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Research Brief

In vitro evaluation of the effects of cysticidal drugs in the *Taenia crassiceps* cysticerci ORF strain using the fluorescent CellTracker CMFDA

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ABSTRACT

Using a murine model of cysticercosis caused by the *Taenia crassiceps* ORF strain, we developed a fluorescent quantitative evaluation of the action of two well known anti-helminthic drugs: albendazole sulfoxide and praziquantel. The fluorescence emitted by a biotransformed CellTracker Probe known as CellTracker Green CMFDA in the vesicular fluids of cysticerci was estimated, and the results were compared with macroscopic observations of the parasites. The pharmacological EC_{50} value of each drug and changes in the level of biotransformation of the fluorescent tracker caused by the drugs could be easily calculated. These drug-induced changes in biotransformation could be related to changes in the GSH/GSSG ratio of parasites. Both the cysticercosis murine model and the CMFDA biotransformation assay could be used as an *in vitro* screening method to evaluate potential or well known cysticidal drugs.

1. Introduction

An *in vitro* assay can be a very useful tool for screening anti-parasitic drugs and may have a variety of applications, including the identification of any potentially interesting new compounds. The success of an *in vitro* assay depends on a well adapted model and a screening method that does not require visual inspection and is not time-consuming nor expensive (Hemphill et al., 2009). The murine metacestode model *Taenia crassiceps* ORF strain (Palomares-Alonso et al., 2007, 2009a; Palomares et al., 2004, 2006) has features that would allow it to be used to create a successful *in vitro* drug-screening assay. It has been considered useful for performing studies applicable to the cysticerci of *T. solium* (Brehm and Spiliotis, 2008; Willms and Zurabian, 2009) and has been shown to be suitable for establishing pharmacological parameters of the well known drugs with clinical use, such as albendazole, praziquantel and nitazoxanide (Jung et al., 2008; Palomares-Alonso et al., 2009a; Palomares et al., 2004, 2006) or for the *in vitro* evaluation of experimental substances with potential cysticidal action (Palomares-Alonso et al., 2009a). Despite these features, there is no guarantee that observed changes in the behavior or the morphology of cysticerci is directly related to its physiological or metabolic state. As a result, further evaluations are necessary. Thus, visual changes in parasites after an *in vitro* drug treatment are only semi-qualitative because they are based on the ability of the examiner and subtle changes in the organisms that must be analyzed at the ultrastructural level (Hemphill et al., 2009; Palomares et al., 2004, 2006).

The viability of cells can be evaluated using fluorescent dyes (Haugland, 2005), known as CellTracker Probes, that serve as indicators of the metabolic activity of cells or represent the physiological status of the organism. These fluorescent probes, such as the CellTracker Green 5-chloromethylfluorescein diacetate reagent (CMFDA), have been useful for developing a rapid and objective method for testing fungal susceptibly to drugs (Arunmozhi Balajee et al., 2005), for evaluating the glutathione cellular content in rat thymocytes treated with thimerosal (Ueha-Ishibashi, 2004) and for evaluating the behavior of the parasites during an *in vivo* cestode infection of copepods (Kurtz et al., 2002). There are several properties that account for the effectiveness of this vital fluorescent probe: in its initial, non-fluorescent state, it can freely diffuse through the cellular plasma membrane; once inside the cell, a detoxification process through the glutathione metabolic pathway, which can only be executed by living cells, results in the conjugation of glutathione to the compound, and the resulting product is an impermeable and brightly fluorescent probe (Haugland, 2005).

One aim of this study was to quantitatively evaluate the biotransformation of CMFDA in a fluorescent CellTracker in *T. crassiceps* cysticerci grown *in vitro* and to determine, by quantitative fluorescent measurements, if this biotransformation can be

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altered by the addition of two cysticidal drugs, albendazole sulfoxide and praziquantel. Another objective was to evaluate the biotransformation activity of metacestodes and to determine if this assay, based upon drug-induced alterations of the biotransformation of the fluorescent probe, could be adapted for establishing pharmacological parameters, such as the concentration that produced 50% effect (EC_{50}), of drugs used against these parasites.

2. Materials and methods

2.1. Parasites

The ORF strain of *T. crassiceps* cysticerci were harvested from the peritoneal cavity of BALB/c female mice infected 3 months after inoculation with cysticerci. Parasites were maintained by serial passage in mice through intraperitoneal injection with 30 ± 5 metacestodes. Mice were killed after 12 weeks of infection, and the parasites were harvested from the peritoneal cavity of the mice in sterile conditions and washed 3× in PBS, pH 7.2. The metacestodes were then maintained *in vitro* as described below. This study was approved by the local ethics committee.

2.2. In vitro culture of parasites

After recovering the cysticerci from mice, the metacestodes were maintained *in vitro* in RPMI 1640, pH 7.2, supplemented with 3.4 mM HEPES, 11.9 mM sodium bicarbonate and an antibiotic–antimycotic solution (10,000 UI/mL penicillin, 10 μg/mL streptomycin and 2.5 μg/mL of amphotericin B). All cell culture reagents were from GIBCO BRL LIFE TECHNOLOGIES (Grand Island, NY, USA). Parasites were maintained at 37 °C, 5% CO_2 and 90% relative humidity in 4 ml of culture medium in microwell plates (NUNC, Roskilde, Denmark), and the culture medium was replaced every 24 h. During culture, parasites were monitored for integrity, motility and morphological aspect using a microdissection microscope (4× magnification).

2.3. Chemicals and reagents

Albendazole sulfoxide (ABZSO) was synthesized and kindly donated by Dr. Rafael Castillo of Facultad de Química (UNAM, México) according to a standard procedure (Soria-Arteche et al., 2005). Praziquantel (PZQ) was obtained from Sigma Chemical Co. (St Louis, Mo, USA) (assay 99%).

2.4. Labeling of cysticerci with CMFDA

The parasites were treated with CellTracker CMFDA (Molecular Probes, Eugene, Or, USA) as follows. Briefly, after incubation of parasites in the presence or absence of drugs, 2 ml of the supernatant medium was substituted with 2 mL of fresh medium containing 6 μM of CMFDA, and the parasites were incubated for 45 min as described for *in vitro* culture. The supernatant medium with CMFDA was then replaced with fresh medium, and the parasites were incubated for 30 min as described above. Finally, the culture supernatant was replaced with a similar volume of 3.7% formaldehyde, and the parasites were incubated for 15 min at room temperature. Fixed parasites were recovered, washed three times with PBS pH 7.2 and stored at 4 °C in light-proof boxes until use.

2.5. Determination of CMFDA fluorescence in cysticerci

Two strategies were followed to quantify the fluorescence due to CMFDA. The initial strategy involved quantifying the intensity of all of the parasites using a Molecular Imager ChemiDoc XRS +System device (BioRad, Hercules, Cal, USA) containing a 520DF30 62 mm filter for GFP/SYBR Gold/fluorescence attached to a supercooled high-resolution CCD camera and a computer with a QuantityOne 1-D Analysis Software V. 4.6 (BioRad). Each group of cysticerci were considered as if they were ready for colony counting. The other strategy for fluorescence quantification was performed as follows. Briefly, after washing, each formaldehyde-fixed cyst was punctured with a 25 G syringe needle, and the extracted fluid was centrifuged at 9503*g* for 30 min at 4 °C. The two fractions obtained after centrifugation were the supernatant, which corresponded to the vesicular fluid (VF), and the pellet, which corresponded to the bladder wall (BW). For the fluorescence measurement, 25 μL of the supernatant was added to 2.5 mL of distilled water kept at RT, and the sample was kept under constant magnetic stirring. Measurements (counts of fluorescence/s, considered as Arbitrary Units of Fluorescence) were performed in a PTI spectrofluorometer (Photon Technology International, Birmingham, NJ, USA) using excitation at 490 nm and detection at 515 nm. Distilled water was used as a blank solution. To estimate the best and specific excitation wavelength for fluorescent CMFDA in VF, comparisons were performed between diluted VF and the blank solution that were scanned using several wavelengths, ranging from 450 to 550 nm.

2.6. Evaluation by microscopy

To observe changes in the parasites, macroscopic observations were done using an Olympus SZX7 inverted stereoscopic light microscope coupled to an epifluorescence device that contained a mercury lamp (100 W) and a 510 nm filter (Olympus Corporation, Sinyuho, Japan). Images were taken with a Kodak Z700 photographic camera, and the contrast of the resulting JPEG images were adjusted using Adobe Photoshop program V 7.0.

2.7. Cysticidal activity

Groups of 30 ± 5 cysts were incubated *in vitro* as described and evaluated after a single drug exposure over the course of 11 days. Parasites were added to 2.5-cm diameter multiwell plates (NUNC) with 4 mL of culture medium that contained the drug for evaluation. Estimations of EC_{50} were performed at selected concentrations for each drug: 0.01, 0.025, 0.1, 0.25 and 1.25 μg/mL for ABZSO; 0.001, 0.003, 0.01, 0.03, 0.1 and 0.3 μg/mL for PZQ. To maintain a constant concentration of each drug, the culture medium was changed every day. Stock solutions of ABZSO and PZQ were prepared in dimethyl sulfoxide (DMSO)/ethanol and ethanol, respectively. For the EC_{50} determination, the dilutions of ABZSO and PZQ were prepared in culture medium. For each experiment, the appropriate controls were groups of cysts incubated in the culture medium containing 0.0125% DMSO/0.075% ethanol for ABZSO and 0.01% ethanol for PZQ.

All experiments were performed in triplicate with a corresponding control consisting of the parasites without drugs. The results were processed using the software GraphPad PRISM V. 4.0.2. A non-linear correlation analysis with variable slope was used for the EC_{50} determinations. EC_{50} values were estimated at 95% confidence limits and were calculated by the log concentration–response curves compared to the fluorescence intensity of Arbitrary Units of Fluorescence (AUF) versus log drug concentration.

2.8. CMFDA biotransformation by cysticerci

For the estimation of the biotransformation of the CMFDA from a non-fluorescent to a fluorescent probe due the metabolic activity of the cysts, parasites that did not receive a drug treatment (control group) were considered to have the capability to transform the CellTracker reagent. After registering the intensity of the