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Research paper

Papaya latex supernatant has a potent effect on the free-living stages of equid cyathostomins in vitro

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ABSTRACT

The control of equid gastrointestinal nematodes in developed countries, in particular the cyathostomins, is threatened by high levels of anthelmintic resistance. In recent years, there has been increasing interest in the evaluation of traditional 'ethnoveterinary' medicines as alternatives to chemical anthelmintics. The cysteine proteinases (CPs), a group of enzymes derived from fruits such as papaya (*Carica papaya*), pineapple (*Ananas comosus)* and figs (*Ficus* spp.), have shown good efficacy against adult stages of a range of parasitic nematodes, in vitro and in vivo. The efficacy of CPs against cyathostomins remains to be explored. In this study, the efficacy of a crude preparation of CPs, papaya latex supernatant (PLS), against the free-living stages of cyathostomins was evaluated using two in vitro tests, the egg hatch test (EHT) and the larval migration inhibition test (LMIT). It was demonstrated that PLS had a potent effect in the EHT, with EC-50 values in the range of 0.12–0.22 μM. At concentrations above 6.25 μM the eggs did not develop, below this concentration the L1 developed but they lost integrity of the cuticle upon hatching. These effects were inhibited by pre-incubation of PLS with the CP inhibitor L-*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino butane) (E64), indicating that CPs were responsible for the anti-parasitic activity. A dose-dependent inhibition of migration of third stage larvae (L3) in the LMIT was demonstrated at higher concentrations of PLS, with EC-50 values in the range of 67.35–106.31 μM. Incubation of PLS with E64 prior to use in the LMIT did not reverse the anti-migratory effect, suggesting that CPs were not responsible for the reduced migration of cyathostomin L3 and that PLS also contains an additional active compound. This is the first report of PLS and/or CPs showing activity against the free-living stages of a parasitic helminth. In addition, it suggests that cyathostomins are highly sensitive to the effects of CPs and further evaluation of their efficacy against parasitic stages and in vivo are strongly indicated.

1. Introduction

Anthelmintic resistance in veterinary parasitic helminths is a global problem, with increasing reports of multi-class resistance. (Geurden et al., 2014; Kaplan et al., 2012; Kaplan and Vidyashankar, 2012; Molento et al., 2008). Novel anthelmintic compounds that have reached the animal health market in the last decade include monepantel, derquantel and emodepside; currently, these have limited licencing across host species and are expensive. In addition resistance has already been reported to monepantel, just two years after its commercial launch (Scott et al., 2013; Van den Brom et al., 2015).

Gastrointestinal (GI) nematodes of equids are no exception in that effective control is threatened by high levels of anthelmintic resistance (Nielsen et al., 2014; Peregrine et al., 2014). The most prevalent and pathogenic GI nematodes of equids in developed countries are the cyathostomins (Kaplan and Vidyashankar, 2012; Love et al., 1999); a group of clade V nematodes belonging to the superfamily Strongyloidea (Lichtenfels et al., 1998). Their pathogenic effect ranges from non-specific weight loss to colic and larval cyathostominosis. The latter is a potentially fatal colitis caused by the mass emergence of larvae from the large intestinal

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wall (Lyons et al., 2000; Murphy and Love, 1997; Peregrine et al., 2006; Uhlinger, 1991).

The three broad spectrum classes of anthelmintic that are licensed to control cyathostomins are the benzimidazoles (BZs), the tetrahydropyrimidines and the macrocylic lactones (MLs,). The MLs, ivermectin and moxidectin, are the most potent and widely used anthelmintics in horses (Allison et al., 2011; Nielsen et al., 2006; Reinemeyer and Rohrbach, 1990). In developed countries, there is widespread resistance in cyathostomin populations to BZs (Kaplan et al., 2004; Lester et al., 2013; Lind et al., 2007; Traversa et al., 2012; Traversa et al., 2007; Traversa et al., 2009) and resistance to pyrantel is common in some regions (Lyons et al., 2001; Nielsen et al., 2013; Stratford et al., 2014; Traversa et al., 2012; Traversa et al., 2007; Traversa et al., 2009). More recently, ML resistance, measured primarily as a reduction in strongyle egg reappearance period, has been reported in several regions (Canever et al., 2013; Milillo et al., 2009; Molento et al., 2008; Nareaho et al., 2011; Relf et al., 2014; Traversa et al., 2009; Trawford and Burden, 2009). None of the new anthelmintic classes that have been licensed for use in small ruminants and companion animals are likely to be licensed in equids (Epe and Kaminsky, 2013).

The threat of anthelmintic resistance has led to increased efforts in the search for novel anti-nematode compounds. In addition to screening large libraries of synthetic compounds, there has been increasing interest in natural products which have been used historically as traditional ethnomedical/veterinary treatments. One promising group of compounds are the cysteine proteinases (CPs) derived from the fruits of plants such as papaya (*Carica papaya*), pineapple (*Ananas comosus*) and figs (*Ficus* spp.). These have been used for centuries for the treatment of human ascarid and hookworm infections (Berger and Asenjo, 1939, 1940; Caldwell and Caldwell, 1929; Fernan-Nunez, 1927; Mueller and Mechler, 2005; Robbins, 1930). In recent years, their efficacy against a number of veterinary parasitic nematodes and cestodes, in vitro and in vivo, has been reported (Buttle et al., 2011; Levecke et al., 2014; Mansur et al., 2014a, b; Satrija et al., 1994; Satrija et al., 1995; Stepek et al., 2005; Stepek et al., 2006, 2007a, b, c). Generally, these compounds appear to be widely effective and hold promise as novel broad spectrum anthelmintics. The CPs have not yet been tested in cyathostomins. Here, the efficacy of an extract of *C. papaya,* papaya latex supernatant (PLS), was evaluated against eggs and third stage larvae (L3) of cyathostomins in vitro using the egg hatch test (EHT) and the larval migration inhibition test (LMIT), respectively.

2. Materials and methods

2.1. Preparation of PLS extract

The PLS used in this study was prepared as previously described (Buttle et al., 2011) at the University of Nottingham and contained the CPs, chymopapain, glycyl endopeptidase, caricain and papain in order of increasing relative abundance (Buttle et al., 1990). The extract was aliquoted into individual vials and sent on dry ice to the University of Liverpool, where it was immediately stored at −80 °C. The extract was thawed before use and the molar active CP concentration measured using enzyme active site titration adapted from Barrett et al. (1981) and Zucker et al. (1985). In brief, PLS was incubated with increasing concentrations of the CP specific inhibitor, L-*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino butane) (E64) (Sigma Aldrich, UK), with 4mMol L-cysteine (Sigma Aldrich, UK) as a reducing agent and N-α-benzoyl-arginyl-*p*-nitroanilide (BAPNA) (Bachem Ltd, UK) as a substrate. As CPs and E64 bind in a 1:1 molar ratio, the concentration of E64 which completely inhibited CP activity was taken to be equal to the concentration of PLS in the assay, thus allowing calculation of the concentration in the stock PLS solution. For use in in vitro tests, stock PLS was made up to 12 mM, serially diluted in 5 mM (EHT) or 10 mM (LMIT) L-cysteine (Sigma Aldrich, UK), pH 7.0, to give working concentrations. The final concentrations in the test were; 0.10, 0.20, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5 and 25 μM for the EHT, and 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200 and 400 μM for the LMIT. The optimal concentrations and pH of L-cysteine were established in preliminary experiments (Peachey, data not shown).

2.2. Parasite sample collection

2.2.1. Equid strongyle eggs

Freshly voided faecal samples were collected from donkeys at the Donkey Sanctuary UK, and from horses that resided at a yard in the North West of the UK. There was a history of anthelmintic resistance at the Donkey Sanctuary but not at the horse yard, although definitive tests for anthelmintic resistance were not performed as part of this study. The samples were stored anaerobically as previously described by (Coles et al., 1992) before transportation to the laboratory. Samples were used within one week of collection. Eggs were extracted from faecal material by serial filtration through reducing sieve sizes: 2 mm, 750 μm, 325 μm, 150 μm, 75 μm and 38 μm. Retentate from the 38 μm sieve was washed into a beaker and centrifuged at 200*g* for 2 min in 10 ml polyallomer tubes (Beckman Coulter, UK). The supernatant was removed by suction and the pellet re-suspended in saturated sodium chloride solution. This was centrifuged for a further two min at 200*g*. The polyallomer tubes were clamped 1 cm from the top of the supernatant and the upper layer, containing eggs, decanted onto a 38 μm sieve. The eggs were washed and collected in 20 ml distilled water (dH_2O) and used immediately. A subset of eggs from each sample were cultured to L3 stages to confirm that only cyathostomins were present.

2.2.2. Larval samples

Freshly voided faecal samples were collected from donkeys at the Donkey Sanctuary UK and horses from a local yard, wrapped in an airtight plastic sleeve before transportation to the laboratory. Upon arrival, samples were removed from packaging and separated into 100 g balls, which were incubated in aerated plastic sleeves for two weeks at 21 °C. Samples were then soaked in warm tap water for 4 h to allow L3 to migrate into solution. The mixture was filtered through a 1 mm sieve to remove faecal debris before recovery of L3 by the Baermann technique (Baermann, 1917) overnight to provide L3 for use in the tests. A subset of L3 from each sample was examined to confirm that only cyathostomins were present. The L3 were stored at 4 °C and used within eight weeks post-recovery.

2.3. Egg hatch test

The EHT was performed in duplicate on samples from four equids (two horses and two donkeys). The eggs in each sample were enumerated and the concentration adjusted to 1 egg/μl. Next, 1900 μl PLS in 5 mM L-cysteine, at the final concentrations described above, were added in duplicate to a 24-well plate, and 100 μl egg suspension added to each well. Duplicate negative controls for 5 mM L-cysteine and dH_2O and positive controls of thiabendazole at 1.5 μg/ml (Sigma, UK) (made up in dimethyl sulfoxide, final concentration 0.05%) were included, to ensure the test accurately differentiated between live and dead eggs. The plate was incubated for 24 h at 27 °C and the test terminated by the addition of 20 μl Lugols iodine solution (Sigma Aldrich, UK). The number of eggs and first stage larvae (L1) were counted in each well using an inverted microscope (Olympus CK). Percentage egg hatch at each concentration was calculated as follows: (L1/L1 + eggs) × 100. The optimal incubation time and temperature were established in preliminary experiments to allow for >80% hatch in controls at the end