Title
RNA interference of Trypanosoma brucei cathepsin B and L affects disease progression in a mouse model.

Permalink
https://escholarship.org/uc/item/4pk21760

Journal
PLoS neglected tropical diseases, 2(9)

ISSN
1935-2727

Authors
Abdulla, Maha-Hamadien
O'Brien, Theresa
Mackey, Zachary B
et al.

Publication Date
2008-09-24

DOI
10.1371/journal.pntd.0000298

Peer reviewed
RNA Interference of Trypanosoma brucei Cathepsin B and L Affects Disease Progression in a Mouse Model

Maha-Hamadien Abdulla1*, Theresa O'Brien1, Zachary B. Mackey1, Mohamed Sajid1, Dennis J. Grab2, James H. McKerrow1

1 Sandler Center for Basic Research in Parasitic Diseases, California Institute for Quantitative Biomedical Research, University of California San Francisco, San Francisco, California, United States of America, 2 Department of Pediatrics, Division of Infectious Diseases, The Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America

Abstract

We investigated the roles played by the cysteine proteases cathepsin B and cathepsin L (brucipain) in the pathogenesis of Trypanosoma brucei brucei in both an in vivo mouse model and an in vitro model of the blood–brain barrier. Doxycycline induction of RNAi targeting cathepsin B led to parasite clearance from the bloodstream and prevent a lethal infection in the mice. In contrast, all mice infected with T. brucei containing the uninduced Trypanosoma brucei cathepsin B (TbCatB) RNA construct died by day 13. Induction of RNAi against brucipain did not cure mice from infection; however, 50% of these mice survived 60 days longer than uninduced controls. The ability of T. b. brucei to cross an in vitro model of the human blood–brain barrier was also reduced by brucipain RNAi induction. Taken together, the data suggest that while TbCatB is the more likely target for the development of new chemotherapy, a possible role for brucipain is in facilitating parasite entry into the brain.

Introduction

Subspecies of Trypanosoma brucei are the causative agents of human African trypanosomiasis. In vitro studies utilizing both small molecule cysteine protease inhibitors and RNA interference (RNAi) have implicated the Clan CA (papain) family of cysteine proteases as critical to the successful lifecycle of Trypanosoma brucei brucei (T. b. brucei) [1,2]. In vivo studies have demonstrated that cysteine protease inhibitors prolong the lives of mice infected with lethal inocula of trypanosomes [1,3]. There are two distinct Clan CA cysteine proteases identified in the T. brucei genome. Brucipain (aka trypanopain-Tb, rhodesain) is a cathepsin L-like protease responsible for the bulk of protease activity in the organism [2]. Trypanosoma brucei cathepsin B (TbCatB) is a more recently characterized protease that is upregulated in the bloodstream stage of the parasite [2]. In in vitro studies, RNAi of TbCatB produced swelling of the endosome compartment analogous to that seen with class-specific cysteine protease inhibitors [1,2] and led to arrest of trypanosome replication and death. In contrast, knockdown of brucipain by RNAi in vitro produced no detectable phenotypic changes. However, it was hypothesized that this enzyme might play a role in the degradation of mistranslated glycosylphosphatidylinositol (GPI) anchored proteins, VSG turnover, disruption of the blood–brain barrier, or degradation of host immunoglobulin [4,5] While RNAi with cultured parasites can provide important insights into the role of a specific gene product in parasite replication and viability, a role in pathogenesis, as proposed for brucipain, can only be validated in vivo. We show that introduction of RNAi from a tetracycline-inducible promoter can be achieved in vivo in a mouse model of T. b. brucei infection, and show that transcriptional silencing of either of these two proteases alters the course of T. b. brucei infection [6].

Materials and Methods

Bloodstream T. brucei strain 90-13

Bloodstream T. brucei strain 90-13 was electroporated with plasmids containing either brucipain (TbRho), TbCatB, or GFP transgenes [2]. The plasmid used, pZJM, allows transfected organisms to be induced to produce RNAi in the presence of tetracycline. The brucipain RNAi construct used for this study is one of three partial open reading frames (ORF) of brucipain used to down regulate its message in vitro. R1 encodes a cDNA that corresponds to the first 597 nucleotides of brucipain ORF. R2 encodes a cDNA encoding the middle 400 nucleotides of the brucipain ORF and R3 encodes a partial cDNA encoding the last 300 nucleotides of the brucipain ORF. Each of these constructs were capable of efficiently and specifically silencing the mRNA of brucipain in vitro. The same T. b. brucei clones expressim the R1 construct used in a previous study [2]. The TbCatB transgene has been described in detail previously [2]. To generate the GFP transgene, the gene encoding GFP (714 nucleotides) was amplified from the pH-D-HX-GFP vector [7]. Methods for electroporation and selection of stable transformants have been described [2].

Trypanosome culture and infection of mice

Bloodstream form (BSF) 90-13 cells expressing T7 RNA polymerase and tetracycline repressor protein were maintained...
in HMI-9 medium [8]. Five BALB/c mice per group (6–8 weeks old) were infected by intraperitoneal injection with 600 parasites carrying pZJMTbRho, pZJMTbCatB, or pZJMGFP plasmids or with control 90-13 parasites. To rule out any direct effects of doxycycline on the course of trypanosome infection, two additional groups of mice were infected with the parental T. b. brucei strain 90-13. One group was given doxycycline-containing food (200-mg/Kg, Bioserv Corporation, San Diego, CA) and water containing 1 mg/ml doxycycline hyclate (Sigma-Aldrich), food (200-mg/Kg, Bioserv Corporation, San Diego, CA) and water, and another three groups were given doxycycline-containing food and water. The two groups infected with pZJMGFP served as a control for a gene that is not found in the trypanosome. Mice were monitored every other day for weight loss, general appearance, and behavior. Further experiments carried out in a culture system show that brucipain facilitates the migration of parasites across a model of the blood–brain barrier. This suggests that while brucipain is not necessary for the viability of the organisms, it may play a role in infection by allowing parasites to reach the central nervous system and produce the severe second stage of sleeping sickness.

The in vitro model of the human blood–brain barrier (BBB)

We used a human brain microvascular endothelial cell (BMEC) line whose phenotypic expression was stabilized by immortalization with pSVT, a pBR322-based plasmid containing the DNA sequence encoding the simian virus 40 large-T antigen [9]. Similar to the primary human BMEC cell line (XIII) from which they were derived, the transfected human BMECs are positive for FVIII-Rag, transpeptidase [9,10], and express gamma glutamyl transpeptidase [9,10]. Human BMECs were cultured at 37 °C in medium 199 (GIBCO) supplemented with 20% heat-inactivated fetal bovine serum and 1× Glutamax (GIBCO) in a humidified environment of 95% air, 5% CO2. The cells were grown to confluence on 6.5-mm-diameter collagen-coated Costar Transwell inserts with a pore size of 3.0 μm until transendothelial electrical resistance (TEER) measurements exceeded 25 cm2 [11]. For the transmigration study, the parasites were added to the top of the human BMEC-containing inserts. The cultures were incubated with and without tetracycline (100 ng/ml) in triplicate at 37 °C, and the number of parasites present at the bottom chamber were determined by counting aliquots in the Neubauer chamber.

Real-time reverse transcription RT-PCR

Gene transcripts for brucipain were quantified in freshly isolated T. b. brucei from mice infected with pZJMTbRho at five days post infection. Blood was separated in a DEAE-sepharose column as previously described [12]. Total RNA extraction from T. b. brucei was performed using the Trizol reagent (Invitrogen, Carlsbad, CA). RT-PCR, the one-step RT-PCR kit (Invitrogen, Carlsbad, CA), and gene-specific primers forward 5'-ATACGCAAATG-CTTGGTTGA-3' and reverse 5'CGTTGATGTTGCGCA-TAGT-3' were used to amplify brucipain. The relative amount of gene transcripts was calculated using methods previously described [13].

Preparation of trypanosome lysates

Parasites were purified from mice infected with parental 90-13 or pZJMTbRho. As reported previously [12], T. b. brucei from infected mice were harvested by centrifugation, washed once in PBS-containing 1% glucose, and resuspended in lysis buffer (1.0% Triton X-100, 10 mM Tris pH 7.5, 25 mM KCl, 150 mM NaCl, 1 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, 20% glycerol). The lysates were incubated on ice for 20 min and cleared by centrifugation at 16,000 g for 15 min at +4 °C. Protein concentration of was determined by the Bradford assay (Bio-Rad).

Western blots of trypanosome lysates following RNAi induction

Ten µg of trypanosome lysate was resolved by 15% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. After transferring and blocking, the PVDF membranes were incubated with rabbit anti-brucipain antiserum (1:2500 dilution) and anti-TbcatB 1:2000 [14] for 1 h and washed three times for five min with TBST (10 mM Tris, pH 7.4, 150 mM NaCl, 0.4% Tween 20). After the third wash, horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:1,000 dilution) was added to the blots for 1 h. The blots were washed again in the same buffer three times for five min and examined by ECL (Amersham Biosciences).

Radiolabeling of cysteine protease active sites with 125I-labeled inhibitors

Equal amounts of trypanosome lysate (10 µg) were labeled with 125I-DCG-04 [15] in the presence of 2 mM dithiothreitol for 45 min at room temperature and subjected to SDS-PAGE. Quantification of labeled enzymes was determined by Phosphoimager analysis (Molecular Dynamics).

Statistical analysis

Data were analyzed using the Mann-Whitney nonparametric test to determine the statistical difference in spleen weight in induced versus un-induced infected mice. Chi-square analysis was performed to determine the significant difference in survival.

Results/Discussion

The goal of these experiments was to validate the in vitro effects of RNAi on TbcatB in an in vivo disease model of African trypanosomiasis, and to explore a potential role of brucipain as a virulence factor. For safety reasons we conducted the knockdown experiment in the human non-infective strain T. b. brucei which has been traditionally grown and studied in mice. Doxycycline by itself produced no significant alteration (+/− 1 day) in the course of T. b. brucei 90-13 infections (Fig. 1A). Equivalent levels of parasitemia and splenomegaly were observed in mice whether or not they were maintained on a doxycycline-containing diet (not
Figure 1. Survival analysis for mice infected with *T. b. brucei*. (A) Kaplan-Meier survival analysis for mice infected with *T. b. brucei* not transfected with the RNAi construct but given an inducing dose of doxycycline (red) versus standard food and water (blue) (*n* = 5 in each group). (B) Infection with trypanosomes containing the RNAi plasmid for brucipain plus or minus induction by doxycycline. Brucipain RNAi resulted in prolonged survival of three out of the five mice (*p* = 0.004) (experiment was conducted twice with the same result). (C) Infection with parasite containing RNAi plasmid for *TbCatB* plus or minus induction by doxycycline. Note that all five mice infected with parasites in which cathepsin B RNAi was induced survived until the experiment was terminated. (D) Spleen weights in mice infected with cathepsin B RNAi parasites and induced with doxycycline were within normal range compared to uninfected controls.

doi:10.1371/journal.pntd.0000298.g001
shown). The in vivo induction of RNAi against brucipain in *T. b. brucei* did not cure infection, but extended the survival of three out of five mice beyond 60 days (Fig. 1B) the experiment was repeated twice with the same result. All mice infected with trypanosomes having the brucipain transcript knockdown had parasitemia and splenomegaly equivalent to that seen in control mice at the time of their sacrifice (not shown). Splenomegaly (quantified by spleen weight) is a convenient gross pathological marker of disease burden [16]. Analysis of mRNA levels in trypanosomes isolated from infected mice confirmed 60% reduction in the level of brucipain mRNA (Fig. 2A). The level of cathepsin B mRNA was not affected by RNAi induction against brucipain in pZJMTbRho induced parasites (Fig. 2B). Active site labeling of brucipain in trypanosomes purified from mouse blood confirmed 60% reduction in brucipain protease activity (Fig. 3C). Endogenous activity levels of brucipain and cathepsin B, quantified by DCG-04 labeling of purified parasites from mice infected with 90-13 strain, confirmed that brucipain was more abundant than cathepsin B (Fig. 3D), consistent with previously published data [2,14]. A control cell line with an insert of GFP was generated to investigate the role of RNAi plasmid construct itself on the parasites in vivo.

**Figure 2. RNAi reduces expression of brucipain mRNA in parasites isolated from mice.** (A) Evidence that RNAi reduces expression of brucipain mRNA in parasites isolated from mice. Mice infected with the brucipain RNAi-containing plasmids were either induced with doxycycline or left un-induced. (B) RNAi induced against brucipain did not decrease mRNA level for cathepsin B.

doi:10.1371/journal.pntd.0000298.g002

**Figure 3. RNAi reduces brucipain protein and protease activity.** (A) Equal amounts of protein were resolved by 15% SDS-PAGE, stained with anti-rhodesain antibody, and visualized by Western blot. Brucipain protein level is decreased after RNAi induction in pZJMTbRho parasites recovered from infected mice but the level of cathepsin B is not decreased after brucipain RNAi induction in pZJMTbRho parasites. (B) Brucipain activity is also decreased by 60% with brucipain RNAi induction. The level of brucipain activity in pZJMTbRho transfected parasites purified from mice was determined with the active site tag 125I-DCG-04, visualized by autoradiography, and quantified by PhosphorImager analysis. (C) In the absence of RNAi bands of brucipain and TbCatB activity can be identified in purified parasites from mice infected with 90-13 labeled with the active site tag 125I-DCG-04 and visualized by autoradiography.

doi:10.1371/journal.pntd.0000298.g003
TbCatB rescued mice from a lethal experiments [1,2]. In the mouse model of infection, RNAi correlates with the results observed in previous in vitro RNAi infection. Induction of RNAi targeting TbCatB transcripts in vivo stream parasites in an experimental model of trypanosome infection. However, even the modest RNAi knockdown achieved for brucipain in vivo might uncover a more direct role for brucipain in parasite viability; brucipain knockouts are being pursued as strategy to more clearly delineate the role of brucipain. While the residual brucipain activity seen after RNAi induction might be responsible for disease progression in two of the mice shown in [Fig. 1B], an alternative conclusion is that brucipain plays a specific role in Trypanosoma pathogenesis in vivo, but not in parasite viability per se. Nikolskaya et al. [5] showed that a cysteine protease inhibitor, known to target brucipain, blocked the ability of African trypanosomes to cross a model of the blood–brain barrier (BBB) [5]. Using this in vitro model of the blood–brain barrier, we confirmed that brucipain is required for African trypanosomes to effectively cross the brain endothelial barriers.

Figure 4. Parasite traversal across human BMEC. Transwell inserts containing human BMEC (initial TEER = 26.3 Ω) were incubated with 3 × 10^5 pZJMTbRho RNAi trypanosomes (+/− tetracycline) and the number of parasites that crossed the BMEC monolayers into the bottom wells determined. All values represent the mean ± SEM of triplicate determinations. doi:10.1371/journal.pntd.0000298.g004

No difference was seen in mouse pathology or in brucipain or cathepsin B levels with GFP-induced parasites (data not shown). In vivo induction of TbCatB RNAi resulted in survival of all five mice for up to two months post infection, after which time the experiment was terminated (Fig. 1C). Un-induced mice began to die 13 days after infection. No trypanosomes were detected in the blood of mice infected with pZJMTbCatB trypanosomes after induction of RNAi with doxycycline. These mice also had normal spleen weights compared to un-induced controls (Fig. 1D). Control mice with no doxycycline died between day 11 and 15 post infection. The last day on which untreated mice died from the trypanosome infection may vary depending on the exact parasite inoculum received and other host defense and host metabolic factors (Fig. 1A vs 1B).

The demonstration that doxycycline induction of RNAi can be achieved in parasites within an animal model of infection is an important technological advance that should encourage the use of this approach by other investigators. The failure of parasites to establish infection with TbCatB RNAi might have been predicted from in vitro assays. However demonstration in an in vivo model of infection is a significant and necessary validation of the key role of TbCatB in infection. The effect of reducing transcripts for the cathepsin L-like trypanosome protease (brucipain) on the progression of the infection was not predicted from in vitro assays. The effect of brucipain RNAi suggests that the cathepsin L protease might play a role as a virulence factor in in vivo infections, where host tissue tropism and the host immune response add new layers of complexity.

In conclusion, gene-specific RNAi can be induced in bloodstream parasites in an experimental model of trypanosome infection. Induction of RNAi targeting TbCatB transcripts in vivo correlates with the results observed in previous in vitro RNAi experiments [1,2]. In the mouse model of infection, RNAi of TbCatB rescued mice from a lethal T. b. brucei infection, resulting in no splenomegaly and no detectable parasites in blood. While induction of RNAi against brucipain in two independent experiments did not cure mice of their infection, it did significantly prolong the survival of five out of ten mice. Since RNAi led to a 60% reduction of brucipain activity (Fig. 3C), it is still possible that a 100% knockdown might uncover a more direct role for brucipain in parasite viability; brucipain knockouts are being pursued as strategy to more clearly delineate the role of brucipain. However, even the modest RNAi knockdown achieved for TbCatB (quantified in [2]) had a profound negative effect on parasite viability both in vitro [5] and in vivo, suggesting that T. brucei cathepsin B is the more likely target for protease inhibitors as chemotherapy against human African trypanosomiasis [17].

While the residual brucipain activity seen after RNAi induction might be responsible for disease progression in two of the mice shown in [Fig. 1B], an alternative conclusion is that brucipain plays a specific role in Trypanosoma pathogenesis in vivo, but not in parasite viability per se. Nikolskaya et al. [5] showed that a cysteine protease inhibitor, known to target brucipain, blocked the ability of African trypanosomes to cross a model of the blood–brain barrier (BBB) [5]. Using this in vitro model of the blood–brain barrier, we confirmed that brucipain is required for African trypanosomes to effectively cross the brain endothelial barriers. Without tetracycline 3.54E+04 ± 1.41E+03 (mean ± SEM) of the initial brucipain RNAi trypanosome (pZJMTbRho–tet) inoculum crossed the endothelial cell barrier (1–2%) (Fig. 4). This is comparable to those noted for T. b. brucei 427 and TREU 927 in previously published reports [5,11]. However when brucipain RNAi was induced by tetracycline, the number of parasites migrating across the barrier was reduced by 50% (1.10E+03 ± 6.35E+02; p = 0.003). The human BMEC transendothelial electrical resistance (TEER) at the end of the experiment was 30.4 ± 1.2 ohms (p = 0.0002), indicating that barrier integrity was maintained for all T. b. brucei treatment conditions. To rule out any effect of tetracycline on the in vitro BBB model other than induces RNAi, trypanosomes (pZJMTbRho) were pretreated with tetracycline, but the antibiotic was then removed and the parasites incubated with human BMEC overnight. The number of parasites crossing the BMEC was the same as control (with tetracycline), demonstrating that tetracycline has no effect on endothelial cells (data not shown). Experiments were repeated twice with the same result. In summary, the data show that knockdown of brucipain transcripts by RNAi led to reduced protease activity but no effect on parasitemia or splenomegaly. However the prolonged survival of some of the infected mice might be due to inability of the parasite to efficiently enter the CNS.

Author Contributions
Conceived and designed the experiments: MHA ZBM MS DJG JHM. Performed the experiments: MHA TO ZBM MS DJG. Analyzed the data: MHA TO JHM. Wrote the paper: MHA DJG JHM.
References

1. Scory S, Caffrey CR, Stierhof YD, Ruppel A, Steverding D (1999) Trypanosoma brucei: killing of bloodstream forms in vitro and in vivo by the cysteine protease inhibitor Z-phe-ala-CHN2. Exp Parasitol 91: 327–333.
2. Mackey ZB, O’Brien TC, Greenbaum DC, Blank RB, McKerrow JH (2004) A cysteine B-like protease is required for host protein degradation in Trypanosoma brucei. J Biol Chem 279: 48426–48433.
3. Troeberg L, Morty RE, Piko RN, Lonsdale-Eccles JD, Palmer JT, et al. (1999) Cysteine protease inhibitors kill cultured bloodstream forms of Trypanosoma brucei brucei. Exp Parasitol 91: 349–355.
4. Triggs VP, Bangs JD (2003) Glycosylphosphatidylinositol-dependent protein trafficking in bloodstream stage Trypanosoma brucei. Eukaryot Cell 2: 76–83.
5. Nikolskaia OV, de ALAP, Kim YV, Lonsdale-Eccles JD, Fukuma T, et al. (2006) Blood-brain barrier traversal by African trypanosomes requires calcium signaling induced by parasite cysteine protease. J Clin Invest 116: 2739–2747.
6. Lecondier I, Walgraff D, Devaux S, Poelvoorde P, Pays E, et al. (2005) Trypanosoma brucei RNA interference in the mammalian host. Mol Biochem Parasitol 140: 127–131.
7. Hill KL, Hutchings NR, Russell DG, Donehoun JE (1999) A novel protein targeting domain directs proteins to the anterior cytoplasmic face of the flagellar pocket in African trypanosomes. J Cell Sci 112 Pt 18: 3091–3101.
8. Wirtz E, Leal S, Ochatt C, Cross GA (1999) A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in Trypanosoma brucei. Mol Biochem Parasitol 99: 89–101.
9. Stins MF, Prasadaro NV, Zhou J, Arditi M, Kim KS (1997) Bovine brain microvascular endothelial cells transfected with SV40-large T antigen: development of an immortalized cell line to study pathophysiology of CNS disease. In Vitro Cell Dev Biol Anim 33: 245–247.
10. Stins MF, Gilles F, Kim KS (1997) Selective expression of adhesion molecules on human brain microvascular endothelial cells. J Neuroimmunol 76: 81–90.
11. Grab DJ, Nikolskaia O, Kim YV, Lonsdale-Eccles JD, Ito S, et al. (2004) African trypanosome interactions with an in vitro model of the human blood-brain barrier. J Parasitol 90: 970–979.
12. Hamadien M, Bakheit M, Harris RA (2000) Interferon-gamma induces secretion of trypanosome lymphocyte triggering factor via tyrosine protein kinases. Parasitology 120(Pt 3): 201–207.
13. Livak KJ, Schnaittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔC(T) Method. Methods 25: 402–408.
14. Caffrey CR, Hansell E, Lucas KD, Brinen LS, Alvarez Hernandez A, et al. (2001) Active site mapping, biochemical properties and subcellular localization of rhodesain, the major cysteine protease of Trypanosoma brucei rhodesiense. Mol Biochem Parasitol 118: 61–73.
15. Bogoy M, Verheest S, Bellingard-Dubouchaud V, Toba S, Greenbaum D (2000) Selective targeting of lysosomal cysteine proteases with radiolabeled electrophilic substrate analogs. Chem Biol 7: 27–38.
16. Abdulla MH, Lim KC, Sajid M, McKerrow JH, Caffrey CR (2007) Schistosomiasis mansoni: novel chemotherapy using a cysteine protease inhibitor. PLoS Med 4: e14. doi:10.1371/journal.pmed.0040014.
17. Renoil AR, McKerrow JH (2006) Drug discovery and development for neglected parasitic diseases. Nat Chem Biol 2: 701–710.