International Journal of Antimicrobial Agents 50 (2017) 97–100

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Short Communication

Anthelmintic activity of cytochrome P450 inhibitors miconazole and clotrimazole: in-vitro effect on the liver fluke *Opisthorchis felineus*

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ARTICLE INFO

Article history:
Received 12 July 2016
Accepted 31 January 2017

Keywords:
Flatworms
Cytochrome P450
Drug metabolism
Opisthorchis felineus

ABSTRACT

Discovery of drugs for the treatment of opisthorchiasis and schistosomiasis is a high priority. The basic metabolic cytochrome P450 (CYP) system in parasitic flatworms contains a single gene. CYP of the liver fluke *Opisthorchis felineus*, the causative agent of opisthorchiasis, is important for survival of the worm, so it may be a promising target for therapeutics against liver fluke infection.

The aims of this study were: (i) to analyse in-vitro anthelmintic activity of various CYP inhibitors using standard motility and mortality assays against juvenile and adult *O. felineus* worms; and (ii) to characterize their anthelminthic effects. Azole inhibitors (ketoconazole, miconazole, triadimenol, clotrimazole and 4-phenyl imidazole) and other inhibitors of haem-containing enzymes (disulfiram, metyrapone, benzyl isothiocyanate, and ticlopidine) were tested. This study revealed that inhibitors of haem monooxygenase enzymes possess anthelmintic activity. The most effective anthelmintic agents against the newly excysted metacercariae (NEM) were the antifungal agents miconazole [concentration to reduce the response by 50% (IC_{50}) 0.79 μM] and clotrimazole (IC_{50} 1.25 μM), both approved by the US Food and Drug Administration. The activity of miconazole and clotrimazole was comparable to that for praziquantel (IC_{50} 0.98 μM). In addition, 100% mortality was observed among NEM after 1 d of treatment with 10 μM miconazole, after 3 d of treatment with 10 μM clotrimazole, or after 7 d of treatment with 40 μM ketoconazole. When various CYP inhibitors were tested on adult worms, clotrimazole, miconazole and ketoconazole were found to be the most effective (IC_{50} 13–20 μM). It is speculated that CYP may represent a promising drug target for combined treatment with other anthelmintic agents. The use of inhibitor–drug combinations may improve the action of standard anthelmintic agents.

1. Introduction

Opisthorchiasis and clonorchiasis caused by food-borne *Opisthorchiidae trematodes* represent a substantial public health problem, with 17 million people infected worldwide. *Opisthorchis felineus* (Rivolta, 1884), *Opisthorchis viverrini* (Poirier, 1886) and *Clonorchis sinensis* (Loos, 1907) are three epidemiologically important species of the Opisthorchiidae family (class Trematoda). *C. sinensis* is endemic in China, the Republic of Korea and Northern Vietnam; *O. viverrini* is endemic in Southeast Asia; and *O. felineus* is endemic in Europe and Russia [1].

Human infection results from eating raw or undercooked freshwater cyprinoid fish carrying the metacercariae of the parasite [1,2]. The infection is associated with hepatomegaly, cholangitis, periductal fibrosis and cholecystitis. The International Agency for Research on Cancer considers two helminth infections (*O. viverrini* and *C. sinensis*) to be definite causes of cancer. *O. felineus* is classified as a carcinogen, potentially dangerous for humans [3,4]. The prevalence of *O. felineus* infection in the population of the endemic regions of Western Siberia is 10–45% [1].

At present, praziquantel (PZQ) is the 'drug of choice' for the treatment of opisthorchiasis, clonorchiasis, schistosomiasis and other trematodiases [5,6]. In many endemic regions of the world, PZQ is prescribed as preventive chemotherapy against parasitic infections. Despite the widespread use of PZQ, there are reports of low sensitivity to this agent among trematodes and the appearance of resistant isolates [6]. In addition, PZQ shows low efficacy against juveniles. *Schistosoma mansoni* cercariae and *O. viverrini* and *O. felineus* newly excysted metacercariae (NEM) are less susceptible to PZQ than adults [5,6]. The resistance mechanisms of some isolates and life stages are unknown.

Currently, molecular parasitologists are searching for new agents against trematodiases [7,8] by screening compound libraries and by analysing the parasitic proteins as potential molecular targets for drug discovery.

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http://dx.doi.org/10.1016/j.ijantimicag.2017.01.037
0924-8579/

Cytochrome P450 (CYP) may be a promising target for the development of therapeutic agents against trematodiases. CYPs are a widespread, diverse superfamily of haem mono-oxygenases that participate in drug biotransformation and in the biosynthesis of secondary metabolites and physiologically important molecules such as sterols, fatty acids and prostaglandins [9]. Previously, the authors demonstrated that parasitic flatworms, including liver flukes (Opisthorchiidae, Fasciolidae), blood flukes (Schistosomatidae) and cestodes (Taeniidae), have a single CYP gene [10].

The authors' recent studies indicated that *O. felineus* CYP is active in tissues, and its function is critical for survival of the worm. Additionally, the azole inhibitor ketoconazole decreased CYP enzymatic activity in tissues [11], and ketoconazole altered the phenotype of the adult worm. The specific substrates for this enzyme and the role of CYP in the parasitic lifestyle remain unknown.

The aims of this study were: (i) to analyse in-vitro anthelmintic activity of various CYP inhibitors using standard motility and mortality assays against juvenile and adult *O. felineus* worms; and (ii) to characterize the markers of their anthelminthic actions.

2. Materials and methods

2.1. Hamsters and experimental design

Syrian hamsters (*Mesocricetus auratus*) were purchased from the Puschino Animal Facility (Russia) and bred at the Animal Facility (RFMEFI61914X0005) of the Institute of Cytology and Genetics Siberian Branch of the Russian Academy of Sciences. All of the procedures were in compliance with the 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 (Permit Number 25 of 12.12.2014). Euthanasia was performed by carbon dioxide inhalation, and all efforts were made to minimize suffering.

O. felineus metacercariae were collected from naturally infected fish (*Leuciscus idus*) caught in the Ob River near Novosibirsk (Western Siberia) and extracted accordingly [5]. Animals (male Syrian golden hamsters, aged 6–8 weeks) were orally infected with 70 *O. felineus* metacercariae.

2.2. Drugs

PZQ (Bayer, Leverkusen, Germany), disulfiram, ketoconazole, metyrapone, miconazole, clotrimazole, 4-phenyl imidazole (4PIM) and triademinol (all from Sigma-Aldrich, St Louis, MO, USA) were dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich) to obtain 1 mM stock solutions. Benzyl isothiocyanate (BITC) (Sigma-Aldrich) and ticlopidine (Sigma-Aldrich) were dissolved in 100% methanol (Sigma-Aldrich) to obtain 1 mM stock solutions.

2.3. In-vitro study

NEM were hatched from metacercariae [5]. Flukes were recovered from the livers of hamsters and washed thoroughly with sterile saline (0.9% NaCl). Worms were incubated at 37 °C for 24 h in RPMI 1640 culture medium (Life Technologies, Camarillo, CA, USA) supplemented with 100 units/mL penicillin, 0.1 mg/mL streptomycin and 0.25 μg/mL amphotericin B (Sigma-Aldrich), and 1% glucose in a CO_2 incubator [5,11].

Worms were incubated and their viability was evaluated under an inverted microscope (Axiovert 40CFL, Zeiss, Jena, Germany) equipped with a camera (Axiocam ICC3, Zeiss) after 24 h of treatment (magnification 10–50×). The worms were classified as dead if they had a dark colour and no movement was observed for 2 min [5,8]. To calculate the concentration to reduce the response by 50% (IC_{50}), the following concentrations of compounds were tested: 0.001, 0.01, 0.1, 1, 10, 100 and 500 μM (dilution factor 1:200). The solvent concentration across different compound concentrations was 0.5%. Four to five adult worms and 30–40 NEM were used per well. The experiments were repeated three times with two or three replicates for each concentration. Flukes incubated in the medium were used as controls, with either 0.5% DMSO or 0.5% methanol depending on the compound.

The motility of viable worms was assessed using standard motility tests [5,8]. Logistic regression of the four parameters was used to calculate IC_{50} and standard error values ('drc 3.0-1' R package). Analysis of variance lack-of-fit test was used to study the hypothesis that a proposed regression model fits the data well.

To estimate the mortality rates, NEM were treated with one of the following agents: 10 μM BITC, 10 μM disulfiram, 10 μM miconazole, 10 μM clotrimazole, 10 μM 4PIM, 40 μM ketoconazole or 1% DMSO and incubated at 37 °C for 18 d. The effect of each drug was repeated three times. To estimate the mortality rates, Kaplan–Meier survival curves were built using the 'survival' (v.2.38) R package [11]. The statistical difference in the survival log-rank (Mantel–Haenszel) test between each pair of samples was calculated.

3. Results

Azole CYP inhibitors were used for testing of anthelmintic activity: ketoconazole, miconazole, triadimenol, clotrimazole and

Table 1
IC_{50} values of cytochrome P450 (CYP) inhibitors against newly excysted metacercariae and adult *Opisthorchis felineus* worms.

Compound	Newly excysted metacercariae		Adults		Target human haem-containing enzymes
	IC_{50} value ± SE (μM)	*P*-value	IC_{50} value ± SE (μM)	*P*-value	
Praziquantel	0.98 ± 0.18	0.98	0.47 ± 0.05	0.95	–
Miconazole	0.79 ± 0.18	0.994	20.05 ± 0.09	0.95	2B6, 2C9, 2C19 3A4, 2A6, 2D6 [12]
Clotrimazole	1.25 ± 0.33	0.94	18.03 ± 0.35	0.95	2B6, 2C9, 2C19 3A4, 2A6 [12]
Ketoconazole	16 ± 1.25	0.98	13.77 ± 0.48	0.96	2C9, 3A4 [12]
Benzyl isothiocyanate	16 ± 0.08	0.96	27.2 ± 0.06	0.95	2B1, 2B6, 1A1, 2E1 [9]
4-Phenyl imidazole	368 ± 29	0.96	1050 ± 48	0.95	Indoleamine-2,3-dioxygenase [9]
Triadimenol	220 ± 17.5	0.95	208 ± 45	0.94	Aromatase and CYP51 [13]
Ticlopidine	40 ± 4.2	0.95	147 ± 36	0.93	2C19, 2B6 [9]
Metyrapone	199 ± 13.3	0.98	99 ± 0.51	0.98	3A4, 11 p-hydroxylase [9]
Disulfiram	764 ± 104	0.94	364 ± 62	0.95	2E1 [9]

IC_{50}, concentration to reduce the response by 50%; SE, standard error.
Data are presented as IC_{50} values ± SE ('drc 3.0-1' R package). *P*-value—analysis of variance lack-of-fit test was used to study the hypothesis that a proposed regression model fits the data well ('drc 3.0-1' R package).