Acta Tropica xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Acta Tropica

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

Combined flubendazole-nitazoxanide treatment of cystic echinococcosis: Pharmacokinetic and efficacy assessment in mice

Ceballos Laura[a,*], Elissondo Celina[b,c], Sánchez Bruni Sergio[a], Denegri Guillermo[b,c], Lanusse Carlos[a], Alvarez Luis[a]

[a] Laboratorio de Farmacología, Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET, Facultad de Ciencias Veterinarias, UNCPBA, Campus Universitario, 7000 Tandil, Argentina
[b] Laboratorio de Zoonosis Parasitarias, Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Funes 3250, 7600 Mar del Plata, Argentina
[c] Argentina and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

ARTICLE INFO

Article history:
Received 16 December 2014
Received in revised form 8 April 2015
Accepted 19 April 2015
Available online xxx

Keywords:
Flubendazole
Nitazoxanide
Cystic echinococcosis

ABSTRACT

The current chemotherapy of cystic echinococcosis (CE) is mainly based on the use of albendazole, and the results have been shown to be highly variable. Thus, new and more efficient treatment options are urgently needed. The goals of the current study were: (a) to compare the *ex vivo* activity of flubendazole (FLBZ) and nitazoxanide (NTZ), given either separately or co-administered, against *Echinococcus granulosus* protoscoleces and cysts, (b) to characterize the plasma disposition kinetics of FLBZ administered alone or combined with NTZ in mice; (c) to compare the *in vivo* activity of FLBZ and NTZ (either each alone or as a combined treatment) against secondary CE developed in mice. *Ex vivo drug activity study*: *E. granulosus* protoscoleces and cysts were incubated either with FLBZ, NTZ, or the FLBZ-NTZ combination. Protoscoleces and cyst viability was monitored by the methylene blue exclusion test and scanning electron microscopy (SEM). *Pharmacokinetic study*: Balb/C mice received FLBZ (5 mg/kg) orally either alone or co-administered with NTZ (100 mg/kg). Blood samples were collected up to 12 h post treatment and plasma analyzed for FLBZ/metabolites by HPLC. *Clinical Efficacy study*: following secondary infection, meaning i.p. injection of 1500 *E. granulosus* protoscoleces/animal ($n = 40$), the both drugs were administered by intragastric inoculation on a daily basis for a period of 25 days. Balb/C mice received FLBZ (5 mg/kg, twice a day) alone, NTZ (100 mg/kg, once daily) alone or a combination of both molecules (FLBZ, 5 mg/kg twice a day and NTZ, 100 mg/kg, once daily). Ten untreated animals were used as a control. All animals were killed and the weight of the cysts collected from each animal was recorded. The presence of NTZ did not markedly affect the FLBZ kinetic parameters in mice. FLBZ alone or combined with NTZ induced a reduction ($P < 0.05$) of cyst weight in comparison to the untreated control and NTZ-treated treated mice. The data obtained here indicate that NTZ did not affect hydatid cyst development in mice. Conversely, FLBZ shows an excellent efficacy against CE.

1. Introduction

The neglected tropical diseases (NTD) are diseases, which mainly affect the world's poorest. Although effective chemotherapy and other treatment options are available, these diseases still prevail amongst the poorest populations in the world. Recently, the World Health Organization (WHO) included human cystic echinococcosis (CE) in the list of NTDs (Budke et al., 2009). CE, known as hydatid disease is caused by larval stages (metacestodes) of the *Echinococcus granulosus*-complex. This cestode parasite has a worldwide distribution. The cycle of *E. granulosus* involves predominantly dogs as final hosts, and sheep, pigs, cattle, camels, and accidentally humans as intermediate hosts, in which a slowly expanding hydatid develops. The unilocular cystic lesions are most often located in the liver, but also in the lungs and in other organs and are the causative agent of CE (Eckert and Deplazes, 2004).

Currently, four treatment modalities are in use: 1- surgery, 2-PAIR (puncture, aspiration, injection of protoscolicidal agent, respiration), 3-chemotherapy with the benzimidazole (BZD) compounds albendazole (ABZ) or mebendazole (MBZ), and 4-watch and wait for inactive, clinically silent cysts. The evidence supporting any of the four treatment modalities, from carefully designed

* Corresponding author. Tel.: +54 2494 439850; fax: +54 2494 439850.
E-mail address: ceballos@vet.unicen.edu.ar (C. Laura).

http://dx.doi.org/10.1016/j.actatropica.2015.04.019
0001-706X/

clinical studies, is insufficient, and choosing treatment options for patients remains controversial (Stojkovic et al., 2009). ABZ and MBZ are given at the WHO recommended dosages of 10–15 mg/kg/day (ABZ) and 40–50 mg/kg/day (MBZ). Although chemotherapy was initially recommended for inoperable patients and patients with multiple organ disease, several studies, mainly case series, have been published suggesting that chemotherapy could be an alternative to surgery in patients with uncomplicated cysts, leading to an increased use of chemotherapy over the years (WHO, 2001). However, the response to BZD treatment is frequently unpredictable (El-On, 2003). The large variability observed in the therapeutic success of BZD anthelmintics on CE may be explained by the host immunological status and/or the features of the cysts, including their size and location. Furthermore, the poor/erratic gastrointestinal absorption is a common inconvenience for the systemic availability of orally administered BZD in most species (Lanusse and Prichard, 1993). Even though alternative drugs have been searched for most of the compounds investigated so far did not progress into clinical development (Hemphill et al., 2007).

Flubendazole (FLBZ), a methylcarbamate BZD, is highly active against a broad spectrum of gastrointestinal nematodes in humans and some animal species. Besides, FLBZ has been evaluated for CE treatment in both mouse (Ceballos et al., 2009) and man (Recco et al., 1984). Interestingly, protoscoleces (PSC) incubated with FLBZ were damaged faster than those cultured with ABZ or its active metabolite, ABZ-sulphoxide (ABZSO) (Elissondo et al., 2006).

Nitazoxanide (NTZ), a thiazolide compound, is another broad-spectrum drug, approved for treating human protozoan and helminthic infections (Hemphill et al., 2006). Nothing is known on the possible mode of action of NTZ concerning helminths, however, the enzymes of the anaerobic electron transport could be considered as potential targets (Stettler et al., 2004). There is *in vitro* evidence to support the efficacy of NTZ against metacetodes of *Echinococcus multilocularis* (Stettler et al., 2003) and *E. granulosus* (Walker et al., 2004). Additionally, combined treatment of NTZ with ABZ against *E. multilocularis* cysts had better efficacy than did ABZ alone (Stettler et al., 2004). The rationale behind using this particular anthelmintic preparation is based on the different mechanism of action of each compound.

There is no information on either potential pharmacokinetic (PK) and/or pharmacodynamic (PD) interactions occurring after co-administration of FLBZ and NTZ. A drug interaction refers to the possibility that one drug may alter the intensity of the pharmacological effects of another drug given concurrently. The modified effect may result from a change on the relationship between drug concentration and response of the organism to the drug (PD interaction) or from a change in the concentration of either one or both drugs (PK interaction). In this context, the simultaneous use of FLBZ and NTZ in the treatment of CE could improve the efficacy of the treatment. In fact, after the combination of ABZ and NTZ in mice, a higher plasma drug exposure of ABZ-sulphoxide has been reported (Stettler et al., 2004).

The goal of the current study was to compare the plasma pharmacokinetic behaviour and clinical efficacy of FLBZ administered alone or combined with NTZ on secondary CE developed in mice. These studies are conducted as foundations on the search of alternative treatment of cystic echinococcosis.

2. Materials and methods

2.1. Chemicals

Pure reference standards of FLBZ, reduced-FLBZ (R-FLBZ) and hydrolyzed-FLBZ (H-FLBZ) used to develop the analytical methodology were kindly provided by Janssen Animal Health (Beerse, Belgium). Oxibendazole (OBZ), used as internal standard, was obtained from Sigma Chemical Company (Saint Louis, MO, USA). Nitazoxanide was kindly provided by Roemmers (Buenos Aires, Argentina). HPLC grade acetonitrile and methanol were from Sintorgan S.A. (Buenos Aires, Argentina) and J.T. Baker (New Jersey, USA), respectively. Cargill Inc. (Hammond, IN, USA) kindly supplied us the Hydroxy-propil-β-cyclodextrins (CDs). Low viscosity grade sodium CMC was purchased from Anedra (Buenos Aires, Argentina). Dimethyl sulphoxide (DMSO) was purchased from Biopack S.A. (Buenos Aires, Argentina). Medium 199 was purchased from Gibco (Invitrogen, Buenos Aires, Argentina). The FLBZ solution was prepared according to Ceballos et al. (2009). The NTZ-suspension was prepared by addition of NTZ (0.3%) and carboxymethylcellulose (CMC, 0.5%) in deionized water (pH = 6.0) with shaking for 6 h. The NTZ suspension was vigorously shaken immediately before intragastric administration to mice.

2.2. Protoscoleces collection and culture

PSC of *E. granulosus* were aseptically collected from liver hydatid cysts of infected cattle slaughtered in an abattoir located in the southeast of Buenos Aires province, Argentina. Vitality was assessed by muscular movements (evaluated under light microscope). The culture protocols were carried out using medium 199 supplemented with 100 IU penicillin, 100 μg/mL streptomycin, 4 mg/mL glucose and 20% (v/v) foetal calf serum.

2.3. Experimental animals

2.3.1. Ethics statement

One hundred and twenty eight (128) Balb/C healthy mice (approx. 30 g) were housed in a controlled temperature (21 ± 2 °C), light-cycled (12 h light/dark cycle) room. Food and water were provided *ad libitum*. Animal procedures and management protocols were approved by the Ethics Committee according to the Animal Welfare Policy (act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar) and according to the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington DC, National Academy Press, 2011).

2.4. Experimental design

2.4.1. Ex vivo *drug activity study on PSC and cysts*

FLBZ and NTZ were dissolved in dimethyl sulphoxide (DMSO) at a drug concentration of 1 and 0.1 mg/mL. The two drugs were added to the medium either separately or in combination at the following final concentrations: 10 μg/mL FLBZ, 1 μg/mL FLBZ, 10 μg/mL NTZ, 1 μg/mL NTZ, 10 μg/mL FLBZ + 10 μg/mL NTZ, 1 μg/mL FLBZ + 10 μg/mL NTZ. Both PSC (1500) and cysts (obtained from BALB/c mice, $n = 10$) were placed in Leighton tubes containing cultured medium 199, supplemented with 100 IU penicillin, 100 μg/mL streptomycin and 4 mg/mL glucose. Cultures were performed in 10 mL of incubation medium at 37 °C without changes of medium (Elissondo et al., 2006, 2007). Each experiment was repeated three times.

2.4.1.1. Protoscoleces. PSC incubated with culture medium containing 20 μL DMSO served as controls. Culture tubes were followed microscopically every day to determine the appearance of morphological alterations. Samples of PSC (approximately 90–100 PSC in 180 μL of incubation medium) from each dosing groups and the controls were taken every 5–6 days for viability assessment using the methylene blue exclusion test. Additionally, ultrastructure studies with scanning electron microscope (SEM) were performed.

Please cite this article in press as: Laura, C., et al., Combined flubendazole-nitazoxanide treatment of cystic echinococcosis: Pharmacokinetic and efficacy assessment in mice. Acta Trop. (2015), http://dx.doi.org/10.1016/j.actatropica.2015.04.019