Journal of Microbiological Methods 87 (2011) 169–175

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

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

Exploration of novel in vitro assays to study drugs against *Trichuris* spp.

Angelika Silbereisen, Lucienne Tritten, Jennifer Keiser *

Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Socinstr. 57, 4002 Basel, Switzerland and University of Basel, Petersplatz 1, 4002 Basel, Switzerland

ARTICLE INFO

Article history:
Received 30 June 2011
Received in revised form 12 August 2011
Accepted 16 August 2011
Available online 24 August 2011

Keywords:
Alamar Blue
Microcalorimetry
Motility assay
MTT
Trichuris muris
xCELLigence System

ABSTRACT

Though trichuriasis is a significant public health problem, few effective drugs are available underscoring the need for new drug therapies. For the evaluation of trichuricidal activity of test compounds in vitro an accurate, reliable, sensitive, fast and cheap drug sensitivity assay is essential. The aim of the present investigation was to evaluate the performance of different in vitro drug sensitivity assays in comparison to the standard motility assay. *Trichuris muris* L4 larvae or adult worms were isolated from the intestinal tract from infected female C57BL/10 mice and incubated in the presence of ivermectin, levamisole and nitazoxanide (200, 100 and 50 μg/ml) for 72 h. The health status of the worms was either evaluated microscopically using a motility scale from 0 to 3 (motility assay), by examination of absorbance or emission in response to metabolic activity (MTT (Thiazolyl Blue Tetrazolium Bromide) and Alamar Blue assay), through analysis of absorbance of an enzyme-substrate reaction (acid phosphatase activity assay), by measuring the noise amplitudes (isothermal microcalorimetry and xCELLigence System) or the heat flow (isothermal microcalorimetry) of *T. muris*. The Alamar Blue assay, xCELLigence and microcalorimetry compared favorably to the standard motility assay. These three assays precisely determined the trichuricidal activity of the three test drugs. The acid phosphatase and the MTT assays showed a poorer performance than the motility assay. In conclusion, the colorimetric Alamar Blue in vitro assay is a good alternative to the motility assay to study drug effects against *T. muris* L4 and adults, since it is easy to perform, precise and of low cost.

1. Introduction

The gastrointestinal nematode *Trichuris trichiura* (whipworm) belongs to the soil-transmitted helminths and is a truly neglected parasite though infecting an estimated 604–795 million people worldwide (Bethony et al., 2006; Hotez et al., 2009). In highly endemic areas, children between 5 and 15 are the main age group at risk with a prevalence of up to 90% (Stephenson et al., 2000). Trichuriasis is prevalent in many countries in the tropics and subtropics, especially East Asia, the Pacific islands, sub-Saharan Africa, Latin America and the Caribbean (Bethony et al., 2006). Light *T. trichiura* infections are often asymptomatic, but abdominal pain, bloody diarrhea, anemia and rectal prolapses are common symptoms occurring in severe and chronic stages (Stephenson et al., 2000). The two benzimidazole drugs, albendazole and mebendazole, are intensively used for the treatment and control of soil-transmitted helminth infections, commonly in the framework of mass drug administration programs (Keiser and Utzinger 2010). The World Health Organization recommends a single-dose treatment with either 400 mg albendazole or 500 mg mebendazole, whereas the frequency of treatment intervention depends on the prevalence of the disease (WHO 2006). Due to the fact that the cure rates of both drugs against infections with *T. trichiura* are only moderate (Keiser and Utzinger 2010), there is a need to develop novel therapies.

In order to identify novel trichuricidal drug candidates a reliable, sensitive, fast and accurate in vitro test system is preferred. The current strategy to analyze drug sensitivity in vitro against different nematodes involves using the larval/adult motility assay (Kotze et al., 2004, 2005) which characterizes the worm viability under the microscope by using a motility scale. However, microscopic techniques are often time-consuming, low throughput, labor intensive, have to be carried out by experienced personnel and have a subjective nature. The aim of this study was therefore to evaluate several in vitro drug sensitivity assays using the mouse strain *Trichuris muris* and to compare the performance of these assays with the motility assay. We selected two standard drugs, ivermectin and levamisole, as well as the antiprotozoal drug nitazoxanide for our studies since this drug showed a high trichuricidal activity in previous in vitro tests we performed (Tritten et al., manuscript submitted for publication). Three colorimetric assays (Alamar Blue, MTT (Thiazolyl Blue Tetrazolium Bromide) and acid phosphatase activity assay) as well as two assays based on real-time monitoring of viability and heat flow (xCELLigence System and isothermal microcalorimetry) were included in our study.

Abbreviations: Lev, levamisole; Iver, ivermectin; NTZ, nitazoxanide; HF, heat flow; HFR, heat flow reduction; a.l., amplitude length.

* Corresponding author. Tel.: +41 61 284 82 18; fax: +41 61 284 81 01.
E-mail address: jennifer.keiser@unibas.ch (J. Keiser).

0167-7012/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2011.08.009

Briefly, viability marker assays are non-radioactive colorimetric assays based on the color change of dyes such as Alamar Blue (Raz et al., 1997; O'Brien et al., 2000) and MTT (Hordegen et al., 2006; Townson et al., 2006) to determine the health status of parasites, cells or bacteria. Healthy parasites ingest the dye resulting in a reduction process and color change of the dye due to chemical or enzymatic reactions by the parasite. The underlying principle of the acid phosphatase activity assay is the high acid phosphatase activity of gastrointestinal nematodes within their intestines. Acid phosphatase has been detected in excretory/secretory products of several nematode species, released in the culture medium (Maki and Yanagisawa 1980a, 1980b, 1980c; Moulay and Robert-Gero 1995; Fetterer and Rhoads 2000). The acid phosphatases react with the substrate pNPP (p-nitrophenyl phosphate) present in the medium, forming a yellow water-soluble product with a strong absorbance. The xCELLigence System, mainly used for cell-based assays, has recently been described to be a suitable technique to measure the motility of different helminths in real time in a fully automated high-throughput manner (Smout et al., 2010). Microelectrodes at the bottom of each well of the E-plate measure the impedance, which can be used to express the worm's viability as noise amplitudes. Finally, isothermal microcalorimetry (Yan et al., 2008) examines the heat flow (endo- or exothermic reactions) of biological processes. This method has recently proven to be an excellent tool to study drug effects on *Schistosoma mansoni* and *Fasciola hepatica* (Manneck et al., 2010; Kirchhofer et al., 2011).

2. Materials and methods

2.1. Drugs, chemicals, reagents and media

Ivermectin was purchased from Sigma-Aldrich (Buchs, Switzerland) and levamisole from Fluka (Buchs, Switzerland). Nitazoxanide was kindly obtained from Laboratoria Wolfs (Zwijndrecht, Belgium). Drug stocks (10 mg/ml) were prepared in 100% DMSO (dimethylsulfoxid, Sigma-Aldrich, Buchs, Switzerland) and stored at 4 °C pending use.

Alamar Blue (resazurin sodium salt, 125 mg/l), MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), p-nitrophenyl phosphate (pNPP), sodium acetate anhydrous (NaAc), amphotericin B (250 µg/ml) and penicillin-streptomycin (10,000 units penicillin + 10 mg streptomycin per ml) were purchased from Sigma-Aldrich (Buchs, Switzerland). Sodium hydroxide (NaOH) was purchased from Fluka (Buchs, Switzerland), Hank's Balanced Salt Solution (HBSS 1X, [−] phenol red, [−] calcium, [−] magnesium) and Fetal Calf Serum (FCS) from Gibco (Basel, Switzerland). RPMI medium was prepared using 10.44 g RPMI 1640 (Gibco), 5 g albumax H (Gibco), 5.94 g hepes (Sigma-Aldrich) and 2.1 g sodium bicarbonate (Sigma-Aldrich) in 1 l deionized water.

2.2. Animals and parasite

Three to five weeks old female C57BL/10 mice were purchased from Harlan Laboratories (Blackthorn, England). Mice were allowed to acclimatize for one week before infection. All animals were kept in groups of 10 mice in macrolon cages with free access to water and rodent food pellets (Rodent Blox from Eberle NAFAG, Gossau, Switzerland) and with a 12 hour light/dark cycle according to Swiss Animal Welfare guidelines.

We used the mouse strain *T. muris*, which is an excellent experimental model for whipworm infections (Worley et al., 1962). Embryonated eggs from *T. muris* were kindly provided by Prof. Jerzy Behnke (University Park of Nottingham, United Kingdom) and Prof. Heinz Mehlhorn (University of Düsseldorf, Germany).

Unembryonated eggs released in the feces of infected mice were isolated and purified through flotation (Ghiglietti et al., 1995) with saturated NaCl (359 g/l in distilled water) and cultured in tap water at room temperature. Embryonation of eggs was controlled under the microscope (magnification 80x, Carl Zeiss, Germany). Each mouse was treated with 4 mg/l dexamethasone (Sigma-Aldrich) supplied with the drinking water two days prior to infection until the end of the experiment (Campbell 1968; Wakelin 1970). All animals were orally infected with 400 embryonated *T. muris* eggs (Wakelin 1970; Stepek et al., 2006). The development of L4 larvae in the gastrointestinal tract was completed between days 26 and 28 after infection (p.i.). Adult *T. muris* worms were present in the mice's gut from day 35 p.i. onwards (Fahmy 1954; Stepek et al., 2006). We decided to use in the present work adult worms as well as the larval stage 4 (L4) of *T. muris* because both parasite stages have an ideal size, are robust and easy to handle.

2.3. In vitro studies

Mice were euthanized by exposure to CO_2 on days 26–28 or from day 35 onwards, respectively. The entire intestine was dissected and placed in a Petri dish containing 0.9% NaCl. Next, it was opened longitudinally using forceps under the binocular microscope (magnification 16x) and isolated parasites were transferred into pre-warmed RPMI medium containing 5–10% amphotericin B and 1% penicillin-streptomycin and maintained at 37 °C and 5% CO_2. Unless otherwise mentioned, RPMI medium supplemented with antibiotics was the medium of choice. Ivermectin, levamisole and nitazoxanide were tested in concentrations of 200, 100 and 50 µg/ml (motility assay, Alamar Blue assay, MTT assay and acid phosphatase assay). Drug concentrations of 50 µg/ml were used in the xCELLigence and microcalorimetry assays. In all assays at least three worms incubated in the highest DMSO concentration used in the test and medium blanks served as controls. All experiments were carried out at least in duplicate. The in vitro assays were performed either in 96-well plates, incubating worms in a total volume of 200 µl (100 µl drug solution) or in 48-well plates and 1 ml medium (500 µl drug solution). Parasites were incubated for at least 72 h at 37 °C and 5% CO_2.

2.3.1. Motility assay

Following incubation with the test drugs as described above, the motility of L4 larvae was evaluated under the microscope (magnification 20–80x) after periods of 24, 48 and 72 h using a motility scale from 0 to 3 (0 = dead, 1 = very low motility, 2 = low motility, 3 = normal motility) (Stepek et al., 2006). Worms classified as 1 (very low motility) showed a strongly reduced viability and/or were only able to move on one end. Worms which showed less viability than the controls were recorded as category 2 (low motility).

2.3.2. Alamar Blue assay

Following incubation of 4–6 L4 larvae with drugs for 72 h, larvae were transferred into 200 µl fresh RPMI medium. The motility of worms was examined as described in Section 2.3.1. Ten microliter resazurin was added and the plates were incubated for another 4 h. The fluorescent emission of the reduced dye was measured at 588 nm using a spectrofluorometer (SpectraMax, Gemini XS, Molecular Devices, UK).

2.3.3. MTT assay

Four L4 larvae were placed into 100 µl medium within a 96-well plate. One hundred microliters of the drug solution to be tested was added and the plate was incubated for 72 h. The motilities of worms were documented. The worms were then carefully transferred into 100 µl fresh medium. In a next step, 100 µl MTT reagent (6 mg/ml in deionized water) was added per well and the plate was incubated for 1 h at 37 °C and 5% CO_2. To solubilize the formazan crystals, the larvae were transferred into a new 96-well plate containing 200 µl 100% DMSO and incubated for another hour. Then the worms were removed and the plate agitated gently to homogenize the solution.