

Anthelmintic metabolism in parasitic helminths: proteomic insights

PETER M. BROPHY*, NEIL MACKINTOSH and RUSSELL M. MORPHEW

Parasitology Group, Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, Aberystwyth, Ceredigion, Wales SY23 3FG, UK

(Received 6 January 2012; revised 16 April 2012; accepted 30 April 2012; first published online 10 July 2012)

SUMMARY

Anthelmintics are the cornerstone of parasitic helminth control. Surprisingly, understanding of the biochemical pathways used by parasitic helminths to detoxify anthelmintics is fragmented, despite the increasing global threat of anthelmintic resistance within the ruminant and equine industries. Reductionist biochemistry has likely over-estimated the enzymatic role of glutathione transferases in anthelmintic metabolism and neglected the potential role of the cytochrome P-450 superfamily (CYPs). Proteomic technologies offers the opportunity to support genomics, reverse genetics and pharmacokinetics, and provide an integrated insight into both the cellular mechanisms underpinning response to anthelmintics and also the identification of biomarker panels for monitoring the development of anthelmintic resistance. To date, there have been limited attempts to include proteomics in anthelmintic metabolism studies. Optimisations of membrane, post-translational modification and interaction proteomic technologies in helminths are needed to especially study Phase I CYPs and Phase III ABC transporter pumps for anthelmintics and their metabolites.

Key words: Proteomics, helminth, anthelmintic, anthelmintic resistance, xenobiotic.

INTRODUCTION

Parasitic helminth worms have major economic impacts on the global livestock industry and chemical anthelmintics, in the absence of commercial vaccines, remain the cornerstone of their control (Keiser and Utzinger, 2010; Sutherland and Leathwick, 2011). Unfortunately, there is increasing evidence that parasitic worms are becoming resistant to many anthelmintics and this represents a major threat to global food security. In attempts to meet the challenge of controlling parasite infection, whilst maintaining anthelmintic longevity, many practices that aim to reduce the selection pressure for anthelmintic resistance are being promoted. The aim of control strategies for ruminant gastrointestinal nematodes is to reduce the risk of disease by controlling the level of infection in the host. Therefore, whilst grazing management practices may contribute to minimising infection levels, the use of broad-spectrum anthelmintics has been the primary method to control these parasites in livestock for over 50 years (Wolstenholme *et al.* 2004). Similarly, with fluke, where the aim is not only to reduce levels of infection, but to target the highly pathogenic juvenile stages, anthelmintics are the choice control method.

There are currently four classes of broad-spectrum anthelmintics for treatment of nematodes of

ruminants; the benzimidazoles (BZs); the imidathiazoles/ tetrahydropyrimidines (levamisole, LEV), the macrocyclic lactones (MLs), including ivermectin (IVM) and moxidectin (MOX) and the amino-acetonitrile derivatives (AAD) (Wolstenholme *et al.* 2004; Kaminsky *et al.* 2008). For liver fluke a number of products are licensed with efficacy against adult fluke. However, the pro-drug triclabendazole (TCBZ) is a novel BZ that demonstrates excellent efficacy against the highly pathogenic juvenile fluke stages, as well as adults, and is, therefore, usually the drug of choice (Brennan *et al.* 2007). Thus, historically, the introduction of relatively inexpensive drugs onto the market has led to their widespread and frequent use, a further intensification of livestock production systems and, in turn, made possible the production of cheap and plentiful food for a growing world population (Wolstenholme *et al.* 2004). Not surprisingly, anti-parasitic agents hold the greatest percentage share of all products in the animal health market (McKellar and Jackson, 2004).

In contrast, surprisingly, despite this global market and reality of widespread anthelmintic resistance within the ruminant and equine industries, not one fully resolved biochemical pathway(s) used by a parasitic helminth to bio-transform a commercial anthelmintic has been published. Potential detoxification routes within parasite tissues for anthelmintics were possibly resolved along with host pathways during the compound development phase, and it may not be commercially feasible to release datasets. However, the absence or unavailability of these

* Corresponding author: IBERS, Aberystwyth University, Aberystwyth SY23 3FG, pmb@aber.ac.uk Tel: +44(0) 1970 622332

datasets potentially curtail a molecular handle to measure drug resistance arising via the pharmacokinetic or non-drug target route. This information void also potentially hinders future strategies for parasitic helminth drug development by others. For example, targeted co-inhibition of a parasite detoxification protein could, in theory, increase the efficacy of an anti-parasite drug ('resistance reversal') and a working understanding of parasite detoxification pathways could allow for building selectivity into lead compound design.

The lack of progress towards understanding the mode of action, metabolism and detoxification of many anthelmintic classes is also likely fragmented as a legacy of the random drug discovery route. The research focus has been on the mechanisms of anthelmintic resistance at the presumed target protein/gene. To this end, significant advances have been made using this targeted one-gene approach, particularly with Benzimidazole resistance (Prichard *et al.* 2007). The recent success in understanding the importance of detoxification mechanisms in pesticide resistance in field insects (Muller *et al.* 2008) and large-scale protein changes under drug treatment in the malarial protozoan parasite *Plasmodium falciparum* (Prieto *et al.* 2008) using genomic and functional genomics-led approaches supports a similar strategy for understanding anthelmintic metabolism in parasitic worms.

An anthelmintic (if metabolised and not secreted unchanged), in principle, can be neutralised or bio-transformed, in series or independently, by three protein level defence systems, termed Phase I to Phase III (Table 1; reviewed by Cvilink *et al.* 2008). In brief, Phase I can add a reactive group, such as hydroxyl group, to the anthelmintic or uncover such a group on the toxic compound. In Phase II, a toxin is joined (conjugated) with a low weight endogenous component via insertion, such as the peptide glutathione, in order to increase solubility (polarity) and usually reduce reactivity. In Phase III, conjugated toxins can be further processed (metabolised) prior to being excreted by protein pumps or sequestered by abundant hydrophobic ligand binding proteins.

However, the few studies that have been published at the metabolite and protein level tentatively suggest that parasitic helminths may have a reduced capacity to neutralise external toxins (xenobiotics) compared to their mammalian hosts (for reviews see Barrett *et al.* 1997; Cvilink *et al.* 2009). Confirmation of this finding could prove a double edged sword for anthelmintic treatment. Firstly, reduced xenobiotic metabolism in parasitic helminths provides a limited template for the design of novel parasite-activated compounds (pro-drugs). In contrast, less pharmacokinetic capacity (non-drug target resistance routes) provides the parasitic helminth with fewer opportunities than their mammalian hosts to reduce the internal concentration of compound, and the

parasite is less likely to innately possess or develop pharmacokinetic-based resistance mechanisms. Understanding the relative contribution of basic pharmacokinetic capacity compared to pharmacodynamic mechanisms (drug target-based routes) to anthelmintic resistance is an important step to resolve in support of new control strategies in field parasite population (Cvilink *et al.* 2009).

To this end, a comparative mining of the genome of the Onchocercidae filarial nematode *Brugia malayi* suggests this particular tissue-dwelling nematode has relatively low pharmacokinetic capacity compared to the free-living nematode *Caenorhabditis elegans*. This finding suggests that natural selection favours broad detoxification defences in the free-living environment compared to a micro-host environment (Lindblom and Dodd, 2009), and not supporting the suitability of free-living helminths as anthelmintic response models. Induction of drug metabolising enzymes on drug exposure is the *norm* in organisms, but proteins clearly cannot be induced on exposure to anthelmintics if their genes are lost and not present in the genome. The downside for parasitic helminth control is that increased level of anthelmintic dosing will not lead to improved efficacy if target mutation or pharmacodynamics is found to be driving resistance development in parasitic helminth populations (Alvarez *et al.* 2005).

To date, only a few experimental studies designed to track even partially the biotransformation of an activated anthelmintic within tissues of parasitic helminths have been published. These few investigations have only probed the *in vitro* ability of crude helminth extracts or isolated enzymes to potentially metabolise or interact with sometimes a parent anthelmintic compound. The problem of picking '*most-likely.*' enzymes and '*most-likely.*' anthelmintic form is that this compound might be bio-transformed by the enzyme, but it is not confirmed if this is an *in vivo* detoxification or if the selected study parasite enzyme is part of only a minor route for detoxification/biotransformation of only a minor pharmacologically active metabolite.

Thus, no integrated Phase I to Phase III case study on a confirmed active anthelmintic metabolite appears to have been completed in a parasitic helminth. In addition, no studies have been published providing parasite protein biomarker panels that could be used to measure anthelmintic success or failure at the molecular dipstick level.

In principle, proteomics offers the opportunity with the support of genomics, reverse genetics (such as RNA interference [RNAi]) and pharmacokinetic studies to capture a global snap-shot of a parasite's response to anthelmintic challenge at the protein level, without pre-empting which enzyme activities or binding proteins are likely to be involved. This review re-visits anthelmintic metabolism in parasitic helminths using recently released genomics

Table 1. The three potential phases of anthelmintic detoxification, adapted from Barrett (1997).

Phase I (Bioactivation)	Phase II (Conjugation with)	Phase III (Elimination)
Oxidation	Glutathione	Metabolism
Reduction	Glucuronides	Excretion
Hydrolysis	Methyl Groups	Sequestration
	Acetyl Groups	
	Amino Groups	
	Sulfate	
	Phosphate	
	Thiosulfate (Rhodanese)	

information and assesses the insight that experimental proteomics has made in the anthelmintic metabolism research area to date.

POTENTIAL PHASE I ANTHELMINTIC METABOLISM CAPACITY IN HELMINTHS

Traditionally, Phase I detoxification of chemical stressors in parasitic helminths was deemed via biochemically based studies to be associated more with reductive and hydrolytic-based metabolism and less by oxidative xenobiotic metabolising enzymes (XME). Genomics also predicts that hundreds of potential Phase I detoxification hydrolases and reductases can be present in helminth genomes (Dieterich *et al.* 2008).

However, in vertebrates, and it appears most other invertebrates, the major Phase I route is oxidative via the cytochrome P450 Superfamily (CYPs). The inability of biochemical techniques to detect the usually major CYP activities in isolated microsomal preparations of adult helminths, in hindsight unfairly, raised the relative importance of reductive and hydrolytic transformations (Precious and Barrett, 1989). In insect pests, CYPs are established as a major route for insecticide resistance (Daborn *et al.* 2002). CYP activity has been demonstrated in range of parasitic protozoa (Barrett, 1998) and the structure of a CYP protein resolved binding to drugs from two trypanosomes (Chen *et al.* 2010).

Limited CYP-type biochemical level activity is detectable in nematode larval stages, but it is not yet confirmed whether this activity is simply metabolic house-keeping or detoxification (Kerboeuf *et al.* 1995; Kotze *et al.* 2006). The few experimental studies completed in digeneans suggest that adult schistosomes, at least, have relatively high CYP-like activity. In *Schistosoma mansoni*, several antibodies to rat microsomal CYPs are shown to cross-react with schistosome extracts (Saeed *et al.* 2002). An inhibitor known to disrupt CYP function in other systems was found to enhance the susceptibility of a triclabendazole (TCBZ)-resistant *F. hepatica* isolate to TCBZ as judged by morphological tegumental surface scanning electron microscopy measurements (Devine *et al.* 2010), although

inhibition with the same CYP system inhibitor reduced TCBZ-sulphoxide formation equally in TCBZ-resistant and -sensitive isolates (Alvarez *et al.* 2005).

Genomics also supports the possibility that biochemical assays might have been a 'red herring.' for CYP activity in adult parasitic helminths. Recent genomic and transcriptomics studies predict synthesis of many protein forms of the CYP superfamily within helminths. To date, the number of CYP genes ranges from 198 in the genome of the insect parasitic nematode *Pristionchus pacificus* (Dieterich *et al.* 2008) to 27 CYP genes/partial genes in the plant parasitic nematode *Meloidogyne incognita* (Bird *et al.* 2009).

CYP has now been extensively investigated in the free-living model nematode *C. elegans*, with 75 predicted CYP genes within 18 families, with CYPs found to be up-regulated on xenobiotic exposure (Kulas *et al.* 2008) and anthelmintic exposure under genome wide transcript level analysis (Laing *et al.* 2010). In addition, the gene encoding a receptor central to the regulation of CYP activity throughout the animal kingdom has been found in *C. elegans* (Lindblom and Dodd, 2009). Importantly, the key protein involved in CYP activity, NADP cytochrome P450 reductase has been identified in nematode genomes and nine CYP genes in *C. elegans* have altered phenotypes on large-scale RNAi gene knock-down experiments (Simmer *et al.* 2003).

CYP function is deemed essential for normal cellular metabolism in other organisms. In addition, it is predicted to be present in helminth genomes. Both points suggest CYP protein level absence will either reflect technical interference, the possibility that CYPs only have specific low level house-keeping metabolic functions (activities not associated with anthelmintic assault) or a unique biochemistry of adult helminths replacing CYP function. Others have suggested the absence is due to a limited access to oxygen required for CYP function (Barrett, 1998), although this seems unlikely as CYP function is universally required for essential house-keeping, and helminths have other functional oxidase enzymes. However, the possibility remains that alternative uncharacterized xenobiotic

peroxidase(s) to CYP are expressed in adult parasitic helminths (Barrett, 1997). To this end, a number of candidate anthelmintic detoxification enzymes have been identified from studies on parasite reactive oxygen species (ROS) defence systems (reviewed by Cvilink *et al.* 2009).

An inhibitor addition strategy was also used to show that another less well characterized Phase I system, flavin mono oxygenase system (FMO), is apparently important in TCBZ metabolism in microsomes of the digenean *F. hepatica* (Alvarez *et al.* 2005). The FMO gene family is smaller than the CYP superfamily and present in almost all phyla that have been examined (Cvilink *et al.* 2009).

The anthelmintics albendazole (ABZ) and TCBZ are subject to Phase I sulphoxidation in a number of parasite helminths (Cvilink *et al.* 2009), possibly by different enzymes in different cellular locations. Furthermore, *in vivo*, significant levels of Phase I oxidation of TCBZ-sulphoxide to the inactive TCBZ-sulphone is detectable in *F. hepatica*, with greater conversion in TCBZ-resistant *F. hepatica* isolate compared to TCBZ-sensitive isolate (Robinson *et al.* 2004). This is evidence of a new non-drug target resistance route in liver fluke (Alvarez *et al.* 2005; Brennan *et al.* 2007). Phase I anthelmintic reduction has also been widely reported in nematodes (Cvilink *et al.* 2008; Kohler and Bachmann, 1981) and cestodes (Barrett, 1997). Thus, overall, there is fragmented information at the protein level of Phase I detoxification capacity in parasitic helminths of medical and veterinary importance.

POTENTIAL PHASE II ANTHELMINTIC METABOLISM CAPACITY IN PARASITIC HELMINTHS

The majority of biochemically based Phase II studies have been limited to glutathione conjugation via glutathione transferases (GSTs), with only a few detailed investigations on other potential conjugation routes by glucose, sulphate, phosphate and N-acetylation (reviewed in Cvilink *et al.* 2009). Genomic studies, such as in *C. elegans* and *P. pacificus* predict the presence of a range of proteins capable of Phase II detoxification such as UDP-glucuronosyltransferases and sulphotransferases (Dieterich *et al.* 2008; Reichert and Menzel, 2005).

GST is usually present in relatively high levels in the cytosol of many adult parasitic helminths. An interesting exception is lower GST levels in tissue-dwelling filarial nematodes (Brophy *et al.* 1990b) possibly correlating with suggested genome level absence of detoxification capacity. There are many GST forms within multi-GST families as would be expected of a xenobiotic defence protein superfamily, such as 54 GSTs in *P. pacificus* and 51 GSTs in *C. elegans* (Van Rossum *et al.* 2001).

Many GSTs (as other detoxification proteins) also have house-keeping metabolic roles. For example, GSTs are possibly hydrophobic ligand transport proteins (Brophy and Barrett, 1990), and their relatively high levels required for transport will be highlighted in proteomic studies, but this is not a detoxification role. Thus, global proteomics will often bring to prominence the abundant soluble GSTs that are relatively easy to assay and purify natively and express as recombinants. However, a highly effective detoxification enzyme might be present in levels undetectable by global proteomic studies, with its identification requiring sub-proteomic investigations, such as following an initial affinity chromatography step; in this manner a new GST form was found in *F. hepatica* (Chemale *et al.* 2006).

There is no mechanistic level evidence that GSTs actually conjugate anthelmintics, despite the number of GST-based reductionist biochemical investigations and identifications in proteomic studies. The only suggestion is that schistosome GST is involved in the biotransformation of the anthelmintic dichlorvos (O'Leary and Tracy, 1991). However, the parent anthelmintic substrate is usually tested on GST, not a pro-drug activated form or a substrate modified by parasite Phase I metabolism. There is ample evidence, including proteomic leads, that isolated native or recombinant forms of helminth GSTs can bind to parent anthelmintic structures (Brophy *et al.* 1990a). To date, only one study tracked the binding of a pro-drug (triclabendazole sulphoxide) to *F. hepatica* proteins following comparative global proteomics (Chemale *et al.* 2010). Loss-of-function phenotype RNAi protocols have been optimised in the free-living nematode *C. elegans* for the GST superfamily (LaCourse *et al.* 2008) and offers opportunity to link GST to function under external anthelmintic stress in culture. However, in the *C. elegans* model at least there appears up-regulation of GST forms, at the protein level, to potentially compensate for down-regulation of other GST forms (LaCourse *et al.* 2008). This study also showed that proteomics can track the success of RNAi knockdown experiments (termed Prot. RNAi).

In addition, further to the lack of demonstration of anthelmintic conjugation with glutathione, there is also limited evidence that parasitic helminths can Phase III metabolise glutathione conjugates, with only low activities of one enzyme detectable by enzymic assay in adult nematodes (cysteine conjugate β -lyase, Adcock *et al.* 1999). The focus on the importance of GST in the pre-genomics area has possibly deflected and discouraged other important Phase II investigations. For example, Phase II glucose conjugation of toxins mainly occurs in plants and not animals, but has been uncovered in parasitic nematodes (Cvilink *et al.* 2008) and

with novel glucoside metabolites of albendazole produced in the nematode model *C. elegans* (Laing *et al.* 2010).

POTENTIAL PHASE III ANTHELMINTIC METABOLISM CAPACITY IN HELMINTHS

Anthelmintic conjugates from Phase I and Phase II, following further potential Phase III enzyme-directed modification would likely be removed from cells at membranes by ATP-binding cassette (ABC)-dependent transport family or sequestered by an abundant binding protein. Additionally, a range of hydrolyase enzymes that could further process Phase II metabolites have been biochemically detected in parasitic helminths (Adcock *et al.* 1999; Cvilink *et al.* 2009).

ABC transporters are found in both prokaryotes and eukaryotes and are associated with multidrug resistance. Genomic studies have predicted many ABC transporters in nematodes, with 129 genes in *P. pacificus* and 56 genes in *C. elegans* respectively (Dieterich *et al.* 2008). In parasitic helminths the experimental research effort has been directed towards the well-characterized P-glycoprotein member of the ABC transporters. For example, studies in *F. hepatica* show that TCBZ-resistant isolates accumulate less TCBZ and its major active metabolite than TCBZ-sensitive isolates, suggesting differences in rates of efflux pumping and presumed to be via P-glycoprotein. In addition, indirect evidence with inhibitors indicated that ABC transport pumps, including P-glycoprotein, in *F. hepatica* are associated with TCBZ resistance as reversal of 'resistance' occurred with inhibition. P-glycoprotein ABC pumps are also associated with anthelmintic resistance in nematodes (reviewed by Alvarez *et al.* 2006).

Sequestration is deemed an alternative of excretion and, to date, only descriptive studies of this route have been undertaken in helminths. Binding of toxins by abundant helminth ligand-binding proteins has been described for both GST (discussed above) and lipid binding proteins (LBPs). LBPs (Barrett *et al.* 1997) have clearly been demonstrated to bind anthelmintics using purified or recombinant protein forms and both GST and LBP can be present at concentrations of up to 50 μM . The binding of anthelmintic may either reduce the effective drug concentration, rendering the buffered compound more likely to be metabolised by a complex or, conversely, may simply disrupt GST/LBP house-keeping functions. One novel example of sequestration may be the binding of toxic ingested haem from the host to GST proteins from blood-feeding gastro-intestinal nematodes (Van Rossum *et al.* 2004). In addition, metallothioneins bind toxic metals and have been extensively investigated and identified in the *C. elegans* model (Hoeckner *et al.*

2011), but interaction with anthelmintics have not yet been investigated.

PROTEOMICS AS A TOOL TO UNCOVER ANTHELMINTIC METABOLISM PATHWAYS

Next-generation sequencing of helminth transcriptomes and genomes can reveal potential pathways for anthelmintic metabolism. However, gene annotation based on previous biochemical studies in other systems will not reveal novel parasitic helminth detoxification processes. Metabolome and proteomic level studies are required to help confirm the detoxification status of a protein and associated pathways. A new metabolite and protein level drive is required to realise the potential of the post-genomic era and support the development of new interactive databases such as HelmCoP, Helminth Control and Prevention (Abubucker *et al.* 2011). Thus, information from genomes should not simply be used to clone and express 'likely enzymes' for assessing anthelmintic metabolism and return to reductionist biology approaches.

As highlighted above, proteomics, unlike classical reductionist biochemistry, has the potential to reveal systematically the complete (soluble and membrane) dynamic protein response (relative expression, modifications and interactions) in a parasitic helminth to an environmental challenge (Barrett *et al.* 2000). To date, most proteomic investigations in parasitic helminths have been descriptive in technically straightforward soluble fractions without environment challenge to address a physiological question. These pioneering proteomic studies showed that helminth proteins could be identified (Jefferies *et al.* 2001), without the luxury of a freely available annotated complete protein database, as is available for the model nematode *C. elegans* for example (Jones *et al.* 2010). Despite this success, there remains a dearth of transcript and gene prediction data for helminths making many potential proteomics experiments extremely challenging, but possible with available databases (Millares *et al.* 2012).

Descriptive proteomics studies have supported understanding of candidate anthelmintic metabolism proteins, such as the soluble GST superfamily. The GST complement of first the model nematode *C. elegans* (Van Rossum *et al.* 2001) and, more recently, in *F. hepatica* (Chemale *et al.* 2006) was resolved via a sub-proteomics approach. In the 2006 study, the authors incorporated two affinity chromatography steps, 2DE proteomics and peptide sequencing to resolve new GST family members. The *Fasciola*-based study, in parallel with probing an EST dataset, identified two new forms of GST (Chemale *et al.* 2006). The disadvantage of affinity chromatography-based sub-proteomic approaches

Table 2. Mining of experimental parasitic helminth proteomic data from publications in refereed journals for evidence of potential anthelmintic metabolising, sequestration and efflux pump proteins

Phase	Protein Identified	Organism	Proteomic Localisation				Reference	
			Somatic	ES Products	Tegument	Other		
PHASE I								
CYPs	Predicted Similar to Cytochrome P450	<i>A. caninum</i>	—	x	—	—	Mulvenna <i>et al.</i> 2009a	
Peroxidases	Thioredoxin Peroxidase 2	<i>C. sinensis</i>	—	x	—	—	Ju <i>et al.</i> 2009	
	Thiol Anti-oxidant Protein	<i>E. friedi</i>	—	x	—	—	Bernal <i>et al.</i> 2006	
	Thioredoxin Peroxidase	<i>F. hepatica</i>	x	x	x	x	Jefferies <i>et al.</i> 2001; Chemale <i>et al.</i> 2010; Hernandez <i>et al.</i> 2010; Moxon <i>et al.</i> 2010; Wilson <i>et al.</i> 2011	
	Putative peroxidase	<i>O. viverrini</i>	—	—	x	—	Mulvenna <i>et al.</i> 2010	
	Thioredoxin Peroxidase 2	<i>O. viverrini</i>	—	x	—	—	Mulvenna <i>et al.</i> 2010	
	Thioredoxin Peroxidase	<i>S. mansoni</i>	x	—	—	x	Braschi <i>et al.</i> 2006; Mathieson and Wilson 2010	
	Thioredoxin Peroxidase	<i>S. japonicum</i>	—	—	—	x	Mulvenna <i>et al.</i> 2009b	
	Thioredoxin Peroxidase	<i>E. granulosus</i>	—	x	—	—	Chemale <i>et al.</i> 2003	
	Thioredoxin Peroxidase	<i>E. multilocularis</i>	x	—	—	—	Wang <i>et al.</i> 2009	
	Thioredoxin Peroxidase	<i>M. corti</i>	x	—	—	—	Laschuk <i>et al.</i> 2011	
	Peroxiredoxin	<i>C. constaricensis</i>	x	—	—	—	Rebello <i>et al.</i> 2011	
	Thioredoxin Peroxidase	<i>H. polygyrus bakeri</i>	x	—	—	—	Morgan <i>et al.</i> 2006; Hewitson <i>et al.</i> 2011	
		Tryparedoxin Peroxidase 2	<i>L. braziliensis</i>	x	—	—	—	Cuervo <i>et al.</i> 2007
		Thioredoxin Peroxidase	<i>T. circumcincta</i>	—	x	—	—	Craig <i>et al.</i> 2006
Reductases	Carbonyl reductase	<i>F. hepatica</i>	—	—	—	x	Moxon <i>et al.</i> 2010	
	Carbonyl reductase	<i>S. mansoni</i>	—	x	—	—	Knudsen <i>et al.</i> 2005	
	Aldo/Keto Reductase	<i>S. mansoni</i>	—	—	—	x	Mathieson and Wilson 2010	
	Aldo/Keto Reductase	<i>M. corti</i>	x	—	—	—	Laschuk <i>et al.</i> 2011	
	Aldo/Keto Reductase Family 1	<i>T. circumcincta</i>	—	x	—	—	Craig <i>et al.</i> 2006	
	Oxidoreductase, Aldo/Keto Reductase Family	<i>B. malayi</i>	—	x	—	—	Bennuru <i>et al.</i> 2009	
Hydrolases	Amidase Domain	<i>F. hepatica</i>	—	—	x	—	Wilson <i>et al.</i> 2011	
	Phosphodiesterase	<i>S. mansoni</i>	—	—	x	—	Braschi <i>et al.</i> 2006	
	Protein Phosphatase 2C	<i>L. braziliensis</i>	x	—	—	—	Cuervo <i>et al.</i> 2007	
FMOs	Electron Transfer Flavoprotein	<i>S. mansoni</i>	—	—	x	—	Braschi <i>et al.</i> 2006	
	Flavin-binding Monooxygenase-like Family Protein	<i>B. malayi</i>	—	x	—	—	Bennuru <i>et al.</i> 2009	
	NADH:Flavin oxidoreductase/NADH Oxidase	<i>L. braziliensis</i>	x	—	—	—	Cuervo <i>et al.</i> 2007	
PHASE II								
GSTs	GST	<i>C. sinensis</i>	—	x	—	—	Ju <i>et al.</i> 2009	

	Mu class GST	<i>E. caproni</i>	—	x	—	—	Sotillo <i>et al.</i> 2010
	GST	<i>E. friedi</i>	—	x	—	—	Bernal <i>et al.</i> 2006
	Mu class GST	<i>F. hepatica</i>	x	x	x	x	Jefferies <i>et al.</i> 2001; Chemale <i>et al.</i> 2006; Morphey <i>et al.</i> 2007; Chemale <i>et al.</i> 2010; Moxon <i>et al.</i> 2010; Hernandez <i>et al.</i> 2010; Wilson <i>et al.</i> 2011
	Sigma class GST	<i>F. hepatica</i>	x	x	x	—	Chemale <i>et al.</i> 2006; Wilson <i>et al.</i> 2011; LaCourse <i>et al.</i> 2012
	GST	<i>O. viverrini</i>	—	x	—	—	Mulvenna <i>et al.</i> 2010
	GST Sm28	<i>S. bovis</i>	—	x	x	—	Perez-Sanchez <i>et al.</i> 2006
	GST Sm28	<i>S. mansoni</i>	x	x	—	x	Curwen <i>et al.</i> 2004; Knudsen <i>et al.</i> 2005; Mathieson and Wilson 2010
	GST Sm26	<i>S. mansoni</i>	x	—	x	x	Curwen <i>et al.</i> 2004; Braschi <i>et al.</i> 2006; Mathieson and Wilson 2010
	GST28	<i>S. japonicum</i>	—	—	—	x	Mulvenna <i>et al.</i> 2009b
	GST26	<i>S. japonicum</i>	—	—	—	x	Mulvenna <i>et al.</i> 2009b
	GST	<i>E. multilocularis</i>	x	—	—	—	Wang <i>et al.</i> 2009
	GST	<i>A. caninum</i>	—	x	—	—	Mulvenna <i>et al.</i> 2009a
	GST	<i>H. contortus</i>	x	x	—	—	Yatsuda <i>et al.</i> 2003; Millares <i>et al.</i> 2010; Yan <i>et al.</i> 2010; Hart <i>et al.</i> 2012
	GST	<i>H. polygyrus bakeri</i>	x	—	—	—	Hewitson <i>et al.</i> 2011
UDP-Glucuronosyltransferases	—	—	—	—	—	—	—
Methyltransferases	Methyltransferase, putative	<i>B. malayi</i>	—	x	—	—	Bennuru <i>et al.</i> 2009
	Skb1 methyltransferase family protein	<i>B. malayi</i>	—	x	—	—	Bennuru <i>et al.</i> 2009
	F25B4 .1	<i>H. contortus</i>	x	—	—	—	Millares <i>et al.</i> 2012
	Aminomethyltransferase						
Sulfotransferases	Thiosulfate sulfuryltransferase	<i>S. ratti</i>	—	x	—	—	Soblik <i>et al.</i> 2011
Glyoxylase	—	—	—	—	—	—	—
PHASE III							
ATP-Binding Cassette (ABC) Transporters/MDRPs	ABC-Type MDTs	<i>F. hepatica</i>	x	—	—	—	Hernandez <i>et al.</i> 2010
	Na/K Transporting ATPase beta subunit	<i>S. mansoni</i>	—	—	x	—	Braschi <i>et al.</i> 2006
	Plasma Membrane Calcium ATPase	<i>S. mansoni</i>	—	—	x	—	Braschi <i>et al.</i> 2006
	ABC Transporter ATP Binding Protein	<i>S. japonicum</i>	x	—	—	—	Cheng <i>et al.</i> 2005
	ATP-binding cassette sub-family E	<i>B. malayi</i>	—	x	—	—	Bennuru <i>et al.</i> 2009
	ABC transporter transmembrane region	<i>B. malayi</i>	—	x	—	—	Bennuru <i>et al.</i> 2009
	ABC Transporter	<i>C. constaricensis</i>	x	—	—	—	Rebello <i>et al.</i> 2011

Table 2. (Cont.)

Phase	Protein Identified	Organism	Proteomic Localisation					Reference
			Somatic	ES Products	Tegument	Other		
Lyases Lipid Binding Proteins	—	—	—	—	—	—	—	—
	FABP I, II & III	<i>F. hepatica</i>	x	x	—	—	—	Jefferies <i>et al.</i> 2001; Morphew <i>et al.</i> 2007; Chemale <i>et al.</i> 2010; Wilson <i>et al.</i> 2011
	FABP	<i>O. viverrini</i>	—	x	—	—	—	Mulvenna <i>et al.</i> 2010
	FABP 15	<i>S. bovis</i>	—	x	x	—	—	Perez-Sanchez <i>et al.</i> 2006
	SM14	<i>S. mansoni</i>	—	x	x	—	—	Knudsen <i>et al.</i> 2005; Braschi <i>et al.</i> 2006
Lipid Binding protein	FABP	<i>S. mansoni</i>	x	—	—	x	—	Curwen <i>et al.</i> 2004; Mathieson and Wilson 2010
	Lipid Binding protein	<i>A. caninum</i>	—	x	—	—	—	Mulvenna <i>et al.</i> 2009a
	Lipid Binding protein	<i>H. contortus</i>	x	—	—	—	—	Millares <i>et al.</i> 2012
	Lipid Binding protein	<i>H. polygyrus bakeri</i>	x	—	—	—	—	Morgan <i>et al.</i> 2006
	FABP	<i>H. polygyrus bakeri</i>	x	—	—	—	—	Morgan <i>et al.</i> 2006; Hewitson <i>et al.</i> 2011
Fatty Acid Retinoid Binding Protein	FABP	<i>S. rattii</i>	—	x	—	—	—	Soblik <i>et al.</i> 2011

is that although proteins can be concentrated by selection away from other cellular proteins, all the proteins of interest need to bind to an affinity matrix in order to reveal the full expressed complement which is not always accomplished. However, sub-proteomics studies such as those based on membrane fractions, could probe for the expression of Phase I (CYPs) or Phase III (P-glycoprotein ATP pump) proteins but are yet to be completed due to the technical complexities associated with analysing membrane fractions.

With the success of RNAi, initially in nematodes and more recently in flatworms, the ability to delineate anthelmintic metabolising or interacting proteins or protein superfamilies can be achieved by combining RNAi with proteomics (LaCourse *et al.* 2008). The combination of both techniques should allow targeted experiments to unravel the complexities of anthelmintic metabolism.

To date, the findings from many descriptive proteomics studies have not been fully realised despite identifying a large variety of proposed xenobiotic detoxification proteins in parasitic helminths (Table 2). Gel-based proteomics is not focused on normalising data and more abundant soluble/xenobiotic detoxification proteins are more readily identified, such as the GSTs, hydrophobic ligand-binding proteins and peroxidases. Interestingly, proteins with detoxification roles are detected in helminth excretory and secretory products (Table 2), suggesting near surface detoxification in a xenobiotic rich host environment. However, descriptive proteomics studies could be enhanced quantitatively to further benefit the understanding of anthelmintic metabolism. Coupling the global separation of helminth proteins to partial least squares–discriminant analysis (PLS-DA) offers the possibility to group proteins and compare innate metabolic pathways in isolates. This approach has been used to protein fingerprint *F. hepatica* isolates in order to develop training sets to support the identification of anthelmintic resistant isolates from those susceptible to treatment (MacKintosh, 2011) and has the potential to identify new biomarkers.

Investigative comparative proteomics has already provided some new insights to support understanding of anthelmintic metabolism. There are many potential routes for a parasite to reduce the concentration of a toxic anthelmintic in their cells: reduce uptake, increase excretion, change or lose a target, not activating a pro-drug and via toxin detoxification pathways. As a case study, a proteomics experiment with defined isolates of *F. hepatica* exposed to TCBZ provided evidence following recombinant expression that a fatty acid binding protein (FABP) was a new type of Phase III anthelmintic sequestration protein associated with resistant isolates while providing further evidence of the role of Mu class GST in

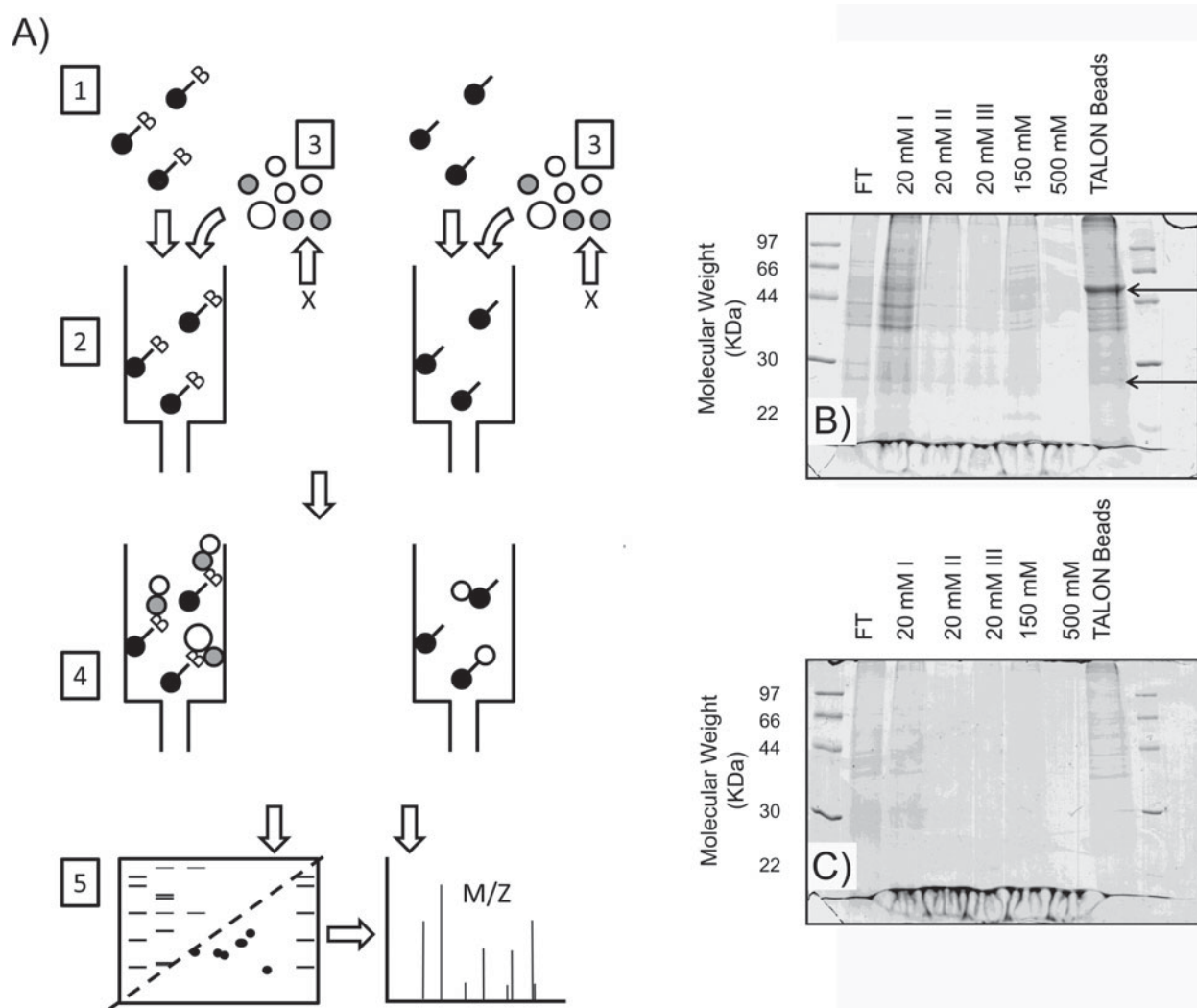


Fig. 1. Understanding anthelmintic action using 'pull down' proteomic methods. A) Schematic representation of 'pull down' methods and their application to studying anthelmintic action where the 'bait' (1) can either be a protein of interest or an anthelmintic. The bait, attached to a solid substrate, can be loaded into a column (2) before being allowed to interact with a protein lysate (3); or the bait and lysate can be pre-incubated before loading into a column. At this stage, if using a protein as bait, an anthelmintic (X) can be introduced to disrupt potential interactions. The interactions are washed substantially to remove non-specific binding partners (4) and then eluted and analysed directly via 1D or 2D SDS-PAGE followed by mass spectrometry or directly by mass spectrometry using gel free methods (5). This method has been demonstrated in B) and C) to investigate protein interactions of *C. elegans* (wild type N2) beta-tubulin (Ben-1) using Dynabeads[®] TALON[®] Co²⁺ affinity purification as the solid support incubated with wild type N2 protein lysate. B) Interactions of wild type N2 Ben-1 Beta-tubulin with N2 lysate purified with Dynabeads[®] TALON[®]. C) Interactions of wild type N2 lysate and Dynabeads[®] TALON[®] only. FT – flow through. 20 mM I-III – Imidazole washes 1–3 containing 20 mM imidazole. 150 mM and 500 mM correspond to 150 mM and 500 mM imidazole washes. TALON beads represents the Dynabeads[®] TALON[®] boiled in SDS buffer. Both B) and C) 12% SDS PAGE Coomassie blue stained. Arrows are *C. elegans* Ben-1 (upper arrow) and Sorbitol dehydrogenase (lower arrow) a known interacting partner of the tubulin complex (Morphew, unpublished).

binding of anthelmintics (Chemale *et al.* 2010). This single proteomics experiment highlighted the dynamics of protein response to anthelmintic exposure and supports previous reductionist biochemistry in independent laboratories that when the data are combined suggested broad TCBZ-induced changes in liver fluke. RNAi assays will confirm the role of FABP in liver fluke anthelmintic response and nucleic acid sequencing studies will reveal if

any markers of TCBZ resistance are found in the FABP gene.

CHALLENGES TO PROTEOMICS TECHNOLOGIES TO SUPPORT UNDERSTANDING OF ANTHELMINTIC METABOLISM

To date, anthelmintic response and metabolism studies have also been neglected by proteomics

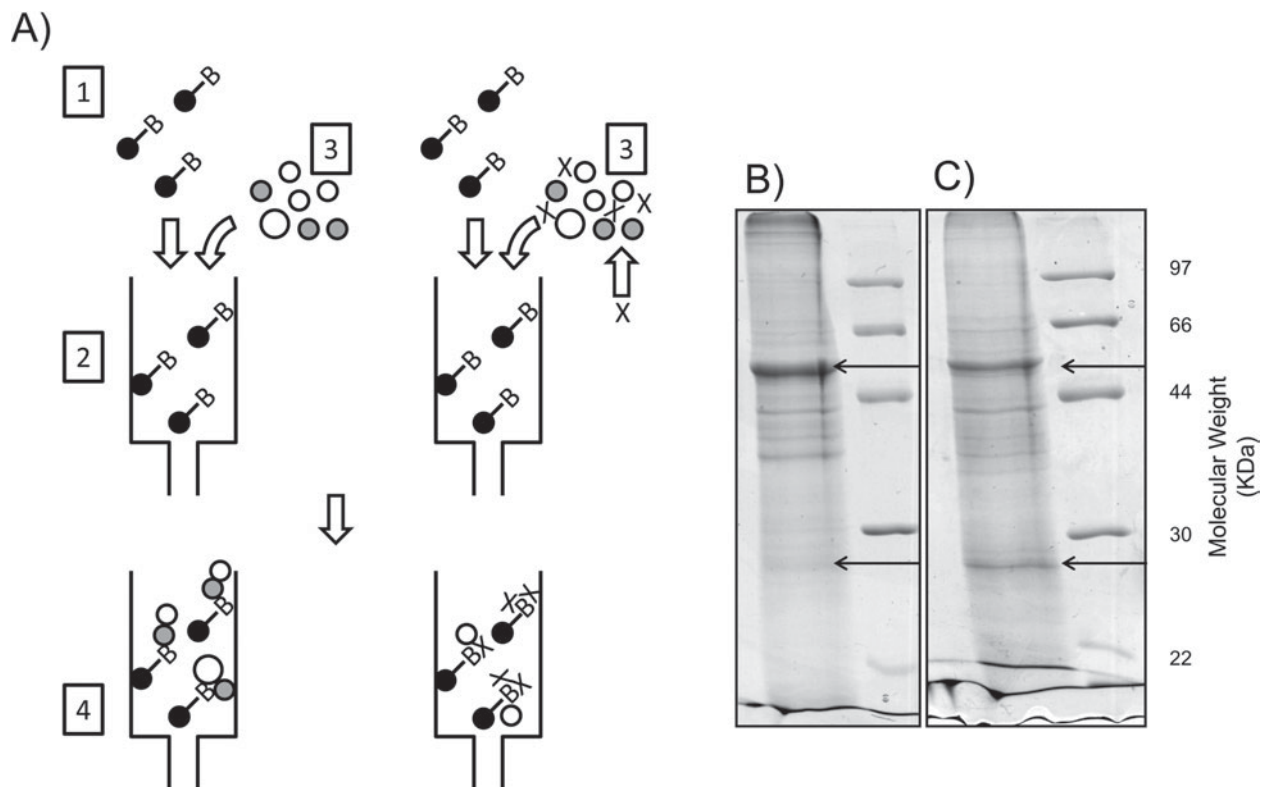


Fig. 2. Anthelmintic disruption of protein-protein interactions. A) Schematic representation of 'pull down' proteomics to study anthelmintic action using a known interacting protein as 'bait' (1). The protein bait, attached to a solid substrate, can be loaded into a column (2) before being allowed to interact with a protein lysate (3) with and without the presence of anthelmintic (X). Protein interactions formed with and without anthelmintic (4) can be analysed as previously detailed (Fig. 1). This is demonstrated in B) and C) showing protein-protein interactions of *C. elegans* (wild type N2) beta-tubulin (Ben-1) interrupted by benzimidazoles. Ben-1 bound to Dynabeads[®] TALON[®] Co²⁺ were incubated with wild type N2 protein lysate in the presence of benzimidazole (B) and absence of benzimidazole (C) to look for disruption of protein interactions. Both B) and C) 12% SDS PAGE Coomassie blue stained. Arrowed are *C. elegans* Ben-1 (upper arrow) in both B) and C) and Sorbitol dehydrogenase (lower arrow) in C) only (Morphew unpublished).

approaches, with only one study of soluble responses in liver fluke published (Chemale *et al.* 2010).

The success of proteomics is entwined with the delivery of genomes or transcriptome databases that will provide the searchable databases to link function with sequence. In contrast, the success of helminth genomes requires an experimental lead to predict functional annotation. It is predicted that there will be over thirty helminth genomes available at least in draft form within the next five years (Abubucker *et al.* 2011).

Helminth proteomic studies have confirmed the presence of post-translational modifications (PTM) (Jefferies *et al.* 2001; Chemale *et al.* 2003) but, to date, there have been no proteomic studies probing the extent or the functional significance of PTM on helminth proteins. With over four hundred potential modifications, and an average of ten PTMs per protein, it is clear that for a full mechanistic understanding of helminth biochemistry, including anthelmintic metabolism, a significant time investment is required in this area of functional proteomics.

Most cellular processes are completed by proteins that temporarily assemble into multimeric complexes. Thus protein-protein interactions are important in protein function. For example, the blocking of such protein-protein interactions by the anthelmintic group benzimidazoles (Bzs) leads to the disruption of tubulin polymerisation (Prichard *et al.* 2007). At present, experiments on helminth protein complexes associated with anthelmintic metabolism have been investigated using 'pull down' proteomics approaches. For example, using an anthelmintic as a 'bait' has shown some success studying interacting partners of albendazole (Chambers *et al.* 2010). In addition, using known anthelmintic interacting/metabolising proteins, such as GST (Greetham *et al.* 2004) and beta-tubulin (Fig. 1) as 'baits', pools of interacting proteins can be identified. Thus, protein networks associated with anthelmintic metabolism/interaction can be established and the effect of anthelmintic pressure on these interactions examined (Fig. 2).

TAP (tandem affinity purification) methodology provides for isolation of proteins as native complexes

from cells for subsequent proteomics analysis, but in helminths this powerful tool has to date only been used in the model nematode *C. elegans* (Lee and Lee, 2004). As heterologous protein expression of parasitic nematode anthelmintic target proteins has previously been successful in the *C. elegans* host (Kwa *et al.* 1995) then the potential of TAP technology for understanding anthelmintic metabolism, at least in nematodes, is a real possibility.

The main challenge for helminth proteomics remains the detection and measurement of integral membrane-bound proteins, such as those important in Phase I and Phase III anthelmintic metabolism. To date, there has been progress understanding the protein complement in the tegument of digeneans (Mulvenna *et al.* 2010; Castro-Borges *et al.* 2011; Wilson *et al.* 2011). Yet, these flatworm studies have produced only a few recognised Phase I and III proteins potentially involved in anthelmintic metabolism (Table 2). The experimental difficulties with protein solubility and low abundance making integral membrane protein identification a challenge for both gel based and gel free proteomics.

CONCLUSIONS

Anthelmintics are essential for parasitic helminth control but there is fragmented information on drug metabolism in parasites derived from reductionist biology, and possibly red herrings (limited CYP and relative importance of GST). To date, most proteomic studies on parasitic helminths have been on descriptive on technically easy soluble fractions and not focused on addressing a biological question, such as response and protein induction to a specific anthelmintic assault and unravelling any relationship between anthelmintic metabolism to anthelmintic resistance. The new genome information will provide templates to link sequence via function via more functional proteomic investigations. Overcoming the difficult technical challenge of optimisation of membrane proteomics in helminths is essential to effectively study Phase I CYPs and Phase III ABC transporter pumps.

With the current information available on parasite protein complements fragmented and major technical challenges resolving membrane proteins, then the future of protein-level research directed towards anthelmintic metabolism is challenging. However, the availability of new genomes/transcriptomes from parasitic helminths should inspire more parasitic worm laboratories to address the challenges of membrane proteomics analyses, using the information available from specialist membrane proteomic developers. The new sequences provide templates of the capacity of xenobiotic metabolism in parasitic helminths and proteomics will confirm the routes of individual anthelmintic response and inform, stimulate and support those interested

in developing new anthelmintics for neglected diseases. At present, the limited information does suggest addressing challenges will be rewarded as there are differences in detail between the biotransformation routes between parasites and hosts.

ACKNOWLEDGEMENTS

This paper is dedicated to the memory of Professor John Barrett.

PMB and RMM thank the BBSRC and DFID for financial support (grant BBH0092561) and the English Beef and Lamb Executive (EBLEX), Hybu Cig Cymru/Meat Promotion Wales (HCC) and Quality Meat Scotland (QMS) for funding NMK.

REFERENCES

- Abubucker, S., Martin, J., Taylor, C.M. and Mitreva, M. (2011). HelmCoP: An online resource for helminth functional genomics and drug and vaccine targets prioritization. *Plos One* **6** (7), e21832.
- Adcock, H.J., Brophy, P.M., Teesdale-Spittle, P.H. and Buckberry, L.D. (1999). Cysteine conjugate beta-lyase activity in three species of parasitic helminth. *International Journal for Parasitology* **29**, 543–548.
- Alvarez, A.I., Merino, G., Molina, A.J., Pulido, M.M., Mckellar, Q.A. and Prieto, J.G. (2006). Role of ABC transporters in veterinary drug research and parasite resistance. *Current Drug Delivery* **3**, 199–206.
- Alvarez, L.I., Solana, H.D., Mottier, M.L., Virkel, G.L., Fairweather, I. and Lanusse, C.E. (2005). Altered drug influx/efflux and enhanced metabolic activity in triclabendazole-resistant liver flukes. *Parasitology* **131**, 501–510.
- Barrett, J. (1997). Helminth detoxification mechanisms. *Journal of Helminthology* **71**, 85–89.
- Barrett, J. (1998). Cytochrome P450 in parasitic protozoa and helminths. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* **121**, 181–183.
- Barrett, J., Jefferies, J.R. and Brophy, P.M. (2000). Parasite proteomics. *Parasitology Today* **16**, 400–403.
- Barrett, J., Saghir, N., Timanova, A., Clarke, K. and Brophy, P.M. (1997). Characterisation and properties of an intracellular lipid-binding protein from the tapeworm *Moniezia expansa*. *European Journal of Biochemistry* **250**, 269–275.
- Bennuru, S., Semnani, R., Meng, Z., Ribeiro, J.M.C., Veenstra, T.D. and Nutman, T.B. (2009). *Brugia malayi* excreted/secreted proteins at the host/parasite interface: stage- and gender-specific proteomic profiling. *PLoS Neglected Tropical Diseases* **3** (4), e410.
- Bernal, D., Carpena, I., Espert, A.M., De La Rubia, J.E., Esteban, J.G., Toledo, R. and Marcilla, A. (2006). Identification of proteins in excretory/secretory extracts of *Echinostoma friedi* (Trematoda) from chronic and acute infections. *Proteomics* **6**, 2835–2843.
- Bird, D.M., Williamson, V.M., Abad, P., Mccarter, J., Danchin, E.G.J., Castagnone-Sereno, P. and Opperman, C.H. (2009). The genomes of root-knot nematodes. *Annual Review of Phytopathology* **47**, 333–351.
- Braschi, S., Curwen, R.S., Ashton, P.D., Verjovski-Almeida, S. and Wilson, A. (2006). The tegument surface membranes of the human blood parasite *Schistosoma mansoni*: A proteomic analysis after differential extraction. *Proteomics* **6**, 1471–1482.
- Brennan, G.P., Fairweather, I., Trudgett, A., Hoey, E., McCoy, Mcconville, M., Meaney, M., Robinson, M., Mcferran, N., Ryan, L., Lanusse, C., Mottier, L., Alvarez, L., Solana, H., Virkel, G. and Brophy, P.M. (2007). Understanding triclabendazole resistance. *Experimental and Molecular Pathology* **82**, 104–109.
- Brophy, P.M. and Barrett, J. (1990). Glutathione transferase in helminths. *Parasitology* **100**, 345–349.
- Brophy, P.M., Crowley, P. and Barrett, J. (1990a). Detoxification reactions of *Fasciola hepatica* cytosolic glutathione transferases. *Molecular and Biochemical Parasitology* **39**, 155–162.
- Brophy, P.M., Crowley, P. and Barrett, J. (1990b). Relative distribution of glutathione transferase, glyoxalase-I and glyoxalase-II in helminths. *International Journal for Parasitology* **20**, 259–261.

- Castro-Borges, W., Simpson, D.M., Dowle, A., Curwen, R.S., Thomas-Oates, J., Beynon, R. J. and Wilson, R. A. (2011). Abundance of tegument surface proteins in the human blood fluke *Schistosoma mansoni* determined by QconCAT proteomics. *Journal of Proteomics* **74**, 1519–1533.
- Chambers, E., Ryan, L. A., Hoey, E. M., Trudgett, A., McFerran, N. V., Fairweather, I. and Timson, D. J. (2010). Liver fluke beta-tubulin isotype 2 binds albendazole and is thus a probable target of this drug. *Parasitology Research* **107**, 1257–1264.
- Chemale, G., Morphew, R., Moxon, J.V., Morassuti, A.L., Lacourse, E. J., Barrett, J., Johnston, D. A. and Brophy, P. M. (2006). Proteomic analysis of glutathione transferases from the liver fluke parasite, *Fasciola hepatica*. *Proteomics* **6**, 6263–6273.
- Chemale, G., Perally, S., Lacourse, E. J., Prescott, M. C., Jones, L. M., Ward, D., Meaney, M., Hoey, E., Brennan, G. P., Fairweather, I., Trudgett, A. and Brophy, P. M. (2010). Comparative proteomic analysis of triclabendazole response in the liver fluke *Fasciola hepatica*. *Journal of Proteome Research* **9**, 4940–4951.
- Chemale, G., Van Rossum, A. J., Jefferies, J. R., Barrett, J., Brophy, P. M., Ferreira, H. B. and Zaha, A. (2003). Proteomic analysis of the larval stage of the parasite *Echinococcus granulosus*: Causative agent of cystic hydatid disease. *Proteomics* **3**, 1633–1636.
- Chen, C.-K., Leung, S. S. F., Guilbert, C., Jacobson, M. P., Mckerrow, J. H. and Podust, L. M. (2010). Structural characterization of CYP51 from *Trypanosoma cruzi* and *Trypanosoma brucei* bound to the antifungal drugs posaconazole and fluconazole. *PLoS Neglected Tropical Diseases* **4** (4), e651.
- Cheng, G. F., Lin, J. J., Feng, X. G., Fu, Z. Q., Jin, Y. M., Yuan, C. X., Zhou, Y. C. and Cai, Y. M. (2005). Proteomic analysis of differentially expressed proteins between the male and female worm of *Schistosoma japonicum* after pairing. *Proteomics* **5**, 511–521.
- Craig, H., Wastling, J. M. and Knox, D. P. (2006). A preliminary proteomic survey of the *in vitro* excretory/secretory products of fourth-stage larval and adult *Teladorsagia circumcincta*. *Parasitology* **132**, 535–543.
- Cuervo, P., De Jesus, J. B., Junqueira, M., Mendonca-Lima, L., Gonzalez, L. J., Betancourt, L., Grimaldi, G., Jr., Domont, G. B., Fernandes, O. and Cupolillo, E. (2007). Proteomic analysis of *Leishmania (Viannia) brasiliensis* by two-dimensional gel electrophoresis and mass spectrometry. *Molecular and Biochemical Parasitology* **154**, 6–21.
- Curwen, R. S., Ashton, P. D., Johnston, D. A. and Wilson, R. A. (2004). The *Schistosoma mansoni* soluble proteome: a comparison across four life-cycle stages. *Molecular and Biochemical Parasitology* **138**, 57–66.
- Cvilink, V., Lamka, J. and Skalova, L. (2009). Xenobiotic metabolizing enzymes and metabolism of anthelmintics in helminths. *Drug Metabolism Reviews* **41**, 8–26.
- Cvilink, V., Skalova, L., Szotakova, B., Lamka, J., Kostianen, R. and Ketola, R. A. (2008). LC-MS-MS identification of albendazole and flubendazole metabolites formed *ex vivo* by *Haemonchus contortus*. *Analytical and Bioanalytical Chemistry* **391**, 337–343.
- Daborn, P. J., Yen, J. L., Bogwitz, M. R., Le Goff, G., Feil, E., Jeffers, S., Tijet, N., Perry, T., Heckel, D., Batterham, P., Feyereisen, R., Wilson, T. G. and French-Constant, R. H. (2002). A single P450 allele associated with insecticide resistance in *Drosophila*. *Science* **297**, 2253–2256.
- Devine, C., Brennan, G. P., Lanusse, C. E., Alvarez, L. I., Trudgett, A., Hoey, E. and Fairweather, I. (2010). Inhibition of cytochrome P450-mediated metabolism enhances *ex vivo* susceptibility of *Fasciola hepatica* to triclabendazole. *Parasitology* **137**, 871–880.
- Dieterich, C., Clifton, S. W., Schuster, L. N., Chinwalla, A., Delehaunty, K., Dinkelacker, I., Fulton, L., Fulton, R., Godfrey, J., Minx, P., Mitreva, M., Roeseler, W., Tian, H., Witte, H., Yang, S.-P., Wilson, R. K. and Sommer, R. J. (2008). The *Pristionchus pacificus* genome provides a unique perspective on nematode lifestyle and parasitism. *Nature Genetics* **40**, 1193–1198.
- Greetham, D., Morgan, C., Campbell, A. M., Van Rossum, A. J., Barrett, J. and Brophy, P. M. (2004). Evidence of glutathione transferase complexing and signaling in the model nematode *Caenorhabditis elegans* using a pull-down proteomic assay. *Proteomics* **4**, 1989–1995.
- Hart, E. H., Morphew, R. M., Millares, P., Wolf, B. T., Brophy, P. M. and Hamilton, J. V. (2012). The soluble proteome phenotype of ivermectin resistant and ivermectin susceptible female *Haemonchus contortus* compared. *Veterinary Parasitology* (In press).
- Hernandez-Gonzalez, A., Valero, M. L., Sanchez Del Pinto, M., Oleaga, A. and Siles-Lucas, M. (2010). Proteomic analysis of *in vitro* newly excysted juveniles from *Fasciola hepatica*. *Molecular and Biochemical Parasitology* **172**, 121–128.
- Hewitson, J. P., Harcus, Y., Murray, J., Van Agtmaal, M., Filbey, K. J., Grainger, J. R., Bridgett, S., Blaxter, M. L., Ashton, P. D., Ashford, D. A., Curwen, R. S., Wilson, R. A., Dowle, A. A. and Maizels, R. M. (2011). Proteomic analysis of secretory products from the model gastrointestinal nematode *Heligmosomoides polygyrus* reveals dominance of Venom Allergen-Like (VAL) proteins. *Journal of Proteomics* **74**, 1573–1594.
- Hoekner, M., Dallinger, R. and Stuerzenbaum, S. R. (2011). Nematode and snail metallothioneins. *Journal of Biological Inorganic Chemistry* **16**, 1057–1065.
- Jefferies, J. R., Campbell, A. M., Van Rossum, A. J., Barrett, J. and Brophy, P. M. (2001). Proteomic analysis of *Fasciola hepatica* excretory-secretory products. *Proteomics* **1**, 1128–1132.
- Jones, L. M., Staffa, K., Perally, S., Lacourse, E. J., Brophy, P. M. and Hamilton, J. V. (2010). Proteomic analyses of *Caenorhabditis elegans* dauer larvae and long-lived daf-2 mutants implicates a shared detoxification system in longevity assurance. *Journal of Proteome Research* **9**, 2871–2881.
- Ju, J.-W., Joo, H.-N., Lee, M.-R., Cho, S.-H., Cheun, H.-I., Kim, J.-Y., Lee, Y.-H., Lee, K.-J., Sohn, W.-M., Kim, D.-M., Kim, I.-C., Park, B. C. and Kim, T.-S. (2009). Identification of a serodiagnostic antigen, legumain, by immunoproteomic analysis of excretory-secretory products of *Clonorchis sinensis* adult worms. *Proteomics* **9**, 3066–3078.
- Kaminsky, R., Ducray, P., Jung, M., Clover, R., Rufener, L., Bouvier, J., Weber, S. S., Wenger, A., Wieland-Berghausen, S., Goebel, T., Gauvry, N., Pautrat, F., Skripsky, T., Froelich, O., Komoin-Oka, C., Westlund, B., Sluder, A. and Maeser, P. (2008). A new class of anthelmintics effective against drug-resistant nematodes. *Nature* **452**, 176–180.
- Keiser, J. and Utzinger, J. (2010). The drugs we have and the drugs we need against major helminth infections. In *Advances in Parasitology, Vol 73: Important Helminth Infections in Southeast Asia: Diversity and Potential for Control and Elimination, Pt B*, Vol. 73 pp. 197–230.
- Kerboeuf, D., Soubieux, D., Guilluy, R., Brazier, J. L. and Riviere, J. L. (1995). *In vivo* metabolism of aminopyrine by the larvae of the helminth *Heligmosomoides polygyrus*. *Parasitology Research* **81**, 302–304.
- Knudsen, G. M., Medzihradzky, K. F., Lim, K. C., Hansell, E. and Mckerrow, J. H. (2005). Proteomic analysis of *Schistosoma mansoni* cercarial secretions. *Molecular & Cellular Proteomics* **4**, 1862–1875.
- Kohler, P. and Bachmann, R. (1981). Intestinal tubulin as possible target for the chemotherapeutic action of mebendazole in parasitic nematodes. *Molecular and Biochemical Parasitology* **4**, 325–336.
- Kotze, A. C., Dobson, R. J. and Chandler, D. (2006). Synergism of rotenone by piperonyl butoxide in *Haemonchus contortus* and *Trichostrongylus colubriformis* *in vitro*: Potential for drug-synergism through inhibition of nematode oxidative detoxification pathways. *Veterinary Parasitology* **136**, 275–282.
- Kulas, J., Schmidt, C., Rothe, M., Schunck, W.-H. and Menzel, R. (2008). Cytochrome P450-dependent metabolism of eicosapentaenoic acid in the nematode *Caenorhabditis elegans*. *Archives of Biochemistry and Biophysics* **472**, 65–75.
- Kwa, M. S. G., Veenstra, J. G., Vandijk, M. and Roos, M. H. (1995). Beta-tubulin genes from the parasitic nematode *Haemonchus contortus* modulate drug-resistance in *Caenorhabditis elegans*. *Journal of Molecular Biology* **246**, 500–510.
- Lacourse, E. J., Perally, S., Hernandez-Viadel, M., Wright, H. A. and Brophy, P. M. (2008). A proteomics approach to quantify protein levels following RNA interference: Case study with glutathione transferase superfamily from the model metazoan *Caenorhabditis elegans*. *Journal of Proteome Research* **7**, 3314–3318.
- Lacourse, J. E., Perally, S., Morphew, R. M., Moxon, J. V., Prescott, M. C., Dowling, D., O'Neill, S. M., Williams, D. J. L., Kipar, A., Hetzel, U., Hoey, E., Zafra, R., Buffoni, L., Arevalo, J. P. and Brophy, P. M. (2012). Sigma class glutathione transferase of the liver fluke *Fasciola hepatica*. *PLoS Neglected Tropical Diseases* **6** (5), e1666.
- Laing, S. T., Ivens, A., Laing, R., Ravikumar, S., Butler, V., Woods, D. J. and Gilleard, J. S. (2010). Characterization of the xenobiotic response of *Caenorhabditis elegans* to the anthelmintic drug albendazole and the identification of novel drug glucoside metabolites. *Biochemical Journal* **432**, 505–514.
- Laschuk, A., Monteiro, K. M., Vidal, N. M., Pinto, P. M., Duran, R., Cervenanski, C., Zaha, A. and Ferreira, H. B. (2011). Proteomic survey of the cestode *Mesocestoides corti* during the first 24 hours of strobilar development. *Parasitology Research* **108**, 645–656.
- Lee, W. C. and Lee, K. H. (2004). Applications of affinity chromatography in proteomics. *Analytical Biochemistry* **324**, 1–10.
- Lindblom, T. H. and Dodd, A. K. (2009). Xenobiotic detoxification in the nematode *Caenorhabditis elegans*. *Journal of Experimental Zoology Part A-Ecological Genetics and Physiology* **311A**, 312–312.
- Mathieson, W. and Wilson, R. A. (2010). A comparative proteomic study of the undeveloped and developed *Schistosoma mansoni* egg and its contents: The miracidium, hatch fluid and secretions. *International Journal for Parasitology* **40**, 617–628.

- McKellar, Q. A. and Jackson, F. (2004). Veterinary anthelmintics: old and new. *Trends in Parasitology* **20**, 456–461.
- Millares, P., Lacourse, E. J., Perally, S., Ward, D., Prescott, M. C., Hodgkinson, J., Brophy, P. M. and Rees, H. H. (2012). Proteomic profiling and protein identification by MALDI-TOF mass spectrometry in unsequenced parasitic nematodes. *Plos One* **7**, (3), e33590.
- Morgan, C., Lacourse, E. J., Rushbrook, B. J., Greetham, D., Hamilton, J. V., Barrett, J., Bailey, K. and Brophy, P. M. (2006). Plasticity demonstrated in the proteome of a parasitic nematode within the intestine of different host strains. *Proteomics* **6**, 4633–4645.
- Morphew, R. M., Wright, H. A., Lacourse, E. J., Woods, D. J. and Brophy, P. M. (2007). Comparative proteomics of excretory-secretory proteins released by the liver fluke *Fasciola hepatica* in sheep host bile and during *in vitro* culture ex-host. *Molecular and Cellular Proteomics* **6**, 963–972.
- Moxon, J. V., Lacourse, E. J., Wright, H. A., Perally, S., Prescott, M. C., Gillard, J. L., Barrett, J., Hamilton, J. V. and Brophy, P. M. (2010). Proteomic analysis of embryonic *Fasciola hepatica*: Characterization and antigenic potential of a developmentally regulated heat shock protein. *Veterinary Parasitology* **169**, 62–75.
- Mueller, P., Warr, E., Stevenson, B. J., Pignatelli, P. M., Morgan, J. C., Steven, A., Yawson, A. E., Mitchell, S. N., Ranson, H., Hemingway, J., Paine, M. J. I. and Donnelly, M. J. (2008). Field-caught permethrin-resistant *Anopheles gambiae* overexpress CYP6P3, a P450 that metabolises pyrethroids. *Plos Genetics* **4** (11), e1000286.
- Mulvenna, J., Hamilton, B., Nagaraj, S. H., Smyth, D., Loukas, A. and Gorman, J. J. (2009a). Proteomic analysis of the excretory/secretory component of the blood-feeding stage of the hookworm, *Ancylostoma caninum*. *Molecular & Cellular Proteomics* **8**, 109–121.
- Mulvenna, J., Moertel, L., Jones, M. K., Nawaratna, S., Lovas, E. M., Gobert, G. N., Colgrave, M., Jones, A., Loukas, A. and Mcmanus, D. P. (2009b). Exposed proteins of the *Schistosoma japonicum* tegument. *International Journal for Parasitology* **40**, 543–554.
- Mulvenna, J., Sripa, B., Brindley, P. J., Gorman, J., Jones, M. K., Colgrave, M. L., Jones, A., Nawaratna, S., Laha, T., Suttiaprapa, S., Smout, M. J. and Loukas, A. (2010). The secreted and surface proteomes of the adult stage of the carcinogenic human liver fluke *Opisthorchis viverrini*. *Proteomics* **10**, 1063–1078.
- O'leary, K. A. and Tracy, J. W. (1991). *Schistosoma mansoni* - glutathione S-transferase-catalyzed detoxication of dichlorvos. *Experimental Parasitology* **72**, 355–361.
- Pérez-Sánchez, R., Ramajo-Hernández, A., Ramajo-Martín, V. and Oleaga, A. (2006). Proteomic analysis of the tegument and excretory-secretory products of adult *Schistosoma bovis* worms. *Proteomics* **6**, S226–S236.
- Precious, W. Y. and Barrett, J. (1989). The possible absence of cytochrome P-450 linked xenobiotic metabolism in helminths. *Biochimica et Biophysica Acta* **992**, 215–222.
- Prichard, R. K., Von Samson-Himmelstjerna, G., Blackhall, W. J. and Geary, T. G. (2007). Foreword: Towards markers for anthelmintic resistance in helminths of importance in animal and human health. *Parasitology* **134**, 1073–1076.
- Prieto, J. H., Koncarevic, S., Park, S. K., Yates, J., Iii and Becker, K. (2008). Large-scale differential proteome analysis in *Plasmodium falciparum* under drug treatment. *Plos One* **3** (12), e4098.
- Rebello, K. M., Barros, J. S. L., Mota, E. M., Carvalho, P. C., Perales, J., Lenzi, H. L. and Neves-Ferreira, A. G. C. (2011). Comprehensive proteomic profiling of adult *Angiostrongylus costaricensis*, a human parasitic nematode. *Journal of Proteomics* **74**, 1545–1559.
- Reichert, K. and Menzel, R. (2005). Expression profiling of five different xenobiotics using a *Caenorhabditis elegans* whole genome microarray. *Chemosphere* **61**, 229–237.
- Robinson, M. W., Lawson, J., Trudgett, A., Hoey, E. M. and Fairweather, I. (2004). The comparative metabolism of triclabendazole sulphoxide by triclabendazole-susceptible and triclabendazole-resistant *Fasciola hepatica*. *Parasitology Research* **92**, 205–210.
- Saeed, H. M., Mostafa, M. H., O'connor, P. J., Rafferty, J. A. and Doenhoff, M. J. (2002). Evidence for the presence of active cytochrome P450 systems in *Schistosoma mansoni* and *Schistosoma haematobium* adult worms. *FEBS Letters* **519**, 205–209.
- Simmer, F., Moorman, C., Van Der Linden, A. M., Kuijk, E., Van Den Berge, P. V. E., Kamath, R. S., Fraser, A. G., Ahringer, J. and Plasterk, R. H. A. (2003). Genome-wide RNAi of *C. elegans* using the hypersensitive rrf-3 strain reveals novel gene functions. *Plos Biology* **1**, 77–84.
- Soblik, H., Younis, A. E., Mitreva, M., Renard, B. Y., Kirchner, M., Geisinger, F., Steen, H. and Brattig, N. W. (2011). Life cycle stage-resolved proteomic analysis of the excretome/secretome from *Strongyloides ratti*-identification of stage-specific proteases. *Molecular & Cellular Proteomics* **10**, (12): M111.010157.
- Sotillo, J., Luz Valero, M., Sanchez Del Pino, M. M., Fried, B., Guillermo Esteban, J., Marcilla, A. and Toledo, R. (2010). Excretory/secretory proteome of the adult stage of *Echinostoma caproni*. *Parasitology Research* **107**, 691–697.
- Sutherland, I. A. and Leathwick, D. M. (2011). Anthelmintic resistance in nematode parasites of cattle: a global issue? *Trends in Parasitology* **27**, 176–181.
- Van Rossum, A. J., Brophy, P. M., Tait, A., Barrett, J. and Jefferies, J. R. (2001). Proteomic identification of glutathione S-transferases from the model nematode *Caenorhabditis elegans*. *Proteomics* **1**, 1463–1468.
- Van Rossum, A. J., Jefferies, J. R., Rijsewijk, F. A. M., Lacourse, E. J., Teesdale-Spittle, P., Barrett, J., Tait, A. and Brophy, P. M. (2004). Binding of hematin by a new class of glutathione transferase from the blood-feeding parasitic nematode *Haemonchus contortus*. *Infection and Immunity* **72**, 2780–2790.
- Wang, Y., Cheng, Z., Lu, X. and Tang, C. (2009). *Echinococcus multilocularis*: Proteomic analysis of the protoscolices by two-dimensional electrophoresis and mass spectrometry. *Experimental Parasitology* **123**, 162–167.
- Wilson, R. A., Wright, J. M., De Castro-Borges, W., Parker-Manuel, S. J., Dowle, A. A., Ashton, P. D., Young, N. D., Gasser, R. B. and Spithill, T. W. (2011). Exploring the *Fasciola hepatica* tegument proteome. *International Journal for Parasitology* **41**, 1347–1359.
- Wolstenholme, A. J., Fairweather, I., Prichard, R., Von Samson-Himmelstjerna, G. and Sangster, N. C. (2004). Drug resistance in veterinary helminths. *Trends in Parasitology* **20**, 469–476.
- Yan, F., Xu, L., Liu, L., Yan, R., Song, X. and Li, X. (2010). Immunoproteomic analysis of whole proteins from male and female adult *Haemonchus contortus*. *Veterinary Journal* **185**, 174–179.
- Yatsuda, A. P., Krijgsveld, J., Cornelissen, A., Heck, A. J. R. and De Vries, E. (2003). Comprehensive analysis of the secreted proteins of the parasite *Haemonchus contortus* reveals extensive sequence variation and differential immune recognition. *Journal of Biological Chemistry* **278**, 16941–16951.