Characterisation of benzimidazole binding with recombinant tubulin from *Giardia duodenalis*, *Encephalitozoon intestinalis*, and *Cryptosporidium parvum*

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Abstract

The binding kinetics of several benzimidazole compounds were determined with recombinant tubulin from benzimidazole-sensitive and -insensitive organisms. This study utilised the naturally occurring high efficacy of the benzimidazoles for the parasitic protozoa *Giardia duodenalis* and *Encephalitozoon intestinalis*, and low efficacy with *Cryptosporidium parvum*. Direct kinetic analysis of the benzimidazole–β-tubulin interaction was performed using a fluorescence-based quenching method to determine the apparent association (k_on) and dissociation (k_off) rate constants from which the affinity constant (K_a) was calculated. The binding kinetics were determined with recombinant β- and γ-tubulin from the parasitic protozoa with several benzimidazole R₂-carbamate analogues. The affinity constant for the binding of several benzimidazoles with β-tubulin from benzimidazole-sensitive protozoa was found to be significantly greater than binding to β-tubulin from benzimidazole-insensitive protozoa. Additionally, the high affinity of several benzimidazole derivatives (albendazole, fenbendazole, mebendazole) for monomeric β- and heterodimeric γβ-tubulin from benzimidazole-sensitive protozoa was also clearly demonstrated. The affinity constants determined with β-tubulin from *G. duodenalis* and *E. intestinalis* also supported the observed in vitro efficacy of these compounds. The binding characteristics of the benzimidazoles with the highest in vitro efficacy (albendazole, fenbendazole, mebendazole) was reflected in their high association and slow dissociation rates with the β-tubulin monomer or dimer from benzimidazole-sensitive protozoa compared with insensitive ones. Benzimidazole-bound γβ-tubulin heterodimers also had a significantly lower rate of microtubule assembly compared with benzimidazole-free γβ-heterodimers. The incorporation of benzimidazole-bound γβ-heterodimers into assembling microtubules was shown to arrest polymerisation in vitro although the addition of benzimidazole compounds to assembled microtubules did not result in depolymerisation. These findings indicate that a benzimidazole-β-tubulin cap may be formed at the growing end of the microtubule and this cap prevents elongation of the microtubule.

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Keywords: Benzimidazoles; Tubulin; Equilibrium association constant; *Giardia duodenalis*; *Cryptosporidium parvum*; *Encephalitozoon intestinalis*; Microtubules

1. Introduction

The anti-protozoal activity of the benzimidazoles, which were initially developed as anthelmintics, has been observed in vitro against a number of parasitic protozoa including *Giardia duodenalis* [1–4] and *Encephalitozoon intestinalis* [5–7]. This activity has also been demonstrated in vivo for the treatment of giardiasis [8–9] and microsporidiosis [10–12]. *G. duodenalis* and *E. intestinalis* are both significant opportunistic parasites [13,14] which are also recognised as significant

Abbreviations: IC₅₀, 50% inhibitory concentration; K_a, equilibrium association constant/affinity constant; k_on, dissociation rate constant; k_off, association rate constant; MBS, MES buffered saline; Me₂SO, dimethyl sulphoxide
waterborne pathogens [14,15]. They cause different severities of gastrointestinal symptoms [14,16], which are generally self-limiting but can become life-threatening in immunocompromised individuals (e.g. transplantation, HIV positive) [17,18]. At present, the most effective therapy for both these parasites is the benzimidazole group of compounds, particularly albendazole [3,5,12,19]. Interestingly, several other enteric parasites have been found to be insensitive to the benzimidazole compounds, including Cryptosporidium parvum [20]. It remains unclear what the molecular basis of sensitivity to these compounds is in particular parasites.

The mechanism underlying the mode of action of the benzimidazoles has been most extensively studied with the parasitic nematode Haemonchus contortus [21–23]. These studies have resulted in the proposal that the benzimidazoles bind to the β-tubulin monomer prior to dimerisation with α-tubulin which blocks subsequent microtubule formation [24]. More specifically, it has been proposed that the benzimidazoles bind to a high-affinity binding site on the β-tubulin monomer [25]. One of the limiting factors in further characterising the binding kinetics of the benzimidazoles has been the lack of techniques to either extract homogenous tubulin from protozoan parasites or produce correctly folded and biologically active recombinant tubulin.

In order to address these problems and to further investigate the molecular basis of the mode of action of benzimidazoles in protozoa, we have utilised an E. coli-based protein expression system to produce biochemically active and assembly-competent α- and β-tubulin from G. duodenalis, E. intestinalis, and C. parvum [26]. The recombinant tubulin produced by this method had a purity of greater than 95% and was free of post-translational modifications (e.g. polyglutamylation). When assembled into microtubules in vitro they were free of accessory proteins (e.g. microtubule associated proteins), which made them ideal for benzimidazole-binding assays.

In this study, the binding affinity of several benzimidazoles has been determined for recombinant tubulin in both a monomeric and dimeric state. This analysis was performed using a fluorescence quenching method based on measuring the amount of intrinsic fluorescence from tryptophan residues. This fluorescence is blocked when drugs bind directly with or close to these amino acids. This technique has been used by several investigators to determine the binding kinetics of the anti-mitotic compounds colchicine [27–30] and taxol [31,32] although more recently it has been applied to Leishmania tubulin to assay dinitroaniline binding [33]. The kinetic analysis involved determining the apparent association rate (k_on) and dissociation rate (k_off) from which the association equilibrium constant (K_a) was calculated. The rate and equilibrium constants were determined for a number of benzimidazole compounds with recombinant α-, β-, and αβ-tubulin to investigate whether their differential efficacy in vitro relates to binding to monomeric or dimeric tubulin.

The benzimidazole compounds used in this study were R2-carbamate analogues with the same basic structure of 1,2-diaminobenzene (Fig. 1). These compounds were albendazole, albendazole-sulphoxide, fenbendazole, mebendazole, oxibendazole, and parbendazole. Thiabendazole was also studied as a non-R2-carbamate analogue (Table 1). All these benzimidazoles have previously been demonstrated to have a broad range of activity against G. duodenalis [1–4] and E. intestinalis [5–7], while being inactive against C. parvum [20], and were therefore predicted to display selectivity of benzimidazole binding with β-tubulin from these parasites.

2. Materials and methods

2.1. Expression of protozoan tubulin in E. coli

The expression of α- and β-tubulin genes from G. duodenalis, E. intestinalis, and C. parvum has been previously described [26]. The recombinant tubulin was maintained in MES buffered saline (MBS) buffer containing 0.1 M 2-[(N-morpholino)ethanesulphonic acid (MES) pH 6.5, 0.2 M NaCl, 0.05% NaN, with protease inhibitors 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μM leupeptin, and 0.5 μM pepstatin A. The protein samples were quantified using a Bio-Rad Protein assay [34] and analysed by SDS-PAGE and immunoblotting as previously described [26].

2.2. Dimerisation and polymerisation of recombinant tubulin monomers

The recombinant tubulin monomers (5 μM) were dimerised and polymerised in buffer containing 0.1 M MES.
Beecham Pharmaceuticals) were prepared in 100% Me$_2$SO. ibendazole, parbendazole, and thiabendazole (SmithKline
albendazole-sulphoxide, fenbendazole, mebendazole, ox-
2.3. Determination of benzimidazole binding kinetics
were tested in quadruplicate. The benzimidazoles were added at high molar concentrations (1 M) to microtubules
assembled as described above. Any change in turbidity ac-
added at high molar concentrations (1 M) to microtubules
zoles de-polymerise assembled microtubules, they were also
was tested in quadruplicate. To determine if the benzimida-
ments were made in triplicate and each drug concentration
were studied at 37°C-tubulin (2 μM/tubulin) was measured following incubation at 37°C.
The apparent association constant ($k_a$) was calculated as $F_{max} - F_1 = AL^{-1}e^{-kt}$
where $F_{max}$ is the maximum protein fluorescence, $F_1$ is the protein fluorescence at time $t$, $A$ is the amplitude of the bind-
ing phase, $k_{on}$ is the apparent association constant at time $t$. The apparent association constant ($k_{on}$) for each benzimidazole tested was calculated as $k_{on} = \frac{\alpha}{[benzimidazole]}$
where $\alpha$ is the slope of the semi-logarithmic binding plot and [benzimidazole] is the benzimidazole concentration. The equilibrium association constant/affinity constant ($K_a$) was obtained from the ratio of the association ($k_{on}$) and dissocia-
tion ($k_{off}$) rates.

2.3. Determination of benzimidazole binding kinetics
Stock solutions of the benzimidazoles, albendazole, albenzodazole-sulphoxide, fenbendazole, mebendazole, ox-
ibendazole, parbendazole, and thiabendazole (SmithKline Beecham Pharmaceuticals) were prepared in 100% Me$_2$SO. The amount of binding by the benzimidazoles to α-, β-, and αβ-tubulin (2 μM) from G. duodenalis, E. intestinalis, and C. parvum was measured following incubation at 37°C for 30 min. The kinetics of benzimidazole binding to tubu-
lin were studied at 37°C under pseudo-first-order conditions
where the benzimidazoles were present in a large excess over tubulin. Initial measurements were made at 5-, 10-, 15- and 20-fold molar excess of each benzimidazole to determine that a 10-fold benzimidazole excess was sufficient with 2 μM tubulin to obtain pseudo-first-order conditions. Specifically, to determine the association rates tubulin monomers or het-
erodimers (2 μM) in MBS buffer were incubated with dif-
fent benzimidazoles (0–20 μM) or 5% Me$_2$SO (negative control) at 37°C for 30 min in quadruplicate. Aliquot were removed at 5 min intervals over the 30 min period for fluores-
cence analysis. The rate of dissociation of each benzimidazole from β-tubulin was determined using benzimidazole-β-
tubulin complexes, which had attained a steady-state of bind-
ing as assessed by spectrofluorometry. The dissociation con-
stants were determined by measuring the time-dependent in-
crease in fluorescence from the tubulin–benzimidazole com-
plexes as the different benzimidazoles were released from
their binding sites following a 200-fold-dilution of the com-
plex in MBS buffer [27,29,30]. Dissociation curves were plot-
ted and analysed using non-linear regression to determine the
dissociation constant ($k_{off}$). This analysis also accounted for
the proportion of tubulin–benzimidazole complexes that did
not dissociate after 15 min.

All the fluorescence measurements were performed us-
ing a Perkin-Elmer luminescence spectrometer LS50 with an
excitation wavelength of 280 nm and emission wavelength
of 340 nm (initially tested over 300–400 nm range). The flu-
orescence measurements were corrected for inner-filter ef-
fects according to the method described by Mertens and Kagi
[35] as $F_{corr} = \frac{F_{obs}}{1 + \frac{320}{A_{320}}}$
where $F_{obs}$ and $F_{corr}$ are the observed and corrected fluo-
rescence values and $A_{320}$ are the absorbances at the
excitation and emission wavelength, respectively. The asso-
ciation kinetics were analysed by mono-exponential and bi-
exponential fits and based on the lowest $R^2$-value and spread
of residuals the best fit was mono-exponential. The associ-
ation and dissociation data was analysed using a non-linear curve fitting program (Prism, GraphPad Software) and a one-
site binding equation. The association rate ($k_{on}$) was calcu-
lated from the change in fluorescence ($F_{max} - F_1$) taking into
account the rate of change in fluorescence following the ad-
dition of the benzimidazoles and these results were graphed
semi-logarithmically against time.
Apparent association rates \( (k_{on}) \) which were calculated from the slope of these plots (Table 3). The benzimidazoles, albendazole, fenbendazole, and mebendazole, had the highest \( k_{on} \) values of \( 1.27 \times 10^9 \), \( 1.15 \times 10^9 \), and \( 1.09 \times 10^9 M^{-1} s^{-1} \), apparent association rates \( (k_{on}) \) which were calculated from the slope of these plots (Table 3). The benzimidazoles, albendazole, fenbendazole, and mebendazole, had the highest \( k_{on} \) values of \( 1.27 \times 10^9 \), \( 1.15 \times 10^9 \), and \( 1.09 \times 10^9 M^{-1} s^{-1} \),
respectively. Albendazole-sulphoxide, oxibendazole, and parbendazole produced $k_{on}$ values of 4.97 × 10^3, 4.28 × 10^3, and 3.59 × 10^3 s^-1, respectively. The lowest-affinity benzimidazole tested was thiabendazole which had an association rate of 4.23 × 10^2 M^-1 s^-1. These results indicate there was a significant difference in the association rates between the highest and lowest affinity benzimidazoles ($a = 0.05$, $t_{value} = 7.5 > t_{0.05}, 3, 2.353$). Similar results were obtained with recombinant E. intestinalis β-tubulin (Table 4).

The average association constants ($k_{on}$) for albendazole with α-, β-, and δ- tubulin from G. duodenalis, E. intestinalis, and C. parvum were also determined (Table 2). These results clearly illustrate the high affinity of albendazole for monomeric β-tubulin and heterodimeric αβ-tubulin from the two benzimidazole-sensitive parasites, G. duodenalis and E. intestinalis. The association constants for G. duodenalis and E. intestinalis β-tubulin or αβ-tubulin were 10^5 M^-1 s^-1 compared with 10^4 M^-1 s^-1 for the benzimidazole-insensitive C. parvum. These results also indicate that albendazole has a very low binding affinity (10^3 M^-1 s^-1) for α-tubulin from all three parasites.

The dissociation rate of the benzimidazole–tubulin complexes was determined by measuring the increase in intrinsic tubulin fluorescence following release of the benzimidazoles from the benzimidazole–tubulin complexes as a result of a 200-fold dilution of the complexes in MBS buffer. The dissociation rate was measured as an increase in relative fluorescence values as a result of a decrease in the quenching effect from the dissociating benzimidazole compounds which occurred in a benzimidazole-specific manner. This method has been used previously to determine the dissociation kinetics of colchicine analogues from colchicine–tubulin complexes [27,29,30]. The resulting dissociation curves (Fig. 4) were used to determine the dissociation rates ($k_{off}$) with β-tubulin from G. duodenalis (Table 3), E. intestinalis (Table 4), and C. parvum (Table 5). The rate of dissociation ($k_{off}$) was 17.6, 16.1, or 15.3 s^-1 (G. duodenalis) and 17.1, 16.5, or 17.6 s^-1 (E. intestinalis) for albendazole, fenbendazole, and mebendazole, respectively.

The benzimidazoles albendazole-sulphoxide, oxibendazole, and parbendazole had comparatively slower dissociation rates of 19.8, 21.3, or 21.0 s^-1 (G. duodenalis) and 23.7, 21.5, or 24.2 s^-1 (E. intestinalis). The lowest affinity benzimidazole, thiabendazole, had a dissociation rate of 32.5 s^-1 (G. duodenalis) and 23.7, 21.5, or 24.2 s^-1 (E. intestinalis), respectively. These results indicate that the rate of dissociation ($k_{off}$) for all the benzimidazole compounds was inversely proportional to the binding affinity ($k_{on}$) with the highest dissociation rate being observed with the lowest affinity benzimidazole, thiabendazole. Conversely, the slowest dissociating benzimidazoles were albendazole, fenbendazole, and mebendazole, which had the highest apparent association rates of the compounds tested.

3.2. Comparative binding affinities of benzimidazoles with protocortubulin

To investigate the relationship between the strength of binding to tubulin and the chemical nature of the benzimidazole compound, the affinity constant ($K_a$) was calculated by the ratio of the association ($k_{on}$) and dissociation ($k_{off}$) rates. The association constant for each benzimidazole compound tested with β-tubulin from G. duodenalis (Table 3), E. intestinalis (Table 4), and C. parvum (Table 5) were determined. Using this method, there was a clearly identifiable high affinity ($K_a > 10^4$ M^-1) of the benzimidazole compounds with β-tubulin from G. duodenalis and E. intestinalis, which was not observed with C. parvum. In considering the affinity constant of the benzimidazoles tested with β-tubulin from the sensitive organisms, G. duodenalis and E. intestinalis, there was no significant difference between the affinity rates for any of the benzimidazoles ($a = 0.05$, $t_{value} = 1.914 < t_{0.05, 3, 2.353}$). However, when the affinity constants of the benzimidazole-insensitive C. parvum was compared with those of G. duodenalis or E. intestinalis, all the benzimidazoles tested had a significantly lower affinity for C. parvum β-tubulin ($a = 0.05$, $t_{value} = 42.9 > t_{0.05, 3, 2.353}$).

The differential affinity of the benzimidazole derivatives was used to distinguish them as having either a high-, medium-, or low-affinity for β-tubulin, which was determined using a comparison of the binding kinetic and affinity constants for seven benzimidazoles with recombinant C. parvum β-tubulin (Table 5).

<table>
<thead>
<tr>
<th>Benzimidazole derivative</th>
<th>$k_{on}$ (M^-1 s^-1)</th>
<th>$k_{off}$ (s^-1)</th>
<th>$K_a$ (M^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albendazole</td>
<td>4.76 ± 1.13 × 10^2</td>
<td>173 ± 23.4</td>
<td>2.75 × 10^2</td>
</tr>
<tr>
<td>Fenbendazole</td>
<td>4.32 ± 1.03 × 10^2</td>
<td>159 ± 23.5</td>
<td>2.71 × 10^2</td>
</tr>
<tr>
<td>Mebendazole</td>
<td>3.88 ± 0.78 × 10^2</td>
<td>187 ± 23.6</td>
<td>2.07 × 10^2</td>
</tr>
<tr>
<td>Albendazole-sulphoxide</td>
<td>3.23 ± 0.45 × 10^2</td>
<td>132 ± 20.4</td>
<td>2.45 × 10^2</td>
</tr>
<tr>
<td>Oxibendazole</td>
<td>2.88 ± 0.57 × 10^2</td>
<td>167 ± 16.9</td>
<td>1.72 × 10^2</td>
</tr>
<tr>
<td>Parbendazole</td>
<td>2.53 ± 0.36 × 10^2</td>
<td>144 ± 18.2</td>
<td>1.75 × 10^2</td>
</tr>
<tr>
<td>Thiabendazole</td>
<td>2.85 ± 0.52 × 10^2</td>
<td>157 ± 17.3</td>
<td>1.82 × 10^2</td>
</tr>
</tbody>
</table>
observed between the two benzimidazole-sensitive organisms (Tables 3 and 4) and was not observed with C. parvum (Table 5). In this way, albendazole, fenbendazole, and mebendazole were regarded as high-affinity benzimidazoles with \( K_a \) values of \( 10^{3} \text{M}^{-1} \) with G. duodenalis and E. intestinalis. Lower affinities were observed with albendazole-sulphoxide, oxibendazole, and parbendazole (G. duodenalis and E. intestinalis \( K_a \) \( 10^{6} \text{M}^{-1} \)), which were termed medium-affinity benzimidazoles. Thiabendazole was regarded as a low-affinity benzimidazole compared with the six other compounds as it had a \( K_a \) value of \( 10^{7} \text{M}^{-1} \) (G. duodenalis) and \( 10^{8} \text{M}^{-1} \) (E. intestinalis). The trend in binding affinities was observed for monomeric \( \beta \)-tubulin from G. duodenalis and E. intestinalis but not C. parvum for which very low binding affinities were determined (\( K_a \) \( 10^{3} \text{M}^{-1} \)) for all the benzimidazoles tested.

3.3. Inhibition of microtubule assembly by benzimidazole compounds

The recombinant \( \alpha \) and \( \beta \)-tubulin monomers from all three parasites wereimerised and the \( \alpha \beta \)-tubulin heterodimers polymerised to form microtubules. The effect of the benzimidazoles on the amount of polymerisation or microtubule assembly was monitored spectrophotometrically over a 30-min period (Fig. 5). With G. duodenalis and E. intestinalis \( \alpha \beta \)-tubulin, the highest affinity benzimidazoles (albendazole, fenbendazole, mebendazole) had the greatest inhibitory effect on polymerisation compared with the lower affinity benzimidazoles (albendazole-sulphoxide, oxibendazole, and parbendazole). The lowest affinity benzimidazole (thiabendazole) had the least effect on polymerisation, particularly during the initial 10-min assembly phase.

The microtubule assembly results for G. duodenalis were not significantly different to those of E. intestinalis. Conversely, none of the benzimidazoles tested significantly reduced the rate of assembly of C. parvum microtubules (results not shown). With C. parvum \( \alpha \beta \)-tubulin all seven benzimidazoles tested reduced the final amount of microtubules assembled by 5–10% without affecting the rate at which maximum assembly occurred (i.e., slope of assembly phase, 3–10 min).

Significantly, when excess molar concentrations of albendazole, fenbendazole, mebendazole were added to microtubules assembled in the absence of benzimidazoles, there was no decrease in turbidity or absorbance indicating they did not de-polymerise the microtubules under these conditions.

4. Discussion

The mechanism underlying the activity of the benzimidazole compounds against parasitic protozoa was investigated in this study. The binding affinities of several benzimidazole R2-carbamate analogues were determined with monomeric \( \alpha \) and \( \beta \)-tubulin and heterodimeric \( \alpha \beta \)-tubulin from benzimidazole-sensitive (G. duodenalis, E. intestinalis) or benzimidazole-insensitive (C. parvum) organisms. This study identified two important aspects of the mechanism of action of the benzimidazole analogues in these parasites. Firstly, regardless of the chemical composition of the benzimidazole compound tested, they have an indistinguishable affinity for monomeric \( \beta \)-tubulin and dimeric \( \alpha \beta \)-tubulin of benzimidazole-sensitive organisms. Secondly, they have a demonstrably higher affinity for \( \beta \)-tubulin from benzimidazole-sensitive organisms when compared with a benzimidazole-insensitive organism.

Interestingly, the R2-derivatives tested in this study produced distinctive binding affinities with G. duodenalis and E. intestinalis recombinant \( \beta \)-tubulin. The affinity constant (\( K_a \)) divided the benzimidazoles tested into high-affinity (albendazole, fenbendazole, mebendazole), medium-affinity (albendazole-sulphoxide, oxibendazole, parbendazole), or low-affinity (thiabendazole) derivatives. The binding affinities determined in this study also reflected the in vitro efficacy of these compounds against G. duodenalis [1–4], E. intestinalis [5–7], and C. parvum [20]. The high-affinity (albendazole, fenbendazole, mebendazole) and medium-affinity (albendazole-sulphoxide, oxibendazole, parbendazole) benzimidazoles have also been reported to have the highest efficacy against G. duodenalis with IC50 values between 50
and 160 mg/ml [2,4] and E. intestinalis with IC₅₀ values between 1 and 10 ng/ml [6,7]. These binding constant results, therefore, suggest that at least some part of their efficacy is due to their differential binding affinity for β-tubulin which is related to the chemical group present in the R₂ side-chain of the benzimidazole derivative.

The relationship between the association (kₐ) and dissociation (kₐ) rates of these benzimidazole derivatives was demonstrated to be inversely proportional. This suggests that the lower in vitro efficacy of the rapidly dissociating benzimidazoles (albendazole-sulphoxide, oxibendazole, parbendazole, and thiabendazole) may be partly due to their inability to form stable benzimidazole-β-tubulin complexes with the converse being true of the higher efficacy benzimidazoles (albendazole, fenbendazole, mebendazole). This relationship is also reflected in the effect each benzimidazole was found to have on the polymerisation of αβ-heterodimers into microtubules. One of the proposed mechanisms of action of the benzimidazoles is through binding to the microtubule, which destabilises the lattice structure of the tubule, subsequently resulting in its disintegration via depolymerisation [36].

The findings of this study partially support this model although via a different mechanism. The addition of the different benzimidazoles to assembling αβ-tubulin heterodimers had a benzimidazole-dependent effect on both the rate and amount of microtubules assembled for the benzimidazole-sensitive parasites. That is, the highest affinity benzimidazoles (albendazole, fenbendazole, mebendazole) decreased both the rate and amount of microtubule assembly, which was observed to decrease with the medium- and low-affinity derivatives. The benzimidazoles were also observed to inhibit the assembly of G. duodenalis and E. intestinalis microtubules (albendazole, fenbendazole, mebendazole) while having no demonstrable effect on the polymerisation of C. parvum heterodimers. Similar findings have been reported with albendazole and recombinant αβ-tubulin from H. contortus [37] although studies with mebendazole have suggested a different mechanism of action in H. contortus [38] to the mechanism of action in the organisms utilised in this study.

The microtubule assembly assay also demonstrated that none of the benzimidazole compounds tested were able to depolymerise assembled microtubules although these tubules were naturally depolymerised by increasing the assembly temperature above 37°C. This indicates that whilst the microtubules were capable of depolymerisation this was not promoted or favoured by the presence of the benzimidazole derivatives tested. These results indicate that the benzimidazole-binding site on β-tubulin from sensitive protozoa is located at or near the inter-heterodimer interface as the presence of the benzimidazoles is sufficient to arrest or inhibit microtubule assembly. Taken together, this study of the in vitro binding kinetics provides a model of benzimidazole-β-tubulin binding which has not been clearly identified previously. This is particularly true when considering the molecular basis of benzimidazole-sensitivity and -insensitivity. The results of this study indicate that a benzimidazole-β-tubulin cap may be formed at the growing or assembling ends of the microtubules and this cap prevents elongation or polymerisation of the microtubule. Whether the inhibition of assembly is due to steric hindrance, blockage of GTP hydrolysis, or another mechanism is unclear from this study. The model proposed by this study is that the benzimidazole binding site on β-tubulin involves a region where the amino acid residues are different between benzimidazole-sensitive protozoa, such as G. duodenalis and E. intestinalis, and insensitive protozoa, such as C. parvum. These results also indicate that it is likely all the benzimidazoles tested interact with the same binding site on β-tubulin but the strength of this binding is partially dependent on the chemical composition of the R₂ group of the benzimidazole derivatives.

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