A study of tribendimidine effects in vitro and in vivo on the liver fluke *Opisthorchis felineus*  

Maria Y. Pakharukova 1,2*, Vladimir A. Samsonov 3, Elena A. Serbina 4 and Viatcheslav A. Mordvinov 1

**Abstract**

**Background:** The food-borne liver fluke *Opisthorchis felineus* is an epidemiologically important species and the causative agent of opisthorchiasis across an extensive territory of Eurasia. For decades, treatment of opisthorchiasis has been based on praziquantel. Tribendimidine could be an alternative drug that has been successfully tested for *Opisthorchis viverrini* and *Clonorchis sinensis* infections. We aimed to assess tribendimidine effects in comparison with praziquantel in vivo and in vitro against the liver fluke *Opisthorchis felineus*.

**Results:** In this study we (i) calculated half-maximal inhibitory concentrations (IC50) by motility tests against *O. felineus* adults and newly excysted metacercariae after tribendimidine treatment in vitro; (ii) determined whether tribendimidine and PZQ effects on adult liver flukes are dependent on or mediated by white blood cells; and (iii) tested in vivo the anthelmintic activity of tribendimidine on juvenile and adult worms. We found that the efficiency of tribendimidine in vitro was similar (IC50 = 0.23 μM for newly excysted metacercariae and 0.19 μM for adult worms) to that of praziquantel (IC50 0.98 μM for newly excysted metacercariae and 0.47 μM for adult worms). The treatment of adult worms in vivo with praziquantel or tribendimidine at 400 mg/kg resulted in a 76% and 77.2% reduction, respectively, in the worm burden during chronic infection.

**Conclusions:** The differences between WBR values after PZQ and TBN treatment were not significant, thus tribendimidine was as effective as praziquantel against *O. felineus* liver flukes. Given the broad-spectrum activity of tribendimidine and efficacy against *O. felineus*, this drug may be a promising candidate for the treatment of opisthorchiasis felinea and other liver fluke infections.

**Keywords:** Food-borne trematodes, *Opisthorchis felineus*, Tribendimidine, in vivo, in vitro, Hamster model

**Background**

The liver fluke *Opisthorchis felineus* is a member of the triad of epidemiologically important species of food-borne trematodes (*O. felineus*, *O. viverrini* and *C. sinensis*), and the causative agent of opisthorchiasis felinea over an extensive territory across Eurasia [1–3]. The prevalence of *O. felineus* infection in the population of the endemic regions of Western Siberia is 10–45% [1, 2]. Human infection results from eating raw or undercooked freshwater cyprinoid fish carrying metacercariae of the parasite [1, 2]. Metacercariae excyst in the duodenum, and the juvenile parasites ascend into the bile ducts, where development into adult worms proceeds over the course of four weeks. The adult liver flukes reside within the bile ducts and gall-bladder [2] and shed eggs that enter the gastrointestinal tract and are released with feces.

The clinical manifestations and pathology induced by chronic infection with *O. felineus*, *O. viverrini* and *C. sinensis* liver flukes are similar [4, 5]. The liver flukes induce several hepatobiliary disorders, including hepatomegaly, cholangitis, periductal fibrosis, chronic inflammation, liver abscesses [2, 3, 6] and potentially cholangiocarcinoma. The International Agency for Research on Cancer classifies the flukes of the family
Opisthorchiidae (O. viverrini and C. sinensis) as group 1 agents and major risk factors for cholangiocarcinoma [4].

To date, the only drug of choice for the treatment of opisthorchiasis, clonorchiasis, schistosomiasis and other fluke infections has been praziquantel (PZQ). PZQ disrupts calcium ion homeostasis and antagonizes voltage-gated calcium channels. Drug discovery for trematode infections has a high priority [7] to avoid the development of resistance to PZQ in the future. Studies on alternative drugs for the treatment of liver fluke infections have shown that tribendimidine (TBN) is a good candidate [8–10]. It is a symmetrical diamidine derivative of amidantel and was developed in China for use in humans in the mid-1980s [11]. TBN is a cholinergic agonist that is selective for nicotinic acetylcholine receptors. TBN has a broad-spectrum activity against intestinal nematodes. The efficiency of TBN against trematodes infection is variable [9, 12]. The drug is effective against O. viverrini and C. sinensis infections, but ineffective against S. mansoni infection [9]. To our knowledge, TBN has never been tested against O. felineus infection.

The aims of the present study were to (i) test in vitro anthelmintic activity of TBN by calculating IC50 values via motility tests on juvenile and adult O. felineus worms; (ii) assess if TBN and PZQ effects on liver flukes are dependent on or mediated by white blood cells; and (iii) test in vivo the anthelmintic activity of TBN on juvenile and adult worms.

Methods

Compounds

TBN [((E,1′E)-N′,N′-((4,4′-(1E,1′E)-(1,4-phenylenebis-methan-1-yl-1-ylidene))bis(azan-1-yl-1-ylidene))bis(4,1-p phenylene))bis[N,N-dimethylacetimidamide]] was synthesized and provided by the Vorozhtsov Institute of Organic Chemistry of the Siberian Branch of the Russian Academy of Sciences (Novosibirsk, Russia). Spectroscopic characteristics confirmed the structure of the compound (purity 99%).

For in vitro tests, PZQ (Sigma-Aldrich, St Louis, USA) and TBN were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to obtain 1 mM stock solutions.

For in vivo experiments, PZQ and TBN were prepared as a suspension in an aqueous solution of 7% Tween 80 (v/v) and 3% ethanol (v/v) before oral administration (10 ml/kg) and were administered at 400 mg/kg body weight.

Animals and infection

Syrian hamsters (Mesocricetus auratus) were purchased from the Animal Facility of the ICG SB RAS. Before infection, the animals were allowed to acclimate for one week at our animal facility. They were kept in groups of maximum three hamsters per cage, with free access to water and rodent food pellets. Euthanasia was performed by carbon dioxide inhalation, and every effort was made to minimize suffering. Opisthorchis felineus metacercariae were collected from naturally infected fish (Leuciscus idas) caught in the Ob River near Novosibirsk (Western Siberia) and extracted accordingly [13].

For in vivo experiments, 83 male Syrian hamsters (aged 6–8 weeks) were chosen randomly. The animals were orally infected with 75 O. felineus metacercariae.

WBC extraction from blood

Blood samples from infected hamsters were collected by the cardiac puncture with anticoagulant (EDTA). Next, 0.3% gelatin (Sigma-Aldrich) in PBS was added to the blood samples in a 3:1 ratio, and the samples were incubated for 4–6 h at 37 °C. This step allowed the separation of blood into an upper plasma layer containing WBCs and a lower (red-blood-cell layer). The upper layer was collected and centrifuged at 2000× g for 5 min. The supernatant was aspirated and the pellet resuspended in PBS. This step was repeated three times. Finally, WBCs were resuspended in RPMI 1640 medium (Life Technologies, Foster City, USA) and counted.

In vitro activity

Newly excysted metacercariae (NEM) were prepared from metacercariae according to a previously published protocol [13]. Adult worms were recovered from the livers of hamsters infected three months earlier and then worms were thoroughly washed with sterile saline solution (0.9% NaCl). For calculation of the half-inhibitory concentration (IC50), we tested the following concentrations of compounds: 0.001, 0.01, 0.1, 1, 10 and 100 μM. The DMSO concentration across different compound concentrations was 0.5% v/v. As control groups, we used flukes incubated in the medium with 0.5% DMSO. Thus, four to five adult worms or 30–40 NEM per well of a 12-well culture plate were analyzed. The worms were incubated at 37 °C for 24 h in the RPMI 1640 medium (Life Technologies) supplemented with 100 U/ml penicillin, 0.1 μg/ml streptomycin, 0.25 μg/ml amphotericin B (Sigma-Aldrich), 1% glucose and the corresponding concentration of a drug [13, 14].

After 24 h of treatment with one of the drugs, viability of the worms was evaluated under an inverted microscope (Axiolab 40CFL, Carl Zeiss, Jena, Germany) equipped with a camera (Axiocam ICC3, Zeiss) (magnification 10–50×). The experiments were repeated three times with two replicates for each concentration. The motility of viable worms was assessed on a motility scale from 0 to 3: 3, very active (similar movements as the control flukes); 2, active (reduced motility when compared to the control; however, the entire body still moving); 1, reduced viability (only movements of the oral sucker); and 0, non-motile
(non-motile for 2 min) [8, 13, 14]. The IC₅₀ value is defined as the concentration of a drug required to decrease the mean motility of a worm to 50% at the 24-h time point. Four-parameter logistic regression was used to calculate IC₅₀ and standard error values (R package drc 3.0-1) [15]. The ANOVA lack-of-fit test was conducted to test the hypothesis that a proposed regression model fits the data well.

To assess the combinatorial activity of the anthelmintics and immune system, freshly extracted adult worms (3 months post-infection) were incubated overnight in the medium. After that, adult worms were incubated in the medium with TBN (0.5 μM) or PZQ (0.5 μM) and freshly extracted 4 × 10⁵ WBCs for 7 days. As control groups, we used flukes incubated in the medium with 0.5% DMSO. Mortality rates were evaluated each day. The worms were classified as dead if they had a dark color and no movement was observed for 2 min [13]. To estimate mortality rates, Kaplan-Meier survival curves were built by means of the survival (v.2.38) R package. Finally, statistical difference in survival log-rank (Mantel-Haenszel test) within each pair of samples was calculated.

### In vivo activity

The hamsters were subdivided into three batches for experimental treatment. In each batch, hamsters were infected with metacercariae extracted from the same batch of fish. In two batches, hamsters were treated 1 month post-infection (corresponding to infection with adult *O. felineus* worms) and 15 days post-infection (corresponding to infection with juvenile *O. felineus*). In the remaining batch, the hamsters were treated 3 months post-infection, which corresponds to chronic infection.

- **Groups of 5–7 hamsters** were treated with a single drug dose *via* oral administration. PZQ and TBN was administered at 400 mg/kg body weight. Ten days after the treatment, the hamsters were placed in new cells; whole-stool samples were collected after 3 subsequent days, and 1 g of each stool was analyzed by means of Mini Parasep concentrators (Apacor, Wokingham, UK). The number of eggs per gram of stool (EPG) was determined. Egg reduction rates (ERR) were calculated as follows: (1 − Arithmetic Mean EPG at follow-up)/Arithmetic Mean EPG in control × 100 [10].

Worms remaining in the hepatobiliary system on day 14 post-treatment were counted after killing the hamsters by CO₂ asphyxiation. Drug activity was expressed as a worm burden reduction (WBR) as described elsewhere [8, 16]. Briefly, WBRs were calculated as follows: (a − b)/a × 100, where a is the average worm count in the control group upon dissection, and b is the average worm count in the treated group upon dissection. The significance of WBR and ERR was evaluated by the Mann-Whitney U-test and Fisher’s exact test in the STATISTICA 6.0 software (Statsoft Inc, Tulsa, USA).

### Results

#### In vitro activity

Evaluation of the compounds by standard motility tests against NEM revealed that IC₅₀ of TBN (IC₅₀ = 0.23 ± 0.059 μM; ANOVA lack-of-fit test: RSS = 0.02, df = 3, P = 0.0001) was similar to that of PZQ (IC₅₀ 0.98 ± 0.18 μM; ANOVA lack-of-fit test: RSS = 0.008, df = 3, P = 0.02; Table 1). When the drugs were tested on adult worms, TBN effects (IC₅₀ 0.19 ± 0.1 μM; ANOVA lack-of-fit test: RSS = 0.006, df = 7, P = 0.0003) were also similar to that of PZQ (IC₅₀ = 0.47 ± 0.05 μM; ANOVA lack-of-fit test: RSS = 0.009, df = 3, P = 0.05; Table 1).

The mortality of adult flukes was different between the PZQ and TBN treatment groups (Mantel-Haenszel log-rank test: χ² = 15.8, df = 1, P < 0.0001). The mortality of adult worms in the TBN group was 40% after 7 days of treatment, as compared to 80% mortality in the PZQ group after 7 days of treatment (Fig. 1a, b).

To assess if TBN and PZQ effects on liver flukes are depending on or mediated by white blood cells, adult worms were co-cultivated in the media together with WBCs freshly extracted from the blood of the infected animals and worm mortality was assessed each following day. We found that the combined action of the drug and the immune compounds caused a much greater effect on the mortality of adult helminths. In particular, the mortality of adult worms in the combined group of PZQ and WBCs and TBN + WBCs (Mantel-Haenszel log-rank test: χ² = 4.8, df = 1, P = 0.0289; Fig. 1a) in comparison with the mortality of worms treated with PZQ (Fig. 1a).

We observed a similar effect under the TBN treatment. Thus, the mortality of helminths treated with TBN and WBCs significantly increased (Mantel-Haenszel log-rank test: χ² = 18.7, df = 1, P < 0.0001; Fig. 1b) when compared with the mortality in the TBN group (Fig. 1b).

It should be noted that the mortality of adult flukes was not different between both combined groups PZQ + WBCs and TBN + WBCs (Mantel-Haenszel log-rank test: χ² = 2.3, df = 1, P = 0.131). WBCs had no effect on the mortality of adult worms in the TBN group after 7 days of treatment (Fig. 1a, b).

#### Table 1 IC₅₀ values of tribendimidine and praziquantel against newly excysted metacercariae and adult *Opisthorchis felineus* worms

| Compound          | NEM (μM) | Adults (μM) |
|-------------------|----------|-------------|
| Tribendimidine (TBN) | 0.23 ± 0.059 | 0.19 ± 0.1 |
| Praziquantel (PZQ)    | 0.98 ± 0.18 | 0.47 ± 0.05 |

For calculation of the half-maximal inhibitory concentration (IC₅₀), we tested the following concentrations of compounds: 0.001, 0.01, 0.1, 1, 10 and 100 μM.

Data are presented as IC₅₀ values ± standard error (R package drc 3.0-1)

Abbreviation: NEM, newly excysted metacercariae
DMSO-treated helminths (Mantel-Haenszel log-rank test: $\chi^2 = 0.9$, df = 1, $P = 0.337$).

**In vivo activity**

We assessed the effect of TBN at different stages of the disease, specifically 15-day and 1-month infections, which are considered acute opisthorchiasis, and 3 month chronic opisthorchiasis. The treatment of hamsters 15 days post-infection with TBN at 400 mg/kg resulted in a WBR of 90.7% (Mann-Whitney U-test: $U = 0$, df = 4, $P = 0.009$; Table 2). The treatment of hamsters 1 month post-infection with TBN at 400 mg/kg caused a WBR of 90% (Mann-Whitney U-test: $U = 0$, df = 4, $P = 0.009$; Table 2).

Treatment with PZQ at 400 mg/kg 1 month post-infection resulted in a WBR of 76.5% (Mann-Whitney U-test: $U = 0$, df = 6, $P = 0.002$). According to Fisher’s exact test ($P = 0.278; 0.447$), the differences between WBR values after PZQ and TBN treatment 1 month post-infection were not significant.

Treatment of hamsters with chronic infection (3 months post-infection) with TBN at 400 mg/kg caused a WBR of 76% (Mann-Whitney U-test: $U = 0$, df = 4, $P = 0.009$; Table 2). For comparison, treatment of hamsters with chronic infection with PZQ resulted in a WBR of 77.2% (Mann-Whitney U-test: $U = 0$, df = 10, $P = 0.014$; Table 2). The differences in worm reduction rates between acute and chronic infection for TBN were not significant (Fisher’s exact test: $P = 0.305$).

**Fig. 1** Kaplan-Meier survival curves for PZQ- (a) and TBN-treated (b) adult worms. A set of Kaplan-Meier survival curves (out of three independent experiments) is shown. $P$-values were obtained by the survival log-rank (Mantel-Haenszel) test (R package survival v.2.38) within each pair of samples. *A significant difference between groups (treatment vs treatment + WBCs), #A significant difference between groups (treatment vs vehicle DMSO), **(###) $P < 0.01; *** (###) $P < 0.001$. Abbreviations: DMSO: dimethylsulfoxide; TBN: tribendimidine; PZQ: praziquantel; WBCs: white blood cells

**Table 2** Worm burden reduction values obtained after treatment of hamsters harboring *O. felineus* with tribendimidine or praziquantel

| Treatment of chronic infection (3 months) | Number of animals | Mean no. of worms ± SD | WBR (%) | Mean EPG ± SD | ERR (%) |
|-----------------------------------------|-------------------|------------------------|---------|---------------|--------|
| Control                                 | 13                | 33 ± 10                | –       | 1863 ± 1006   | –      |
| TBN, 400 mg/kg                          | 5                 | 7 ± 4                  | 76      | 927 ± 620     | 51.0   |
| PZQ, 400 mg/kg                          | 11                | 8 ± 5                  | 77.2    | nd            | nd     |
| Treatment of acute infection (1 month)   |                   |                        |         |               |        |
| Control$^1$                             | 5                 | 19 ± 2                 | –       | 863 ± 279     | –      |
| Control$^2$                             | 6                 | 34 ± 9                 | –       | nd            | –      |
| TBN$^1$, 400 mg/kg                      | 5                 | 2 ± 4                  | 90      | 299 ± 244     | 65.4   |
| PZQ$^2$, 400 mg/kg                      | 7                 | 8 ± 6                  | 76.5    | nd            | nd     |
| Treatment of acute infection (15 days)   |                   |                        |         |               |        |
| TBN$^1$, 400 mg/kg                      | 5                 | 2 ± 2                  | 90.7    | 463 ± 281     | 46.3   |

Superscript numbers denote data relative to the corresponding control group

*Abbreviations: TBN, tribendimidine; PZQ, praziquantel; SD, standard deviation, WBR, worm burden reduction rate, EPG, eggs per gram of feces, ERR, egg reduction rates, nd, not determined*
Discussion
In this study, we compared the anthelmintic activity of TBN and PZQ on the experimental model of the liver fluke infection caused by O. felineus. Our data indicate that the efficacy of TBN is similar to that of PZQ in vitro and in vivo on different stages of the disease: at one and three months after the infection. TBN efficacy against O. felineus are in compliance with the data shown on O. viverrini [8–10]. The IC\textsubscript{50} for TBN in vitro against adult O. viverrini was shown to be 0.05 \( \mu \text{g/ml} \) [8]. A similar efficacy of a single dose in vivo has been shown in studies against O. viverrini: 400 mg/kg of TBN treatment was sufficient for 95.7% WBR in hamsters four weeks after the infection [9]. High efficacy of TBN has also been demonstrated on an experimental model of clonorchiasis (4–6 weeks of C. sinensis infection): a 150 mg/kg dose was sufficient for 91.5% WBR, and 240 mg/kg resulted in 99.4% WBR [17].

High cure rates were observed with TBN (90%) and PZQ (90%) among O. viverrini-infected participants with low-intensity infections [10]; however, several studies have shown lower efficacy rates for TBN and PZQ in the treatment of moderate and heavy infections (44% for TBN and 56% for PZQ) [12, 18]. Thus, TBN has efficacy comparable to that of PZQ in the treatment of liver fluke infections. Nevertheless, TBN was shown to have a good safety profile and caused fewer adverse events [10] compared with PZQ when tested on participants with O. viverrini infection. In particular, in one clinical trial, participants treated with PZQ were about four times more likely to have an adverse event than those treated with TBN [10]. All adverse events in the TBN group were mild except for nausea. Moderate adverse events were reported in the PZQ group, including vertigo, nausea, fatigue, abdominal cramps and vomiting [10].

We also demonstrated that apparently there is an immunity-dependent lethal effect of TBN on the liver flukes O. felineus. The mortality of helminths in the combined group of TBN and WBCs significantly increased in contrast to that of worms treated with TBN alone. Although both substances have completely different chemical structures (suggesting that they must act on different molecular targets), they have similar effects on the appearance of helminths. In particular, both drugs cause helminth immobilization and paralysis and damage the tegument [17, 19]. TBN acts as a cholinergic agonist of the B subtype and L subtype of nicotinic acetylcholine receptors [11]. In contrast, the precise mechanism of action of PZQ has not yet been elucidated (reviewed in [20]). Exposure of worms to PZQ causes a massive influx of calcium, contraction of the musculature, disruption of the tegument, and effects on calcium channels (reviewed in [20]). The disruption of the tegument presumably allows immune cells of the host to attack the parasite's tissues and organs (reviewed in [20]). Accordingly, simultaneous administration of WBCs with PZQ in our study resulted in a higher mortality rate than that of PZQ alone (Fig. 1a). These findings are in agreement with the data on schistosomiasis. It has been reported that immune effector mechanisms may be synergistically involved in the action of PZQ on worms [21]. In particular, administration of rabbit anti-Schistosoma mansoni antiserum simultaneously with PZQ yielded a greater WBR relative to PZQ alone.

The tegument of trematodes is a multinuclear syncyti-um that is approximately 4 \( \mu \text{m} \) thick and has several vital functions, including protection from immune cells of the host, absorption of nutrients, ion transport and communication with the underlying nervous system. The tegument is in direct contact with muscle fibers, ensuring an instantaneous reaction of the muscles to external stimuli, such as mechanical pressure, an ion gradient, or a gradient of nutrients [22]. Furthermore, the ability to damage the tegument has been described for other anthelmintics, such as artemether [17], artesunate [23] and mefloquine [24]. It should be noted that many anthelmintic agents act on different molecular targets but have the same effect: tegumen-tal damage. This fact does not mean a direct effect of the drugs on a molecular target within the tegument but rather an indirect effect through disturbances in the physiology of helminths and damage to the parasite's tissues and organs.

Mortality in vitro in combined groups of adult worms treated with TBN + WBCs and PZQ + WBCs was almost the same. These findings are in agreement with the results from our in vivo study, where respective single oral doses of TBN and PZQ resulted in similar anthelmintic effects.

Conclusions
For decades, treatment of the liver fluke infection caused by Opisthorchis felineus has been based on PZQ. Our findings on an experimental model showed that TBN is also an efficient drug for therapy of this pathology. Both in vivo and in vitro experiments demonstrated its high efficacy against O. felineus. Hence, it can be a potential candidate for the treatment of the disease.

Abbreviations
DMSO: Dimethylsulfoxide; EPG: Eggs per gram of feces; ERR: Egg reduction rate; IC\textsubscript{50}: Half-inhibitory concentration; PZQ: Praziquantel; TBN: Tribendimidine; WBCs: White blood cells; WBR: Worm burden reduction

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
VAS synthesized tribendimidine and confirmed its purity. MYP developed the study protocol and analysis of the results. EAS was responsible for animal work. VAM provided study oversight. All authors participated in drafting the manuscript, contributed to its revisions and approved the final manuscript.

Ethics approval
All procedures were in compliance with EU Directive 2010/63/EU for animal experiments. The animals were kept and treated according to the protocols approved by the Committee on the Ethics of Animal Experiments of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (ICG SB RAS; permit number: 42 of 25.05.2018).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Author details
1Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (SB RAS), 10 Lavrentiev Ave, Novosibirsk 630090, Russia. 2Novosibirsk State University, 2 Pirogov Str, Novosibirsk 630090, Russia. 3Vorozhtsov Institute of Organic Chemistry of SB RAS, Novosibirsk, Russia. 4Institute of Systematics and Ecology of Animals, SB RAS, Novosibirsk, Russia.

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