patients [45]. The Cmax for SQV (1.4 µM) is lower than the IC50 observed in our experiment. This tells us its clinical relevance can only be achieved through repeated daily doses given for longer treatment duration or by giving it in combination with

RTV [45]. The application of RTV and SQV, which are among the first generation of HIV-1 protease inhibitors, might be considered as the drawback of our study since this generation of drugs is limited by low bioavailability and high pill burden to patients [46]. The results can, however, lay a preliminary background for further research using those clinically highly relevant HIV-1 protease inhibitors.

Conclusion

The HIV-1 protease inhibitors, RTV and SQV, were relatively effective against *T. brucei* cells. The standard drug, pentamidine, was observed to be slow acting but most effective when compared with RTV and SQV. Clinically, however, the drug pentamidine, with all its contraindications, acts only to the 1st stage of the disease. Studies indicate that brucipain, a cysteine protease of *T.b. gambiance*, plays an important role during transendothelial migration of the parasite through the human blood-brain barrier. Based on our current findings, the capacity of RTV and SQV to inhibit the cysteine-and metalloproteases in *T. brucei* cells brings a bright future in the prevention and/or treatment of the cerebral/fatal stage of HAT using these two PIs. Since they are clinically approved and registered drugs, the possibility of applying these agents in HAT patients can also be cost-effective and a short cut solution. Even though it may not be easy to infer *in vitro* experimental results into clinical applications, RTV, unlike SQV, can be clinically relevant as its IC_{50} is below the C*max* (14.8 μM) of the serum concentration achievable during the standard dosing of patients. However, these preliminary results should be supported with additional enzyme kinetics studies, specific enzyme-substrate analysis, lead optimization, and other molecular interventions prior to stepping up into further inferences.

Ethics Approval and Consent to Participate

Not applicable

Consent for Publication

Not applicable

Availability of Data and Material

All data generated or analyzed during this study are included in this published article.

Authors' Competing Interests

The authors declare that they have no competing interest.

Funding

There was no funding available to undertake this study.

Authors' Contributions

All authors read and approved the final version of the manuscript. NW: Conceived, designed, performed the experiments, analyzed the data and wrote the paper. GB: Conceived and designed experiments, contributed reagents/materials/data tools and edited the paper.

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References

1. Keita M, Bouteille B, Enanga B, Vallat JM, Dumas M (1997) Trypanosoma brucei brucei: a long-term model of human African trypanosomiasis in mice, meningoencephalitis, astrocytosis, and neurological disorders. Exp parasitol 85: 183-192.
2. Franco JR, Simarro PP, Diarra A, Jannin JG (2014) Epidemiology of human African trypanosomiasis. Clin Epidemiol 6: 257-275.
3. WHO (2006) Human African trypanosomiasis (sleeping sickness): Epidemiological update. Wkly Epidemiol Rec 81: 71-80.
4. WHO (2013) Control and surveillance of human African trypanosomiasis. World Health Organization technical report series 1-237.
5. Simarro PP, Jannin J, Cattand P (2008) Eliminating human African trypanosomiasis: where do we stand and what comes next? PLoS Medicine 5: e55.
6. Burri C (2014) Chemotherapy against human African trypanosomiasis: is there a road to success? Parasitol 137: 1987-994.
7. Kuzoe FAS, Schofield CJ (2005) Strategic review of traps and targets for tsetse and African trypanosomiasis control. UNICEF/UNDP/World Bank/ WHO TDR.
8. Büscher P, Cecchi G, Jamonneau V, Priotto G (2017) Human African trypanosomiasis. Lancet 390: 2397-2409.
9. WHO Fact Sheet (2015) Trypanosomiasis, human African (sleeping sickness).
10. Ettari R, Tamborini L, Angelo IC, Grasso S, Schirmeister T, et al. (2013) Development of rhodesain inhibitors with a 3-bromoisoxazoline warhead. ChemMedChem 8: 2070-2076.
11. Nkemngu NJ, Nkemgu-Njinkeng J, Rosenkranz V, Wink M, Steverding D (2002) Antitrypanosomal activities of proteasome inhibitors. Antimicrob Agents Chemother 47: 3036.
12. Rosenthal PJ (2004) Cysteine proteases of malaria parasites. Int J Parasitol 34: 1489-1499.
13. Shen HB, Chou KC (2009) Identification of proteases and their types. Analyt Biochem 385: 153-160.
14. Caffrey CR, Schanz M, Nkemngu NJ, Nkemgu-Njinkeng J, Brush M, et al. (2002) Screening of acyl hydrazide proteinase inhibitors for antiparasitic activity against Trypanosoma brucei. Int J Antimicrob Agents 19: 227-231.
15. Mott BT, Ferreira RS, Simeonov A, Jadhav A, Ang KKH, et al. (2010) Identification and optimization of inhibitors of Trypanosomal cysteine proteases: cruzain, Rhodesian, and TbCatB. J Medi Chem 53: 52-60.
16. den Blaauwen T, Andreu JM, Monasterio O (2014) Bacterial cell division proteins as antibiotic targets. Bioorg Chem 55: 27-38.
17. Vermelho AB, Branquinha MH, D'Ávila-Levy CM, Luis A, Dias EPS, et al. (2010) Biological roles of peptidases in trypanosomatids. Open Parasit J 4: 5-23.
18. Steverding D, Sexton DW, Wang X, Gehrke SS, Wagner GK, et al. (2012) Trypanosoma brucei: chemical evidence that cathepsin L is essential for survival and a relevant drug target. Int J Parasitol 42: 481-488.
19. McKerrow JH, Engel JC, Caffrey CR (1999) Cysteine protease inhibitors as chemotherapy for parasitic infections. Bioorg Med Chem 7: 639-644.

20. Pérez B, Teixeira C, Gomes JRB, Gomes P (2013) Development of Plasmodium falciparum protease inhibitors in the past decade (2002-2012). Curr Med Chem 20: 3049-3068.
21. Lima APCA, Rei FCG, Costa TFR (2013) Cysteine peptidase inhibitors in trypanosomatid parasites. Curr Med Chem 20: 3152-3173.
22. Ndao M, Beaulieu C, Black WC, Isabel E, Vasquez-Camargo F, et al. (2014) Reversible cysteine protease inhibitors show promise for a Chagas disease cure. Antimicrob Agents Chemother 58: 1167-1178.
23. Ndao M, Nath-Chowdhury M, Sajid M, Marcus V, Mashiyama ST, et al. (2013) A cysteine protease inhibitor rescues mice from a lethal Cryptosporidium parvum infection. Antimicrob Agents Chemother 57: 6063-6073.
24. Savoia D, Allice T, Tovo PA (2005) Antileishmanial activity of HIV protease inhibitors. Int J Antimicrob Agents 26: 92-94.
25. Santos LO, Marinho FA, Altoé EF, Vitório BS, Alves CR, et al. (2009) HIV aspartyl peptidase inhibitors interfere with cellular proliferation, ultrastructure and macrophage infection of Leishmania amazonensis. PloS One 4: e4918.
26. Parikh S, Gut J, Istvan E, Goldberg DE, Havlir DV, et al. (2005) Antimalarial activity of human immunodeficiency virus type 1 protease inhibitors. Antimicrob Agents Chemother 49: 2983-2985.
27. Valdivieso E, Rangel A, Moreno J, Saugar JM, Cañavate C, et al. (2010) Effects of HIV aspartyl-proteinase inhibitors on Leishmania spp. Exp Parasitol 126: 557-563.
28. Andrews KT, Fairlie DP, Madala PK, Ray J, Wyatt DM, et al. (2006) Potencies of human immunodeficiency virus protease inhibitors in vitro against Plasmodium falciparum and in vivo against murine malaria. Antimicrob Agents Chemother 50: 639-648
29. Parikh S, Liu J, Sijwali P, Gut J, Goldberg DE, et al. (2006) Antimalarial effects of human immunodeficiency virus type 1 protease inhibitors differ from those of the aspartic protease inhibitor pepstatin. Antimicrob Agents Chemother 50: 2207-2209.
30. Perteguer MJ, Gómez-Puertas P, Cañavate C, Dagger F, Gárate T, et al. (2013) Ddi1-like protein from Leishmania major is an active aspartyl proteinase. Cell Stress Chaperones 18: 171-181.
31. Worku N, Mossie A, Stich A, Daugschies A, Trettner S, et al. (2013) Evaluation of the In vitro efficacy of artemisia annua, Rumex abyssinicus, and Catha edulis forsk extracts in cancer and Trypanosoma brucei Cells. ISRN Biochem 2013: 910308.
32. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.
33. Hu X, Beeton C (2010) Detection of functional matrix metalloproteinases by zymography. J Vis Exp 8: 2445.
34. Hotez PJ (2008) Forgotten people, forgotten diseases. The neglected tropical diseases and their impact on global health and development. Emerg Infect Dis 15: 510-511.
35. Mesu VKBK, Kalonji WM, Bardonneau C, Mordt OV, Blesson S, et al. (2018) Oral fexinidazole for late-stage African Trypanosoma brucei gambiense trypanosomiasis: a pivotal multicentre, randomised, non-inferiority trial. Lancet 391: 144-154.
36. Li F, Hua SB, Wang CC, Gottesdiener KM (1996) Procyclic Trypanosoma brucei cell lines deficient in ornithine decarboxylase activity. Mol Biochem Parasitol 78: 227-236.
37. Blum J, Nkunku S, Burri C (2001) Clinical description of encephalopathic syndromes and risk factors for their occurrence and outcome during melarsoprol treatment of human African trypanosomiasis. Trop Med Int Health 6: 390-400.
38. Andreani G, Lodge R, Richard D, Tremblay MJ (2012) Mechanisms of interaction between protozoan parasites and HIV. Curr Opin HIV AIDS 7: 276-282.
39. Santos LO, Garcia-Gomes AS, Catanho M, Sodre CL, Santos ALS, et al. (2013) Aspartic peptidases of human pathogenic trypanosomatids: perspectives and trends for chemotherapy. Curr Med Chem 20: 3116-3133.
40. Lonsdale-E JD, Mpimbaza GW (1986) Thiol-dependent proteases of African trypanosomes. Analysis by electrophoresis in sodium dodecyl sulfate/polyacrylamide gels co-polymerized with fibrinogen. Eur J Biochem 155: 469-473.
41. Proto WR, Coombs GH, Mottram JC (2013) Cell death in parasitic protozoa: regulated or incidental? Nat Rev Microbiol 11: 58-66.
42. Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H, et al. (2005) The genome of the African trypanosome Trypanosoma brucei. Science 309: 416-422.
43. Sangenito LS, Menna-Barreto RFS, Oliveira AC, d'Avila-Levy CM, Branquinha MH, et al. (2018) Primary evidence of the mechanisms of action of HIV aspartyl peptidase inhibitors on Trypanosoma cruzi trypomastigote forms. Int J Antimicrob Agents 52: 185-194.
44. Uzcátegui NL, Carmona-Gutiérrez D, Denninger V, Schoenfeld C, Lang, F, et al. (2007) Antiproliferative effect of dihydroxyacetone on Trypanosoma brucei bloodstream forms: cell cycle progression, subcellular alterations, and cell death. Antimicrob Agents Chemother 51: 3960-3968.
45. Nsanzabana C, Rosenthal PJ (2011) In vitro activity of antiretroviral drugs against Plasmodium falciparum. Antimicrob Agents Chemother 55: 5073-5077.
46. Alfonso Y, Monzote L (2011) HIV protease inhibitors: Effect on the opportunistic protozoan parasites. Open Med Chem J 5: 40-50.