

Abstract of Dissertation

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Title : Towards development of effective chemotherapy for babesiosis: Targeting dihydro-orotate dehydrogenase to suppress the growth of *Babesia* parasites

(バベシア原虫のジヒドロオロト酸デヒドロゲナーゼを標的としたバベシア症に対する有効な治療法の開発)

Abstract

Babesia is a haemotropic protozoal parasite of the genus *Babesia*, order Piroplasmida, phylum Apicomplexa. *Babesia* infection is one of the most common infections of free-living animals worldwide. The present study is focusing on *Babesia bovis*, the most virulent erythrocytic protozoa causing bovine babesiosis and *B. caballi*, *Theileria equi*, the agents causing equine piroplasmosis (EP). Infection with *B. bovis* is fatal due to its pathogenesis and neurological symptoms. Infection with *B. caballi* and *T. equi* in equids significantly impact on international movement of horses. Economic losses in bovine babesiosis results from mortality, ill-thrift, abortions, reduction of milk/meat production and draft power as well as expensive control measures (such as acaricide treatments, purchase of vaccines and therapeutics). Particularly, economic losses associated with EP are significant and include the cost of treatment, especially in acutely infected horses, in abortion in the last trimester of gestation, loss of performance, death, and restrictions in meeting international requirements related to exportation or participation in equestrian sporting events. The use of common chemotherapeutic drugs for bovine babesiosis, namely imidocarb dipropionate has been reported to predispose milk and meat to drug residues and diminazene aceturate causes adverse side effects and induce drug resistance for bovine babesiosis. While, treatment of EP in donkeys are particularly sensitive to imidocarb and treated animal have high mortality rates. Swelling and necrosis at diminazene aceturate injection sites as well as primary signs of intoxication such as respiratory distress and depression have been reported. Amicarbalide, euflavin and tetracycline have also been reported as other treatments but do not completely eliminate the parasites. Buparvaquone and combination with arteether result in temporary clearance of EP infection in donkeys but has not been administrated in horses. Moreover, EP is often resistant to most of therapeutic agents. The lack of commercial vaccine suggests that the intensive search for new drug targets and new chemotherapeutic compounds as strategy to combat bovine babesiosis and EP is required.

The pyrimidine biosynthesis pathway is essential for RNA, DNA, glycoproteins and phospholipids biosynthesis, which are important for division and growth of cells. Six enzymes of *de novo* pyrimidine synthesis pathway have been identified from *B. bovis* homogenates, indicating self pyrimidines production ability. Dihydroorotate dehydrogenase (DHODH) is the fourth enzyme in *de novo* pyrimidine biosynthesis pathway that catalyzes the oxidation of dihydroorotate to orotate. DHODH can be inhibited by naphthoquinone analog, and its derivatives. Atovaquone (ATV), a naphthoquinone analog and approved anti-malarial drug, leflunomide (LFN), an antirheumatic drug and brequinar (Breq), an immunosuppressive agent, have been identified as DHODH inhibitors. Furthermore, triazolopyrimidine derivatives have been evaluated on *P. falciparum* and showed promising inhibitory effects on parasite growth. Inhibition of DHODH results in reduced levels of uridine 5' monophosphate (UMP), which is an essential pyrimidine precursor. This DHODH enzyme is currently a drug target for treatment of malaria, toxoplasmosis, leishmaniasis, but is yet not characterized in bovine babesiosis and EP. This study aimed to characterize *B. bovis* DHODH (BboDHODH) and assess its potential as a chemotherapeutic target for treatment of bovine babesiosis. Furthermore, application of this new chemotherapeutic target on EP have also been evaluated.

B. bovis DHODH (BboDHODH) was characterized. Bioinformatic analysis, amplification, cloning, recombinant protein production, detection of native enzyme and measurement of the enzymatic properties were conducted. DHODH inhibitor including atovaquone (ATV), brequinar (Breq), leflunomide (LFN) and 7-hydroxy-5-[1,2,4] triazolo [1,5,a] pyrimidine (TAZ) were evaluated on the recombinant rBboDHODH enzymatic properties and on the growth of *B. bovis* *in vitro*. *T. equi* DHODH (TeDHODH) amino acid was compared to BboDHODH. Effect of DHODH inhibitors were evaluated on the growth of *T. equi* and *B. caballi* *in vitro*.

Bioinformatic analysis showed that BboDHODH is a homologue enzyme among apicomplexa parasites. The amplified and sequenced BboDHODH gene has an open reading frame of 1,248 bp encoding 416 amino acids with a predicted 44-kDa molecular weight. Comparison of BboDHODH with *Bos Taurus* DHODH (BosDHODH) amino acid sequence showed 42% similarity. Interestingly, the Thr103 (FMN binding site) and Thr281 (substrate binding site) observed in the parasite enzyme (BboDHODH) were replaced by Ser117 and Ser282 in the host enzyme (BosDHODH). These suggest that it has different amino acids at the FMN binding site and substrate binding site from host enzyme. Moreover, the phylogenetic analysis showed that *B. bovis*, *T. equi* and *T. orientalis* DHODHs belong to the same cluster, which was distinct from mammalian DHODHs. Characterization of BboDHODH showed that this enzyme has DHODH 2 like region which belong to class II DHODH enzyme, it was detected in *B. bovis* lysates and located in parasite mitochondria. The recombinant BboDHODH was successfully expressed as soluble protein with 42.4-kDa. rBboDHODH was an active enzyme exhibited enzymatic properties with specific activity of 475.7 ± 245 Unit/mg and the kinetic constant for this enzyme revealed K_m values of $276.2 \mu\text{M}$ and $94.41 \mu\text{M}$ for L-dihydroorotaic acid (L-DHO) and decylubiquinone (Q_o), respectively. rBboDHODH relative activity in the presence of $1 \mu\text{M}$ ATV, 1 mM LFN, 0.1 mM Breq and 1 mM TAZ was 19.1%, 51.5%, 106.5% and 101.0%, respectively. These showed that ATV and LFN significantly reduced the enzymatic properties of the recombinant protein while Breq and TAZ had no effect on recombinant enzymatic activity. Moreover, ATV and LFN significantly inhibited the growth of *B. bovis* 48 hrs post treatment. These inhibitors did not affect the bovine RBCs. Atovaquone treated parasites were supplemented with ORA and UMP. Supplementation of ORA at 25, 50 and $100 \mu\text{M}$ in ATV treated parasite cultures led to $1.20 \pm 0.2\%$, $1.50 \pm 0.3\%$ and $1.36 \pm 0.4\%$ parasitemia, respectively. On the

other hand, supplementation of UMP at 25, 50 and 100 μ M in ATV treated parasite cultures led to $1.15 \pm 0.3\%$, $1.26 \pm 0.2\%$ and $1.93 \pm 0.4\%$ parasitemia, respectively. These results suggest that supplementation with ORA or UMP prevent the parasite starvation by probably providing pyrimidine precursors needed for *B. bovis* survival. Inhibition with ATV led to lowering the level of ORA and UMP production resulting in non-division and no growth of the parasites. Comparison of *T. equi* DHODH (TeDHODH) with other *Babesia* DHODH amino acid sequence showed that TeDHODH was similar to other *Babesia* parasites DHODH. Detection of native DHODH in *T. equi* and *B. caballi* by mice anti BboDHODH showed their similar molecular weight on western blotting. The mice anti rBboDHODH cross-reacted with *T. equi* and *B. caballi* parasites lysates. This suggests that DHODHs among *Babesia* parasites were homologous enzyme and might exhibit similar role.

DHODH inhibitors which had inhibited the growth of *B. bovis* also effect on the growth of *T. equi* and *B. caballi* *in vitro*. This is an advantage of targeting DHODH enzyme for *Babesia* spp. Infection because it may be a multi-species target. Inhibitory effect of DHODH inhibitor showed that ATV, Breq and LFN significantly inhibited the growth of *T. equi* and *B. caballi* from 96 and 72 hrs, respectively. ATV is the most effective compound in this study with an IC_{50} value that is at nano molar level and comparable with diminazene aceturate (Di). It was effective against *B. bovis* with IC_{50} 2.38 ± 0.53 nM, *T. equi* 28.18 ± 16.77 nM and *B. caballi* 127.73 ± 3.38 nM. ATV is more effective on *B. bovis* than *T. equi* and *B. caballi* among protozoa parasites. On the other hand, Breq was effective on *T. equi* and *B. caballi* but 2 folds more effective on *B. caballi* than *T. equi*. However, when compared to ATV high concentration of Breq is needed for clearing the parasites *in vitro*, as it showed IC_{50} range from 5-10 μ M. LFN significantly inhibited rBboDHODH enzymatic activity to 50%. However, the IC_{50} of LFN was higher than observed for Di. LFN was less effective on *B. bovis*, *B. caballi* and *T. equi* compared with ATV, Breq and Di. Nevertheless, further modification on the basis of LFN structure might improve the potency, selectivity and specificity of this compound against *Babesia* parasites.

In summary, DHODH is a homologue enzyme among *Babesia* parasites (*B. bovis*, *B. caballi* and *T. equi*). The available DHODH inhibitors were effective against the growth of *B. bovis*, *B. caballi* and *T. equi* *in vitro* suggesting that DHODH could be a potential chemotherapeutic target for treatment of bovine babesiosis and EP.