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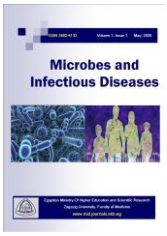

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Original article

Resistance mutations impair benzimidazole efficacy against *Ascaridia galli* by altering β-tubulin interactions

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ABSTRACT

Background: The emergence of benzimidazole resistance among helminths of veterinary and public health importance has been of grave concern. Benzimidazole resistance is caused by mutations (i.e., F167Y, E198A, and F200Y) that occur in the β-tubulin genes of helminths thereby affecting the structure of the expressed protein. This research aims to determine the effects of these canonical resistance-associated mutations on the *in silico* binding of *A. galli* β-tubulin and benzimidazole drug ligands. **Methods:** The β-tubulin amino acid sequence of *A. galli* was retrieved and edited to contain the resistance-associated mutations. These were used to model the tertiary structure of the protein. The *in silico* docking studies used the modeled proteins with benzimidazole drug ligands (i.e., Fenbendazole, Mebendazole, Oxfendazole, and Albendazole). The binding affinities (kcal/mol) and docking positions were examined. **Results:** The results show that resistance-associated mutations caused alterations in the binding interactions between the *A. galli* β-tubulin and benzimidazole drug ligand. The E198A mutation caused slight reductions in binding affinities and changes in the binding positions indicating its potential role in conferring benzimidazole resistance. Molecular dynamics simulations revealed that wild-type and mutated *A. galli* β-tubulins share comparable binding behavior with Fenbendazole. The binding free energies show that the E198A mutation caused decreased binding efficacy, potentially causing resistance. **Conclusion:** The E198A mutation causes weakening of the β-tubulin-benzimidazole interactions indicating its crucial role in conferring resistance in *A. galli.* The emergence of these benzimidazole resistance-associated mutations should be assessed in field isolates as they may negatively affect the efficacy of pharmacological interventions.

Introduction

Ascaridia galli is recognized as the most widespread parasitic nematode that affects chickens globally [1]. It is particularly prevalent in laying hens kept in free-range, organic, and conventional farming conditions due to the parasite's simple life cycle [2]. Targeted treatment has been touted as an effective deworming control of these parasitic worms. However, concerns have been raised regarding the emergence of resistance against anthelmintics due to protracted implementations of deworming [3]. Benzimidazole resistance has been a rising concern in nematodes of veterinary and public health concern [4, 5]. In *A. galli*, several researches have been conducted revealing that these poultry nematodes have developed reduced sensitivity against benzimidazole drugs [6, 7]. It is important to note, however, that none of these

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isolates had the resistance-associated mutations [6]. This reduced sensitivity to benzimidazole drugs, particularly Fenbendazole, has also been observed in *Ascaridia dissimilis* infecting turkeys [8]. Resistance in this important parasitic nematode has caused concerns regarding its economic implications [9]. A recent study of human and swine ascarids also reports no occurrence of these mutations in isolates from multiple countries [10]. In contrast, studies on *A. lumbricoides* have shown the presence of these mutations post-treatment [11,12]. To date, canonical mutations associated with benzimidazole resistance have yet to be reported among *A. galli* in the peer-reviewed literature.

Benzimidazole resistance is caused by single nucleotide polymorphisms (SNPs) that occur in the β-tubulin genes of parasitic helminths [13]. These mutations result in amino acid substitutions that alter the structure of the protein, thereby inhibiting the efficient binding of the benzimidazole drug ligand [14,15]. The canonical mutations associated with benzimidazole resistance include those that occur at codons 167 (TTC, TTT/Phenylalanine/F → TAC, TAT/Tyrosine/Y), 198 (GAG, GAA/Glutamine/Q → GCG, GCA/Alanine/A) and 200 (TTC/ Phenylalanine/F → TAC/Tyrosine/Y) [4,16]. These mutations have been reported to occur in soil-transmitted helminths affecting humans and animals [11,17], parasitic helminths of ruminants [18], and have been extensively studied in laboratory nematodes [19, 20]. However, there have been contradicting results concerning their actual roles in conferring resistance [21,22]. Hence, further research from multiple disciplines (e.g., *in vivo, in vitro,* and *in silico*) on this matter has been called upon to decipher the precise mechanism of resistance in each parasitic hookworm species [23].

The emergence of benzimidazole resistance in helminths of public and veterinary health concerns presents an opportunity to tackle the issue from multiple vantage points. Recently, *in silico* methods have been employed to assess the contributions of resistance-associated mutations in disrupting benzimidazole ligand binding [24–26]. Hence, this research applies *in silico* methods to determine the effects of canonical resistance mutations in the interaction between *A. galli* β-tubulin and benzimidazole drug ligands.

Materials and Methods

Homology Modeling of *A. galli* β-tubulin proteins

The nucleotide coding sequence of *A. galli* β-tubulin was obtained from the UniProt Server (ID No. R4VG92) (https://www.uniprot.org/uniprotkb). The 450 amino acid sequence is the lone reported complete β-tubulin sequence for its species thus far [27]. Amino acid substitutions that are associated with benzimidazole resistance in nematodes of veterinary health concern (i.e., F167Y, E198A, and F200Y) [13, 16] were encoded into the peptide sequence via BioEdit Sequence Alignment Editor version 7.2.5 [28]. Both wild-type and mutated β-tubulins underwent homology modeling via SWISS-MODEL (https://swissmodel.expasy.org/). A previously described β-tubulin crystal structure with a ligand bound in the colchicine binding site was used as the template (PDB ID No. 6fkj, chain B). This was done to ensure that the binding pocket remained accessible to the drug ligand during *in silico* docking.

Structural Quality Assurance Checks

The modeled proteins were downloaded in the PDB format. The UCLA-DOE LAB — SAVES v6.0 (https://saves.mbi.ucla.edu/) platform was used to assess the quality of the predicted protein's structure. Specifically, Verify3D and PROCHECK Ramachandran plots were used. A score of more than 80% in Verify3D means that the predicted 3D structure is compatible with its sequence and therefore is considered for downstream application. Moreover, Ramachandran plots that showed more than 90% of the sequence are in their most favored phi and psi backbones dihedral angles signifying a good quality protein structure. The ProSA (Protein Structure Analysis) server was used to pinpoint errors in the predicted structure (https://prosa.services.came.sbg.ac.at/prosa.php). β-tubulin structures with z scores of more than -5.0 were deemed to be of good quality.

To determine the resemblance of the modeled wild-type and mutated *A. galli* β-tubulins with that of the defined crystal structure, superimposed structural analysis was done using the PDB Pairwise Structure Alignment Tool (https://www.rcsb.org/alignment). Another defined β-tubulin crystal structure was used in this analysis (PDB ID No. 1jff, chain B). The following results were evaluated: RMSD, TM-score, Identity,

Aligned Residues, Sequence Length, and Modeled Residues.

In silico Docking

The docking of the modeled *A. galli* β-tubulins was done using AutoDock Vina [29] in PyRx-Virtual Screening Tool version 0.8. (https://pyrx.sourceforge.io/). After adding polar hydrogens, these proteins were imported into PyRx and were set as docking macromolecules. Ligand molecules were obtained from PubChem [30] (https://pubchem.ncbi.nlm.nih.gov/). Fenbendazole (A) (PubChem Compound ID: 3334), Oxfendazole (B) (PubChem Compound ID: 40854), Mebendazole (C) (PubChem Compound ID: 4030), and Albendazole in the form of Albendazole sulfoxide (D) (PubChem Compound ID: 83969) were used as benzimidazole drug ligands. These ligands were uploaded to PyRx, and assigned as ligands, and energy was minimized.

In silico docking was performed by setting the center of the 25 Å surface diameter docking grid at position 200, similar to the protocol reported by Jones et al. (2022) (Coordinates: X=15.6; Y=57.5; Z=33.1). The docking grid contained all the canonical resistance-associated mutations. Docking results with the lowest RMSD (preferably 0) were downloaded as PDB files. The β-tubulin-benzimidazole ligand interactions, both 3D and 2D, were visualized, inspected, and documented using BIOVIA Discovery Studio Visualizer version 21.1.0.20298 (https://discover.3ds.com/discovery-studio-visualizer-download). The docking binding affinities (ΔG) in kcal/mol were also evaluated.

Additionally, to determine the binding pocket volume of the modeled proteins, these were uploaded to the CAVER Web Server v.1.2 (https://loschmidt.chemi.muni.cz/caverweb/). Binding pockets that included the canonical resistance-associated residues (i.e., 167, 198, and 200) were selected. The 3D representation of the binding pocket and its corresponding volume in $Å^3$ were documented.

Molecular Dynamics

The behavior of the β-tubulin and Fenbendazole complexes was investigated using a 50 ns molecular dynamics (MD) simulation performed on the WebGro server (https://simlab.uams.edu/index.php). The GROMOS96 43a1 force field was employed within a triclinic system solvated with SPC water and neutralized with 0.15 M NaCl. The system was first energy minimized using the steepest descent algorithm for 5000 steps. Subsequently, NVT (constant volume, constant temperature) and NPT (constant pressure, constant temperature) equilibration steps were performed at 300 K and 1 atm pressure, respectively, using the Leap-frog integrator. The production MD simulation ran for 50 ns, recording data at every 1000th step (1000 frames). The Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) plots were constructed to analyze the dynamics of the protein-ligand complex. The MM-GBSA (Molecular Mechanics-General Born Surface Area) and MM-PBSA (Molecular Mechanics-Poisson-Boltzmann Surface Area) binding free energies were determined using fastDRH [31] (http://cadd.zju.edu.cn/fastdrh/overview/).
Rescoring was done using ff19SB with OPC water model force field for receptors and GAFF2 force field for the Fenbendazole ligands.

Results

Modeling of β-tubulin proteins

The quality assurance checks of the predicted wild-type and mutated *A. galli* β-tubulins showed that all were modeled with high quality. All predicted proteins had scores of more than 80% in Very3D **(Figure 1A)**. Also, all had more than 90% of their residues in their favored conformation as depicted by the Ramachandran Plots **(Figure 1B)**. Further, all models had ProSA z-scores of more than -9.0, which indicated high modeling structural quality **(Figure 1C)**. The pairwise structural analysis showed that all predicted *A. galli* β-tubulins were of high structural resemblance to its mammalian homolog that was determined via crystallography **(Figure 2)**.

***In silico* docking of β-tubulin with benzimidazole drug ligands**

The introduction of benzimidazole resistance-associated mutations to *A. galli* β-tubulin resulted in reduced predicted binding pocket volume **(Figure 3)**. β-tubulin with the E198A and F200Y mutations caused marked reductions in the binding pocket volume compared to the wild-type protein. Moreover, the canonical resistance mutations caused several alterations in the ΔG between the protein and the benzimidazole drug ligands. Of these ligands, Mebendazole showed the highest ΔG in both wild-type and mutated β-tubulin. Meanwhile, Albendazole had the lowest. On the whole, the introduction of the resistance-associated mutations

caused an increase in the ΔG which means that having these mutations results in better binding attraction between the drug and the protein target **(Figure 4)**. Reduction in ΔG, alluding to resistance, was only observed in docking experiments with E198A.

Aside from alterations in ΔG, resistance-associated mutations in the *A. galli* β-tubulins also caused changes in the binding interactions of the benzimidazole drug ligands **(Figures 5 and 6)**. These changes include alterations in key interactions like loss of important hydrogen bonds, emergence of unfavorable acceptor-acceptor interactions, and changes in the types of bonds formed. It is important to note that among the locations containing canonical resistance mutations, position 198 formed the most interaction with various benzimidazole drug ligands. In this position, changing glutamic acid (E) to alanine (A) caused the loss of hydrogen bonds observed in other docking poses, changes in the types of bonds formed (e.g., hydrogen bond to pi-covalent bonds), and unfavorable interactions **(Figure 5)**. This indicates that amino acid substitutions in this position caused by resistance may negatively affect binding efficacy with the drug.

Molecular dynamics simulations

The results from the 50 ns molecular dynamics simulations revealed similar protein-ligand behavior and interactions between the β-tubulin and Fenbendazole (FBZ). The RMSD plot showed that all complexes stabilized at around 10 ns, but the trajectory of the complex with the E198A mutation had higher deviation values from 20 to 50 ns simulation time **(Figure 7A)**. Analysis of the β-tubulin backbone of the wildtype and mutated proteins revealed similar flexibility profiles indicating that the mutations do not significantly affect the protein's structural integrity **(Figure 7B)**. The binding free energies calculation through MM-PBSA and MM-GBSA revealed that all canonical mutations decreased the binding efficiency of β-tubulins and FBZ. Moreover, the E198A mutation caused a marked reduction in binding energy, indicating its role in conferring benzimidazole resistance *A. galli*.

Figure 1. Quality assurance checks of modeled *Ascaridia galli* β-tubulin isotype A proteins. The Verify3D (A) and PROCHECK Ramachandran Plots (B) (https://saves.mbi.ucla.edu/), and ProSA (C) (https://prosa.services.came.sbg.ac.at/prosa.php) assessments showed that the modeled proteins are of high quality and therefore suited for downstream *in silico* studies.

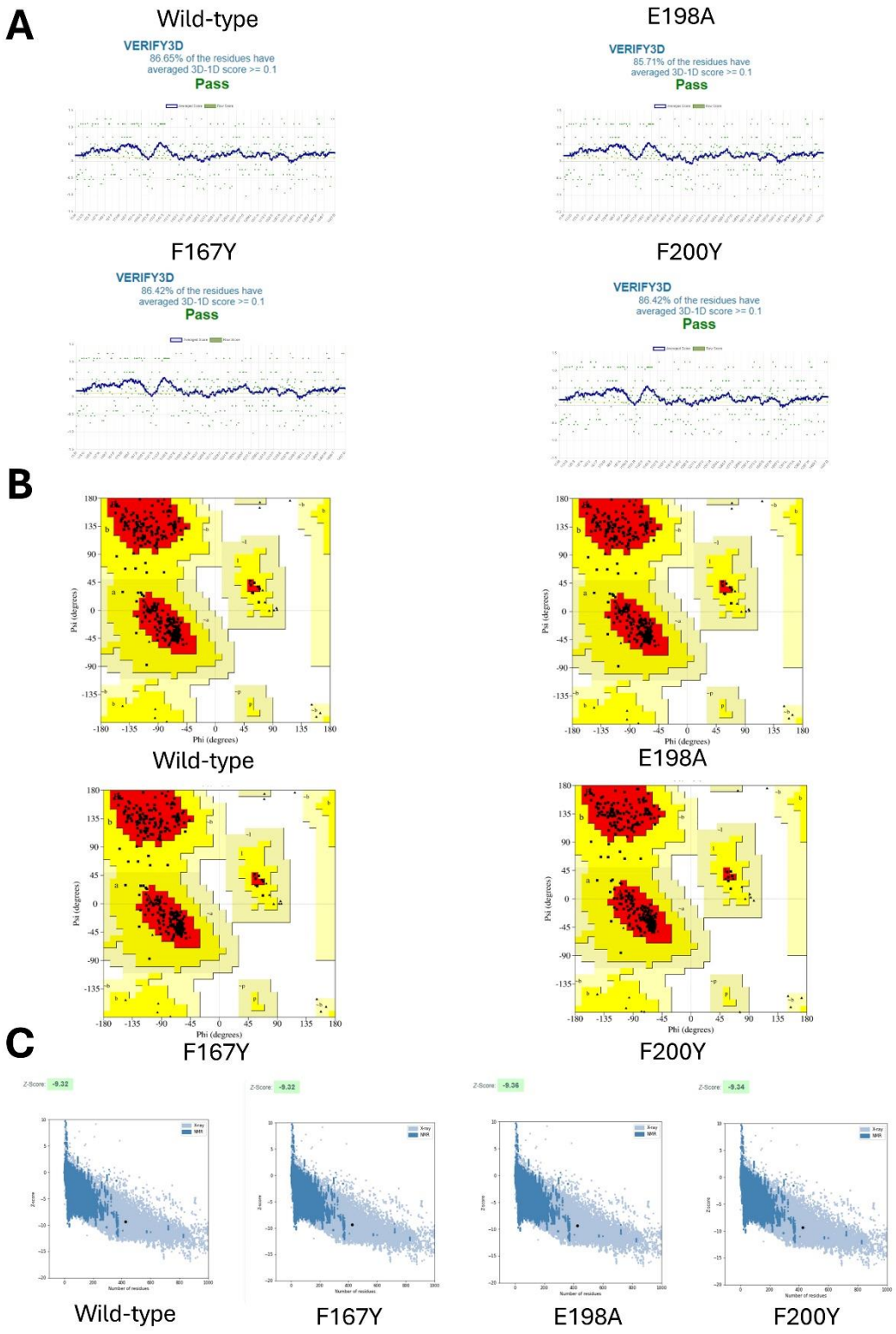
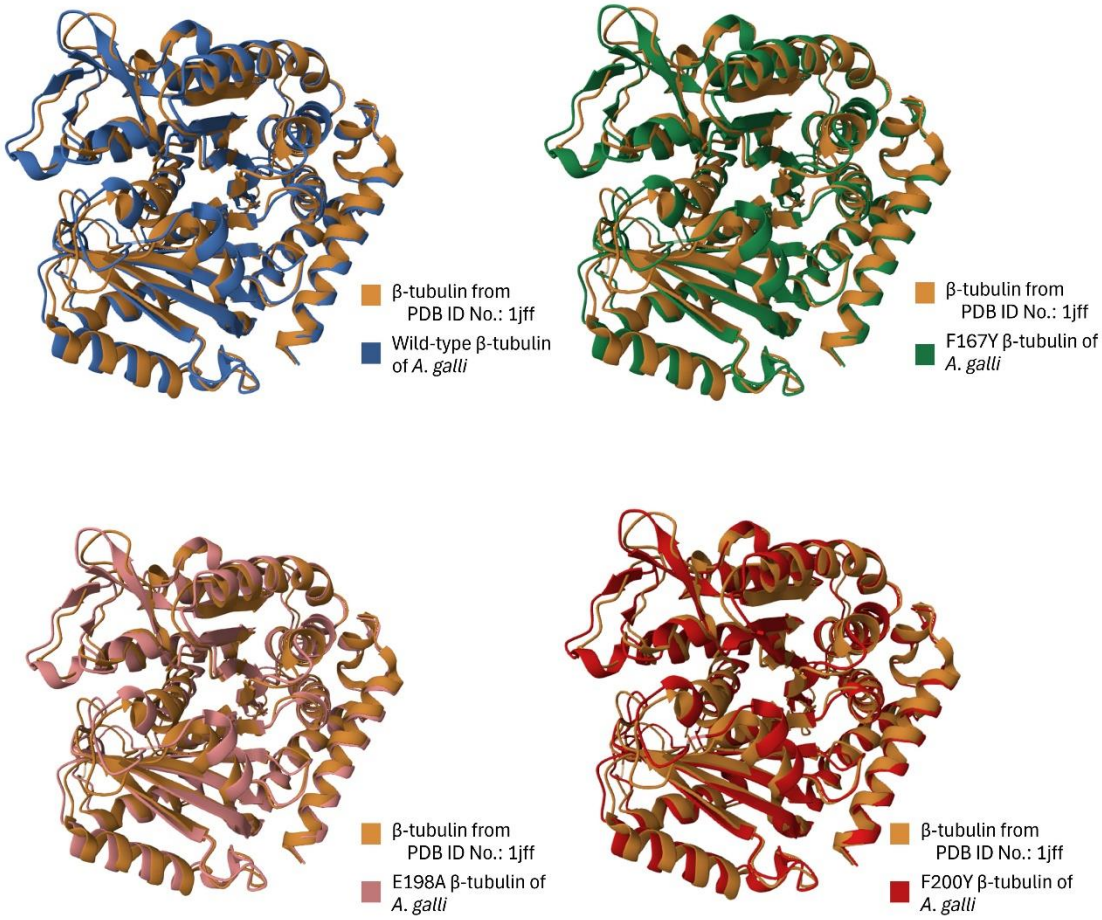
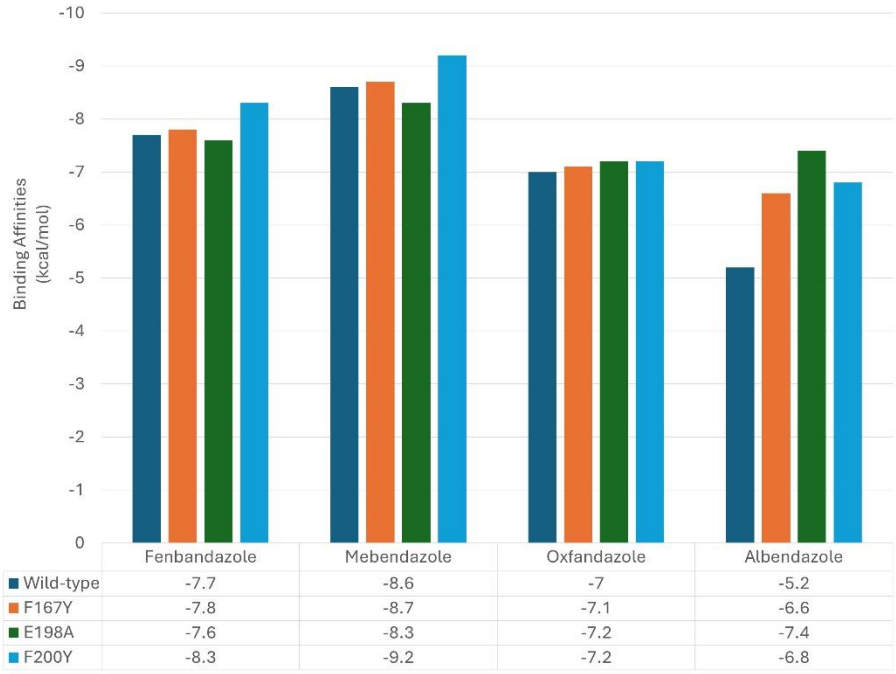


Figure 2. Pairwise structural analysis of the modeled *Ascaridia galli* β-tubulin isotype A proteins. The modeled β-tubulins were highly similar to the defined crystal structure of its mammalian counterpart. The PDB Pairwise Structure Alignment Tool was used for this analysis (https://www.rcsb.org/alignment



Entry	Chain	RMSD	TM-score	Identity	Aligned Residues	Sequence Length
1JFF β-tubulin	B	-	-	-	-	445
Wild-type	A	1.68	0.96	92%	424	427
F167Y	A	1.68	0.96	92%	424	427
E198A	A	1.68	0.96	92%	424	427
F200Y	A	1.68	0.96	92%	424	427

Figure 3. Estimation of binding pocket volume of the modeled wild-type and mutated *A. galli* β-tubulins. The benzimidazole resistance-associated mutations caused diminished binding pocket volume potentially hindering drug ligand binding. This assessment was done using Caver Web v.1.2 (https://loschmidt.chemi.muni.cz/caverweb/).



	Fenbandazole	Mebendazole	Oxfandazole	Albendazole
Wild-type	-7.7	-8.6	-7	-5.2
F167Y	-7.8	-8.7	-7.1	-6.6
E198A	-7.6	-8.3	-7.2	-7.4
F200Y	-8.3	-9.2	-7.2	-6.8

Figure 4. Binding affinities (ΔG) of the *in silico* docking of benzimidazole drug ligands with the modeled *A. galli* β-tubulin proteins. The results show that all canonical resistance-associated mutations (i.e., F167Y, E198A, and F200Y) increased the ΔG between the protein and different drug ligands.

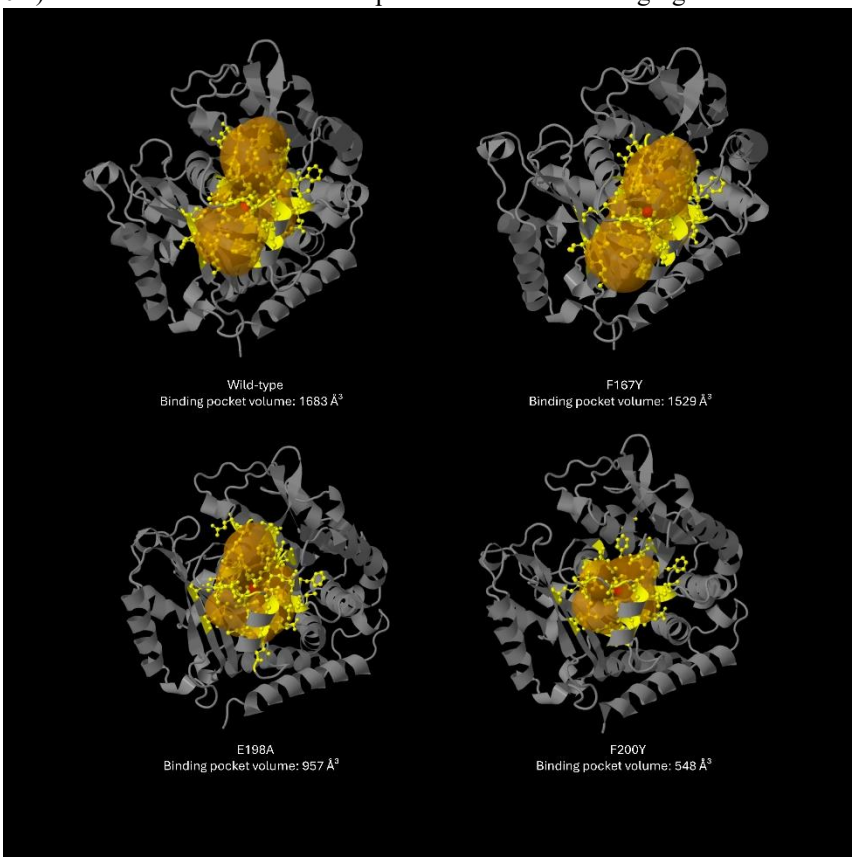


Figure 5. The 3D and 2D representations of amino acid interactions of the docking experiments for the wild-type and F167Y mutated β-tubulins. Frequent drug ligand interactions with amino acid positions 198 (a canonical resistance mutation locus) and 165 were observed implicating their potential role in conferring resistance. Docking poses were inspected and documented using BIOVIA Discovery Studio Visualizer (https://discover.3ds.com/discovery-studio-visualizer-download).

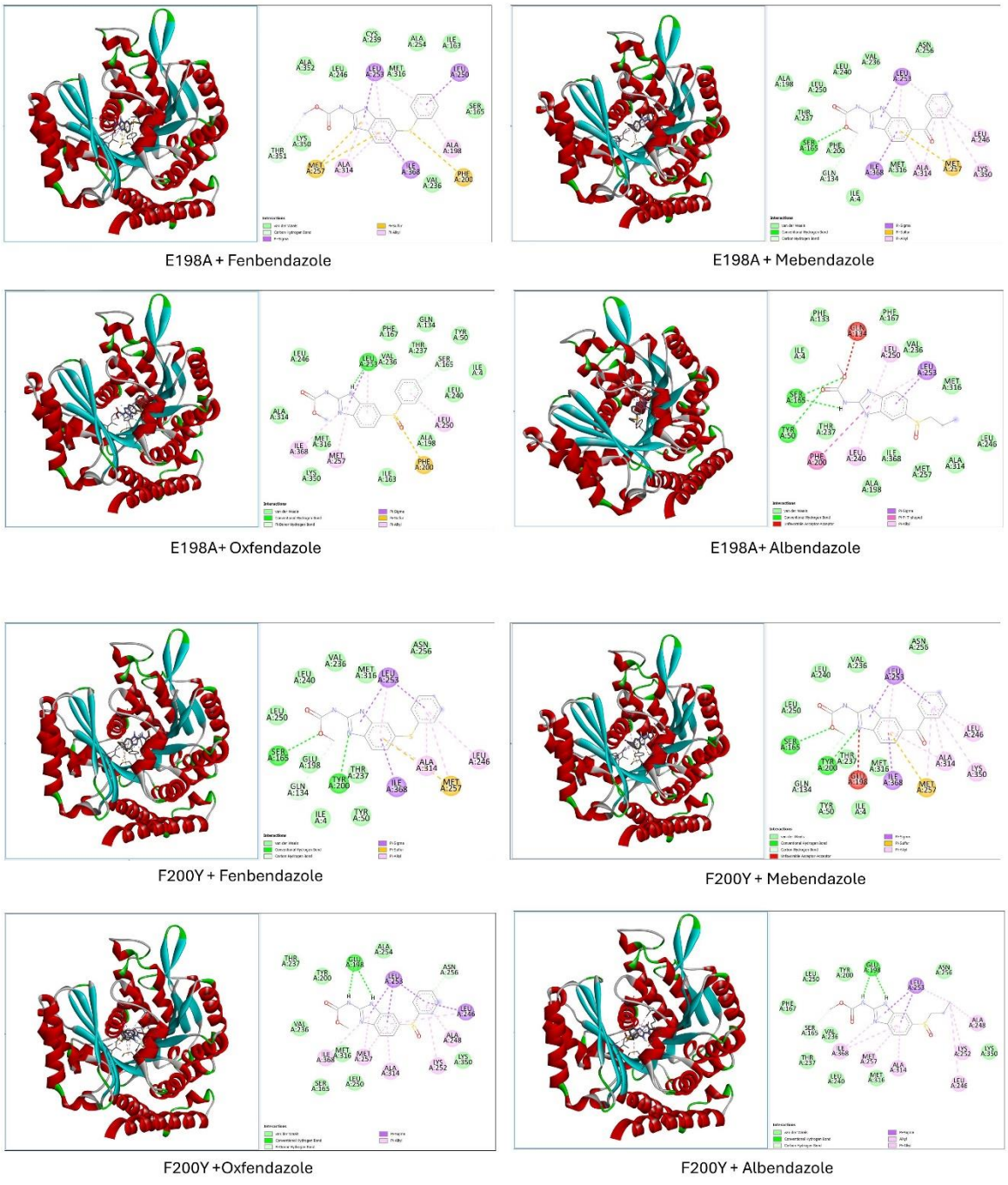


Figure 6. The 3D and 2D representations of amino acid interactions of the docking experiments for the E198A and F200Y mutated β-tubulins. The introduction of Alanine at position 198 resulted in losses of key bonds with the benzimidazole drug ligands. Docking poses were inspected and documented using BIOVIA Discovery Studio Visualizer (https://discover.3ds.com/discovery-studio-visualizer-download)

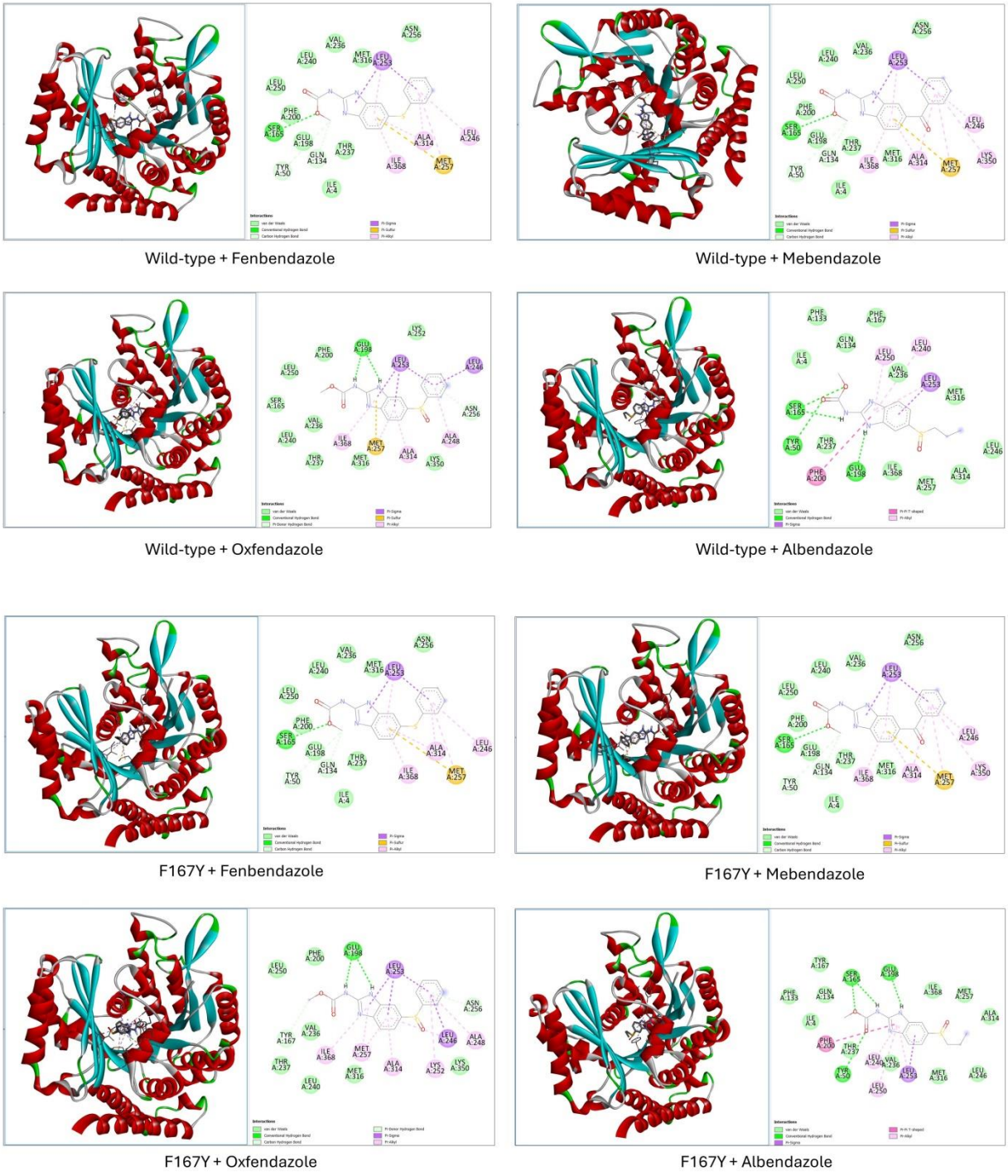
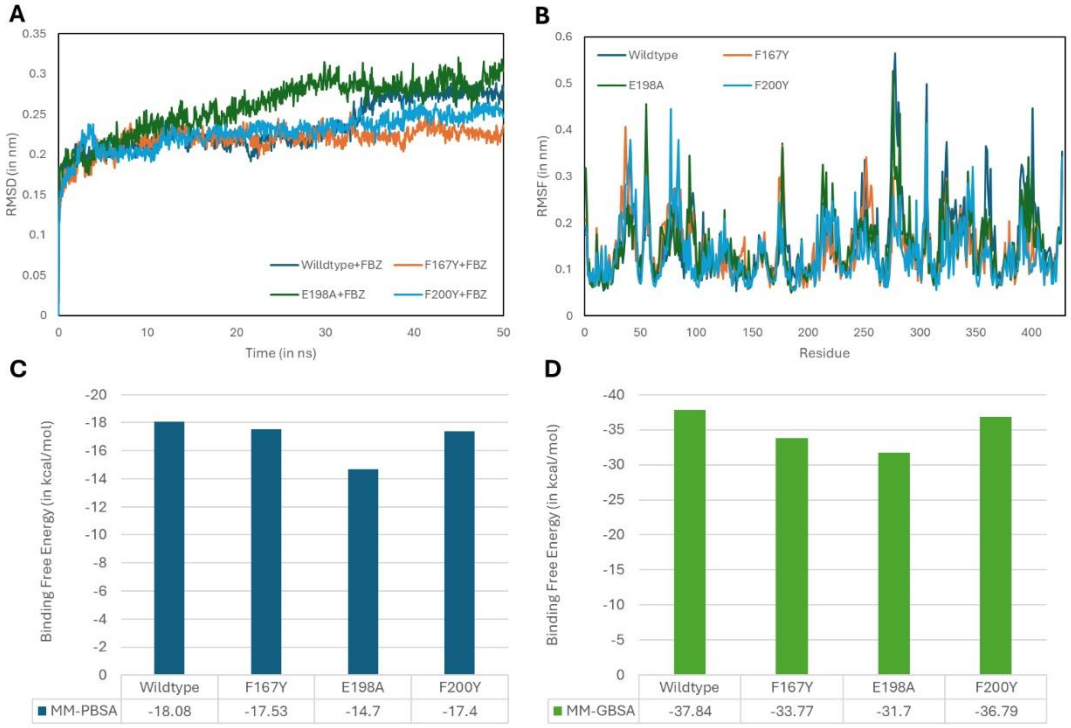


Figure 7. Molecular dynamics simulation results and binding free energies calculation. The RMSD (A) and RMSF (B) plots show that the β-tubulin-FBZ complexes behave similarly, and that wildtype and mutated protein backbone share similar flexibility profiles. The MM-PBSA (Molecular Mechanics-Poisson-Boltzmann Surface Area) (C) and MM-GBSA (Molecular Mechanics-General Born Surface Area) (D) binding free energies show that the E198A mutation caused marked decreases energetically between the β-tubulin-FBZ interactions.



Discussion

This investigation aimed to assess the consequences of canonical benzimidazole resistance-associated mutations (i.e., F167Y, E198A, and F200Y) on the binding of *A. galli* β-tubulin with benzimidazole drugs. The introduction of these resistance-associated mutations causes several alterations in the protein-ligand interactions. The results show that resistance-associated mutations cause alterations in binding affinity and positions. Specifically, mutations at position 198 may be integral in conferring benzimidazole resistance in *A. galli*. In general, the mutations caused an increase in ΔG, except in proteins with E198A. Moreover, the loss of key interactions, the emergence of interaction unfavored interactions, and the changes in the types of bonds formed were particularly noted in β-tubulins with the same mutations. Both wild-type and mutated proteins share the same structural motion and flexibility dynamics profile. These results reveal some interesting insights into benzimidazole resistance in *A. galli.*

The ΔG affinity alterations caused by the resistance-associated mutations observed in this study have also been reported in other peer-reviewed papers. Increased binding energies brought about by canonical resistance mutations have been reported in *silico* docking studies in *Haemonchus contortus* and *Trichinella spiralis* [24,32]. Specifically, the increase in ΔG by the introduction of F200Y and the decrease caused by E198A mutations in this study have been noted in *H. contortus* [32]. A similar trend was observed in *Trichuris trichiura* and *Ancylostoma duodenale* [25]. In contrast, *in silico* reports in other ascarid nematodes like *Ascaris lumbricoides* have reported the opposite—E98A caused improved binding affinity while the F167Y and F200Y caused a

diminishing effect [26]. The results of this research with the aforementioned one from the literature point out that the introduction of canonical resistance-associated mutations causes alterations in the binding efficacy between the β-tubulin protein and the benzimidazole drug ligand.

The significant role played by amino acid residue at position 198 in the β-tubulin-benzimidazole drug ligand interaction, as observed in this research, is also echoed in previously published papers. Docking studies conducted by [26] showed that among members of the *Ascaridomorpha* infraorder, the residues at position 198 form numerous hydrogen and noncovalent interactions with albendazole. The same was also observed when they studied these interactions in soil-transmitted helminths of humans and animals, equine and ruminant nematodes, and parasitic roundworms of fish [25]. Considering these results with those of the present study, it can be inferred that amino acid residues at position 198 are integral in the stable binding of the benzimidazole drug ligand to the *A. galli* β-tubulin protein. As such, mutations in this position may be detrimental in conferring benzimidazole resistance in *A. galli.*

The molecular dynamics analysis showed that the dynamic behavior of the β-tubulin-benzimidazole complexes remains comparable despite the presence of benzimidazole resistance-associated mutations. Similar RMSD and RMSF profiles have been reported in a previous *in silico* study on Ascarid β-tubulins [26]. The significance of the E198A mutation in significantly altering the binding efficiency between β-tubulin and benzimidazole was reaffirmed in this research. A similar finding was found in previously published studies in *H. conturtos* [32]. This *in silico* finding is supported by laboratory studies in *C. elegans* genetically edited to contain the E198A and other mutations within the same position conferred phenotypic resistance when challenged with benzimidazole drugs [33,34]. Our results, when considered with the aforementioned results of other studies, prove that the E198K mutation in the β-tubulin gene confers benzimidazole resistance in *A. galli.*

Conclusion

The emergence of benzimidazole resistance remains a concern in parasitic helminths of public and veterinary concern. The results of this research show that canonical resistance-associated mutations cause alterations in the *in silico* docking of *A. galli* β-tubulin and benzimidazole drug ligands. In particular, the E198A mutation caused a decrease in ΔG and changes in the binding positions. Moreover, the amino acid residues in this position, both wild-type and mutated, caused the most numerous interactions with the benzimidazole drug ligands among the docking simulations. Molecular dynamics studies also point to the key role of the E19A mutation in conferring resistance. These findings indicate that mutations in amino acid position 198 are integral in conferring benzimidazole resistance in *A. galli.* Further research is needed to affirm this finding.

Supplementary material

Not applicable.

Data availability

Data generated in this study can be made available upon request from the corresponding author.

Acknowledgments

Not applicable.

Ethical standards

This research does not require ethical approval as it did not use animal or human subjects.

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