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A possible model of benzimidazole binding to β-tubulin disclosed by invoking an inter-domain movement☆

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Abstract

Although it is well established that benzimidazole (BZMs) compounds exert their therapeutic effects through binding to helminth β-tubulin and thus disrupting microtubule-based processes in the parasites, the precise location of the benzimidazole-binding site on the β-tubulin molecule has yet to be determined. In the present study, we have used previous experimental data as cues to help identify this site. Firstly, benzimidazole resistance has been correlated with a phenylalanine-to-tyrosine substitution at position 200 of *Haemonchus contortus* β-tubulin isotype-I. Secondly, site-directed mutagenesis studies, using fungi, have shown that other residues in this region of the protein can influence the interaction of benzimidazoles with β-tubulin. However, the atomic structure of the αβ-tubulin dimer shows that residue 200 and the other implicated residues are buried within the protein. This poses the question: how might benzimidazoles interact with these apparently inaccessible residues? In the present study, we present a mechanism by which those residues generally believed to interact with benzimidazoles may become accessible to the drugs. Furthermore, by docking albendazole-sulphoxide into a modelled *H. contortus* β-tubulin molecule we offer a structural explanation for how the mutation conferring benzimidazole resistance in nematodes may act, as well as a possible explanation for the species-specificity of benzimidazole anthelmintics.

Keywords: Benzimidazole; Tubulin; Benzimidazole resistance; Domain movement; *Haemonchus contortus*; *Fasciola hepatica*

1. Introduction

The benzimidazoles (BZMs) are an important group of microtubule inhibitors which have found widespread use as broad-spectrum anthelmintics, showing high efficacy against a range of nematode, trematode and cestode parasites. It is well established that BZMs bind directly to the β-tubulin of nematodes, interfering with microtubule dynamics and consequently disturbing microtubule-based processes in these helminths [1,2]. However, the nature of the BZM-binding site on the protein remains to be characterised. A review by Prichard [3] proposes a particular mode of BZM binding in nematode β-tubulin; although, it has been recognised that this is not consistent with the atomic structure of the β-tubulin molecule [4].

To address the question of the exact nature of the BZM-binding site, we begin with the observation that a number of naturally occurring β-tubulin mutations have been shown to be associated with BZM resistance in parasitic nematodes. The best documented of these is the phenylalanine-to-tyrosine substitution at position 200 on the β-tubulin isotype-I molecule of *Haemonchus contortus* [5]. In addition, the same mutation of *H. contortus* β-tubulin isotype-II can also confer BZM resistance [3]. Similarly, a

☆ The model structure generated in the current study is available from the RCSB (PDB Accession number, 1oj0).

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phenylalanine-to-tyrosine or histidine substitution at position 167 is also found in BZM-resistant trichostrongylid nematodes [3,6].

In a second, alternative and experimental approach to the question, a photoactive fenbendazole derivative was used by Nare et al. [7]. The drug reacted with β-tubulin to label leucine 65 and phenylalanine 81. These results are in conflict with the analysis of β-tubulin genes from BZM-resistant nematodes, which had highlighted phenylalanine 200 [3,5].

Overall, the most convincing evidence for the identification of residues on the β-tubulin molecule associated with BZM resistance comes from a third approach, involving site-directed mutagenesis studies [8] which were interpreted in terms of the three dimensional structure of β-tubulin [9]. Most β-tubulin mutations affecting BZM sensitivity were seen to cluster within a small volume of the molecular structure: residues 6, 50, 134, 165, 167, 198, 200 and 257 form a cluster within a compact region of the folded protein. These amino acids are believed to interact with BZM-type drugs in β-tubulin from *Aspergillus nidulans* [10,11]. Specifically, the mutation of alanine 165 to valine causes resistance to thiabendazole, but supersensitivity to benomyl, carbendazim and nocodazole [10]. Since, thiabendazole and carbendazim differ only at their carbon-2 positions, Jung and Oakley deduced that this position of the drug interacts directly with β-tubulin residue 165 of *A. nidulans*. Similarly, mutation of β-tubulin phenylalanine 167 to a tyrosine has been shown to confer resistance to carbendazim and nocodazole, but benomyl supersensitivity in *Saccharomyces cerevisiae* [12]. These workers concluded that the increased benomyl sensitivity is due to hydrophobic bonding between the butylcarbamyl substituent at carbon-1 of benomyl with the aromatic nucleus of phenylalanine 167. As the side-chains of residues 165 and 167 (and residue 200) lie in close proximity to each other in the β-tubulin molecule, the wider cluster of residues in which they occur may be a good candidate for the BZM-binding site, indeed this region has been implicated in microtubule stability [17]. However, in the atomic structure of tubulin [9] the cluster is buried within the protein (Fig. 1), and would thus be inaccessible to BZMs. How then do the BZMs gain access to these residues?

The β-tubulin structure is proposed to be composed of three domains: N-terminal (residues 1–201), intermediate (residues 202–371) and C-terminal (residues 372–427) [9]. The cluster of implicated residues lies at the interface between the N-terminal domain and the intermediate domain. Relaxation of these domains relative to each other would widen the gap between the N-terminal domain and the intermediate domain, thus increasing the accessibility of the implicated residues.

Such domain movement may occur on dimer dissociation into the constituent α- and β-tubulin monomers. Support for this proposal comes from the comparison of the atomic structures of tubulin and the bacterial tubulin homologue, FtsZ, [13,14]. FtsZ is considered to be an approximate model for the conformation of the tubulin monomer. In this structure, the N-terminal domain is rotated by approximately 11° away from the intermediate domain [13]. GTP hydrolysis by polymerised tubulin has also been proposed to be associated with a similar conformation change, leading to microtubule depolymerisation [14].

In the current communication, we integrate information from studies on FtsZ and tubulin to present an alternative model to that of Prichard [3] for a putative BZM-binding site within *H. contortus* β-tubulin that reconciles the results of the site-directed mutagenesis studies with the locations of the naturally occurring β-tubulin mutations. In doing so, we also present an explanation of how BZMs could gain access to an otherwise buried binding site by invoking a β-tubulin domain rotation similar to that seen in FtsZ. A possible structural explanation for the species-specificity of the BZMs is also given together with clarification of why benzimidazole resistance in the parasitic nematodes is associated with the presence of a tyrosine residue at position

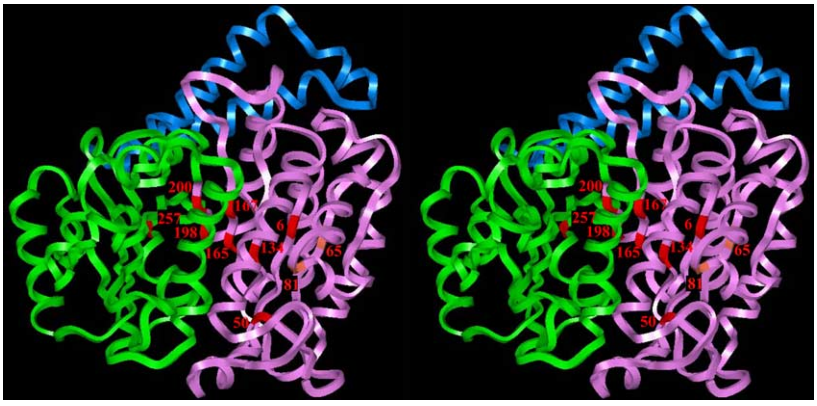

Fig. 1. Ribbon stereodiagram of porcine β-tubulin (1jff) showing residues associated with BZM resistance. Those residues identified by site-directed mutation are highlighted and numbered in red (H6, Y50, Q134, N165, F167, E198, Y200 and M257) and those derived from photoaffinity labelling experiments are shown in orange (L65 and F81). In the main, these residues lie in the interior of the β-tubulin fold, only two are solvent accessible, the total Connolly surface area for the SDM residues is 60 Å^2 (40 Å^2 for Met257 and 20 Å^2 for Phe167). The three β-tubulin domains are coloured as follows: N-terminal—purple, intermediate—green and C-terminal—blue.

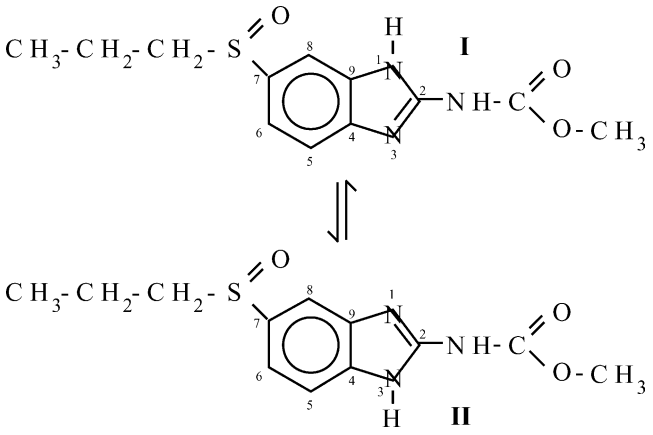

Fig. 2. 2D chemical structure of albendazole sulphoxide tautomers. Tautomer II was used throughout the study as a H-bond could be formed between the N2 proton of ABZ-SO and the backbone carbonyl oxygen of residue 236 of β-tubulin. No potential partner was present for the corresponding N1 proton, either inter- or intra-molecularly.

200 of the β-tubulin isotype-I molecule. In these studies, we have used a model of the most prevalent albendazole metabolite, the sulphoxide, ABZ-SO (Fig. 2).

2. Methodology

2.1. Generation of helminth β-tubulin and albendazole sulphoxide models

All molecular modelling was carried out using the Insight II, Biopolymer and Discover software from Accelrys (CA), running on a Silicon Graphics O_2 workstation. Using the β-tubulin sequences of *Fasciola hepatica* [15] and *H. contortus* isotype *β12–16* [16] model structures were generated from the β-subunit of the refined bovine αβ-tubulin dimer atomic structure [17], which is available from the Brookhaven Data Bank (PDB Accession number, 1JFF). The alignment of these sequences is shown in Fig. 3. As the identity of the sequences was high and no gaps were observed in the alignment, the helminth proteins were modelled by residue replacement, followed by relaxation of the structure using molecular mechanics with the CVFF force field (Discover, Accelrys, CA). All minimisations were continued until the derivatives were <0.005 kcal/mol Å. A molecular structure for albendazole sulphoxide (ABZ-SO) was built using the Biopolymer software. A number of conformations of the propyl moiety of tautomer II (see Fig. 2) were examined using Discover with the CVFF force field, a structure of low PE which was conformationally compatible with the β-tubulin cavity was used throughout for ABZ-SO.

2.2. Docking albendazole sulphoxide into H. contortus β-tubulin isotype β12–16

The unit cell of the tubulin structure used to generate the models of the β-tubulins of *H. contortus* and *F. hepatica* contains an αβ-heterodimer. It has been observed that the monomeric bacterial tubulin homologue, FtsZ, has a slightly different domain orientation from the tubulin dimer. In order to create the approximate conformation of the β-tubulin monomer, the N-terminal domain of the *H. contortus* isotype *β12–16* β-tubulin model was rotated by 11° with respect to the intermediate domain, as is seen in the monomeric FtsZ structure. This was achieved by alteration of the psi bond of residue cysteine 201 (from −80.5° to −91.5°). In order to prevent clashes between the C-terminal domain and the rotated N-terminal domain, the C-terminal domain was also rotated by a similar amount by alteration of the psi bond of residue alanine 382 (from −65.0° to −54.0°). Root mean square deviation (R.M.S.D.) analysis of the C_α atoms was subsequently performed on the protein to compare the overall fold of the structure in the unrotated (''closed'') and the rotated (''open'') conformations.

The minimised ABZ-SO molecule was manually docked into the cleft formed by rotation of the N-terminal domain. The position of the drug was guided by the aromatic side-chains of phenylalanine 167 and phenylalanine 200, mutations of which are known to cause BZM resistance in parasitic nematodes. The orientation of the drug in the binding site was dictated by edge-to-face aromatic interactions and also the potential for hydrogen-bond formation with neighbouring residues (valine 236, glutamate 198 and serine 165). The side-chains of phenylalanine 167, phenylalanine 200, isoleucine 202, methionine 233 and leucine 253 were rotated slightly (<40° alteration in chi-1 value) in order to accommodate the drug in the local environment. The ''open'' *H. contortus* monomeric β-tubulin model was minimised a second time with the ABZ-SO docked within its structure. In this instance, the built structure was minimised in three stages: initially acting only on those residues <4 Å from the drug, then increasing the limit to include those <6 Å from the drug and then finally those <10 Å from the drug.

2.3. Binding site area analysis

In order to compare the surface area for ligand interaction within the putative ABZ-SO binding sites of porcine, fluke and *H. contortus* β-tubulin models, the Connolly surface (1.4 Å probe) of those residues making direct contact (<3.5 Å) with the docked drug was calculated. The surface area of the ABZ-SO molecule was calculated in a similar manner. The surface area calculations were carried out for the proteins in the ''closed'' conformation and at various degrees of rotation (from 0° to 20°) of the cysteine 201 psi angle, these intermediate models were not individually minimised. The residues used to calculate the surface area of the putative binding site were: histidine 6, phenylalanine 20, glutamine 134, threonine 136, serine 165, phenylalanine 167, glutamate 198, phenylalanine 200, valine 229, methionine 233, glycine 235, threonine 237, leucine 240, leucine 250, leucine 253 and methionine 257.

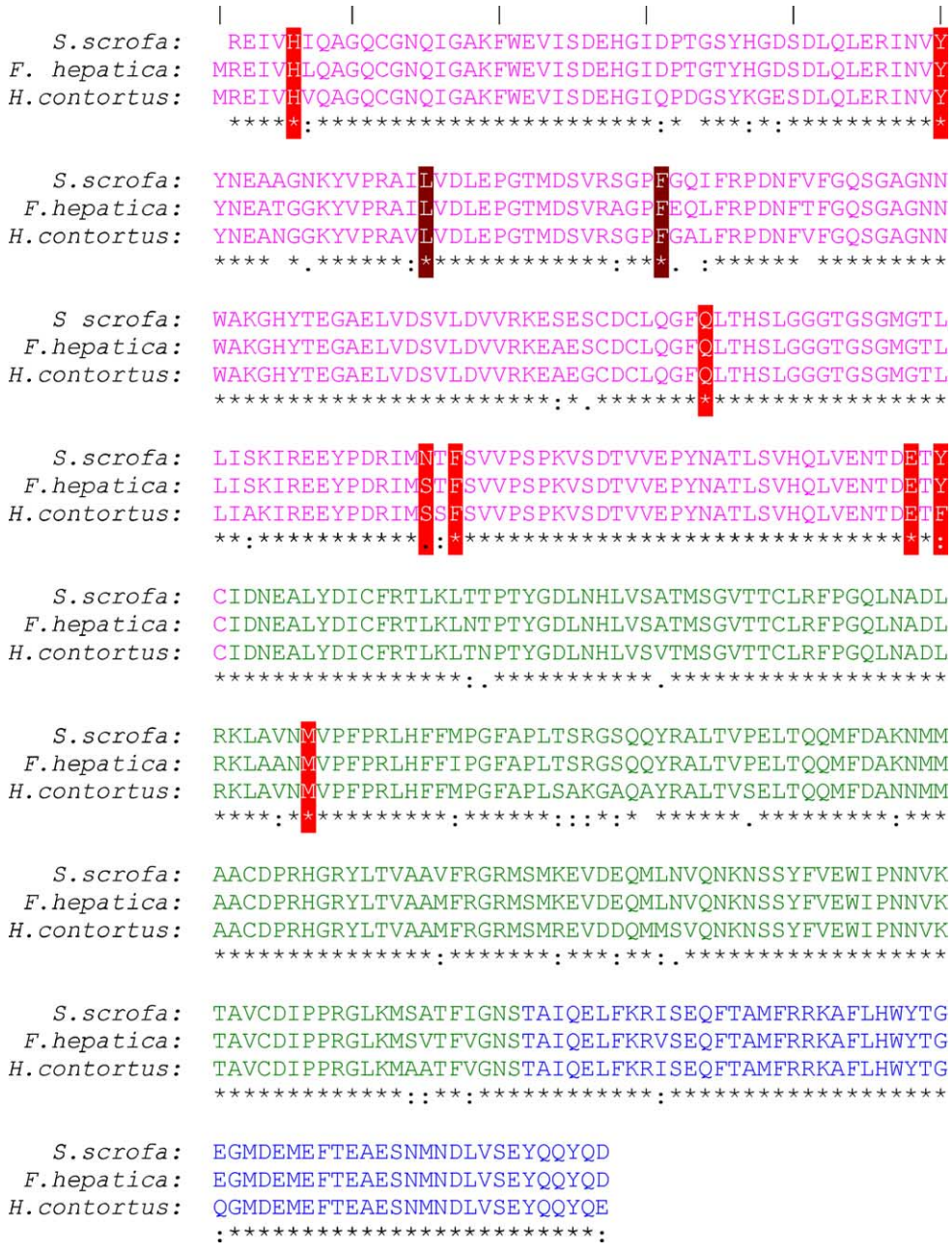

Fig. 3. Alignment of pig (*S. scrofa*), *F. hepatica* and *H. contortus* β-tubulin partial sequences used for generating the homology models. 96% and 92% identities were found between the two helminth and the pig sequence, respectively. The colouring scheme follows that of Fig. 1.

3. Results

3.1. Generation of a monomeric (“open”) β-tubulin model for docking studies

The solvent-accessible surface for the minimised structure of ABZ-SO was found to be 303 Å^2. Residues 6, 50, 134, 165, 167, 198, 200 and 257 of *H. contortus* β-tubulin form the putative ABZ-SO binding site (see below). The solvent-accessible surface area of these residues in the initial conformation of *H. contortus* β-tubulin generated by homology modelling from the porcine αβ-dimer (the “closed” conformation) was 77 Å^2. The corresponding surface areas of porcine and fluke β-tubulins were 22 Å^2 and 40 Å^2, respectively.

By invoking an inter-domain movement between the N-terminal and intermediate domains of the β-tubulins these values increase (Fig. 4), with a plateau being reached at approximately 11° of rotation for each molecule (the “open” conformation). The accessible surface areas under these circumstances increased to 188 Å^2 for *H. contortus*, 149 Å^2 for fluke and 143 Å^2 for porcine β-tubulin. The R.M.S.D. value for the rotated *H. contortus* model as measured against FtsZ was 2.3 Å.

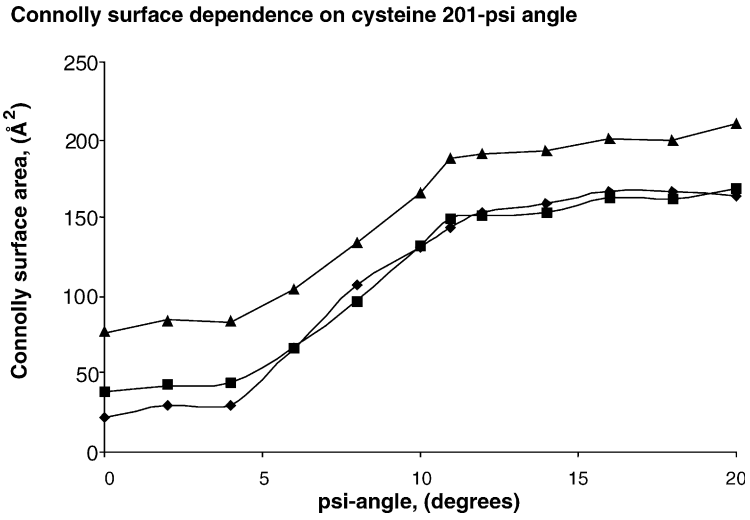

Fig. 4. The dependence of the Connolly surface area of the cleft of porcine (◆-◆), fluke (■-■) and *H. contortus* (▲-▲) β-tubulins on the rotation of the psi C_α–N bond angle of residue cysteine 201. The cleft surface area of the *H. contortus* model is much greater than those of the fluke or porcine models, both in the "closed" and "open" conformations. *Note:* the surface area does not increase significantly following rotations of the N-terminal domain by more than 11°.

3.2. Docking of albendazole sulphoxide

Following inter-domain rotation to expose residues of *H. contortus* β-tubulin known to interact with BZM-type drugs (residues 6, 50, 134, 165, 167, 198, 200 and 257) [5,6,10–12], ABZ-SO was manually docked adjacent to these residues and successfully minimised (Fig. 5). The drug was placed within a pocket formed by β-tubulin strands S5 and S6 and helix H7. Minimisation resulted in the "open" structure being returned to a more "closed" conformation, as judged by psi N–C_α bond angle of residue cysteine 201 being rotated from −92° to −86°, the original value of −81° was not attained due to the presence of the drug "wedged" between the N-terminal and intermediate domains. Comparison of residues close to the drug (<6 Å) in the ABZ-SO/β-tubulin complex with the starting "closed' β-tubulin gave a value of 0.39 Å R.M.S.D. (C_α trace).

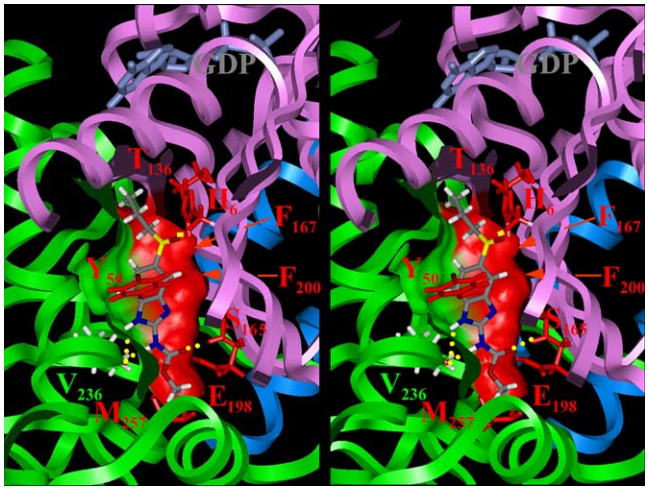

Fig. 5. Proposed docking of the ABZ-SO molecule into the cleft of the *H. contortus* β-tubulin isotype *β12–16* model. The ribbon is coloured according to the scheme of Fig. 1. The cut-away Connolly surface of the proposed binding site is coloured green except for regions contributed by those residues implicated in BZM resistance by site-directed mutagenesis or through the occurrence of natural mutations. H-bonds are shown as dotted yellow lines. A GDP molecule, bound in the same location as in the porcine structure, is shown in gray at the top of the figure. The albendazole molecule is coloured according to the standard scheme except that all carbon atoms are coloured dark gray to provide contrast against the β-tubulin surface.

3.3. Analysis of drug/β-tubulin interactions in various species

Only two species-dependent residue differences occur within the binding sites of porcine, fluke and *H. contortus* β-tubulin. Firstly, residues 165 and 200 vary in the different organisms (see Table 1). Based on the structure of the porcine molecule, the side-chains of residues 165 and 200 are orientated towards each other and in some, but not all, of the organisms these residues can form a hydrogen-bond across the base of the putative binding cleft. In the case of porcine β-tubulin, the phenyl hydroxyl of tyrosine 200 may act as a hydrogen-bond donor in forming a bond with the side-chain carbonyl oxygen of asparagine (Fig. 6). However, in *F. hepatica*, the residue at position 165 is a serine, potentially capable of acting as a H-bond acceptor, but whose side-chain oxygen, at 5 Å, is beyond the normal hydrogen-bonding distance to the tyrosine 200 phenolic hydroxyl. The potential donor residue at position 200 in *H.*

Table 1
Inter-species residue variation at positions 7, 64, 165 and 200 of β-tubulin

Position	Pig	*F. hepatica*	*H. contortus β12–16*
7	Ile	Leu	Val
64	Ile	Ile	Val
165	Asn	Ser	Ser
200	Tyr	Tyr	Phe

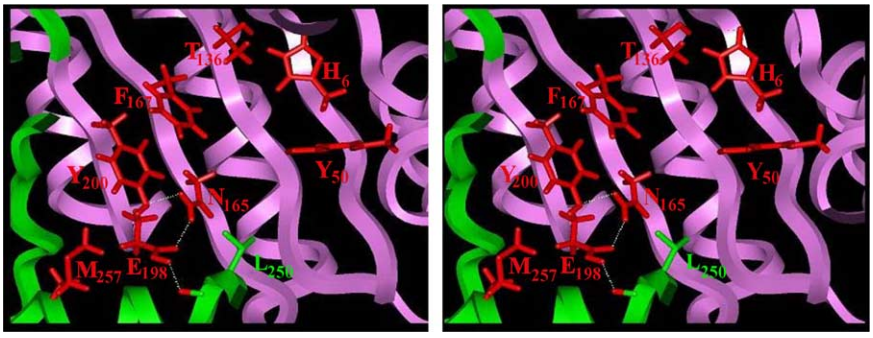

Fig. 6. Detail from the putative BZM-binding site in the porcine β-tubulin molecule. A number of H-bonds (dotted white lines) are possible at the base of the cleft: asparagine 165 ←tyrosine 200; asparagine 165 → glutamate 198 and glutamate 198 →leucine 250. These interactions may stabilise the pocket and prevent the entry of the carbamate moiety of ABZ-SO. Furthermore, as the conserved H-bond between glutamate 198 and the backbone carbonyl oxygen of leucine 250 bridges the N-terminal and intermediate domains, this interaction may oppose any domain movement facilitating the entry of the drug into the cleft. Although, this bond is also possible in the *H. contortus* β-tubulin model, the potential stabilising effects of the asparagine 165 → glutamate 198 and asparagine 165 ← tyrosine 200 interactions will not occur in this organism.

contortus β-tubulin isotype *β12–16* (phenylalanine rather than tyrosine) offers no possibility of forming a hydrogen-bond with residue 165. The strength of these hydrogen-bonds could be ranked with the susceptibility of the organisms to BZM-binding, i.e. pig < fluke < nematode, and would suggest that ease of access to the lower part of the binding site may be correlated with functional BZM-binding.

The second inter-species residue difference potentially involved in BZM-binding occurs at positions 7 and 64, near the other end of the binding site. Although, in all three species these residues are aliphatic and hydrophobic in character (see Table 1), there are differences in the sizes of the side-chains (pig = fluke > nematode) which may enable more favourable BZM binding to the nematode β-tubulin once the N-terminal domain ''re-closes'' around the bound drug. These residues are adjacent to the conserved histidine 6, which has the potential for forming a hydrogen-bond with the ABZ-SO sulphoxide oxygen.

4. Discussion

Although, it has been widely accepted that benzimidazoles bind to β-tubulin the detail of this interaction has been obscure. In this study, we present a model that may clarify this issue.

4.1. Modelling a BZM-binding site in β-tubulin

The use of molecular modelling has proved to be a valuable tool in the characterisation of tubulin–ligand binding sites, such as those of taxol and colchicine [18,19]. This approach has also been applied to the current study in an attempt to reconcile the data of a number of workers with regard to the location of the BZM-binding site on the β-tubulin molecule. *H. contortus* β-tubulin isotype *β12–16* was chosen as the best candidate for modelling as it represents a ''BZM-susceptible'' form of the protein and was also used in the cross-linking study of Nare et al. [7]. Due to the highly conserved nature of the sequences of the tubulin family of proteins (>90% identity), it is very likely that the fold of the β-tubulins of *H. contortus* and *F. hepatica* will be virtually identical to that of the bovine tubulin that was used to generate the electron diffraction structure [9]. As a result, only local changes in the modelled structures may be expected to arise from differences in the primary sequences between *H. contortus* isotype *β12–16* and *F. hepatica* β-tubulins as compared with the porcine protein sequence used to derive the starting structure. In our modelling procedure, the complete sequences of the helminth β-tubulins were modelled as a single structurally conserved region.

The atomic structure of the αβ-dimer [9] shows that a number of the amino acids implicated in BZM resistance in a range of organisms form a tight cluster at the interface between the N-terminal and intermediate β-tubulin domains. However, this cluster is buried within the protein and therefore is inaccessible to the solvent. Proteins are dynamic structures and there are several means by which these residues could become accessible to BZMs. Firstly, if the side-chains of some surface residues were to move, then the drugs might be able to access the proposed binding site residues. Such residues include Arg 156 and Leu 265 (K.H. Downing, personal communication). Alternatively, the ''cleft'' between the N-terminal and intermediate domains could be widened on the dissociation of dimeric tubulin into monomers during microtubule dynamics. The structure of monomeric tubulin is unknown at present; however, the atomic structure of the bacterial homologue FtsZ was determined with the unit cell containing only monomeric species [20], in this structure there is a movement of the two domains. An alternative explanation for the alteration in β-tubulin conformation may arise during GTP hydrolysis [14]. Such ligand-mediated conformational change also occurs in other proteins, such as maltose-binding protein (MBP): a bacterial protein responsible for maltose uptake. Following binding of maltooligosaccharides, the protein undergoes a

35° rotation about a hinge region from an ''open'' to a ''closed'' structure [21].

For ligand interaction, a solvent-accessible surface area on the protein surface comparable with that of the drug is required: in the case of ABZ-SO this was 303 Å^2. This is significantly greater than that observed for the BZM interactive cluster of amino acids in any of the ''closed'' structures. Examination of the surface areas of the potential BZM-binding clefts formed by domain movements in the various β-tubulin monomer models shows that the potential binding site is significantly greater in *H. contortus* β-tubulin (188 Å^2) than in the β-tubulins of pig (143 Å^2) and *F. hepatica* (148 Å^2). The fact that these values are less than that of ABZ-SO indicates that the drug may not be completely enclosed by the protein when bound. The choice of the modelled cysteine 201 psi bond rotation to generate a viable ABZ-SO binding site is validated by the observation that the resultant structure is comparable to that of the experimentally determined monomeric FtsZ [20], as judged by the low R.M.S.D. value when the pairs of structures are compared. The choice of 11° was dictated by the fact that no further increase in surface area was obtained by continued rotation (Fig. 4). This 11° inter-domain rotation exposed residues 6, 50, 134, 165, 167, 198, 200 and 257 of β-tubulin. From independent studies, these residues are believed to interact with BZM-type drugs [5,6,10–12]. The additional requirement for rotation of the C-terminal domain helices −11 and −12, to prevent clashes with the N-terminal domain arising from this domain rearrangement, has further implications for the potential mode of action of these drugs, as these C-terminal helices have been proposed to interact with the kinesins [22] which would be consistent with the inhibition of vesicle transport observed in parasites treated with BZMs [23]. An alternative explanation to the direct involvement of this cluster of residues (associated with BZM resistance) with the binding of this class of drugs would be that this region of the β-tubulin structure is critical to the formation of a BZM-binding site elsewhere on the surface of the molecule. However, we did not find any evidence in support of this hypothesis in the present study, possibly as a result of the methodology used or possibly as a result of the limitations on resolution in the starting porcine structure imposed by experimental constraints. Our findings presented here must be, therefore, regarded as speculative.

The putative binding site created by domain separation accommodated the drug without any major steric clashes. The combined model was successfully minimised and this resulted in a slight re-closure of the two domains around the drug molecule, with the cysteine 201 psi angle rotation being reduced from the ''open'' value of 11° to 5° away from the native β-tubulin structure in the dimer. The small movement of the C_α trace of residues adjacent to the drug after minimisation with the ABZ-SO docked into position, shows that the potential BZM-binding cleft created by inter-domain movement can accommodate ABZ-SO without any major re-folding of the molecule, with only limited side-chain re-orientation being required to accommodate the drug. A similar situation has been observed in a cytochrome P450 protein from the myxobacterium, *Sorangium cellulosum.* The overall fold of the P450 protein was very similar before and after binding of the sizeable ligand, epithilone [24]. The fact that a large proportion of the surface area of the proposed BZM-binding site is formed by those residues which have been implicated in BZM resistance by site-directed mutagenesis and the occurrence of natural mutations [5,6,10–12], lends support to the orientation of the drug in the model of the ABZ-SO/*H. contortus* β-tubulin complex.

In the proposed orientation within the putative binding site, ABZ-SO makes a number of hydrogen-bonds with the side-chains and backbone of the surrounding residues within the *H. contortus* β-tubulin model. (Fig. 5) The sulphoxide oxygen of ABZ-SO forms a hydrogen-bond with the proton of one of the imidazole nitrogens of histidine 6 and the amide proton of the drug hydrogen-bonds with the backbone oxygen of valine 236. The side-chains of glutamate 198 and serine 165 also form hydrogen-bonds with the carbonyl oxygen of ABZ-SO. Furthermore, leucine 250, leucine 253 and methionine 257 form a hydrophobic pocket which interacts with the methyl group of the carbamate moiety. Hydrophobic interactions between the BZM-ring structure and the phenyl rings of phenylalanine 167, phenylalanine 200 and tyrosine 50 are also possible. The optimised structure of the ABZ-SO molecule in the complex with β-tubulin almost completely retains the planar conformation of free ABZ-SO which facilitates its accommodation within the binding cleft in the *H. contortus* model and suggests that no undue strain has been introduced on docking.

4.2. Selective efficacy of BZMs

Comparison of the potential ABZ-SO binding sites within porcine, fluke and nematode β-tubulin models provides a structural explanation for the species-specificity of the BZMs. Part of the reason that BZM anthelmintics have no effect on mammals (when used at normal veterinary therapeutic doses) may lie firstly in terms of the surface area of the binding site and secondarily in terms of the potential for hydrogen-bonding within the site itself. The site within the ''open'' conformation of *H. contortus* BZM-susceptible β-tubulin has a larger surface area than those of the porcine and fluke β-tubulin models; therefore, the BZMs may be more easily accommodated by the nematode β-tubulin BZM-binding site than that of either the fluke or pig. More specifically, within the putative BZM-binding site, the only residues that differ between the pig, *H. contortus* and *F. hepatica* β-tubulin, are residues 165 and 200. The atomic structure of porcine β-tubulin shows that the side-chains of these amino acids, which lie towards the base of the cleft, can potentially interact with each other (Fig. 6). *H. contortus β12–16* has a serine at position 165 and a phenylalanine at position 200. There is no possibility of hydrogen-bonding between these two

residues, so the base of the BZM-binding cleft is unobstructed. However, porcine β-tubulin has asparagine and tyrosine at positions 165 and 200, respectively. The side-chains of these amino acids can form a hydrogen-bond across the base of the cleft. This may ''close off'' the hydrophobic pocket into which the terminal methyl of the ABZ-SO carbamate group fits in our model. In this way, BZM binding to porcine β-tubulin could be impaired, thus explaining the refractoriness of mammalian β-tubulin to BZM-type drugs. *F. hepatica* β-tubulin may be seen as an intermediate between the porcine and nematode proteins as the fluke has a serine at position 165 and a tyrosine at position 200. These residues may also form a weak hydrogen-bond across the base of the binding cleft. This may explain the apparent lack of activity of the classical BZMs against liver fluke. The hierarchy of hydrogen-bonding capabilities between residues 165 and 200 in the different β-tubulins: pig > fluke > nematode, is in agreement with the efficacy of the drugs against these organisms: at normal therapeutic doses, ABZ-SO has no effect on pigs, it is only active against adult liver flukes at elevated doses and is highly active against nematodes. This activity against mature flukes may result from the varying expression of β-tubulin isotypes between juvenile and adult *F. hepatica* or may be due to differential exposure to the drug in the distinctive niches occupied by flukes of varying maturity.

4.3. Significance of residue 200 in BZM resistance

The phenylalanine-to-tyrosine mutation at position 200 of *H. contortus* β-tubulin isotype-I has long been known to confer resistance to BZM anthelmintics [5]. This substitution is believed to cause a reduction of the high-affinity binding of the BZMs to the nematode β-tubulin molecule [2,3]; although, these authors did not give a precise mechanism to explain this effect. In structural terms, this mutation represents only a very conservative change, as the only difference between the two residues is the presence of a *p*-oxygen atom in the case of tyrosine. However, in functional terms, the presence of tyrosine at position 200 facilitates the possible formation of a H-bond at the base of the BZM binding cleft, a phenomenon that phenylalanine at this position cannot carry out. Thus, in BZM-resistant *H. contortus*, the tyrosine 200 side-chain may H-bond with that of serine 165. As in the fluke model, this interaction potentially ''closes off'' the hydrophobic pocket at the base of the cleft and so drug binding is impaired. This is in agreement with the data of Lacey et al. [26] which suggest that the biochemical mechanism of BZM resistance in *H. contortus* is due to the inability of the drugs to form stable BZM–tubulin complexes. Consequently, it is likely that it is the interaction of tyrosine 200 with the neighbouring residue 165 that confers BZM resistance in parasitic nematodes such as *H. contortus* just as it does in *F. hepatica*.

4.4. Other insights provided by the model

It has been suggested that BZMs bind to β-tubulin and ''cap'' the microtubule by physically preventing further polymerisation [1,25]. The present study suggests that this is not the case and that drug-induced conformational changes within the β-tubulin molecule result in net microtubule depolymerisation by locking β-tubulin monomers in the ''open'' conformation, thus interfering with heterodimer formation prior to incorporation into microtubules. Alternatively, on the basis of the difference in the atomic structures of tubulin and the bacterial protein FtsZ, it has been suggested that GTP hydrolysis by β-tubulin already polymerised in microtubules may result in a change in the orientation of the N-terminal and intermediate domains [14]. These domain movements effectively convert the straight, protofilament-forming conformation of the tubulin dimer into the curved depolymerising state. This occurs under normal conditions and is part of the continual cycle of microtubule polymerisation and depolymerisation. Detrich et al. [27] proposed that phenylalanine 200 (rather than a tyrosine at this position) might elevate the activation energy barrier for GTP hydrolysis-induced conformational change. In doing so, the presence of phenylalanine at position 200 can oppose the conformational changes normally induced by GTP hydrolysis, rendering the structure more rigid and promoting tubulin polymerisation through stronger lateral interactions between protofilaments. In short, phenylalanine 200 may stabilise β-tubulin in a conformation that is favourable for polymerisation. Conversely, we propose that BZM binding may oppose the stabilising effects of this residue and lead to microtubule depolymerisation by ''wedging'' the two domains apart, thus mimicking the domain movements normally induced by GTP hydrolysis. This would render the microtubule unstable so that subsequent depolymerisation of the structure occurs. We propose that this effect is particularly prominent in the BZM-susceptible, phenylalanine 200-containing, *H. contortus* β-tubulin and lies at the origin of this organism's sensitivity to BZMs. Other effects of BZM binding in this region are possible. The GTP-binding site on the β-tubulin molecule is located at the mouth of the proposed BZM-binding cleft, consequently drug binding may alter the interaction of GTP with the protein. As polymerisation of tubulin subunits is a GTP-dependent process, the binding of BZMs may indirectly prevent this via the disruption of GTP hydrolysis. Other indirect effects on the cytoskeleton, such as the disruption of motor proteins, are also possible as mentioned above.

BZM-resistant nematodes have a tyrosine residue rather than a phenylalanine at position 200 of the isotype-I β-tubulin [5]. If Detrich et al. [27] are correct regarding the structural significance of phenylalanine 200, the microtubules of BZM-resistant nematodes would be inherently less stable than their BZM-susceptible counterparts. This may be a pleiotropic consequence of selection as drug-

resistant organisms are often left at a disadvantage; this usually takes the form of a reduction in biological fitness, as is evident in praziquantel-resistant *Schistosoma mansoni* [28].

The BZM-derivative, TCBZ, is unusual amongst this group of drugs as it is fasciolid-specific. The location of the TCBZ-binding site on its proposed target molecule, fluke β-tubulin, however, has yet to be determined although preliminary studies indicate that TCBZ will not fit into the BZM-binding site described in this paper. Consequently, binding studies and molecular modelling may be applied to address this issue, and will be the subject of further communications.

Note added in proof

In vindication of our proposal that inter-domain movement giving an 'open' subunit structure may be important to accommodate benzimidazole binding and microtubule disruption, we note a recent publication [29] which reports the crystal structure of two tubulin heterodimers complexed with colchicine and the stathmin-like domain of RB3. These authors report that when the structure of this complex is compared with that of protofilament tubulin obtained without RB3, the intermediate domains are seen to rotate away from the N- and C-terminal domains by 11° in both the α- and β-tubulin subunits in the complex. They associate this rotation with the microtubule disrupting action of RB3 through both secondary structure and inter-subunit movements in tubulin oligomers.

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