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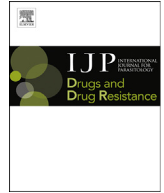

In vitro flubendazole-induced damage to vital tissues in adult females of the filarial nematode *Brugia malayi*

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ABSTRACT

The use of a microfilaricidal drug for the control of onchocerciasis and lymphatic filariasis necessitates prolonged yearly dosing. Prospects for elimination or eradication of these diseases would be enhanced by availability of a macrofilaricidal drug. Flubendazole (FLBZ), a benzimidazole anthelmintic, is an appealing candidate macrofilaricide. FLBZ has demonstrated profound and potent macrofilaricidal effects in a number of experimental filarial rodent models and one human trial. Unfortunately, FLBZ was deemed unsatisfactory for use in mass drug administration (MDA) campaigns due to its markedly limited oral bioavailability. However, a new formulation that provided sufficient bioavailability following oral administration could render FLBZ an effective treatment for onchocerciasis and LF. This study characterized the effects of FLBZ and its reduced metabolite (FLBZ-R) on filarial nematodes *in vitro* to determine the exposure profile which results in demonstrable damage. Adult female *Brugia malayi* were exposed to varying concentrations of FLBZ or FLBZ-R (100 nM–10 μM) for up to five days, after which worms were fixed for histology. Morphological damage following exposure to FLBZ was observed prominently in the hypodermis and developing embryos at concentrations as low as 100 nM following 24 h exposure. The results indicate that damage to tissues required for reproduction and survival can be achieved at pharmacologically relevant concentrations.
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1. Introduction

The debilitating diseases onchocerciasis and lymphatic filariasis (LF) are major causes of long term disability and impede socio-economic development in endemic countries (WHO, 1995; WHO, 2010). Despite the magnitude of the problem, there is optimism about prospects for the elimination of onchocerciasis (Basanez et al., 2006; Mackenzie et al., 2012) and eradication of LF, with the World Health Organization targeting it for elimination by 2020 (WHO, 2010). To increase the likelihood that this goal will be achieved, it is important to address the challenges inherent to current chemotherapeutic strategies used in these programs. The drugs employed in mass drug administration programmes are principally microfilaricidal agents, and also limit reproduction; this strategy reduces transmission and the development of pathology in onchocerciasis but necessitates annual or twice-yearly dosing for many years. An effective and safe macrofilaricide would clearly shorten the time required to reach program goals. In addition, a macrofilaricide would have the benefit of reducing pathology in LF, in which the characteristic sequelae of elephantiasis and hydrocele are initiated by adult worms residing in lymphatic vessels.

Flubendazole (FLBZ), a benzimidazole (BZ) anthelmintic, is an appealing prospective macrofilaricide for use in onchocerciasis and LF. First developed for gastrointestinal (GI) nematodes of animals, FLBZ was found to be potent and efficacious for this indication (Bradley et al., 1983). Subsequently, FLBZ was approved for the treatment of human intestinal parasites (Horton, 1990), an indication for which it is also highly effective (Yangco et al., 1981; Kan, 1983). What is most appealing in the current context is the very high macrofilaricidal efficacy attained in experimental filarial rodent models (Denham et al., 1979; Mak, 1981; Mackenzie and Geary, 2011) and in a human trial in onchocerciasis (Dominguez-Vazquez et al., 1983).

Early *in vitro* studies of BZ anthelmintic effects focused on GI nematodes. Ultrastructural observations of *Ascaris suum*

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6 h following exposure to mebendazole (Borgers and DeNollin, 1975; Borgers et al., 1975) showed a loss of microtubule structures in intestinal cells. Further exposure resulted in decreased glycogen content, depletion of apical secretory granules, and accumulation of secretory granules near the Golgi, associated with swelling and disruption of microvilli (Borgers and DeNollin, 1975; Borgers et al., 1975; Atkinson et al., 1980). FLBZ-induced damage to reproductive organs of filariae has also been reported (Howells and Delves, 1985; Cárdenas et al., 2010).

Other investigators reported similar findings after FLBZ exposure in culture of *Toxocara canis* and *A. suum*, including vacuolization of the musculature, female gonadal tissue, intestine, and, to a lesser degree, the hypodermis (Hanser et al., 2003). Swelling of intestinal cell endoplasmic reticulum and complete disruption of intestinal cells occurred. Following FLBZ treatment of infected animals, loss of intestinal microtubules from cells in the GI tract of the filarial nematodes *Brugia malayi* and *Litomosoides sigmodontis* was observed, using transmission electron microscopy, when the parasites were recovered as soon as 6 h post-dosing (Franz et al., 1990). Increasingly severe damage to other tissues, including the hypodermis and reproductive tissues, was observed as time after dosing increased.

FLBZ is efficacious in humans infected with *Onchocerca volvulus* (Dominguez-Vazquez et al., 1983; Mackenzie and Geary, 2011). Recent efforts have been made to develop a new formulation of FLBZ that would enable oral dosing rather than the parenteral routes used in previous studies (Ceballos et al., 2014; Longo et al., 2014). Definition of the pharmacokinetic profiles needed for efficacy with an orally-bioavailable formulation would be facilitated by knowledge of the time-concentration exposure profiles at which FLBZ is detrimental to the survival of adult filariae. The present study examines time- and concentration-dependent morphological changes in *B. malayi* adult females caused by exposure to FLBZ *in vitro*.

2. Methods

2.1. Parasites

Adult female *B. malayi* were isolated from the peritoneal cavity of jirds (*Meriones unguiculatus*) > 120 days post-infection as described (Moreno and Geary, 2008; Bennuru et al., 2009). Briefly, recovered adult worms were washed three times with warm (37 °C) RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Sigma–Aldrich Corp., St. Louis, MO, USA; hereafter referred to as RPMI).

Adult females were exposed to varying concentrations of FLBZ or its reduced metabolite (FLBZ-R) (10 nM, 100 nM, 1 μM, 10 μM; Epichem Pty Ltd, Murdoch, WA, Australia) over a period of 24, 48, 72, 96 or 120 h, with media changes every 24 h. FLBZ and FLBZ-R solutions were prepared by dissolving the respective drug in 100% DMSO, and added to RMPI to a final DMSO concentration of 0.1%. Control RPMI contained an equivalent percent of DMSO. Three females were cultured in 1 mL RPMI at 37 °C, 5% CO_2 and 95% humidity in each concentration. All worms were fixed for subsequent histological analysis.

Parasite isolation and culture was conducted at the Filariasis Research Reagent Resource Center in Athens, GA, USA.

2.2. Assessment of parasite motility

Parasite motility was assessed visually under light microscopy. Motility was scored as either: immotile, with no motion during the observation period; slightly motile, where only twitching of the head and/or tail was observed; moderately motile, with slow sinusoidal movements; or highly motile and comparable to the drug-free control. Each sample was observed for at least one minute for scoring of motility.

2.3. Histological preparation

B. malayi were fixed in glutaraldehyde (5% in 0.1 M sodium cacodylate buffer, pH 7.2; five worms in 1 mL) for a minimum of 48 h in preparation for histological processing. Worms from each treatment were combined into groups and coiled prior to embedding in Histogel (FisherScientific), which allowed visualization of various anatomical regions in multiple worms on a single slide. Dehydration, clearing, and vacuum infiltration with paraffin were completed using a Sakura VIP tissue processor. Parasites were then embedded in paraffin with a ThermoFisher HistoCentre III embedding station. A Reichert Jung 2030 rotary microtome was used to cut 4–5 micron sections, which were dried at 56 °C for 2–24 h. Slides were stained with haematoxylin and eosin prior to examination under light microscopy (60 and 100× magnification).

2.4. Assessment of worm damage

Sections were assessed independently by three parasitologists familiar with filarial nematode morphology, including one board certified pathologist/parasitologist (CDM); a second board certified pathologist was also consulted in planning and developing the system (DWA). Worms from two independent experiments were examined for damage to the following tissues: body wall, including cuticle, hypodermis and longitudinal muscle; intestine; and reproductive tract, including the uterine wall and embryonic stages (classified as early [ovary, oocytes, early morulae, late morulae] or late [sausage, pretzel, microfilariae]); and pseudocoelomic space. To aid the comparative analysis of drug-derived effects, tissues were classified into four categories of damage: no damage (0), minor (1), moderate (2), severe (3). This damage score was determined by assessing tissues for nuclear and cytoplasmic distortions, cellular size and shape, membrane integrity, accumulation of debris, and distortion of overall anatomical integrity (Fig. 1).

Two methods of analysis were performed. The first method adhered to classical techniques used by histopathologists to determine tissue damage, in which all sections on a slide were surveyed, interpreted and translated into a single damage score for each tissue type. The second method involved scoring damage in each tissue type for each worm section on a slide. These scores were then averaged for all sections on the slide to obtain the damage score.

3. Results

3.1. Quality of sections

Control worms retained well-preserved tissue structure for up to 72 h in culture. Noticeable loss of normal condition occurred in control groups after 96 h, seen as an increase in vacuolization in the intestine and hypodermis. A high degree of variability in this latter morphological change was observed among worms in the same treatment group, as well as along the length of an individual worm. Two independent experiments were conducted in an attempt to reduce this variability. Multiple transverse sections were assessed to enable estimation of the proportion of the specimen which was damaged. Samples from the 96 and 120 h incubations were excluded from further analyses to eliminate the influence of this

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