Studies on development of novel diagnostic methods and discovery of chemotherapeutic agents against animal African trypanosomosis

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Abstract of Dissertation

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Title : Studies on development of novel diagnostic methods and discovery of chemotherapeutic agents against animal African trypanosomosis

(動物アフリカトリパノソーマ感染症に対する新規診断法及び化学療法薬開発に関する研究)

Abstract

Animal African trypanosomosis (AAT) AAT is mainly caused by *T. congolense*, *T. vivax* and *T. brucei*. In wild animals, these parasites cause relatively mild infections while in domestic animals they cause a severe, often fatal disease. All domestic animals can be affected by AAT and the symptoms are fever, listlessness, emaciation, hair loss, discharge from the eyes, edema, anemia, and paralysis. For control of AAT, it is important to block the transmission routes for controlling the spread of infection. Therefore, the development of reliable and sensitive diagnostic tests and effective drugs for trypanosome infection is essential for the control of AAT. The overall objective of this study was to develop novel diagnostic methods of AAT and to evaluate new drug targets of African trypanosomes to facilitate drug discovery, with specific objectives as follows: 1) To identify and characterize *T. congolense* novel antigen for application in the serodiagnosis (chapter 1); 2) To establish and evaluate the potential use of TcIL3000.0.25950 (TcP46) gene from *T. congolense* in semi-nested PCR assay (chapter 2); and, 3) To evaluate the trypanocidal activities of the compounds targeting the different components of endoplasmic reticulum associated protein degradation (ERAD) pathway (chapter 3).

In chapter 1, I have identified one *T. congolense* protein as a new candidate serodiagnostic antigen. The TcP46 is expressed 5.36 times higher in metacyclic forms than

epimastigote forms. The complete nucleotide sequences of TcP46 contained an open reading frame of 1,218 bp. Southern blot analysis indicated that at least two copies of the TcP46 gene were tandemly-arranged in the *T. congolense* genome. The recombinant rTcP46 was expressed in *Escherichia coli* as a GST fusion protein. Western blot analysis and confocal laser scanning microscopy revealed that the native TcP46 protein is expressed in the cytoplasm during all life-cycle stages of the parasite. Moreover, an ELISA based on rTcP46 detected the specific antibodies as early as 8 days post-infection from mice experimentally infected with *T. congolense*. No cross-reactivity was observed in the rTcP46-based ELISA against serum samples from cattle experimentally infected with *Babesia bigemina*, *B. bovis* and *Anaplasma marginale*. The results revealed that this antigen is specific for *T. congolense* infection, indicating that it could be a potential antigen for detection of antibodies against *T. congolense*. Indeed, the TcP46 gene shared a low nucleotide sequences identity with *T. b. brucei* and no match with the other *Trypanosoma* spp. and apicomplexan parasites detected by BLAST. Therefore, the TcP46 gene may be a candidate target for developing a specific and sensitive molecular diagnostic method to screen the *T. congolense* infection.

In chapter 2, I developed a semi-nested polymerase chain reaction (semi-nPCR) for the detection of *T. congolense* using oligonucleotide primers designed from a tandemly-arranged TcP46 gene. Two sets of primers produced the expected amplifications of 816 bp (external primers) and 590 bp (internal primers) with the *T. congolense* DNA, whereas no amplifications were observed with the genomic DNA extracted from the other hemoprotozoan parasites including the *B. bovis*, *B. bigemina*, *B. canis*, *T. evansi*, *Theileria annulata*, *Th. orientalis*, *Neospora caninum*, and *Toxoplasma gondii*. Thereafter, the sensitivity of the method was determined as 0.7 pg purified DNA or 10 trypanosomes ml^{-1}. Furthermore, the semi-nPCR could detect the presence of the parasites as early as one day post infection (d. p. i.) in experimentally *T. congolense*-infected mice blood, which was more sensitive than the conventional buffy coat technique as 6 d. p. i. and the previously reported PCR test based on the internal transcribed spacer 1 (ITS1) of rDNA (Kin-PCR) as 3 d. p.i. In addition, I screened 96 bovine and 29 canine blood DNA samples from South Africa by the present semi-nPCR method and Kin-PCR. The results revealed that the 28.1% and 17.2% were positive for cattle and dog samples, respectively. Sequence analysis using partial TcP46 gene revealed that the South African isolates shared 98.5% nucleotide sequence identity with that of *T. congolense* IL3000 strain. Altogether, the semi-nPCR method was

suitable for epidemiological studies on *T. congolense* infection. It revealed higher prevalence for *T. congolense* infection in cattle and dogs than the Kin-PCR method. Moreover, the present study provided new evidence to confirm that the *T. congolense* is a wide epidemic species that infected cattle and dogs in South Africa. The results also demonstrated a high prevalence of *T. congolense* in South Africa calling for the need to design effective control programs in the further study.

In chapter 3, I validated endoplasmic reticulum associated protein degradation (ERAD) pathway in *T. bruce. brucei* and *T. congolense* as a drug target. I evaluated the *in vitro* activity of 9 inhibitors that targeted the different components of ERAD pathway against bloodstream forms of *T. b. brucei*, *T. congolense* and normal human foreskin fibroblast (HFF) cells. All the compounds exhibited trypanocidal activity at different levels, dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) was the most potent inhibitor in both *T. b. brucei* (IC_{50}=0.03 ± 0.01 nM) and *T. congolense* (IC_{50}=0.11 ± 0.01 nM), while LY-374973 was the least potent inhibitor for *T. b. brucei* (IC_{50}=24,400 ± 14,619 nM) and LY-411575 (IC_{50}=18,367 ± 2,904 nM) was the least potent inhibitor for *T. congolense*. However, some of these inhibitors also displayed cytotoxicity towards HFF cells. The tested inhibitors showed different selectivity indices (SI) for *T. b. brucei* and *T. congolense*. 17 DMAG exhibited higher selectivity for both *T. b. brucei* (SI=145,100) and *T. congolense* (SI=39,572). 16F16 exhibited lowest selectivity for both *T. b. brucei* (SI=0.63) and *T. congolense* (SI=0.51). According to these results, targeting the ERAD pathway may be a new strategy in the treatment of trypanosome infection. Thus, this study provides a basis for development of new trypanocidal drugs targeting ERAD pathways of the parasites.

In conclusion, I identified a recombinant antigen, TcP46, for developing serodiagnostic methods to detect *T. congolense* infection and showed that this antigen is specific to *T. congolense* infection. ELISA based on the rTcP46 showed that it could be a marker for both early and chronic stages of *T. congolense* infection. Therefore, rTcP46-ELISA could be a promising diagnostic method. Subsequently, the semi-nPCR assay based on the TcP46 gene specifically detected *T. congolense* at low parasitermias in parasite culture and mouse models. The semi-nPCR also could be used as a reliable and potential tool for epidemiological survey of the *T. congolense*-infected animals. Additionally, growth inhibition effects of a panel of known inhibitors targeting the components of ERAD pathway were evaluated in trypanosome species. The effective growth inhibitions of parasites were observed in *T. b. brucei* and *T. congolense in vitro*. Therefore, the ERAD pathway is a

promising drug target in African trypanosomes.

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