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ORIGINAL PAPER

Liver fluke β-tubulin isotype 2 binds albendazole and is thus a probable target of this drug

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Abstract Albendazole is a benzimidazole drug which can be used to treat liver fluke (*Fasciola hepatica*) infections. Its mode of action is believed to be the inhibition of microtubule formation through binding to β-tubulin. However, *F. hepatica* expresses at least six different isotypes of β-tubulin, and this has confused, rather than clarified, understanding of the molecular mechanisms of benzimidazole drugs in this organism. Recombinant *F. hepatica* β-tubulin proteins were expressed in, and purified from, *Escherichia coli*. These proteins were then used in pull-down assays in which albendazole was covalently linked to Sepharose. β-Tubulin isotype 2 was pulled down in this assay, and this interaction could be reduced by adding competing albendazole. Molecular modelling of β-tubulin isotypes suggests that changes in the side change conformations of residue 200 in the putative albendazole binding site may be important in determining whether, or not, a particular isotype will bind to the drug. These results, together with previous work demonstrating that albendazole causes disruption of microtubules in the liver fluke, strongly suggest that β-tubulin isotype 2 is one of the targets of this drug.

Abbreviations

ABZ	Albendazole
ABZ.SO	Albendazole sulphoxide
IPTG	Isopropyl-β-D-thiogalactopyranoside
rmsd	Root mean square deviation
TCBZ	Triclabendazole
VdW	Van der Waal's

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Introduction

Parasitism by the liver fluke, *Fasciola hepatica*, is a problem of increasing importance in both humans and farm animals. Up to 17 million humans are infected (mostly in the developing world) with further 250 million considered to be at risk (Robinson and Dalton 2009; Mas-Coma et al. 2009a). Global agricultural losses were estimated at $3 billion per year in 1994 (Boray 1994). The increasing levels of infection are due to two main factors. Warmer, wetter summers in temperate climates have favoured the intermediate host (the snail *Galba truncatula*; Kenyon et al. 2009; Mas-Coma et al. 2009b), and resistance to the main drug of choice, triclabendazole (TCBZ), has been reported in many countries and regions (Thomas et al. 2000; Moll et al. 2000; Fairweather 2005; Brennan et al. 2007). Liver fluke

infections can also be treated with other benzimidazoles, such as albendazole (ABZ, Fig. 1).

Both ABZ and TCBZ are believed to target β-tubulin subunits in the fluke's microtubule cytoskeleton. Evidence in support of this hypothesis comes from observations that the drugs (and their sulphoxide metabolites) disrupt microtubules and microtubule-based processes (e.g. vesicle transport and cell division; Robinson et al. 2002; Buchanan et al. 2003; Fairweather 2005; Halferty et al. 2009; Fairweather 2009) and that in vitro exposure of flukes to the microtubule-disrupting drug tubulozole-C causes similar morphological effects to those seen with triclabendazole sulphoxide (Robinson et al. 2003). Immunocytochemistry revealed disruption to tubulin organisation in the fluke's tegumental syncytium (Robinson et al. 2002; Buchanan et al. 2003; McConville et al. 2006). However, there is no experimental evidence for the direct interaction of TCBZ and tubulin.

ABZ has been shown to disrupt mammalian microtubule formation, both in vitro and in vivo (Solana et al. 1998; Chu et al. 2009). Furthermore, ABZ interaction with β-tubulin is well established in a variety of fungi, protozoa and helminths (Fetterer 1986; Lacey and Prichard 1986; Lubega and Prichard 1990; Lubega and Prichard 1991; Jimenez-Gonzalez et al. 1991; Cruz and Edlind 1997; Oxberry et al., 2001; MacDonald et al. 2004; Henriquez et al. 2008). The interaction of ABZ, but not TCBZ, with crude liver fluke tubulin preparations has been demonstrated experimentally by displacement of radio-labelled colchicine (Fetterer 1986). However, the adult fluke expresses at least six different isotypes of β-tubulin (Robinson et al. 2001; Ryan et al. 2008). It is unlikely that ABZ binds to all these molecules with equal affinity. This lack of knowledge of the molecular target (or targets) of ABZ hinders our understanding of the molecular mechanisms of action of this drug in the liver fluke. Here, we aimed to identify which isotype(s) interact with ABZ by chemically immobilising ABZ onto a solid matrix and using this to "pull down" recombinant liver fluke β-tubulin isotypes. ABZ was chosen for these experiments because it has been shown to have a higher affinity (compared to the sulphoxide metabolite, ABZ.SO) for β-tubulin from *Haemonchus contortus*, *Giardia duodenalis*, *Encephalitozoon intestinalis* and *Cryptosporidium parvum* (Lubega and Prichard 1991; MacDonald et al. 2004). Furthermore, ABZ has greater potency in the inhibition of liver fluke egg hatching than ABZ.SO (Alvarez et al. 2009), and molecular modelling suggests that both ABZ and ABZ.SO bind to β-tubulin in a similar manner (Robinson et al. 2004).

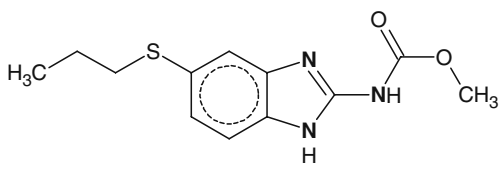

Fig. 1 The structure of albendazole. The potential sites of attachment to the resin are shown in *bold*. Note that either of the two nitrogen groups in the benzimidazole ring can be protonated and that the amide nitrogen is likely to be less reactive than those in the ring

Materials and methods

Construction of expression vectors for liver fluke β-tubulin isotypes

The coding sequences of liver fluke β-tubulin isotypes (Ryan et al. 2008) were amplified by PCR and cloned into pET-46 Ek/LIC (Merck, Nottingham, UK) using the manufacturer's protocol for ligation-independent cloning. This vector adds sequence at the 5'-end of the coding sequence encoding the amino acids MAHHHHHHVDDDDK. Correct insertion of the coding sequences was verified by restriction digestion and DNA sequencing of the entire insert (MWG Biotech, Ebersburg, Germany and Fusion Antibodies, Dunmurry, UK).

Expression and purification of liver fluke β-tubulin proteins in *Escherichia coli*

Expression plasmids were transformed into competent *E. coli* HMS174(DE3) (Merck). Single colonies from these transformations were picked and grown overnight, shaking at 37°C in 5 ml of Luria–Bertani (LB) medium supplemented with 100 $\mu g ml^{-1}$ ampicillin. These overnight cultures were diluted into 1 l of LB (supplemented with 100 $\mu g ml^{-1}$ ampicillin) and grown, shaking at 37°C until $A_{600\ nm}$ reached between 0.6 and 1.0 (typically 3–4 h). The cultures were then induced by the addition of IPTG to a final concentration of 1 mM and grown overnight at 30°C. After this time, the cultures were harvested by centrifugation (4,200×*g* at 4°C for 15 min), resuspended in approximately 20 ml of buffer R (50 mM Hepes–OH, pH 7.5, 150 mM NaCl, 10% (*v*/*v*) glycerol) and frozen at −80°C until required.

Cell suspensions were thawed, and guanidine hydrochloride was added to a final concentration of 6 M. The suspensions were then sonicated on ice (three pulses at 100 W for 30 s each with 15 s in between for cooling) and centrifuged (27,000×*g* at 4°C for 30 min). The supernatant was applied to a nickel-agarose column (1 ml His-Select, Sigma) which had been previously equilibrated in buffer A (50 mM Hepes–OH, pH 7.5, 500 mM NaCl, 10% (*v*/*v*) glycerol, 6 M guanidine hydrochloride). Once the sample had passed through the column under gravity, the column

was washed with 20 ml of buffer B (50 mM Hepes–OH, pH 7.5, 8 M urea). This wash was followed by 20 ml of buffer C (buffer B supplemented with 100 mM NaCl) and 20 ml of buffer D (buffer B supplemented with 1 M NaCl and 5 mM imidazole). Recombinant liver fluke tubulins were eluted with three 2-ml aliquots of buffer E (buffer C supplemented with 200 mM imidazole). These aliquots were dialysed overnight at 4°C against buffer R supplemented with 2 mM dithiothreitol. Tubulin-containing fractions were identified by 10% (*w*/*v*) SDS-PAGE, divided into 50–100-μl aliquots and stored frozen at −80°C.

Preparation of ABZ-substituted sepharose

N-Hydroxysuccinimidyl-Sepharose 4 Fast Flow (Sigma) was prepared by mixing 200 μl of the resin with 100 μl coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.4). The resin was then pelleted by centrifugation (20,000×*g* for 2 min) and washed in cold 1 mM HCl for 30 min. After this time, the mixture was centrifuged, the supernatant removed and the resin resuspended in 500 μl of deionised water. This mixture was centrifuged, the supernatant removed and the pellet suspended in 100 μl of coupling buffer. ABZ (750 μM in DMSO) was added to the resin to a final volume of 1 ml and mixed gently overnight at 22°C. (for primary amines a coupling time of a few hours would be sufficient; however, given the greater difficulty of coupling secondary amines, an extended time was used). The resin was separated by centrifugation, resuspended in 100 μl coupling buffer and stored at 4°C until required. The extent of coupling to the resin was estimated to be 95% by comparing absorbance values at 340 nm of the ABZ solution before ($A_{340\ nm}$=0.11) and after ($A_{340\ nm}$=0.057) the coupling reaction. This wavelength was chosen as the solution had a non-saturating absorbance before coupling and a non-zero absorbance afterwards.

Pull-down assays

ABZ-substituted sepharose (10 μl, suspended in coupling buffer) was mixed with protein solution (50 pmol dissolved in 10 μl of buffer R) and agitated gently overnight at 22°C. The reaction mixtures were centrifuged (20,000×*g* for 2 min), and the supernatant removed and mixed with 10 μl of gel loading buffer (125 mM Tris–HCl, pH 6.8, 4% (*w*/*v*) SDS, 20% (*v*/*v*) glycerol, 1% (*w*/*v*) dithiothreitol, 0.002% (*w*/*v*) bromophenol blue). The pellet was washed three times in 50 μl of buffer R and then mixed with 10 μl of gel loading buffer. Competition experiments were carried out with 750 μM ABZ present in the initial reaction mixture. Assays were analysed by resolving the supernatant and washed pellet fractions by 10% (*w*/*v*) SDS-PAGE. An initial screen was carried out with all six recombinant β-tubulin isotypes. The best interaction detected in this screen was then confirmed by replication and competition experiments. This approach conserved both ABZ–Sepharose and the recombinant proteins, thus enabling all experiments to be carried out with the same batch of reagents.

Molecular modelling of β-tubulin isotypes

All molecular modelling was carried out using the Insight II, biopolymer and Discover Software from MSI Technologies, Inc (CA), running on a Silicon Graphics (Fremont, CA, USA) O_2 workstation. Using residues 1–427 of the *F. hepatica* β-tubulin isotype 1 sequence (accession number CAO79607), model structures were generated from the β-subunit of the porcine αβ-tubulin dimer atomic structure (PDB accession number 1TUB (Nogales et al. 1998)). These sequences were modelled initially by residue replacement. Van der Waal's (VdW) clashes were identified and side chain rotamer searches used to minimise them followed by relaxation of the structure using molecular mechanics with the consistent valence force field (Discover, MSI Technologies, Inc, CA). Further optimisation was carried out, with the α-carbon atoms fixed, by molecular dynamics at 300 K for 100 ps. The SHAKE algorithm was used to speed up the calculation. Explicit water was not used, instead a distance-dependent dielectric was used ($\varepsilon=8r$). A conformation at 76 fs had the lowest potential energy and was subjected to energy minimisation, again with the α-carbon atoms fixed. All minimisations were calculated until derivatives were <0.001 kcal/mol Å. Using residues 1–427 from the sequences for *F. hepatica* β-tubulins 2 and 3 (accession numbers CAO79608 and CAO79609), models were generated from the β-tubulin 1 model by residue replacement. VdW clashes were identified and side chain rotamer searches used to minimise them. Restrained minimisations were performed and continued until the derivatives were <0.001 kcal/mol Å. The .pdb files of the three models are provided as Electronic Supplementary Materials to this paper.

Results and discussion

Liver fluke β-tubulins can be expressed in *E. coli* and purified in a soluble form

Initial experiments to purify recombinant liver fluke β-tubulins under native conditions (using methods similar to those described previously using this expression vector system (Pathmanathan et al. 2008)) resulted in no soluble β-tubulin in the fractions eluted from the column (data not shown). Consequently, a protocol involving extraction and purification of proteins from *E. coli* under denaturing conditions was adopted, followed by dialysis to remove

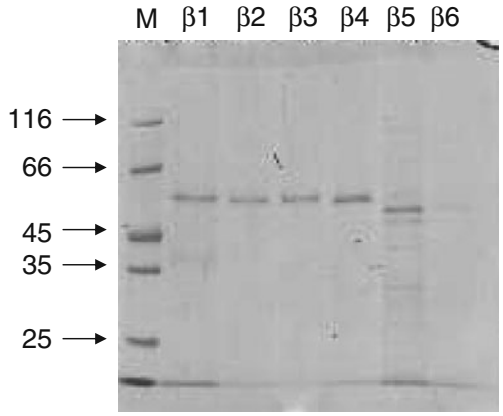

Fig. 2 Purified, recombinant *F. hepatica* β-tubulin isotypes. The sizes (in kilodaltons) of molecular mass markers (*M*) are shown on the *left*. Note that β-tubulin isotypes 5 and 6 have slightly lower molecular masses than isotypes 1–4 and thus run faster in SDS-PAGE. Minor contaminants were observed in some of the preparations, especially β5. However, as these proteins were to be used in pull-down assays (which select for the protein of interest, effectively purifying it further), these preparations were considered sufficiently pure

the denaturants and promote refolding. This procedure resulted in soluble protein yields of between 0.1 and 1.0 mg protein per litre of bacterial culture (Fig. 2).

Identification of ABZ binding partners

In order to identify which (if any) of the six known liver fluke β-tubulin isotypes interacts with ABZ, the drug was immobilised onto Sepharose resin. The immobilisation of small molecules for the identification of protein binding partners has been used successfully by others (for example see Marshak et al. 1981; Sellick and Reece 2003). Furthermore, although NHS-coupling chemistry is most commonly used with primary amines (e.g. lysine residues in proteins), there are also documented examples of coupling through secondary amine groups (Cline and Hanna 1987). Therefore, we assume that ABZ was linked through at least one of the three secondary amine groups in the molecule (Fig. 1). Given that there are three potential sites of attachment, it is possible that the molecule was linked in several different conformations and may have

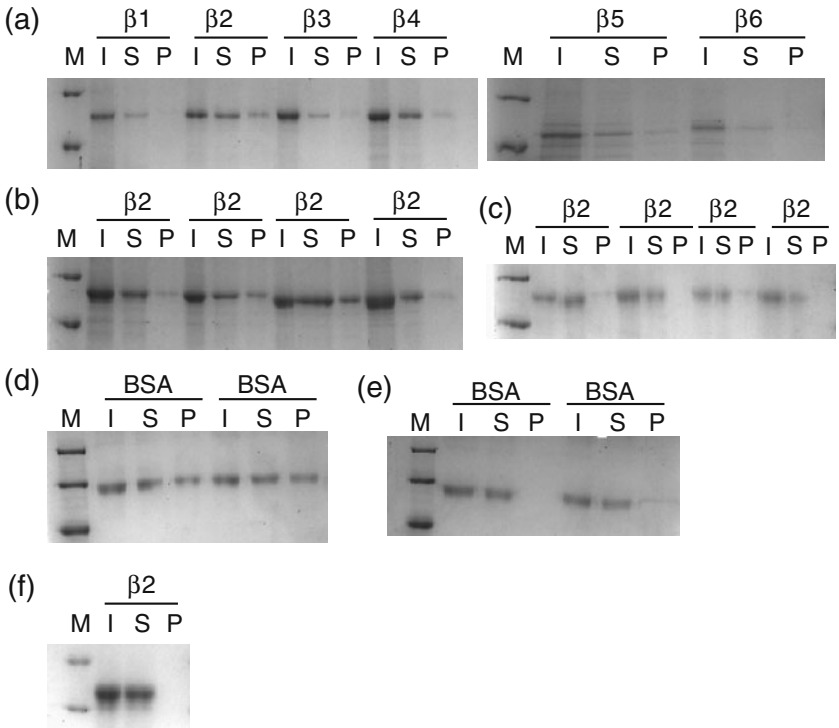

Fig. 3 ABZ interacts with β-tubulin isotype 2 in a pull-down assay. In each experiment, 50 pmol protein was mixed with ABZ–Sepharose in a total reaction mixture of 10 μl. For each experimental condition, the initial material (*I*) before the pull-down experiment, together with the supernatant, i.e. unbound material (*S*), and pellet, i.e. bound material (*P*) after the pull-down, were analysed by 10% (*w*/*v*) SDS-PAGE. Molecular mass markers (*M*) were used to verify the identities of the proteins. In an initial screen (**a**), the ability of the six recombinant β-tubulin isotypes to interact with ABZ–Sepharose was tested. The interaction with β-tubulin isotype 2 was confirmed by repeating this pull-down experiment four times (**b**). This interaction could be largely abolished by competition with ABZ in solution (**c**). ABZ–Sepharose also interacted with BSA (**d**), and this interaction could also be competed with ABZ (**e**). β-Tubulin isotype 2 does not interact with NHS–Sepharose which had been activated and washed in the absence of ABZ (**f**). Molecular mass marker sizes in **a**, **b**, **c** and **f** were 45 and 66 kDa and in **d** and **e** were 45, 66 and 116 kDa

presented different aspects to potentially interacting proteins.

An initial screen using all six recombinant liver fluke β-tubulins revealed that β-tubulin isotypes 2, 3, 4 and 5 interact with ABZ (Fig. 3a). Since isotype 2 was the most strongly interacting protein in this assay (followed by isotypes 4, 3 and 5) further experiments concentrated on this isotype. This interaction could be replicated (Fig. 3b) and could be reduced by adding soluble ABZ as a competitor to the reaction mixture (Fig. 3c). It should be noted that this kind of assay is not highly quantitative, and some variation in the amount pulled down is expected (as seen here). The validity of the methodology was confirmed by incubating immobilised ABZ with bovine serum albumin (BSA). This protein has previously been shown to bind to ABZ using native gel electrophoresis and fluorescence quenching (Chambers et al., 2010). Immobilised ABZ interacted with BSA (Fig. 3d), and this interaction could be competitively reduced using excess, soluble ABZ (Fig. 3e). β-Tubulin isotype 2's interaction was with the immobilised drug and not the matrix as Sepharose which been activated in the absence of ABZ did not bind to the protein (Fig. 3f). Taking these results together, we conclude that that β-tubulin isotype 2 binds specifically to ABZ and is thus a likely target for the drug. It should be noted that our experiments do not rule out the possibility of other targets for ABZ—including other tubulin isotypes as steric hindrance resulting from immobilisation may prevent interaction with some proteins. Furthermore, there is currently no published information on life cycle stage, or tissue-specific expression patterns of the *F. hepatica* tubulin isotypes. Therefore, it is difficult to speculate on the specific physiological effects of ABZ in the fluke.

Determinants of ABZ–tubulin interaction

The broad applicability of ABZ as an anti-parasitic and anti-fungal drug means that there is a considerable body of data to draw on. Furthermore, resistance has been documented in a range of different organisms, and β-tubulin encoding genes from several different resistant organisms have been sequenced. In some cases, for example *G. duodenalis*, no changes were detected in the β-tubulin gene sequence (Upcroft et al. 1996; Arguello-Garcia et al. 2009). A common site of alteration is Phe-200, which is often mutated to tyrosine (Kwa et al. 1994; Elard et al. 1996;

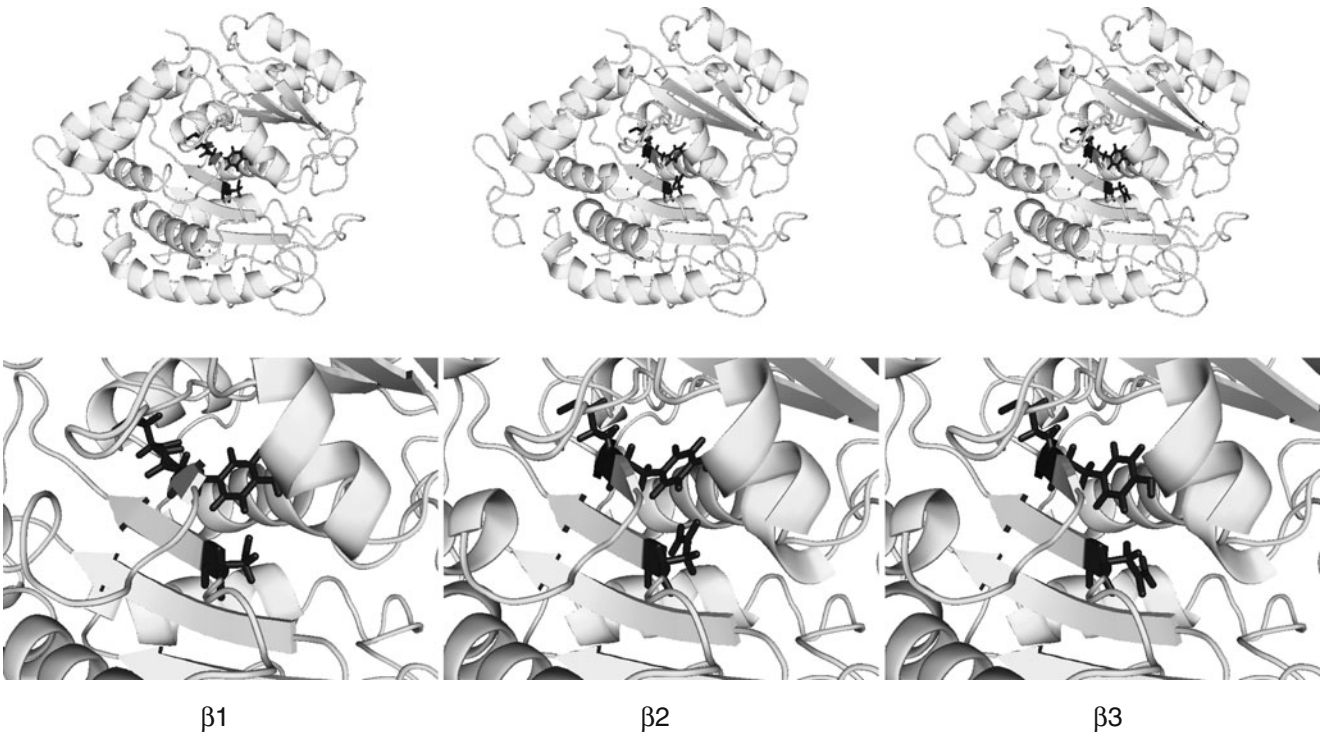

Fig. 4 Modelled structures of residues 1–427 of *F. hepatica* β-tubulin isotypes 1, 2 and 3. The structures were overlaid and visualised using PyMol (DeLano Scientific, Palo Alto, CA, USA; http://pymol.org). The overall folds (*top row*) show a high degree of structural similarity. In the *bottom row*, close-ups of the region are shown including two positions implicated in binding ABZ (residues 165 and 200) and the putative hinge residue (Cys-201). This reveals little alteration in the conformation of the side chain of residue 201 across the three isotypes. However, there are changes in the conformation of residue 200 in isotype 2 and residue 165 in isotype 3. In each model, residues 165, 200 and 201 are shown in *stick representations in black*. As shown, the three residues are in an approximately vertical line with residue 165 at the *bottom*, residue 200 in the *middle* and residue 201 at the *top*

Prichard 2001; Schwab et al. 2005; Hoti et al. 2003; Henriquez et al. 2008). Other sites of importance are His-6, Ala-65, Ala/Asn-165, Phe-167, Glu-198 and Arg-241 (Cruz and Edlind 1997; Schwab et al. 2005; Ghisi et al. 2007; Henriquez et al. 2008; Schwenkenbecher and Kaplan 2009). The solution of the three-dimensional structure of the αβ-tubulin dimer (Nogales et al. 1998; Lowe et al. 2001) has enabled the construction of molecular models of tubulins from helminths. Previously, β-tubulin isotype 1 from the parasitic nematode *H. contortus* has been modelled in complex with ABZ.SO (Robinson et al. 2004). In this model, accommodation of ABZ.SO requires rotation of the psi angle of Cys-201 resulting in the opening of a cleft and exposure of the ABZ.SO binding residues. This open form of β-tubulin may resemble the monomeric, rather than polymerised form, of the molecule. It is reasonable to hypothesise, therefore, that binding to ABZ.SO (or ABZ) stabilises the monomeric form of β-tubulin. This hypothesis is consistent with the observation that ABZ causes depolymerisation of microtubules (Chu et al. 2009; Solana et al. 1998). A tyrosine residue at position 200 could, potentially, form a hydrogen bond with either serine or asparagine at position 165. Formation of this hydrogen bond may block ABZ.SO's access to the binding cleft (Robinson et al. 2004).

In the six known liver fluke β-tubulin sequences, only isotype 1 has a serine–tyrosine pair at positions 165 and 200 (Table 1). If the conclusions drawn from the molecular model are correct (Robinson et al. 2004), then this isotype is unlikely to bind ABZ. However, isotypes 2 and 3 both have an asparagine–tyrosine pair at these positions yet bound to immobilised ABZ in these experiments (Fig. 2). This suggests that there may be additional, as yet unidentified, determinants of ABZ interaction in β-tubulin.

Molecular modelling of β-tubulin isotypes 1, 2 and 3

To understand the differences in ABZ binding between these three isotypes, molecular models were generated based on the structure of pig β-tubulin (Nogales et al. 1998; Lowe et al. 2001). As expected for proteins with highly similar primary sequences, the modelled structures are also almost identical (Fig. 4). The root mean square deviation (rmsd) between the models of β-tubulin isotypes 1 and 2 is 0.901 Å (based on 6,501 equivalent atoms); the rmsd between isotypes 1 and 3 is 0.931 Å (based on 6,522 equivalent atoms), and between isotypes 2 and 3, the rmsd is 0.252 Å (based on 6,541 equivalent atoms). The conformations of the putative hinge residue, Cys-201 (Robinson et al. 2004), are similar in all three models. However, the orientation of the side chain of residue 165 is pointing away from Tyr-200 in isotype 3, whereas it points towards the phenolic ring in isotypes 1 and 2. The orientation of this tyrosine side chain is similar in isotypes 1 and 3, but different in isotype 2 (Fig. 4). In the model of *H. contortus* β-tubulin in complex with ABZ.SO, residues Glu-198, Val-236, Leu-250, Leu-253 and Met-257 make contact with the ABZ moiety (Robinson et al. 2004). Making the reasonable assumption that both ABZ and ABZ.SO bind to tubulin in a similar manner, then it is possible that alterations in the orientations of these residues would affect the interaction. However, examination of these residues in the *F. hepatica* β-tubulin models revealed no substantial differences in their conformations between the three isotypes (data not shown). Although results from molecular modelling should be interpreted with care, this suggests that side chain orientations, as well as primary sequence, may be important in determining the affinity of a particular β-tubulin isotype for ABZ.

Conclusions

The demonstration of interactions between β-tubulin and albendazole, combined with previously published evidence for the drug's ability to disrupt microtubule-based processes, presents strong evidence that β-tubulin isotypes are important molecular targets of ABZ in the liver fluke. Of the isotypes studied here, β2-tubulin isotype 2 appears to have the strongest interaction with ABZ, and this may be the most important interaction pharmacologically. However, many drugs have several physiological targets, and therefore, it would not be surprising if further targets were identified in the future. Nevertheless, this identification of at least one likely target, together with a source of recombinant β-tubulin isotypes and molecular models,

Table 1 Residues in β-tubulin associated with ABZ resistance. β-Tubulin molecules which bind ABZ tend to have the consensus residues at these positions (indicated in the top row). None of the known liver fluke β-tubulin isotypes have the full consensus set of residues

Isotype	His-6	Asn/Ala-165	Phe-167	Glu-198	Phe-200	Arg-241
β1	His	Ser	Phe	Glu	Tyr	Arg
β2	His	Asn	Phe	Glu	Tyr	Arg
β3	His	Asn	Phe	Glu	Tyr	Arg
β4	His	Thr	Phe	Glu	Phe	Arg
β5	Phe	Asn	Phe	Glu	Leu	Arg
β6	Tyr	Cys	Phe	Glu	Phe	Arg

should facilitate the further investigation of the molecular mechanisms of action of this anthelmintic drug.

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References

Alvarez L, Moreno G, Moreno L, Ceballos L, Shaw L, Fairweather I, Lanusse C (2009) Comparative assessment of albendazole and triclabendazole ovicidal activity on *Fasciola hepatica* eggs. Vet Parasitol 164:211–216

Arguello-Garcia R, Cruz-Soto M, Romero-Montoya L, Ortega-Pierres G (2009) *In vitro* resistance to 5-nitroimidazoles and benzimidazoles in *Giardia duodenalis*: variability and variation in gene expression. Infect Genet Evol 9:1057–1064

Boray JC (1994) Diseases of domestic animals caused by flukes. F.A. O, Rome, pp 1–32

Brennan GP, Fairweather I, Trudgett A, Hoey E, McCoy M, McConville M, Meaney M, Robinson M, McFerran N, Ryan L, Lanusse C, Mottier L, Alvarez L, Solana H, Virkel G, Brophy PM (2007) Understanding triclabendazole resistance. Exp Mol Pathol 82:104–109

Buchanan JF, Fairweather I, Brennan GP, Trudgett A, Hoey EM (2003) *Fasciola hepatica*: surface and internal tegumental changes induced by treatment *in vitro* with the sulphoxide metabolite of albendazole ('Valbazen'). Parasitology 126:41–153

Chambers E, Hoey EM, Trudgett A, Fairweather I, Timson DJ (2010) Binding of serum albumin to the anthelmintic drugs albendazole, triclabendazole and their sulphoxides. Vet Parasitol 171(1–2):172–175

Chu SW, Badar S, Morris DL, Pourgholami MH (2009) Potent inhibition of tubulin polymerisation and proliferation of paclitaxel-resistant 1A9PTX22 human ovarian cancer cells by albendazole. Anticancer Res 29:3791–3796

Cline GW, Hanna SB (1987) The aminolysis of *N*-hydroxysuccinimide esters. A structure-reactivity study. J Am Chem Soc 109:3087–3091

Cruz MC, Edlind T (1997) β-Tubulin genes and the basis for benzimidazole sensitivity of the opportunistic fungus *Cryptococcus neoformans*. Microbiology 143:2003–2008

Elard L, Comes AM, Humbert JF (1996) Sequences of β-tubulin cDNA from benzimidazole-susceptible and -resistant strains of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. Mol Biochem Parasitol 79:249–253

Fairweather I (2005) Triclabendazole: new skills to unravel an old(ish) enigma. J Helminthol 79:227–234

Fairweather I (2009) Triclabendazole progress report, 2005–2009: an advancement of learning? J Helminthol 83:139–150

Fetterer RH (1986) The effect of albendazole and triclabendazole on colchicine binding in the liver fluke *Fasciola hepatica*. J Vet Pharmacol Ther 9:49–54

Ghisi M, Kaminsky R, Maser P (2007) Phenotyping and genotyping of *Haemonchus contortus* isolates reveals a new putative candidate mutation for benzimidazole resistance in nematodes. Vet Parasitol 144:313–320

Halferty L, Brennan GP, Trudgett A, Hoey L, Fairweather I (2009) Relative activity of triclabendazole metabolites against the liver fluke, *Fasciola hepatica*. Vet Parasitol 159:126–138

Henriquez FL, Ingram PR, Muench SP, Rice DW, Roberts CW (2008) Molecular basis for resistance of *Acanthamoeba* tubulins to all major classes of antitubulin compounds. Antimicrob Agents Chemother 52:1133–1135

Hoti SL, Subramaniyan K, Das PK (2003) Detection of codon for amino acid 200 in isotype 1 β-tubulin gene of *Wuchereria bancrofti* isolates, implicated in resistance to benzimidazoles in other nematodes. Acta Trop 88:77–81

Jimenez-Gonzalez A, De Armas-Serra C, Criado-Fornelio A, Casado-Escribano N, Rodriguez-Caabeiro F, Diez JC (1991) Preliminary characterization and interaction of tubulin from *Trichinella spiralis* larvae with benzimidazole derivatives. Vet Parasitol 39:89–99

Kenyon F, Sargison ND, Skuce PJ, Jackson F (2009) Sheep helminth parasitic disease in south eastern Scotland arising as a possible consequence of climate change. Vet Parasitol 163:293–297

Kwa MS, Veenstra JG, Roos MH (1994) Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in β-tubulin isotype 1. Mol Biochem Parasitol 63:299–303

Lacey E, Prichard RK (1986) Interactions of benzimidazoles (BZ) with tubulin from BZ-sensitive and BZ-resistant isolates of *Haemonchus contortus*. Mol Biochem Parasitol 19:171–181

Lowe J, Li H, Downing KH, Nogales E (2001) Refined structure of αβ-tubulin at 3.5 Å resolution. J Mol Biol 313:1045–1057

Lubega GW, Prichard RK (1990) Specific interaction of benzimidazole anthelmintics with tubulin: high-affinity binding and benzimidazole resistance in *Haemonchus contortus*. Mol Biochem Parasitol 38:221–232

Lubega GW, Prichard RK (1991) Interaction of benzimidazole anthelmintics with *Haemonchus contortus* tubulin: binding affinity and anthelmintic efficacy. Exp Parasitol 73:203–213

MacDonald LM, Armson A, Thompson AR, Reynoldson JA (2004) Characterisation of benzimidazole binding with recombinant tubulin from *Giardia duodenalis*, *Encephalitozoon intestinalis*, and *Cryptosporidium parvum*. Mol Biochem Parasitol 138:89–96

Marshak DR, Watterson DM, Van Eldik LJ (1981) Calcium-dependent interaction of S100b, troponin C, and calmodulin with an immobilized phenothiazine. Proc Natl Acad Sci USA 78:6793–6797

Mas-Coma S, Valero MA, Bargues MD (2009a) Chapter 2. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv Parasitol 69:41–146

Mas-Coma S, Valero MA, Bargues MD (2009b) Climate change effects on trematodiases, with emphasis on zoonotic fascioliasis and schistosomiasis. Vet Parasitol 163:264–280

McConville M, Brennan GP, McCoy M, Castillo R, Hernandez-Campos A, Ibarra F, Fairweather I (2006) Adult triclabendazole-resistant *Fasciola hepatica*: surface and subsurface tegumental responses to *in vitro* treatment with the sulphoxide metabolite of the experimental fasciolicide compound alpha. Parasitology 133:195–208

Moll L, Gaasenbeek CP, Vellema P, Borgsteede FH (2000) Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in The Netherlands. Vet Parasitol 91:153–158

Nogales E, Wolf SG, Downing KH (1998) Structure of the αβ tubulin dimer by electron crystallography. Nature 391:199–203

Oxberry ME, Geary TG, Winterrowd CA, Prichard RK (2001) Individual expression of recombinant α- and β-tubulin from *Haemonchus contortus*: polymerization and drug effects. Protein Expr Purif 21:30–39

Pathmanathan S, Elliott SF, McSwiggen S, Greer B, Harriott P, Irvine GB, Timson DJ (2008) IQ motif selectivity in human IQGAP1: binding of myosin essential light chain and S100B. Mol Cell Biochem 318:43–51

Prichard R (2001) Genetic variability following selection of *Haemonchus contortus* with anthelmintics. Trends Parasitol 17:445–453

Robinson MW, Dalton JP (2009) Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. Phil Trans R Soc B 364:2763–2776

Robinson MW, Hoey EM, Fairweather I, Dalton JP, McGonigle S, Trudgett A (2001) Characterisation of a β-tubulin gene from the liver fluke, *Fasciola hepatica*. Int J Parasitol 31:1264–1268

Robinson MW, Trudgett A, Hoey EM, Fairweather I (2002) Triclabendazole-resistant *Fasciola hepatica*: β-tubulin and response to *in vitro* treatment with triclabendazole. Parasitology 124:325–338

Robinson MW, Trudgett A, Hoey EM, Fairweather I (2003) The effect of the microtubule inhibitor tubulozole-C on the tegument of triclabendazole-susceptible and triclabendazole-resistant *Fasciola hepatica*. Parasitol Res 91:117–129

Robinson MW, McFerran N, Trudgett A, Hoey L, Fairweather I (2004) A possible model of benzimidazole binding to β-tubulin disclosed by invoking an inter-domain movement. J Mol Graph Model 23:275–284

Ryan LA, Hoey E, Trudgett A, Fairweather I, Fuchs M, Robinson MW, Chambers E, Timson DJ, Ryan E, Feltwell T, Ivens A, Bentley G, Johnston D (2008) *Fasciola hepatica* expresses multiple α- and β-tubulin isotypes. Mol Biochem Parasitol 159:73–78

Schwab AE, Boakye DA, Kyelem D, Prichard RK (2005) Detection of benzimidazole resistance-associated mutations in the filarial nematode *Wuchereria bancrofti* and evidence for selection by albendazole and ivermectin combination treatment. Am J Trop Med Hyg 73:234–238

Schwenkenbecher JM, Kaplan RM (2009) Real-time PCR assays for monitoring benzimidazole resistance-associated mutations in *Ancylostoma caninum*. Exp Parasitol 122:6–10

Sellick CA, Reece RJ (2003) Modulation of transcription factor function by an amino acid: activation of Put3p by proline. EMBO J 22:5147–5153

Solana HD, Teruel MT, Najle R, Lanusse CE, Rodriguez JA (1998) The anthelmintic albendazole affects *in vivo* the dynamics and the detyrosination-tyrosination cycle of rat brain microtubules. Acta Physiol Pharmacol Ther Latinoam 48:199–205

Thomas I, Coles GC, Duffus K (2000) Triclabendazole-resistant *Fasciola hepatica* in southwest Wales. Vet Rec 146:200

Upcroft J, Mitchell R, Chen N, Upcroft P (1996) Albendazole resistance in *Giardia* is correlated with cytoskeletal changes but not with a mutation at amino acid 200 in β-tubulin. Microb Drug Resist 2:303–308