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A highly conserved, inhibitable astacin metalloprotease from *Teladorsagia circumcincta* is required for cuticle formation and nematode development ☆

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Parasitic nematodes cause chronic, debilitating infections in both livestock and humans worldwide, and many have developed multiple resistance to the currently available anthelmintics. The protective collagenous cuticle of these parasites is required for nematode survival and its synthesis has been studied extensively in the free-living nematode, *Caenorhabditis elegans*. The collagen synthesis pathway is a complex, multi-step process involving numerous key enzymes, including the astacin metalloproteases. Nematode astacinsare crucial for *C. elegans* development, having specific roles in hatching, moulting and cuticle synthesis. NAS-35 (also called DPY-31) is a homologue of a vertebrate procollagen C-proteinase and performs a central role in cuticle formation of *C. elegans* as its mutation causes temperature-sensitive lethality and cuticle defects. The characterisation of DPY-31 from the ovine gastrointestinal nematode *Teladorsagia circumcincta* and its ability to rescue the *C. elegans* mutant is described. Compounds with a hydroxamate functional group have previously been shown to be potent inhibitors of procollagen C-proteinases and were therefore examined for inhibitory activity against the *T. circumcincta* enzyme. Phenotypic screening against *T. circumcincta*, *Haemonchus contortus* and *C. elegans* larval stages identified compounds that caused body morphology phenotypes consistent with the inhibition of proteases involved in cuticle collagen synthesis. These compounds correspondingly inhibited the activity of recombinant *T. circumcincta* DPY-31, supporting the hypothesis that this enzyme may represent a potentially novel anthelmintic drug target.

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1. Introduction

Gastrointestinal (GI) nematodes cause chronic debilitating infections in livestock and humans worldwide, having a major economic impact on sheep farming resulting in loss of appetite, weight loss, decreased wool, meat and milk production and death (Zajac, 2006; Roeber et al., 2013). Current treatment is through the use of anthelmintic drugs (McKellar and Jackson, 2004); however, multiple resistance to anthelmintics of the three major classes has now developed in the veterinary parasites (Pomroy, 2006; Papadopoulos et al., 2012). Only a limited number of new drugs with novel modes of action have become available in recent years (Besier, 2007; Epe and Kaminsky, 2013), thereby limiting future prospects for effective control. No vaccines have yet been developed against these infections, although many different molecules have been under investigation for many years as potential vaccine candidates (Dalton and Mulcahy, 2001; Diemert et al., 2008; LeJambre et al., 2008).

All nematodes are surrounded by an external protective structure called the cuticle. The cuticle functions as an exoskeleton and provides protection from the external environment during development, hence its importance for nematode survival (Page et al., 2014). Synthesis of this structure is a complex, multi-step process, involving numerous enzymes (Page and Winter, 2003). The cuticle is largely composed of collagens (Fetterer, 1989; Johnstone, 2000), which are homologous between the free-living nematode, *Caenorhabditis elegans*, and parasitic nematodes such as the major GI nematodes of sheep, *Teladorsagia circumcincta* (Johnstone et al., 1996) and *Haemonchus contortus* (Laing et al., 2013). The process of cuticle biosynthesis has been studied in detail in *C. elegans* (Page and Winter, 2003), with many of the crucial cuticle synthesising enzymes and proteases also present in parasitic nematodes (reviewed in Page et al., 2014), suggesting that

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the cuticle biosynthesis process may be similar between *C. elegans* and its parasitic counterparts.

Protease enzymes are essential for the continued development and survival of nematodes in the host and fall into the following main classes: aspartic, cysteine, metallo-, threonine and serine proteases. The astacin metalloprotease enzymes play an essential role in cuticle biosynthesis in *C. elegans* (Hishida et al., 1996; Davis et al., 2004; Novelli et al., 2004, 2006; Suzuki et al., 2004). These enzymes are structurally distinct zinc metallo-endopeptidases that are characterised by two conserved motifs in the N-terminal astacin domain: the zinc-binding active site (HExxHxxGFxHExxRxDRD) and the methionine-turn (SxMHY) (Bond and Beynon, 1995). Binding of the zinc in the active site is essential for the catalytic activity of the enzyme; this zinc is pentacoordinated in a trigonal–bipyramidal geometry between the three histidine residues in the binding motif, the tyrosine in the methionine-turn and a water molecule (Bode et al., 1992). The first astacin metalloprotease identified was found in the crayfish, *Astacus astacus*, and consisted of a signal peptide, prodomain and a catalytic protease domain containing both conserved catalytic motifs. Astacin metalloproteases containing C-terminal domains of unknown functions, as well as the N-terminal catalytic domain, have now been isolated from a range of organisms including humans, mice, *Drosophila melanogaster* and *C. elegans* (Stöcker et al., 1993; Möhrlen et al., 2003, 2006). The primary role in all species is in development (Bond and Beynon, 1995), such as the hatching and moulting of *C. elegans* (Hishida et al., 1996; Davis et al., 2004; Suzuki et al., 2004). Functional roles for astacin proteases in parasitic nematodes include host tissue penetration by infective L3s (Williamson et al., 2006), cuticle formation and ecdysis (Gamble et al., 1989; Stepek et al., 2010, 2011) and digestion (Gallego et al., 2005).

There are 39 nematode astacin (NAS) metalloproteases expressed in *C. elegans*, which are subdivided into six subgroups (I to VI). Based on expression studies and RNA interference (RNAi), many of these proteases play developmental roles in *C. elegans* (Möhrlen et al., 2003). All the *C. elegans* NAS have a similar domain arrangement: signal peptide, prodomain, N-terminal catalytic astacin domain and may include a combination of the following C-terminal domains: Epidermal Growth Factor (EGF), Complement component Uegf and BMP-1 (CUB) and ThromboSPondin type-1 repeat (TSP-1) (Möhrlen et al., 2003). Removal of the prodomain causes conformational changes to the astacin domain, which results in enzyme activation (Guevara et al., 2010). The functions of the C-terminal domains are largely unknown but these domains, whilst having a non-catalytic purpose, are hypothesised to regulate the catalytic activity of the enzyme, provide its specificity and determine when and where the protein performs its role (Wermter et al., 2007). The subgroup V enzymes, NAS-33 to NAS-38, are found only in nematodes and have a unique nematode-specific C-terminal domain arrangement, consisting of one EGF, one CUB and one TSP-1 domain (Möhrlen et al., 2003). The enzyme NAS-34 is required for embryo hatching (Hishida et al., 1996) and NAS-36 and NAS-37 are both crucial to the moulting process (Davis et al., 2004).

DPY-31 (also known as NAS-35) has similarities to the vertebrate procollagen C-proteinase Bone Morphogenetic Protein-1 (BMP-1), which is critical for the assembly of collagen fibres during cartilage and bone formation through its excision of the C-terminal domain of procollagen to form mature collagen (Li et al., 1996). In *C. elegans*, *dpy-31* is expressed throughout the life-cycle, particularly in the embryonic and larval stages, in most hypodermal cells, as well as the rectal and vulval epithelial cells (Novelli et al., 2004). *Caenorhabditis elegans dpy-31(e2770)* mutants are inviable at the standard growth temperature of 20 °C and partially viable at 15 °C, with survivors displaying severe body morphology defects known as Dumpy (short and fat) (Novelli et al., 2004). Genetic suppressor screens identified the DPY-31 cleavage site in the C-terminus of the essential cuticle collagen SQT-3 (Novelli et al., 2006). The procollagens, such as SQT-3, in *dpy-31(e2770)* mutants therefore remain partially processed and cannot form mature collagens. Thus, DPY-31 plays a crucial role in cuticle formation and the moulting process in *C. elegans*. Earlier work has indicated that hydroxamate-based compounds are highly effective inhibitors of procollagen C-proteinase (Ovens et al., 2000; Fish et al., 2007; Bailey et al., 2008), suggesting that these compounds may also be effective against the nematode DPY-31 enzymes.

Here we identify and characterise the DPY-31 orthologue from the important GI nematode of sheep, *T. circumcincta*. The naturally occurring hydroxamic acid, actinonin (Gordon et al., 1975), and other developed inhibitory compounds containing a hydroxamate functional group (Fish et al., 2007) were screened against recombinant *T. circumcincta* DPY-31 and against larval stages of *T. circumcincta*, *H. contortus* and *C. elegans* to determine their effectiveness as inhibitors of this key developmental enzyme.

2. Materials and methods

2.1. Nematode strains and culture

Caenorhabditis elegans was cultured as described previously (Stiernagle, 2006), with the strains N2 (wild-type), HT1593 (*unc-119*(*ed3*) III) and DR96 (*unc-76*(*e911*) V) provided by the *Caenorhabditis* Genetics Center (CGC), USA. The *C. elegans dpy-31(e2770)* strain was provided by Professor Jonathan Hodgkin, University of Oxford, UK.

Teladorsagia circumcincta MTci2 and *H. contortus* ISE strains were provided by Professor David Knox (Moredun Research Institute, UK). Nematode eggs were extracted from infected sheep faeces using a salt flotation protocol and L1s were allowed to hatch in tap water at approximately 25 °C overnight. These L1s were cleaned free from eggs and faecal matter by passing down a Baermann apparatus overnight. Active, healthy *T. circumcincta* L3s (<6 months old) were exsheathed by exposure to CO_2, as detailed in Halliday et al. (2012), except that the larvae were initially activated at 40 °C in a New Brunswick Scientific, USA (E24 Series) shaker at 60 rpm for 15 min, exposed to CO_2 in a total volume of 5 ml, and then incubated at 40 °C with shaking at 100 rpm for 90 min. The exsheathment progress of a 20 μl sample was monitored under a Zeiss dissecting microscope.

2.2. Preparation of genomic DNA, RNA and cDNA

Genomic DNA was isolated from adult *T. circumcincta* (MTci5) by homogenisation in Proteinase K and repeated phenol:chloroform extractions, as described previously (Stepek et al., 2010). For RNA, adult nematodes, L3s or L1s were disrupted in TRIzol (Life Technologies, UK) using a hand-held Pestle and Motor Mixer (VWR, UK) with 1.5 ml disposable pestles and screw-capped tubes (VWR), and total RNA isolated following the TRIzol (Life Technologies) protocol. cDNA was prepared using an AffinityScript Multiple Temperature cDNA synthesis kit (Agilent Technologies, USA).

2.3. Identification of the coding and genomic sequences of *T. circumcincta dpy-31*

The *C. elegans* DPY-31 protein sequence was used to tBLASTn search the *T. circumcincta* database (M. Mitreva, Washington University School of Medicine, USA, personal communication). The supercontig0014679 and scaffold00169 had the highest homology score, and potential intron–exon splice sites were predicted using GeneWise2 (http://www.ebi.ac.uk/Tools/psa/

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