IJP: Drugs and Drug Resistance 8 (2018) 145–157

Contents lists available at ScienceDirect

IJP: Drugs and Drug Resistance

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

Structural model, functional modulation by ivermectin and tissue localization of *Haemonchus contortus* P-glycoprotein-13

Marion David[a,b,1], Chantal Lebrun[a,1], Thomas Duguet[b], Franck Talmont[c], Robin Beech[b], Stéphane Orlowski[d], François André[d], Roger K. Prichard[b,**], Anne Lespine[a,*]

[a] INTHERES, Université de Toulouse, INRA, ENVT, Toulouse, France
[b] Institute of Parasitology, McGill University, Sainte-Anne-De-Bellevue, Canada
[c] Institute of Pharmacology and Structural Biology, UMR 5089, CNRS, Toulouse, France
[d] CEA, Institut de Biologie Frédéric Joliot, Centre de Saclay, SB2SM, UMR9198 CNRS, I2BC; 91191 Gif-sur-Yvette Cedex, France

ARTICLE INFO

Keywords:
Nematode
Haemonchus contortus
ABC transporters
P-glycoprotein
Ivermectin
Homology modeling

ABSTRACT

Haemonchus contortus, one of the most economically important parasites of small ruminants, has become resistant to the anthelmintic ivermectin. Deciphering the role of P-glycoproteins in ivermectin resistance is desirable for understanding and overcoming this resistance. In the model nematode, *Caenorhabditis elegans*, P-glycoprotein-13 is expressed in the amphids, important neuronal structures for ivermectin activity. We have focused on its ortholog in the parasite, Hco-Pgp-13. A 3D model of Hco-Pgp-13, presenting an open inward-facing conformation, has been constructed by homology with the Cel-Pgp-1 crystal structure. In silico docking calculations predicted high affinity binding of ivermectin and actinomycin D to the inner chamber of the protein. Following *in vitro* expression, we showed that ivermectin and actinomycin D modulated Hco-Pgp-13 ATPase activity with high affinity. Finally, we found *in vivo* Hco-Pgp-13 localization in epithelial, pharyngeal and neuronal tissues. Taken together, these data suggest a role for Hco-Pgp-13 in ivermectin transport, which could contribute to anthelmintic resistance.

1. Introduction

Parasite nematodes cause morbidity in animals and humans and macrocyclic lactones (ML), such as ivermectin (IVM), are important anthelmintic drugs for therapy (Campbell, 2016; Omura, 2016). However, the long-term use of ML has led to the development of drug resistance, challenging therapeutic control (Kaplan and Vidyashankar, 2012).

The multi-drug resistance (MDR) transporters from the ATP-binding cassette (ABC) protein superfamily are involved in the transport of structurally unrelated xenotoxins, and have been recognized as major players in resistance to drugs in mammals, bacteria and parasites (Lage, 2003; Jones and George, 2005; Koenderink et al., 2010). In mammals, P-glycoprotein (MDR1/ABCB1/Pgp) can efflux structurally unrelated drugs, including IVM (Schinkel et al., 1994; Pouliot et al., 1997; Roulet et al., 2003; Lespine et al., 2007). Many Pgps, i.e. full-size transporters of the B sub-family, are expressed in nematodes, and they display fair sequence homologies with human ABCB1. The free-living nematode *Caenorhabditis elegans* expresses sixty ABC proteins, among which fourteen Pgp homologs are localized in different organs and expressed at various stages of development (Zhao et al., 2004). *Haemonchus contortus*, one of the most prevalent pathogen parasitic nematodes in small ruminants, is genetically close to *Caenorhabditis elegans* and its genome has recently been sequenced (Laing et al., 2013) (ftp://ftp.sanger.ac.uk/pub/pathogens/Haemonchus/contortus). In this species, ten homologs of Pgps were identified, and two have been localized: Hco-Pgp-2 in the pharynx, anterior intestine and head neurons, and Hco-Pgp-9.1 in the uterus of females (Godoy et al., 2015a, 2016).

There is indirect but converging evidence that some nematode Pgps can transport ML, in *C. elegans* (Ardelli and Prichard, 2013; Janssen et al., 2013a, 2015) and in *Parascaris equorum* (Janssen et al., 2013b). Some mammalian Pgp inhibitors alter the transport function of Pgps in

Abbreviations: ABC, ATP-binding cassette; ACD, actinomycin D; AH, anthelmintic; BlastP, protein Basic Local Alignment Search Tool; bp, base pairs; Cel, *Caenorhabditis elegans*; Hco, *Haemonchus contortus*; Hsa, *Homo sapiens*; IVM, ivermectin; MDR, multidrug resistance; ML, macrocyclic lactone(s); Mmu, *Mus musculus*; NBD, nucleotide binding domain; PDB, Protein data bank; Pgp, P-glycoprotein; QMEAN, Qualitative Model Energy Analysis; RMSD, root mean square deviation; SNP, single nucleotide polymorphism; TM(D), transmembrane (domain)

* Corresponding author. INRA-INTHERES_UMR 1436, 180, Chemin de Tournefeuille, BP 93173, F-31027, Toulouse Cedex 3, France.
** Corresponding author. Institute of Parasitology, McGill University, Macdonald Campus, 21111, Lakeshore Road, Ste Anne-de-Bellevue, QC, H9X 3V9, Canada.
E-mail addresses: roger.prichard@mcgill.ca (R.K. Prichard), anne.lespine@inra.fr (A. Lespine).
[1] Contributed equally.

https://doi.org/10.1016/j.ijpddr.2018.02.001
Received 31 October 2017; Received in revised form 9 February 2018; Accepted 12 February 2018
Available online 15 February 2018

C. elegans and in the parasitic nematodes *H. contortus*, *Cylicocylus elongatus* and *Dirofilaria immitis* (Kaschny et al., 2015; Godoy et al., 2015a, 2015b, 2016; Mani et al., 2016). Such inhibitors can also improve the susceptibility of nematodes to ML (Bartley et al., 2009; James and Davey, 2009; Lespine et al., 2012; Menez et al., 2016). In addition, increased ML resistance is associated with induction of expression of Pgp genes (James and Davey 2009; Lespine et al., 2012). Recently, using the first crystal structure of a nematode Pgp, Cel-Pgp-1, resolved at a resolution of 3.4 Å (Protein Data Bank code: 4F4C) (Jin et al., 2012), high affinity binding of several anthelmintic drugs, including IVM, on Cel-Pgp-1 has been predicted by *in silico* docking calculations (David et al., 2016).

To understand the respective physiological functions of Pgps in *H. contortus*, it is of interest to consider their sites of expression. In both *C. elegans* and *H. contortus*, IVM resistance has been linked to defects in the morphology of the amphids (Freeman et al., 2003; Urdaneta-Marquez et al., 2014; Menez et al., 2016). *Cel-Pgp-6* and *Cel-Pgp-13* are expressed in the amphids (Zhao et al., 2004). *H. contortus* does not have a homolog of *Cel-Pgp-6*, but it has a homolog of *Cel-Pgp-13*, and it was therefore of interest to characterize *Hco-Pgp-13*. Full-length *Hco-Pgp-13* cDNA was cloned and transfected into *Pichia pastoris* which stably expressed functional Hco-Pgp-13. Based on the crystal structure 4F4C of Cel-Pgp-1 (Jin et al., 2012), the protein was 3D modeled in open inward-facing conformation, which is expected to be competent for substrate uptake, leading to two high-quality, alternative but complementary, structural models. Using *in silico* docking on these models, in combination with *in vitro* ATPase assays on membranes of *Hco-Pgp-13* transfected *P. pastoris*, we show for the first time that actinomycin D (ACD) and IVM specifically bind to Hco-Pgp-13 and modulate its ATPase activity, and could hence be transported by the parasitic Hco-Pgp-13. Furthermore, the expression of Hco-Pgp-13 in the tissues of *H. contortus* larvae and adults was assessed. Our finding of apparent Hco-Pgp-13 expression in digestive, epithelial and neuronal tissues is consistent with a general detoxification function, possibly handling various xenobiotics, in analogy with ABCB1 in mammals. Finally, in the context of anthelmintic drug resistance in parasitic nematodes, Hco-Pgp13 is likely to have a role in IVM resistance.

2. Material and methods

2.1. Parasites

The PF23 strain of *H. contortus* used is susceptible to MLs (Ranjan et al., 2002). Worms were originally supplied by Fort Dodge Animal Health, Princeton, NJ, USA and were maintained by our laboratory. Animals and standardized operating procedures used in this research study were approved (Protocol 3845) and subjected to the guidelines from the Animal Care Committee of McGill University, Canada. Worms were obtained from passages consisting of an artificial infection with the larvae from the previous generation in naive lambs, without anthelmintic exposure. They were then collected from the abomasum of the host and incubated in PBS at 37 °C before storage at -80 °C.

2.2. RNA extraction and reverse transcription

Total RNA was extracted from twenty adult *H. contortus*, homogenized and extracted according to the instruction of the manufacturer (ThermoFisher, Canada). RNA concentration was determined with a Nanodrop photometer IMPLEN® at a wavelength of 260 nm and assessed by gel electrophoresis. Good quality RNA was stored at -80 °C. The reverse transcription to cDNA was performed using the SuperScript® III reverse transcriptase (ThermoFisher, Canada), starting with 1 µg RNA and following the instructions of the manufacturer. The cDNA obtained was stored at -20 °C for further use.

2.3. Amplification of the Hco-Pgp-13 cDNA sequence

A pair of primers, *Hco-Pgp-13*-F1 and *Hco-Pgp-13*-R2 (Suppl. Table S1), were designed using the Geneious software version 5.5.6. (http://www.geneious.com/; PO Box 5677, Wellesley St, Auckland 1141, New Zealand), across the 3' end of the predicted sequence of *Hco-Pgp-13* from the Sanger Institute (ftp://ftp.sanger.ac.uk/pub/pathogens/Haemonchus/contortus) (Laing et al., 2013). A first PCR was run using these primers and the reverse transcribed cDNA of whole adult *H. contortus* as template. A fragment of 3488bp was obtained and sequenced (Genome Quebec Innovation Centre, McGill University, QC, Canada) using eight primers, *Hco-Pgp-13*-F1 to *Hco-Pgp-13*-R8 (Supplementary Table S1).

Four more primers were designed to identify the 5′-end of *Hco-Pgp-13* by nested PCR: the nematode spliced leader sequence SL1 (Blaxter and Liu, 1996), a specific forward primer *Hco-Pgp-13*–F9 (Supplementary Table S1) and two specific reverse primers *Hco-Pgp-13*–R10 and *Hco-Pgp-13*-R11 (Supplementary Table S1). The 887 bp PCR products thus obtained were sequenced and aligned to the first 3488 bp product with MultAlin software (Corpet, 1988), and the overlap of the amplicons confirmed. The full-length sequence was then aligned against the cDNA of the predicted sequence of *Hco-Pgp-13* using MultAlin (Supplementary Figure S1).

2.4. Determination of Hco-Pgp-13 protein sequence, phylogenic analysis and calculation of TMD homologies relative to Cel-Pgp-1

The translation of *Hco-Pgp-13* cDNA into protein sequence was performed using ExPASy – Translate tool (http://web.expasy.org/translate/). The parameters of the protein (molecular weight, length) were calculated with ExPASy – ProtParam (http://web.expasy.org./protparam/). The presence of N- and O-glycosylation motifs was predicted using ExPASy - ScanProsite tool (http://prosite. Expasy. org/), as well as that of consensus motifs, including the Walker A and the less conserved Walker B motif. Multiple sequence alignment of Pgps of various organisms, including Hsa-Pgp (Supplementary Figure S2), was performed using Muscle algorithm under Seaview software (Edgar, 2004a, 2004b). The prediction of amino acids located within the transmembrane bilayer was performed with the Protter tool (Omasits et al., 2014) and the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The visualization of the topology of the full length protein sequence across the plasma membrane was represented using the Protter tool (Fig. 1). The TM helices and eight N-glycosylation motifs were represented as predicted by this program, N518 being the only one not previously predicted by ExPASy - ScanProsite.

A PhyML phylogenetic tree with all Hco-Pgps, all Cel-Pgps, Hsa-Pgp and Mmu-ABCB1a protein sequences (Fig. 2) was constructed using Seaview software from the multiple alignments previously performed with Muscle (Supplementary Figure S2) after removal of NBDs from all the considered protein sequences.

The locations of the TMDs of all the proteins of this alignment were deduced from Cel-Pgp-1 4F4C crystal structure (Jin et al., 2012). The first amino acid of TMD1 of each Pgp was identified as the one aligned to the first amino-acid of TM1 of Cel-Pgp-1, thus excluding the small extra-helix TMa and TMb (Jin et al., 2012) not previously described in other ABC transporters, and considered to be part of the N-terminal region. The similarity and identity percentages of the TMDs of Cel-Pgp-1 with the TMDs of all other Pgps were then determined using BlastP (http://blast.ncbi.nlm. nih. gov/Blast.cgi). TMD1 and TMD2 were considered independently, and the mean of the two values for each Pgp is reported in Table 1.

2.5. Design of specific antibodies against Hco-Pgp-13

The specificity and suitability of various antigens for antibody production against Hco-Pgp-13 were analyzed by GenScript. Among