In vitro Antitheilerial Activity of Paluther (Artemether 80) Against Theileria lestoquardi

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ABSTRACT

Aim: The aim of this study was to screen Artemether 80 for activity against Theileria lestoquardi (Apicomplexa: Theileridae) using buparvaquone as a standard drug.
Study Design: In vitro study under laboratories conditions.
Place and Duration of Study: Veterinary Research Institute, between 2006 and 2008.
Methodology: Artemether 80 was screened for the first time to investigate activity against T. lestoquardi at different concentrations. Blood was collected separately from normal sheep and

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sheep infected naturally with Theileria. Normal lymphocyte cells and lymphocyte cells infected with Theileria were isolated from heparinized blood with Ficoll-paque. Isolated cells were grown in Minimum Essential Medium (MEM), supplemented with 20% calf serum and sub cultured. The parasite was identified with indirect fluorescent antibody test (IFA). A volume of 2.7 ml of lymphoblast cell suspension at concentration of 5x10⁴ cell/ml was distributed in tissue culture plates, and then 0.3 ml of drug at concentrations of 0.1, 1.0, 10 and 100 mg/L was added separately. A volume of 0.3 ml MEM was added to infected untreated control.

**Results:** The *in vitro* antitheilerial activity of Artemether 80 against *T. lestoquardi* 48 h after exposure was 0%, 14%, 30% and 45% at concentrations of 0.01, 0.1, 1.0 and 10 mg/L, respectively as compared with activity of buparvaquone at the same concentrations being 74%, 83%, 92% and 100%, respectively. Both Artemether 80 and buparvaquone caused *in vitro* partial cytotoxic effect at the highest concentrations. Activity and/or partial cytotoxic effect of both drugs caused changes in the morphology of macroscizonts and host lymphoblast cells, decreased the number of macroscizonts/cell, mean number of dividing cells, increased the number of cells with extra cellular parasites.

**Conclusion:** It was concluded that Artemether 80 is slightly effective *in vitro* against *T. lestoquardi*.

Key words: Theileria lestoquardi; artemether 80; buparvaquone; *in vitro* activity.

1. INTRODUCTION

*Theileria lestoquardi* [1] is a tick-borne protozoan parasite of sheep and goats [2] which occurs in South Eastern Europe, Northern Africa, Western and Central Asia [3] and in India [4]. *T. lestoquardi* was diagnosed in 1915 and thought to be a significant disease that affects sheep in Sudan [5-8]. It causes malignant theileriosis in sheep and goats which may be acute, sub acute or chronic. The parasite is transmitted by the ixodid tick *Hyalomma anatolicum* [9] and causes great economic losses in sheep in Sudan [10]. *T. lestoquardi* and *Theileria* species (China) are considered to be highly pathogenic [11,12]. The other species, such as *T. ovis* and *T. separata* are less pathogenic and have lower economic importance than *T. lestoquardi* [3,13].

The most widely method for the control of theileriosis is control of vectors by chemical acaricides. However, control has become less reliable because of acaricides resistance, poor management of tick control and illegal animal movement in many countries [14]. Vaccination using attenuated schizont infected cell lines is widely used for *T. annulata*, while the infection and treatment method using ground up infected ticks as stabilate and tetracycline treatment is being implemented in a number of countries in East and Central Africa for East Coast fever control [15,16]. Chemotherapy is also widely used. Parvaquone, halofuginone and buparvaquone are used to treat *T. parva*, *T. annulata* and *T. lestoquardi* [17,18].

In the present investigation, the antimalarial drug, Artemether 80, was tested for antitheilerial effect using Buparvaquone as a standard antitheilerial drug. Artemether 80 is synthetic antimalarial drug derived from artemisinin. Artemisinin is derived from the leaves and flowers of *Artemisia annua*, also termed *A. annua* L., or Artemisia. Artemisia had long been used for medical purposes in China and was recommended for chills and fevers (symptoms of malaria). The active ingredient, artemisinin, was first isolated by Chinese Scientists in 1972 and named Qinghaosu, as a part of an antimalarial drug discovery program established in response to a request from North Vietnam during the Vietnam War [19-22].

2. MATERIALS AND METHODS

2.1 Paluther® (Artemether 80)

Artemether (80 mg/ml); FAB/MAN Kunming Pharmaceutical corporation-Yunnan, China Lic. Cititech-Beijing-China was used. Artemether 80 is available as a solution (80 g/L) of Artemether in ampoules only for intramuscular administration.

2.2 Butalex™ (Buparvaquone)

Butalex™ (buparvaquone injection 50 g/L); Schering-Plough Animal Health, U.K.) was purchased from a local dealer and used.
2.3 Isolation of *Theileria lestoquardi*

Separation of lymphocytes from a normal healthy sheep, and a sheep infected naturally with *T. lestoquardi* using gradient method with Ficoll-paque was carried [23]. Normal and infected lymphocytes were grown in MEM with glutamine, antibiotics, antifungal using standard procedure [24]. The identity of the parasite was verified using indirect florescent antibody technique (IFA) as described previously [25]. The isolated normal cells had limited multiplication and can’t go for further passages, while infected ones multiplied continuously, and repeatedly sub-cultured till passage 8 which was used for the test.

Artemether 80 (80 g/L) and buparvaquone (50 g/L) were dissolved separately in minimum amount of Dimethyl Sulphoxide (DMSO), diluted to give a concentration of 100 mg/L. Then, 10 fold dilutions were done from each drug to give concentrations of 10, 1.0 and 0.1 mg/L, respectively. The final concentration of DMSO at the highest concentration of the drug (10 mg/L) was 0.01%.

2.4 Screening of Artemether 80 and Buparvaquone for Antitheilerial Activity

Viable lymphoblast cells were counted according to the method described in Flow Laboratories Catalogue (Flow Laboratories Irvine, Scotland, Ayrshire) using Neubauer haemocytometer (Fein Optic Blankburg, G.D.R.). lymphoblasts suspension (2.7ml) containing 50000 cells/ml were distributed into tissue culture plates (Falcon, Corning Glass works-New York) in which clean sterile cover slips were placed. MEM (0.3 ml) was added to the untreated control. In addition, DMSO (0.3 ml) at highest concentration was added to negative control. Artemether 80 and buparvaquone, (0.3ml) of each were added to the respective wells to give a final concentration of 10, 1.0, 0.1 and 0.01mg/L, respectively. The plates were examined under an inverted microscope (Hund Gmbh Wilheimum, Wetzlar, Wilover 30, Germany) for observation of morphology and viability of the lymphoblasts and incubated at 37°C. The experiment was repeated twice. After 24 h each plate was examined visually and microscopically. Forty eight h later, the plates were examined as before. The cover slips were air dried and fixed in absolute methanol for 5 min. Untreated control lymphoblast cells and treated ones were used for viable cell counts and preparation of slides.

2.5 Preparation of Slides

Cell suspension (0.1 ml) was cytocentrifuged to deposit cells directly onto the slides at 600 rpm for 5 min (Cytospin, Shandon, Southern Products Ltd., Astmoo, Run Corn, Cheshire, U.K.). Six slides were prepared from untreated control and from each concentration of the treated lymphoblast cells, air-dried and fixed in absolute methanol for 5 minutes. The slides and cover slips were stained with Giemsa's stain.

The cover slips were used for microscopic description of lymphoblasts and macroschizonts. The slides were used for determination of mean number of lymphoblasts with dead macroschizonts in 50 lymphoblasts, mean number of living and dead macroschizonts in 10 untreated control and 10 treated lymphoblasts, respectively, mean number of lymphoblasts with extra cellular macroschizonts per field, mean number of dividing cells (binucleated and multinucleated) per field while mean number of viable lymphoblast was calculated according to standard method using haemocytometer to exclude dead lymphoblasts stained with Trypan blue. The partial cytotoxic effect was determined by microscopic examination to observe the degenerative changes of the lymphoblasts, and viable cell count.

2.6 Statistical Analysis

The data presented in this study were analyzed using the computer program SPSS (Statistical Packages for Social Science) version 10. The significant difference using (ANOVA) were considered at $P < 0.05$.

3. RESULTS

3.1 Antitheilerial Activity of Artemether 80

The antitheilerial activity of Artemether 80 after 48h exposure was less potent than that of buparvaquone which was used as the standard drug (Table 1).

3.2 Effect of Artemether 80 on Macroschizonts and Lymphoblasts

Effect of Artemether 80 on number of macroschizonts per lymphoblast cell using buparvaquone as a reference drug is shown in Table 1. Morphology of dead macroschizonts due to activity of Artemether 80 revealed dark coloration and degeneration (Fig. 1b). On the
other hand, buparvaquone caused necrosis with loss of structure of the dark macroschizonts (Fig. 1c), compared to untreated control with normal pink macroschizonts (Fig. 1a).

Artemether 80 effects also caused changes in the number of dividing cells (binucleated and multinucleated) at concentrations of 0.1, 1.0 and 10.0 mg/L. Moreover, the number of binucleated and multinucleated cells treated with buparvaquone were significantly \((P<0.05)\) decreased at all concentrations, compared with the control (Table 2). However, the mean number of lymphoblast cells with extra cellular vacuolated cytoplasm (Fig. 2a) while that of Buparvaquone resulted in lymphoblast cells with pyknosis and dark chromatin (Fig. 2b). Also the number of viable cells 48 h after exposure to both Artemether 80 and buparvaquone significantly \((P<0.05)\) decreased as compared with the control.

4. DISCUSSION

Due to the similarity between the life cycle of *Theileria* and *plasmodium malaria*, Artemether 80 was studied to evaluate its activity against *T. lestoquardi*.

The antihelierial activity of Artemether 80 against *T. lestoquardi* was in agreement with previous study which indicated that Artemether *absinthium* had schizontidal activity against *P. falciparum* [26]. The schizontidal activity of Artemether 80 against malaria parasite is due to the destruction of sexual erythrocytic forms of *P. falciparum* and *P. vivax*. The antimalarial activity of artemether is characterized by inhibition of the protein synthesis when trophozoites grow. The mode of action of Artemether 80 against *T. lestoquardi* was unknown. The results of the present study showed that Artemether 80 was less potent than buparvaquone. Buparvaquone (BW720C: Butalex); 2-trans-(4-t-butylcyclohexyl) methyl-3-hydroxy-1, 4-napthoquinone; is the most active compound in the series of hydroxynaphtoquinones which act on the parasite respiration by interfering with electron transport system [27].

![Fig. 1. Theileria lestoquardi lymphoblast with normal pink macroschizonts (a. untreated control), dark pyknotic macroschizonts (b. treated with artemether 80), dark degenerated macroschizonts with loss of structure (C. treated with buparvaquone) after 48 h exposure to drugs at concentration of 1.0 mg/L, Giemsa's stain, (x1000)](image)
Table 1. Mean (± SD) *In vitro* activity of artemether 80 and buparvaquone against *Theileria lestoquardi*

<table>
<thead>
<tr>
<th>Concentration mg/L</th>
<th>Number of lymphoblast cells with dead macroschizonts</th>
<th>Number of lymphoblast cells with living macroschizonts</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Artemether 80</td>
<td>Buparvaquone</td>
<td>Artemether 80</td>
</tr>
<tr>
<td>0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>50.00±0.00</td>
</tr>
<tr>
<td>0.01</td>
<td>0.00±0.00</td>
<td>37.00±0.75*</td>
<td>50.00±0.00</td>
</tr>
<tr>
<td>0.10</td>
<td>7.00±0.00*</td>
<td>41.83±0.75*</td>
<td>43.00±0.63*</td>
</tr>
<tr>
<td>1.00</td>
<td>15.17±0.00*</td>
<td>46.00±0.89*</td>
<td>34.83±0.73*</td>
</tr>
<tr>
<td>10.00</td>
<td>22.50±0.33*</td>
<td>50.00±0.00*</td>
<td>27.50±0.55*</td>
</tr>
</tbody>
</table>

Results are mean ± SD, statistical significance of difference from corresponding control values: *P<0.05

Table 2. Mean effect of artemether 80 and buparvaquone on number of *Theileria lestoquardi* macroschizonts/cell, number of cells with extra cellular macroschizonts and number of dividing cells

<table>
<thead>
<tr>
<th>Concentration mg/L</th>
<th>Number of macroschizonts/cell</th>
<th>Number of cells with extra cellular macroschizonts</th>
<th>Number of dividing cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Artemether</td>
<td>Buparvaquone</td>
<td>Artemether</td>
</tr>
<tr>
<td>0.00</td>
<td>21.23±0.16</td>
<td>21.23±0.14</td>
<td>2.50±0.55</td>
</tr>
<tr>
<td>0.01</td>
<td>21.23±0.15</td>
<td>18.65±0.25*</td>
<td>2.50±0.55</td>
</tr>
<tr>
<td>0.10</td>
<td>20.70±0.09*</td>
<td>18.08±0.16*</td>
<td>2.50±0.55</td>
</tr>
<tr>
<td>1.00</td>
<td>20.20±0.06*</td>
<td>17.28±0.19*</td>
<td>2.83±0.75</td>
</tr>
<tr>
<td>10.00</td>
<td>19.73±0.08*</td>
<td>16.57±0.12*</td>
<td>3.67±1.03*</td>
</tr>
</tbody>
</table>

The data are expressed as mean ± SD. *P<0.05: significantly different from control by ANOVA, n=6
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Fig. 2. *Theileria lestoquardi* lymphoblast cell with vacuolated cytoplasm (a. cytotoxic effect of artemether 80); pyknosis and dark chromatin (b. cytotoxic effect of buparvaquone) after 48 h exposure to drugs at concentration of 10.0 mg/L, Giemsa’s stain, (x1000)

Table 3. Mean in vitro effect of artemether 80 and buparvaquone on number of viable cells

<table>
<thead>
<tr>
<th>Concentration mg/L</th>
<th>Number of viable cells x10^7/ml</th>
<th>48 h before</th>
<th>48h after</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00 (control)</td>
<td>5.00±0.00</td>
<td>6.25±0.35</td>
<td>6.25±0.35</td>
</tr>
<tr>
<td>0.01</td>
<td>5.00±0.00</td>
<td>6.25±0.35</td>
<td>5.50±0.00*</td>
</tr>
<tr>
<td>0.10</td>
<td>5.00±0.00</td>
<td>6.00±0.00</td>
<td>5.50±0.00*</td>
</tr>
<tr>
<td>1.00</td>
<td>5.00±0.00</td>
<td>5.25±0.35*</td>
<td>5.00±0.00*</td>
</tr>
<tr>
<td>10.00</td>
<td>5.00±0.00</td>
<td>4.25±0.00*</td>
<td>4.50±0.00*</td>
</tr>
</tbody>
</table>

The data are expressed as mean± SD. *P<0.05: significantly different from control by ANOVA

There was clear microscopic evidence that Artemether 80 and buparvaquone killed the macroschizonts within the lymphoblasts. Accordingly, the rate of lymphoblast cell multiplication was decreased, because multiplication of lymphoblast cells is simulated by the parasite which is located in the Golgi apparatus of the host cell [28]. As a result, the number of dividing cells and viable cells decreased. Our result is in agreement with a previous study which indicated that the rate of multiplication of *T. annulata* infected cells was markedly reduced as the parasite was eliminated [29].

The number of viable cells/ml in untreated control after 48 h incubation at 37°C insignificantly (P>0.05) increased. However, another author found that the number of cells/ml in infected untreated control increased by three folds [29]. This may reflect differences in growth rates due to inherent genetic and/or environmental conditions. Moreover, *T. parva* infected lymphoid cells in suspension culture used 20% foetal calf serum in growth medium considered that the type and quantity of the serum were critical in their work [30]. However, the number of cells with extra cellular macroschizonts increased. This could be due to the effect of the drugs on the fragility of cell membrane.

The low toxicity of buparvaquone is due to the alkyl-substitution with the bigger volume and strong hydrophobicity [31]. This result may support our findings.

5. CONCLUSIONS

Artemether is an essential drug used for curative treatment of severe malaria. In this study, it was selected for the first time for discovery of its activity against theileriosis under laboratories conditions because buparvaquone: the widely used drug against theileriosis: has a long plasma
half life of at least 7 days and it is not available all the time.

From this study, it is concluded that Artemether 80 has low in vitro activity against *T. lestoquardi*.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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