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Four different benzimidazoles as treatment against Encephalitozoon cuniculi infection in rabbits and how they may bind to β-tubulin

Bachelor's thesis in Chemistry
Supervisor: Huiling Liu
February 2022

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Abstract

Encephalitozoon cuniculi infection in pet and laboratory rabbits is an ongoing problem, that sometimes causes severe disease and death in rabbits. Treatment of the parasitic infection is mostly involving benzimidazoles, more specifically methyl 2-benzimidazole carbamates, to stop or reduce the infection. The binding site of benzimidazoles and the mechanism of action are still controversial and a better understanding of this may help finding novel benzimidazoles that has a stronger effect than the drugs most commonly used today. Fenbendazole is the recommended drug for treatment against the parasite in rabbits and albendazole is embryotoxic and teratogenic and is therefore not recommended. Both fenbendazole and albendazole metabolises to compounds that are effective in reducing the proliferation of *E. cuniculi*. There are not many studies on the effect of oxibendazole and thiabendazole (thiabendazole is not a carbamate) on *E. cuniculi*.

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1 Introduction

Pet and laboratory rabbits are commonly found to be infected with a microsporidian parasite, *Encephalitozoon cuniculi*. Microsporidia are eukaryotic single-cell parasites that are classified as fungi, or a sister group of fungi [1]. They form spores that can infect new hosts. Rabbits get mostly infected by ingestion or inhalation of the spores, often from the infected rabbits' urine. Severity of encephalitozoonosis differs from mild signs to sudden death [2]. *E. cuniculi* is a host generalist; it is found to infect a number of birds and mammals, though the main host is the rabbit [3]. The parasite is also found in humans and is thought to be zoonotic [4], i.e. it is transmitted from animals to humans. *E. cuniculi* infected humans that are immunocompromised can develop disease [5]. Treatment of the disease is therefore not only important to the health of rabbits, but also to the health of humans.

Spore reduction is important to treat an *E. cuniculi* infection. Studies evaluating the efficacy of different treatments are limited, however, the use of certain benzimidazole derivatives, mainly albendazole and fenbendazole, have been shown to have effect. The exact mechanism of action of these drugs is unknown and a better understanding of it may help finding novel benzimidazole drugs with better affinity and efficacy. The goal of this report is to review findings that point to a binding site for benzimidazole drugs and to evaluate which of the four benzimidazoles, fenbendazole, albendazole, oxibendazole and thiabendazole is best at treating encephalitozoonosis in rabbits, based on the binding site discussion and studies on the potency and effect of the benzimidazoles against *E. cuniculi*.

2 Theory

2.1 *Encephalitozoon cuniculi* infection in rabbits

Microsporidia are obligate intracellular parasites, meaning that they can only reproduce inside living cells of the host organism. *E. cuniculi* sporoplasm enters the host cell and develops to a meront, i.e. the proliferative form. The cells then differentiate to different stages ending in mature spores. Rupture of the host cell spreads the *E. cuniculi* spores to other cells of the host, and some spores are excreted via the urine, which can then infect other rabbits [6]. Most infected rabbits are asymptomatic, but in some cases these parasites cause neurological,

renal and/or ocular disease. Neurological symptoms are often vestibular disease, ataxia and/or hemiparesis. Vestibular disease is a sudden disturbance of balance and coordination that can cause a head tilt, known as a typical symptom of *E. cuniculi* infection in rabbits. Ataxia is represented by a lack of muscle control and hemiparesis refers to not being able to or having difficulty to move one side of the body. Renal disease is damage to the kidneys and lesions on the kidneys are common postmortem findings. In some rabbits this causes urinary incontinence. Ocular disease occurs when the rabbit is infected as a fetus in utero. Lesions on the lens can cause pain and blindness. In addition to drugs aimed at eliminating the parasite (benzimidazoles), anti-inflammatory medication and additional symptomatic treatment, dependent on the symptoms of each case, can be necessary when treating *E. cuniculi* infected rabbits[2].

2.2 Benzimidazoles against *Encephalitozoon cuniculi*

2.2.1 Chemistry

Benzimidazole is a bicyclic aromatic compound that is the fusion of benzene and imidazole (figure 2.1). The Benzimidazole nucleus is in several different important bioactive compounds, like vitamin B_{12}. The fact that benzimidazoles are found in naturally occurring compounds and that the benzimidazole nucleus is similar to natural nucleotides, signals their broad spectrum of biological activities. Benzimidazole derivatives were early used as plant fungicides and veterinary anthelmintics. Today, numerous pharmacological properties of different benzimidazoles are known: antimicrobial, antivirus, antiparasite, antihypertensive, antihistaminic, anticancer, anti-inflammatory, antiulcer, hormone modulating, proton pump inhibitors and more[7]. The numbering of the possible substituent positions are given in figure 2.2. The hydrogen in position 1 is acidic and the nitrogen in position 3 is basic, and so the hydrogen readily tautomerizes, making the 6-substituted benzimidazole a tautomer to 5-substituted benzimidazole (figure 2.2). Most biological active benzimidazoles are substituted at position 1, 2 and/or 5(6)[7].

Fenbendazole, albendazole and oxibendazole are methyl 2-benzimidazole carbamates, they have a methyl carbamate group at position 2. The general structure of the three benzimidazoles is illustrated in figure 2.3. Due to the resonance of the amide bond of the carbamate group, they have *cis-trans* isomerism. Fenbendazole, albendazole and oxibendazole have different substituents at position

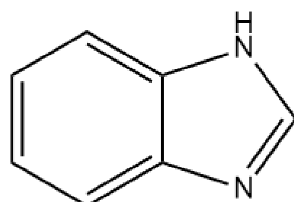

Figure 2.1: Benzimidazole

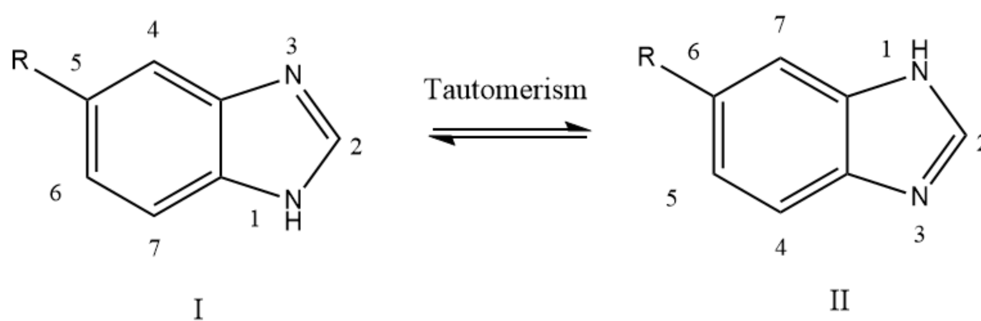

Figure 2.2: Benzimidazole tautomerism

5(6). These are shown in table 2.1. Thiabendazole does not have a carbamate group, but instead a thiazole substituent at position 2 (see figure 2.4).

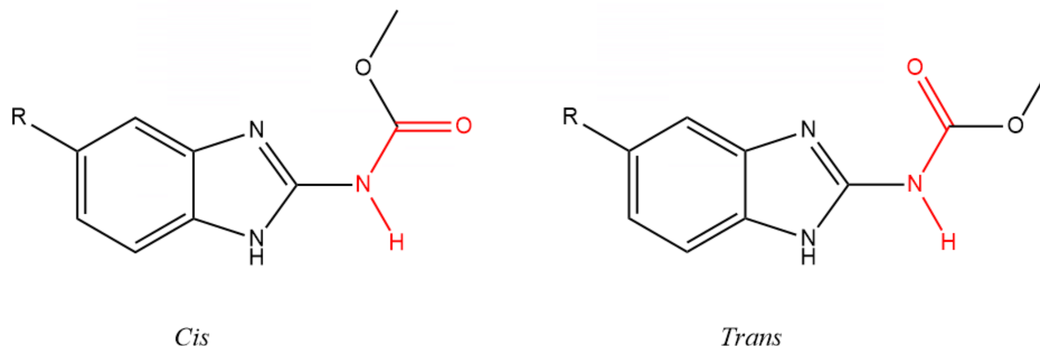

Figure 2.3: *Cis* and *trans* structures of methyl 2-benzimidazole carbamates

Table 2.1: Chemical structure of the 5(6) substituents of the benzimidazole carbamate compounds. For complete structure see figure 2.3

Compound	R-group
Fenbendazole	Ph-S-
Oxfendazole	Ph-(S=O)-
Albendazole	CH_3-CH_2-CH_2-S-
Albendazole sulfoxide	CH_3-CH_2-CH_2-(S=O)-
Oxibendazole	CH_3-CH_2-CH_2-O-

2.2.2 The mode of action of benzimidazoles against *Encephalitozoon cuniculi* in rabbits

The protein β-tubulin has been identified as the target of benzimidazoles. This inhibits microtubule polymerization and force depolymerization. In cells, this causes the inhibition of cell growth, inhibition of proliferation and cell death [8]. An important property of the benzimidazoles used against parasites is that they are selectively toxic to lower eukaryotes and have little effect on mammalian tubulin [9]. Benzimidazoles that have been used against presumptive *E. cuniculi* infection in rabbits are fenbendazole (20 mg/kg orally once a day for 30 days), albendazole (30 mg/kg orally once a day for 30 days) and oxibendazole (30 mg/kg orally once a day the first 7-14 days, then 15 mg/kg for 30-60 days) [10]. Fenbendazole and albendazole are metabolized to their respective sulfoxides, which are

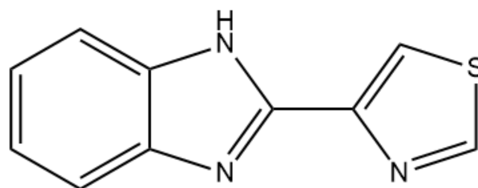

Figure 2.4: Thiabendazole

also known to be antiparasitics. Fenbendazole sulfoxide, (oxfendazole) and albendazole sulfoxide are major metabolites of their respective parent compounds in rabbits[11]. The sulfoxide substituents are shown in table 2.1. The metabolites of oxibendazole are not well known, if at all[12]. The major metabolite of thiabendazole is 5-hydroxythiabendazole (hydroxy group at position 5(6))[13]. 5-hydroxythiabendazole has no anthelmintic activity[14].

Albendazole has been reported to be embryotoxic and teratogenic[6], i.e. toxic to and causing defects in the fetuses. There are reported individual cases with suspected benzimidazole toxicosis in rabbits treated with one of the three methyl benzimidazole carbamates[15]. Thiabendazole has been concluded to not be teratogenic or otherwise harmful to rabbit fetuses at relatively high dosages[16]. Treatment practices advocate the use of fenbendazole for *E. cuniculi* prevention, based on findings by Suter et al. in 2001[17]. Some also advocate fenbendazole as treatment against chronic *E. cuniculi* in rabbits, though no controlled study has confirmed the clinical efficacy of fenbendazole in cases with advanced central nervous system inflammation[6].

2.3 Microtubules

Microtubules are a part of the cytoskeleton and, in eukaryotic cells, they are a vital constituent of the mitotic spindles that pull the chromosomes apart during cell division. Spindle microtubules are the only microtubules identified in microsporidia[18]. Microtubules are polymerized tubulin, forming a hollow cylinder. Tubulin is a dimeric protein consisting of α- and β-tubulin. The arrangement of tubulin in microtubules is such that the α-tubulins in the dimers are towards one direction of the microtubule, and the β-tubulins towards the other direction. The end exposing α-tubulin is the minus end and the end exposing β-tubulin is the plus end. Microtubules and tubulin dimer are illustrated in figure 2.5.

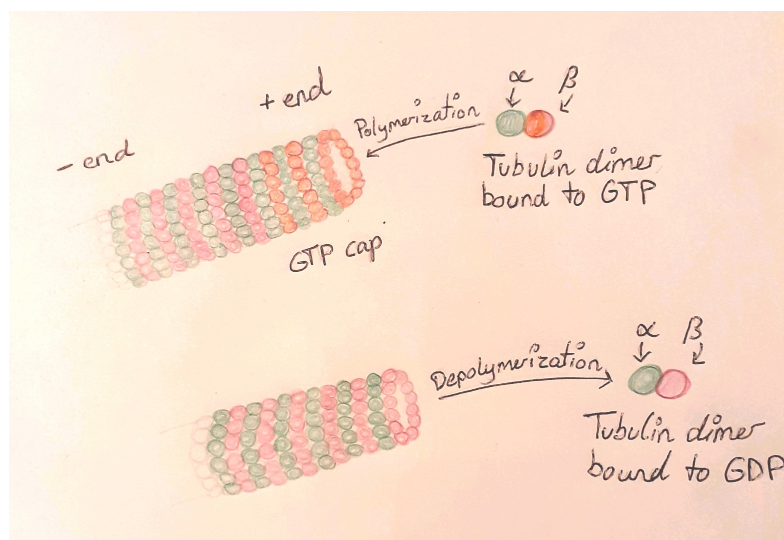

Figure 2.5: Microtubule polymerization (top) and depolymerization (bottom). A GTP cap hinders depolymerization.

Both α- and β-tubulin bind guanosine triphosphate, GTP. GTP is an important source of energy in many biochemical reactions. It is dimeric GTP tubulin that adds to the plus end of the microtubule under polymerization. In cells, the minus end is generally considered to be stable, and not growing. Microtubule with GTP bound tubulin is stable and will not depolymerize. Some time after polymerization, the β-tubulin GTP hydrolyses to GDP, which makes the microtubule less stable. A GTP cap on the plus end ensures that the microtubule does not depolymerize. A loss of the GTP cap results in depolymerization. This is illustrated in figure 2.5. This behaviour of growing and shortening is called dynamic instability[19]. In addition to GTP, there are so-called microtubule-associated

proteins regulating the dynamics of the polymerization/depolymerization.

Colchicine is a microtubule depolymerization agent that is toxic to mammalian cells. β-tubulin is composed of three domains: N-terminal (residues 1-201), intermediate (residues 202-371) and C-terminal (residues 372-427). The colchicine binding site is near the monomer-monomer interface in the intermediate domain in β-tubulin. Colchicine bound tubulin is prevented from adopting a straight structure, which is necessary for assembly to microtubule[20]. Competitive inhibition studies indicates that benzimidazoles bind close to the colchicine binding site[21], and many benzimidazole resistant mutants of helminths and fungi contain mutations in β-tubulin genes that are expressed near the colchicine binding site. The binding site of the benzimidazoles is however still controversial[22].

2.4 β-tubulin residues important for benzimidazole sensitivity

Most studies on the benzimidazole binding site are focusing on the binding of methyl 2-benzimidazole carbamates, and important residues of the β-tubulin protein that when mutated confers resistance to benzimidazoles. The organisms with these natural or artificial mutations are typically helminths or fungi. The organisms are originally susceptible to benzimidazoles and often have similar β-tubulin DNA sequence in the region where the important residues are located. *E. cuniculi* β-tubulin share these similarities[9]. The results and discussion of these studies regarding the binding site can therefore be relevant, despite that they are not specifically about *E. cuniculi* tubulin. The region containing the important residues, that is close to the colchicine binding site, is located between the N-terminal and intermediate domains[23].

Two of the most significant amino acids that when mutated confer benzimidazole resistance in fungi and helminths are residues 200 and 198 of β-tubulin. In most benzimidazole sensitive organisms residue 200 is phenylalanine, F200[9]. There are several examples of phenylalanine to tyrosine mutations, F200Y, that confer resistance in nematodes[24]. The mutation causes the residue to be polar instead of nonpolar. Residue 198 is most often glutamate in sensitive organisms, but also in some resistant organisms (e.g. humans)[9][24]. There are several mutations of residue 198 from glutamate to different amino acids that confer benzimidazole resistance in fungi, and also some in nematodes[25].

Jung and Oakley found that the mutation A165V in the fungi *Aspergillus nidulans* confers resistance to thiabendazole but increased sensitivity to benomyl, carbendazim and nocodazole[26]. Thiabendazole and carbendazim differ only at position 2. Jung and Oalkey therefore concluded that position 2 of benzimidazoles may interact directly to residue 165. Benomyl, carbendazim and nocodazole have carbamate at position 2.

Li et al. found that the F167Y mutation in the fungi *Saccharmomyces cerevisiae* confers resistance to carbendazim and nocodazole, but supersensitivity to benomyl[27]. Benomyl differs from carbendazim and nocodazole on position 1. Carbendazim and nocodazole are unsubstituted while benomyl has a butylcarbamyl substituent. The conclusion was a possible model where the phenyl in phenylalanine has hydrophobic interaction with the benzimidazole core. The mutation makes the amino acid more polar and therefore weakens the bond to the benzimidazole. Li et al. postulated that the benomyl supersensitivity may be due to hydrogen bonding between the hydroxide on tyrosine and the substituent at position 1 of benomyl.

There are more residues than the four mentioned of β-tubulin that seems to be important in the binding of benzimidazoles, also located in the same region.

2.5 Pharmacodynamics

The inhibitory effect of a drug is dependent on several factors; the concentration, the pharmacokinetics, the affinity and the efficacy of the drug. Potency is a measure of the amount of the drug needed to produce an effect of given intensity. It is often expressed as IC_{50}, half maximum inhibitory concentration, i.e. the concentration needed to produce 50% of the drug's maximum effect. Pharmacokinetics is about how long the drug is available to the target and the metabolism of the drug. The affinity is the extent of how readily the drug binds to the binding site of the target. Affinity is an important factor of the potency, which is a measure of the amount of the drug needed to produce an effect of given intensity. The potency is often expressed as IC_{50}, half maximum inhibitory concentration, i.e. the concentration needed to produce 50% of the drug's maximum effect. Efficacy is a measure of the response caused by the drug bound to the target. E_{max} is the maximum effect of the drug. When E_{max} is reached, an increase in concentration will not produce a greater effect.

3 Discussion

3.1 The benzimidazole binding site on β-tubulin

The identification of the benzimidazole binding site on β-tubulin is hampered by the fact that the exact 3D structures of β-tubulin of different organisms are unknown. Two studies, one from 2004, one from 2013, use different structural templates to generate the structure of β-tubulin of specific nematodes. The studies then used molecular docking to place a benzimidazole into their modeled β-tubulin. The results of the two studies and others are discussed below, with emphasis on the binding of the four benzimidazoles of this report and the two relevant sulfoxide metabolites.

3.1.1 2004 study protein template

A study from 2004, by Robinson et al.[23], proposes an orientation of albendazole sulfoxide in a putative binding site in *Haemonchus contortus* β-tubulin containing the residues most thought to interact with benzimidazole. The sulfoxide oxygen (position 5) hydrogen bond with the H-N in H6. Tautomer I (figure 2.2) can hydrogen bond with the carbonyl oxygen of the backbone of V236. The carbonyl oxygen of the carbamate moiety (position 2) forms hydrogen bonds with the side chains of E198 and S165. M257 is part of a hydrophobic pocket which interacts with the methyl group of the carbamate moiety. This orientation also enables hydrophobic interactions between the benzimidazole ring structure and the phenyl rings at F167, F200 and Y50.

A naturally occurring F to T mutation at position 200 in *H. contortus* β-tubulin confers resistance to benzimidazole anthelmintics. Robinson et al. speculates that the added oxygen atom in the mutation forms a hydrogen bond with serine at position 165 and thus closes off the hydrophobic pocket for the benzimidazole ring structure[23].

Minagawa et al. published a study in 2021[22] looking at fission yeast (*Schizosaccharomyces pombe*) microtubules and reconfirmed that a β-tubulin mutant called nda3-TB101 is resistant to benzimidazoles. The mutation differs only from the wild type protein in one amino acid. The tyrosine in position 50 (Y50) in wild type is changed to serine (S50). The study found that the Y50S mutation interfered binding of thiabendazole in vitro, by detecting thiabendazole collected from columns containing either wild type or nda3-TB101 β-tubulin. The flow

of thiabendazole was lower through the nda3-TB101 column than the wild type column. Minagawa et al. suggest that Y50 serves as a wall, containing the benzimidazole inside the binding pocket proposed by Robinson et al. The mutation represents a structural difference, making residue 50 smaller. The benzimidazole may therefore more easily disassociate from the binding site of the mutant, and thereby has lower affinity to the Y50S mutated tubulin. Minagawa et al. did however not prove that residue 50 is directly interacting with benzimidazole and that the mutation is not simply causing structural change to a binding site placed in another region of the protein.

Residue 165 in β-tubulin differs in different benzimidazole susceptible organisms, the amino acids often being serine, alanine, threonine or cysteine[24]. In *E. cuniculi* the residue is cysteine. Alanine is a hydrophobic amino acid, and it can not form hydrogen bonds with the side chain. Therefore the hydrogen bonding which Robinson et al. propose may not be very important, in addition, the side chain of glutamic acid also acts as a hydrogen bond donor to the same carbonyl oxygen of benzimidazole carbamates in this putative binding site. C165 in *E. cuniculi* can however have moderately strong dipole-dipole interactions with benzimidazole carbamate, since the thiol group of cysteine is a moderately good hydrogen bond donor[28]

Robinson et al. did not discuss the interactions between the amino acids of their proposed binding site and other benzimidazoles than albendazole sulfoxide. The only difference between albendazole sulfoxide and albendazole is the lacking sulfoxide oxygen in albendazole. One can therefore assume albendazole will have the same interactions except a weaker bond between H6 and the sulfur of the sulfide substituent, as sulfides are weaker hydrogen bond acceptors than sulfoxides.

Compared to albendazole sulfoxide, fenbendazole has a phenyl group instead of the propyl group in addition to also missing the sulfoxide oxygen. The phenylsulfanyl substituent of fenbendazole has one less rotatable bond than the propylsulfanyl of albendazole. This may make the free binding energy of fenbendazole lower than that of albendazole, because the more flexible molecule (albendazole) will loose more entropy when bound and restricted by the protein compared to a less flexible molecule (fenbendazole), given that the less flexible molecule have a suitable conformation to fit into the binding pocket. How the propyl group of albendazole sulfoxide is oriented in this putative binding site is however not ex-

plained. The metabolite of fenbendazole, oxfendazole, may be assumed to have similar hydrogen bonding as albendazole sulfoxide with residue H6 of β-tubulin.

The ether oxygen of oxibendazole may be expected to be a stronger hydrogen bond acceptor than the sulfur of albendazole and fenbendazole because of the higher electronegativity, but weaker than their sulfoxide metabolites. Ethers are weaker hydrogen bond acceptors than sulfoxides because of the sulfoxide resonance, making the sulfoxide oxygen more negatively charged.

3.1.2 2013 study protein template

Aguayo-Ortiz et al. published a study in 2013[29] using a different protein template than that of Robinson et al. The study focuses on the hydrogen bonds between the amino acids and methyl 2-benzimidazole carbamates from computational data, using the β-tubulin DNA sequence to a nematode (*Trichinella spiralis*). Aguayo-Ortiz et al. considered both the tautomerization and the *cis-trans* isomerism of the methyl 2-benzimidazole carbamates. The binding energies of the methyl 2-benzimidazole carbamates considered in this report were lowest for the tautomer I (figure 2.2) and the *cis* structure of the carbamate group (figure 2.3). It is this isomer that is used when discussing the orientation of the benzimidazoles in the putative binding site. In this orientation, E198 hydrogen bonds with the two H-N at benzimidazole carbamate and T165 hydrogen bonds with the carbonyl oxygen of the carbamate substituent. C165 of *E. cuniculi* β-tubulin can as previously stated also hydrogen bond with the carbamate substituent. Molecular dynamics calculations identified hydrogen bonding between Q134 and the two oxygens of the carbamate. Aguayo et al. suggest that this helps internalizing the benzimidazole carbamate into the binding site and bringing it into contact with E198.

Another study by Aguayo-Ortiz et al.[24] employed their binding site model to study the resistance and susceptibility of different organisms to benzimidazole carbamates. The results suggested that the formation of the two hydrogen bonds with E198 is the main reason for the stabilization of the benzimidazole carbamates in the proposed binding site. Y167 mutation interacts with Q134 and thus may hinder the internalization into the binding site. Y200 mutation interacts with E198 and thus may hinder E198 to interact with benzimidazoles.

In addition, C239 hydrogen bonds with potential hydrogen bond acceptors in substituents at position 5. Residue 239 is also cysteine in *E. cuniculi.* This

orientation will therefore favor benzimidazole carbamates like albendazole sulfoxide before carbendazim. Ethers (oxibendazole) and thioethers (fenbendazole and albendazole) will also have dipole interactions with C239. This indicates that the metabolites of albendazole and fenbendazole are the more active compounds than albendazole and fenbendazole, respectively, which correlates with the study's calculated binding energies: the metabolites had lower binding energy than their respective parent compounds.

Of the three methyl benzimidazole carbamates considered in this report, fenbendazole had the lowest binding energy, followed by albendazole. Oxibendazole had the highest binding energy. Based on the interaction with C239 alone, one may expect that oxibendazole would have lower binding energy than albendazole and fenbendazole, since sulfur is less electronegative and more polarizable than oxygen, causing thioethers to being weaker hydrogen bond acceptors than ethers. Aguayo-Ortiz et al. did not discuss this ordering of binding energy in their study. Only C239 was shown to be interacting with the substituent at position 5 of albendazole, fenbendazole and oxibendazole in the study's illustrated predicted binding modes. However, these results suggests that fenbendazole and albendazole are better choices as tubulin inhibitors in vivo than oxibendazole, mostly because of the seemingly added affinity the sulfoxide metabolites of fenbendazole and albendazole have.

3.1.3 Region close to GTP binding site, residues 138 and 178

Despite many studies' focus on the region close to the colchicine binding site in β-tubulin and the strong correlation between mutations of amino acids in this region and benzimidazole resistance, some point to the existence of an alternative binding site for benzimidazoles. A study from 2010 maintains that benomyl and colchicine can bind to mammalian tubulin simultaneously [30]. This indicates that there is an alternative binding site in β-tubulin for benzimidazoles.

In a study from 2018 Vela-Corcía et al. [31] proposed a different binding site in *Podosphaera xanthii* β-tubulin for methyl 2-benzimidazole carbamates, like benomyl, despite that the E198A mutation confer resistance to methyl benzimidazole carbamates. They found that the mutation induces changes in the structure of β-tubulin, and computational molecular docking of carbendazim (unsubstituted methyl 2-benzimidazole carbamate) indicated a region close to the GTP binding site, with S138 and T178 directly interacting with carbendazim. The study's

computational model showed that the region is structurally altered after the E198A mutation, thus affecting the capability to bind methyl benzimidazole carbamates. Vela-Corcía et al. did not manage to determine if mutations to alanine in residue 138 and 178 conferred resistance to methyl benzimidazole carbamates due to instability of the mutant proteins.

These results reveal the uncertainty of the binding site of benzimidazoles in β-tubulin. There is a fair amount of evidence indicating a binding site close to the colchicine binding site, containing residues like 167, 198 and 200, granting that the actual binding mode is unknown. The fact that there may be another binding site complicates the discussion further. This report will for the most part assume that there is only one binding site for benzimidazoles, which is close to the colchicine binding site.

3.1.4 Thiabendazole versus methyl 2-benzimidazole carbamates

Computational models of putative benzimidazole binding site on β-tubulin are mostly focused on the binding of methyl 2-benzimidazole carbamates, and not other benzimidazoles like thiabendazole. Thiabendazole does not have an extra hydrogen bond donor at the substituent in position 2. This suggests that a binding as proposed by Aguayo-Ortiz et al., but with thiabendazole, may be less favourable due to the loss of an important hydrogen bond to E198. The thiazole group of thiabendazole have the same amount of hydrogen bond acceptors as the methyl carbamate group, but the sulfur of thiazole is a poor acceptor compared to the oxygens of methyl carbamate. If the right orientation is possible, the nitrogen of thiazole can hydrogen bond, similarly to the carbonyl oxygen of methyl carbamate, to residue 198 or 165 as proposed by Robinson et al. or to residue 165 as proposed by Aguayo-Ortiz et al. Thiabendazole and carbendazim have similar topological polar surface area[32] [33], which indicates that the two different position 2 substituents have similar affinity to a given hydrophobic/hydrophilic environment.

3.2 Activity differences of the benzimidazoles against *Encephalitozoon cuniculi*

Most studies on the activity of benzimidazoles against *E. cuniculi* in vivo and in vitro are considering albendazole and/or albendazole metabolites[34]. In vitro, a greater effect is achieved if the inoculation and treatment are done simulta-

neously compared to if the drug is added to established infections[35]. In vivo, albendazole is found to inhibit spore formation, but often just temporarily, as found by Lallo et al. in 2013[36]. Lallo et al. studied the effect of different drugs against *E. cuniculi* infection in immunosuppressed mice. The drugs were albendazole and its metabolite, albendazole sulphoxide and two non-benzimidazole drugs (metronidazole and cyclosporine). The mice treated with albendazole and albendazole sulphoxide were given treatment 400 mg/kg twice daily for 3 weeks and 50 mg/kg daily for 3 weeks, respectively. Lallo et al. found that none of the drugs at the tested dosages were effective in stopping the infection. The benzimidazoles did significantly reduce the infection and the symptoms, though 60 days after the end of treatment, the infection could be seen again.

The findings of Suter et al. in 2001[17] are very important for the treatment practices of encephalitozoonosis in rabbits. They achieved prevention of *E. cuniculi* infection in their prophylactic study, using fenbendazole. *E. cuniculi* free rabbits were given doses of fenbendazole daily for seven days before they were infected with *E. cuniculi* spores, and some time after infection (two and 21 days). The rabbits remained seronegative until 120 days after infection and when the rabbits were euthanized six months after infection, no *E. cuniculi* could be isolated from their brain tissue. In contrast, spores were isolated from all the untreated rabbits in the control group. Their therapeutic study managed to isolate *E. cuniculi* from brain tissue of seven of nine naturally infected, untreated rabbits. No spores were found in the brain tissue of the eight naturally infected rabbits that were treated with 20 mg fenbendazole/kg bodyweight daily for four weeks, though two of them were euthanized after the four weeks because of neurological symptoms consistent with encephalitozoonosis. These results point to that fenbendazole does have a spore reductive effect in rabbits with advanced infection, but does not necessarily stop the infection completely, similar to albendazole.

Franssen et al., 1995[37], studied the susceptibility of *E. cuniculi* to different drugs in vitro, using monolayers of rabbit kidney cells introduced to a suspension containing *E. cuniculi* spores. The benzimidazoles tested in this study were thiabendazole, albendazole and oxibendazole. The study found the three benzimidazoles and fumagillin most effective. Fumagillin has a different mode of action than benzimidazoles). Treatment of 1 day old *E. cuniculi* cultures with the benzimidazoles at 5 µg/ml for 1 day was enough to decimate the parasites. After 8 days of treatment, some individual parasites were still found, but there

were no proliferating colonies and no spores were formed. Including starting of treatment at 1 day old cultures, Franssen et al. also tested the benzimidazole drugs in 8 days old cultures. The maximum inhibitory effect was less for all the drugs on the older *E. cuniculi* cultures. Treatment of 8 days old cultures did not clear the host cells of the parasites, there were remaining colonies and some spore formation. The mean half maximal inhibitory concentration and mean growth inhibition at µg/ml in 1 and 8 days old cultures are listed in table 3.1. The IC_{50} was lowest for oxibendazole and highest for thiabendazole, meaning oxibendazole showed highest potency and thiabendazole showed lowest potency. The IC_{50} was only determined in 1 day old *E. cuniculi* cultures. Oxibendazole and thiabendazole had similar high growth inhibition in 1 day old cultures, and albendazole showed a less strong inhibitory effect. In 8 days old cultures thiabendazole had the strongest effect and oxibendazole had the lowest. Franssen et al. did not discuss the difference in order of effectiveness in 1 and 8 days old cultures.

Table 3.1: The IC_{50} of albendazole, thiabendazole and oxibendazole against 1 day old *E.cuniculi* and the growth inhibition in 1 and 8 days old *E. cuniculi* of the drugs at a concentration of 5 µg/ml.

Compound	IC_{50} (µg/ml)	% Growth inhibition (1 day)	% Growth inhibition (8 days)
Albendazole	0.0044 ± 0.0008	78.4 ± 5.2	63.0 ± 3.1
Oxibendazole	0.0015 ± 0.00015	90.4 ± 3.3	21.8 ± 4.1
Thiabendazole	0.30 ± 0.04	89.0 ± 2.3	71.4 ± 2.1

When used as a treatment of infection in rabbits, the spores have most likely infected the rabbit for more than 1 day, and so the results of the inhibitory effect in 8 days old cultures are maybe more relevant. However, the effect in vivo may be very different from the effect of the different drugs in vitro. Oxibendazole showed a relatively poor inhibitory effect in 8 days old cultures, but a strong effect in 1 day old cultures, and also with high potency. Whether the potency of the drugs also differ from younger and older cultures is unknown. Despite the promising results from the tests done on 1 day old cultures, the results of the 8 days old cultures indicate that oxibendazole may not be very effective against *E. cuniculi* infection in chronic cases. Thiabendazole showed high E_{max} in both 1 and 8 days old cultures, but a low potency. This high E_{max} and low potency may be because of a relatively low affinity to the binding site, but a strong response when thiabendazole is bound to tubulin, i.e. high efficacy.

Thiabendazole seems not to be toxic to rabbits, and so a lower potency than the drugs commonly used against the parasite may not be a problem. If thiabendazole was used to treat rabbits, there would maybe be cases of benzimidazole toxicosis due to thiabendazole, similar to the three benzimidazoles used to treat *E. cuniculi* infections in rabbits. In vivo, thiabendazole is metabolized to an inactive compound, and so the potency may be lowered further, compared to albendazole (and fenbendazole), because of the need for a higher dosage for enough unmetabolized thiabendazole to reach tubulin inside the cells. Research on the toxicity of thiabendazole to rabbits and the potency and efficacy of thiabendazole against *E. cuniculi* infection in rabbits are needed to know if thiabendazole can be used as treatment against encephalitozoonosis.

The two methyl benzimidazole carbamates had more similar high potency, compared to that of thiabendazole. It would be interesting to have computational results of how thiabendazole would fit into putative binding sites and the calculated binding energies, similar to the research done by Aguayo-Ortiz et al. on methyl benzimidazole carbamates, to explain the seemingly difference in affinity. If the binding site was known, the effect of the binding of different benzimidazoles on tubulin could be examined. This could help understanding the different efficacies of the benzimidazoles, which is of course important when considering a drug.

4 Conclusion

Lack of the actual 3D structure of β-tubulin is one reason why the binding site of benzimidazoles is unknown. Different protein templates causes different putative orientations of the benzimidazoles in the same region. There may also be other binding sites of methyl 2-benzimidazole carbamates. The study from 2013 by Aguayo-Ortiz et al. gave the most insight of the binding of different methyl 2-benzimidazole carbamates to their model of β-tubulin, despite not discussing hydrophobic interactions. The added affinity of the sulfoxide metabolites, compared to the thioethers and ether, caused by strong hydrogen bonding, indicates fenbendazole and albendazole as good microtubule inhibitors in vivo. The orientation and affinity of thiabendazole or other thiazole substituted benzimidazoles to the putative binding sites are not mentioned by the different studies discussing the binding site of benzimidazoles to β-tubulin.

Fenbendazole is the most advocated drug for use against *Encephalitozoon cuniculi* in rabbits. The recommendation is highly due to the findings of Suter et al. in 2001. The information and studies available to date points to fenbendazole as the best drug to treat encephalitozoonosis in rabbits.

Albendazole is the most studied benzimidazole against *E. cuniculi.* There have been documented several incomplete responses and relapses in different hosts. The most important reason not to use the drug in rabbits is the toxicity.

The in vivo effect of oxibendazole against *E. cuniculi* and toxicity in rabbits are not well studied. In vitro, oxibendazole has high potency and high effect on young cultures, but low effect on older cultures. The problems of treating *E. cuniculi* infection often include that the infection is not completely stopped, i.e. a not high enough effect of the drug. Oxibendazole seems therefore not to be a solution to that problem.

The susceptibility of thiabendazole in rabbits is also poorly studied. Research on why thiabendazole may have lower affinity and higher efficacy in vitro compared to methyl 2-benzimidazole carbamates, could be useful in understanding the benzimidazole binding site and mechanism of action. The fact that the drugs showed an overall better effectiveness against the younger cultures in vitro, compared to the older, coincides with the fact that benzimidazole drugs have been shown to be effective at preventing *E. cuniculi* infection in vivo, but not so much at treating an established infection. The results of the inhibitory effect from the in vitro study by Franssen et al. allude to thiabendazole as a promising drug against *E. cuniculi* infection, if the pharmacokinetics and potency of the drug will allow it, which may not be the case.

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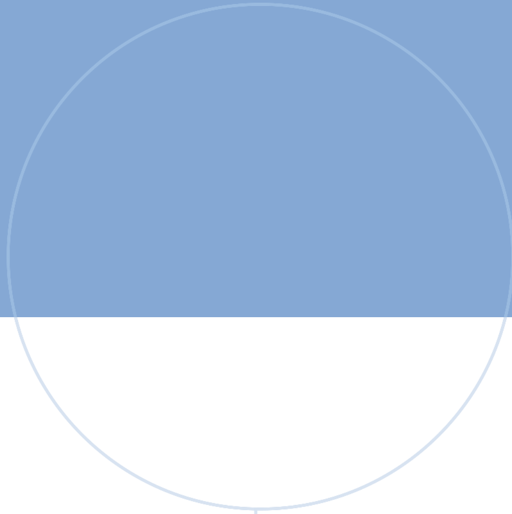
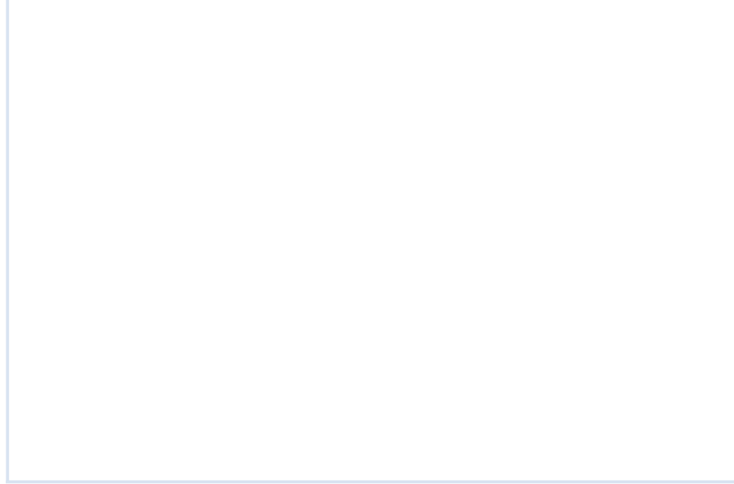

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