

Antifungal benzimidazoles disrupt vasculature by targeting one of nine β -tubulins

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1 **ABSTRACT**

2 Thiabendazole (TBZ) is an FDA-approved benzimidazole widely used for its antifungal and
3 antihelminthic properties. We showed previously that TBZ is also a potent vascular disrupting
4 agent and inhibits angiogenesis at the tissue level by dissociating vascular endothelial cells in
5 newly formed blood vessels. Here, we uncover TBZ's molecular target and mechanism of action.
6 Using human cell culture, molecular modeling, and humanized yeast, we find that TBZ selectively
7 targets only 1 of 9 human β -tubulin isotypes (TUBB8) to specifically disrupt endothelial cell
8 microtubules. By leveraging epidemiological pesticide resistance data and mining chemical
9 features of commercially used benzimidazoles, we discover that a broader class of benzimidazole
10 compounds, in extensive use for 50 years, also potently disrupt immature blood vessels and inhibit
11 angiogenesis. Thus, besides identifying the molecular mechanism of benzimidazole-mediated
12 vascular disruption, this study presents evidence relevant to the widespread use of these
13 compounds while offering potential new clinical applications.

14 INTRODUCTION

15 The vascular system is built by the combination of *de novo* formation of blood vessels by
16 vasculogenesis and the sprouting of new vessels from existing vessels via angiogenesis^{1,2}.
17 Imbalances in angiogenesis underlie a variety of physiological and pathological defects, including
18 ischemic, inflammatory, and immune disorders^{1,3,4}. Indeed, angiogenesis is central to tumor
19 malignancy and cancer progression, as new blood vessels must be established to supply oxygen
20 and nutrients to the growing tumor. Accordingly, inhibition of angiogenesis is now a well-
21 recognized therapeutic avenue¹⁻⁵. Defined angiogenesis inhibitors such as Avastin (FDA approved
22 since 2004) are now in wide use in the clinic and, over the past 30 years, several dozen drugs have
23 been approved or entered clinical trials as angiogenesis inhibitors⁵⁻⁹.

24 In recent years, a new class of anti-vascular drugs, termed vascular disrupting agents (VDAs), have
25 gained attention as potential alternative therapeutics operating by distinct mechanisms¹⁰⁻¹³. Unlike
26 angiogenesis inhibitors which selectively prevent the formation of new blood vessels, VDAs
27 function by dismantling existing vasculature, making them potentially effective for therapies
28 beyond cancer, for example in the treatment or control of macular degeneration and diabetic
29 retinopathies^{14,15}. While several VDAs have shown therapeutic potential, none have yet been
30 approved, with several candidates still in clinical trials^{11,16,17}.

31 Given the lengthy approval process, the failure of many drugs to succeed in clinical trials, and the
32 high costs involved with developing new compounds, drug repurposing offers an attractive
33 alternative for developing new therapies more quickly. We recently developed strategies to exploit
34 data from diverse model organisms to identify both deeply conserved genetic networks as well as
35 small molecules that may manipulate them¹⁸⁻²⁰. This effort identified thiabendazole (TBZ) as both
36 a novel angiogenesis inhibitor and VDA²⁰.

37 TBZ is one of a large class of biologically active benzimidazole compounds that are widely used
38 commercially or clinically, with applications ranging from photographic emulsions and circuit
39 board manufacturing, to serving as one of the most common heterocyclic ring systems used for
40 small molecule drugs²¹. The FDA approved TBZ in 1967 for human use for treating systemic
41 fungal and helminthic infections, but it is more widely used in veterinary settings and in
42 agricultural pesticides and preservatives. However, we found that TBZ also possesses potent

43 vascular disrupting ability, demonstrated *in vitro* in human cell culture and *in vivo* in mice and
44 frogs, including for retarding tumor growth and reducing intratumoral vessel density in preclinical
45 murine xenograft models²⁰.

46 Several other VDAs have been reported to collapse the vasculature by inhibiting microtubule
47 polymerization dynamics *via* binding β -tubulin^{11,16}. Indeed, though the basis for TBZ's vascular
48 disrupting action is unknown, it is proposed that TBZ's fungicidal action is mediated *via* disrupting
49 fungal microtubule assembly and dynamics^{22,23}. In particular, mutations in β -tubulin have been
50 frequently found to confer resistance to TBZ in parasitic/invasive fungal and nematode species²¹⁻
51 ³. However, in humans, TBZ does not generally disrupt cell growth, and even in human umbilical
52 vein endothelial (HUVEC) cells, while it somewhat reduced tubulin protein abundance it did not
53 elicit gross defects in the microtubule cytoskeleton²⁰. At angiogenesis-inhibiting doses, the overall
54 development of TBZ treated animals is normal¹⁸, consistent with TBZ's safety record in humans
55 and veterinary settings²⁴. Therefore, we hypothesized that only certain types of human cells, such
56 as subsets of endothelial cells involved in forming the vasculature, might be uniquely susceptible
57 to TBZ.

58 Here, we experimentally determined TBZ's specific molecular target and cellular mechanism of
59 vascular disrupting activity. We find that TBZ disrupts microtubule growth, with increased
60 potency in endothelial cells. Using predictive molecular modeling, human cell culture, and
61 humanized yeast, we find TBZ predominantly targets only one of nine human β -tubulins,
62 suggesting an explanation for its cell-type specificity. Finally, based on epidemiological data
63 mining and chemical structures, we discovered that a larger family of benzimidazoles—in clinical
64 and commercial use for >50 years—all act as VDAs, disrupting the vasculature in a vertebrate
65 animal model. These newly discovered VDAs include two World Health Organization (WHO)
66 antihelmintics (albendazole and mebendazole) administered for the treatment of human intestinal
67 infections, one broad-spectrum antifungal/antihelmintic (fenbendazole) used to treat farm animal
68 infections, and two banned pesticides (benomyl and carbendazim) used to prevent wild fungal and
69 nematode mediated crop destruction. Knowledge of their vascular disrupting activities should thus
70 inform their use in at-risk individuals (such as during pregnancy) and opens new clinical
71 applications for these compounds.

72

73 RESULTS

74 Thiabendazole disrupts microtubule plus ends in endothelial cells

75 Thiabendazole exhibits broad-spectrum activity against fungal and nematode crop pests²⁵, but
76 prior to demonstration of its VDA activity, it was generally thought to lack activity in tetrapods²⁴.
77 Its binding target and mechanism of vascular action remains poorly understood²⁶, although *in vitro*
78 studies have suggested various benzimidazole compounds inhibit cell growth by interfering with
79 microtubule polymerization^{23,26–28}. Benzimidazole suppressor screens in both *Saccharomyces*
80 *cerevisiae* and *Caenorhabditis elegans* have independently identified resistance mutations
81 occurring in β -tubulin genes, giving some insight into the binding site^{29,30}. The case for a β -tubulin
82 binding site is strengthened by numerous animal and agricultural studies also demonstrating
83 resistance mutations arising repeatedly and independently across multiple parasitic nematode and
84 fungal species infecting farm livestock and crops^{22,27,31–43}.

85 To examine the effects of the microtubule cytoskeleton and dynamics in the presence of TBZ, we
86 examined the localization of GFP-tagged EB1, which labels growing microtubule plus ends and
87 provides a proxy for microtubule dynamics in both endothelial (HUVEC) and non-endothelial
88 (NIH3T3) human cells. Despite the grossly normal architecture of microtubules in TBZ-treated
89 HUVECs, TBZ significantly reduced the accumulation of EB1 at microtubule plus ends (**Fig. 1A',**
90 **B'**) as compared to its control (**Fig. 1A, B**). Importantly, and consistent with the overall normal
91 morphology and patterning of TBZ-treated embryos²⁰, we found that TBZ had a substantially less
92 robust effect on EB1 accumulation at microtubule plus ends in fibroblasts as compared to
93 endothelial cells (**Fig. 1C-E**). These data are consistent with, and provide new insights into,
94 previous studies showing that TBZ's interaction with tubulin interferes with microtubule
95 polymerization in nematodes and fungi^{44,45}.

96 Thiabendazole selectively targets TUBB8 among human β -tubulins

97 Three commonly observed mutations in fungal and nematode β -tubulins (F200Y, E198A, and
98 F167Y) confer resistance to TBZ (**Fig. 2A, B**), suggesting that its binding site is in the vicinity of
99 these residues^{22,27,31–43} (**File S1**). Based on the previously observed benzimidazole suppressor

100 mutations, we used 3D structural modeling to evaluate TBZ's potential binding sites in a fungal
101 β -tubulin. We first constructed 3D homology models of the *Schizosaccharomyces pombe* (fission
102 yeast) wild-type and TBZ-resistant F200Y β -tubulins, based on the previously determined *Ovis*
103 *aries* β -tubulin crystal structures (PDB: 3UT5⁴⁶ and 3N2G⁴⁷) as templates. We computationally
104 refined the structures and then evaluated potential binding modes of TBZ, as detailed in the
105 Methods, using computational docking algorithms to localize TBZ's potential binding sites within
106 the fungal β -tubulin structures (**Fig. S1**). We identified a binding site around F200 to be the most
107 probable (**File S2**). We found that the preferred binding conformations of TBZ in both models
108 (**Fig. S1**) situated close to (but distinct from) the colchicine binding site. These observations were
109 in strong agreement with computational predictions made on parasitic β -tubulins binding
110 benzimidazoles²⁷ and recent crystal structures of other benzimidazole derivatives binding to
111 bovine brain β -tubulins⁴⁸.

112 On measuring the polar contacts and clashing energies of TBZ with tubulin, we found that the
113 wild-type β -tubulin bound to TBZ more favorably with contact energy (-9.9 kcal/mol) as compared
114 to its F200Y counterpart, which showed unfavorable repulsions (+27.6 kcal/mol)(**File S2, S3**). For
115 the wild-type protein, TBZ's polar contacts included E198 and Q134 (**Fig. 2C, S1**). Arene-
116 hydrogen interactions between the drug and protein included contributions from F200, L250, and
117 L253. However, for our F200Y mutant, repulsion was observed in our fixed ligand experiments
118 predominantly caused by unfavorable contacts made with Y200, F240, L250, and L253 (**Fig. S1**).
119 Our analyses suggest F200Y likely forms a hydrogen bond to E198 in the TBZ-resistant mutant,
120 thus constricting the pocket and occluding binding.

121 Unlike fungi, tetrapods have multiple β -tubulin isotypes (here, we use the term isotype to denote
122 the protein products of paralogous genes, in accordance with prior tubulin literature), and their
123 expression varies in different cells and tissue types. For example, human tubulin β I
124 (TUBB/TUBB5) is constitutively expressed in many cells and tissues, whereas β III (TUBB3) is
125 exclusively enriched in neurons and the brain^{49,50}. The specific roles of different β -tubulin isotypes
126 are not yet fully understood, but recent studies indicate that their sequence diversity modulates
127 binding affinity to tubulin-binding drugs and influences microtubule dynamics through distinct
128 interactions with molecular motors^{50,51}. The recurrence of TBZ resistance mutations at the same
129 three loci across diverse fungi and nematodes (**File S1**) led us to hypothesize that human β -tubulin

130 isotypes might have differential sensitivities to TBZ by virtue of incorporating resistant residues
131 at positions 167, 198, and 200, potentially explaining both its tissue-specific effects and generally
132 low toxicity in humans.

133 Indeed, multiple sequence alignment of human and yeast β -tubulin genes indicated that while F167
134 remained conserved across all the human isotypes, positions 198 and 200 were variable (**Fig. 2B**).
135 Moreover, all human β -tubulin isotypes except TUBB1 and TUBB8 contain the F200Y resistance
136 mutation. Because TUBB1 also harbors the other commonly observed E198A suppressor (**Fig.**
137 **2B**), TUBB8 is the only human β -tubulin isotype predicted by sequence to be TBZ-sensitive.
138 Given this variability across isotypes, we next asked how the E198A and F200Y mutations would
139 be expected to affect TBZ's ability to bind at its predicted site in human isotypes.

140 We first evaluated this hypothesis computationally, by constructing 3D homology models for each
141 of the human β -tubulin isotypes in the same manner as for the fungal model (see Methods). We
142 then performed induced-fit docking with TBZ across our human β -tubulin models. Using a TBZ-
143 wild-type fungal β -tubulin complex as a template, we docked TBZ into the same pocket in each of
144 the human isotypes and measured protein-ligand interactions in the superimposed structures. In
145 agreement with our primary sequence based predictions, TBZ fit well into the predicted binding
146 pocket of only TUBB1 and TUBB8, which lack the F200Y mutation. Both showed favorable
147 binding energies of -1.8 and -8.3 kcal/mol, respectively (**File S2**). The large difference in contact
148 energy among these isotypes could be explained by position 198. In TUBB1, alanine occupies
149 position 198, whereas TUBB8 has glutamate, which contributed heavily to the binding energy in
150 all of our simulations when both F200 and E198 were present. Our data suggest that TBZ binding
151 is stabilized by hydrogen bonds with residues Q134 and E198 in the presence of F200 (**Fig. S1,**
152 **S2**). Taken together, our *in silico* studies predicted that TBZ should strongly bind TUBB8 and
153 weakly bind TUBB1, but should not bind any other human β -tubulin isotypes.

154 **Functional assays in human endothelial cells and humanized yeast confirm TBZ specificity** 155 **to human TUBB8**

156 Given TBZ's effects on human vascular endothelial cells and *in vivo* vascular disruption in
157 *Xenopus* embryos²⁰, we wished to test directly if TUBB8-specific binding could explain the
158 compound's effects. We thus asked whether resistance to TBZ could be acquired by simply

159 supplying human β -tubulin isotypes predicted to be resistant. We tested this by two independent
160 assays: (i) by overexpressing specific sensitive or resistant human β -tubulin isotypes in human
161 endothelial cells and (ii) by humanizing Baker's yeast's β -tubulin *TUB2* to enable assays of
162 individual human β -tubulin isotypes.

163 To test if microtubule dynamics in human cells could be significantly restored by supplying
164 resistant β -tubulin isotypes, we singly transfected HUVEC cells with plasmids overexpressing
165 either TUBB4 or TUBB8 and assayed microtubule dynamics by measuring the comet lengths of
166 end-binding protein EB3 (**Fig. 3A**). Compared to untransfected HUVECs, we saw that
167 overexpressing TUBB4 significantly rescued the decrease in comet length observed in TBZ-
168 treated cells (**Fig. 3B**). Transfection with *TUBB8*, by contrast, had no effect (**Fig. 3B**). The
169 differences became very significant after 30 minutes of exposure (**Fig. 3B**).

170 As an independent assay of TBZ action on human tubulins, we turned to humanized yeast, as our
171 previous work showed that of the nine human β -tubulins, only TUBB4 and TUBB8 could
172 functionally replace *TUB2* in *Saccharomyces cerevisiae*⁵². From our modeling and docking data,
173 we hypothesized that yeast strains humanized with TUBB8 would be susceptible to TBZ while
174 humanizing with TUBB4 would confer TBZ resistance. *Saccharomyces cerevisiae* possesses 2 α -
175 tubulins (*TUB1* and *TUB3*) that interact with *TUB2* to form tubulin heterodimers, which in turn
176 oligomerize to form microtubules. Wild-type BY4741 haploid strains are TBZ-resistant. However,
177 previous studies have shown that on deleting *TUB3*, yeast strains become susceptible to
178 benzimidazoles⁵³ likely due to reduced overall α -tubulin stoichiometry or possibly by TBZ
179 occluding *TUB2*'s dimerization with *TUB1* but not *TUB3*. Therefore, we performed all our yeast
180 replacement assays in a *tub3 Δ* background, which yielded a clear growth defect in the presence of
181 TBZ (**Fig. S3**). In order to test the effect of TBZ on human β -tubulin isotypes TUBB4 and TUBB8,
182 we used CRISPR/Cas9 to construct yeast strains with these human isotypes in place of the
183 endogenous *TUB2* and tested them in the presence or the absence of the drug (**Fig. 3A**). We found
184 that strains possessing wild-type *TUB2* and human TUBB8 exhibited slow growth in the presence
185 of TBZ (at conc. 20 μ g/ml). By contrast, the strain humanized with TUBB4, which is predicted to
186 be resistant to TBZ, grew normally in the presence of TBZ (**Fig. 3C, S3A**).

187 Together with our *in silico* docking data, our results in HUVECs and humanized yeast indicate
188 that TUBB8 is uniquely TBZ-sensitive, suggesting in turn that vascular endothelial cells are
189 selectively sensitive to its loss.

190 **Benzimidazole resistance patterns and chemical similarities suggest additional VDAs**

191 Given the plethora of fungal and nematode studies on benzimidazole pesticide resistance in
192 agriculture^{22,33–36,38,40–44,54–71} (**Fig. 4A**), we reasoned that TBZ's molecular mechanism may extend
193 to other commercially used benzimidazole compounds. Indeed, based on our experiments, a simple
194 epidemiological signature should be sufficient to identify other pesticides that likely to function as
195 vascular disrupting agents and angiogenesis inhibitors: (i) the compounds should be selectively
196 toxic to fungal and nematode clades but demonstrate low toxicity in tetrapods, and (ii) sensitive
197 species should specifically gain benzimidazole resistance from F167Y, E198A or F200Y β -tubulin
198 mutations.

199 In order to understand how extensively distributed benzimidazole resistance was, we mined ~40
200 years of literature to identify reported cases of pesticide resistant species seen in wild and parasitic
201 nematodes and fungi. Benzimidazole resistance is a global phenomenon (**Fig. 4A**); across 9 major
202 commercial benzimidazole-based pesticides, we found multiple independent instances of reported
203 resistance across 27 (12 nematodes and 15 fungal) parasitic species (**File S1**), all of which
204 exhibited at least 1 of the 3 signature β -tubulin mutations. These widespread patterns of
205 benzimidazole pesticide resistance suggested at least 9 new candidate VDAs.

206 As a complement to the epidemiological data, we also considered chemical properties by asking if
207 pesticide benzimidazoles shared similar chemical feature profiles relative to other benzimidazoles.
208 We curated >80 commercially available compounds in the benzimidazole class spanning a diverse
209 range including pesticides, fungicides, therapeutics, and preservatives. Upon hierarchical
210 clustering of these benzimidazoles based on their chemical properties computed from JOELib's
211 features matrix^{72,73} (**File S5**), we found that pesticide benzimidazoles generally shared similar
212 chemical properties and clustered together (**Fig. 4B**).

213 **Numerous commercially used benzimidazoles also function as vascular disrupting agents**

214 We next tested if pesticides exhibiting the epidemiological signature and clustering in the same
215 clades by virtue of their chemical features would also specifically inhibit TUBB8 and function as
216 VDAs. We selected 12 commercially used benzimidazole compounds across 2 clusters (**Fig. 4B**).
217 Our list included 2 anthelmintics, both World Health Organization essential medicines
218 (albendazole and mebendazole) prescribed to treat broad-spectrum human intestinal nematode
219 infections; fenbendazole, an anthelmintic prescribed specifically for animals against
220 gastrointestinal nematode parasites; 2 currently banned pesticides, benomyl and carbendazim,
221 formerly used in agriculture; triclabendazole, specifically used to treat liver fluke infections; and
222 5 proton-pump inhibitors (esomeprazole, lansoprazole, omeprazole, pantoprazole, and
223 rabeprazole) used to treat gastrointestinal and stomach acid disorders. The latter set were from a
224 different clade and did not exhibit the epidemiological signature, serving as negative controls.

225 We first took advantage of our humanized yeast strains to rapidly discriminate TUBB8-specific
226 inhibition from general β -tubulin inhibition. We found that 5 of the 12 compounds tested
227 selectively inhibited TUBB8, as evidenced by the growth profiles observed for the humanized
228 strains when cultured in the presence of the drugs (**Fig. 5, S4**). Notably, none of the 5 proton pump
229 inhibitors or colchicine exhibited any tubulin inhibition (**Fig. S4, S5A**), confirming the specificity
230 of the epidemiological signature as a predictor of TUBB8 inhibition. In contrast, triclabendazole
231 was generally toxic, behaving as a pan-isotype inhibitor (**Fig. S5B**).

232 Testing the 5 positive TUBB8-inhibiting compounds in *Xenopus laevis* embryos showed strong
233 vascular disrupting activity for all 5 compounds (**Fig. 5**). As we observed previously for TBZ²⁰,
234 the gross morphology of the treated embryos was largely normal (**Fig. 5**). Thus, this broader class
235 of benzimidazoles do in fact generally act as vascular disrupting agents in vertebrates.

236

237 **DISCUSSION**

238 In the >30 years of therapeutic research efforts in the angiogenesis field only a highly restricted
239 set of drugs have yet been approved¹¹. Given the frequent failure to successfully make it through
240 clinical trials and the high costs and lengthy process associated with developing new compounds,
241 drug repurposing can offer efficient alternatives in developing new patient therapies with

242 accelerated timeframes. This study represents a rather unconventional path to drug repurposing,
243 leveraging a combination of model organisms, humanized yeast, cell culture, molecular modeling,
244 and epidemiological data mining to determine TBZ's molecular target and mechanism of vascular
245 action. Indeed, TBZ was initially identified as a VDA and angiogenesis inhibitor by using a
246 Baker's yeast model of angiogenesis discovered in a computational search for orthologous
247 phenotypes, or phenologs, aimed at exploiting deep evolutionary conservation to prioritize yeast
248 processes relevant to human diseases¹⁸⁻²⁰. Although obviously lacking blood vessels and a
249 circulatory system, yeast nonetheless retains conserved biological pathways and processes relevant
250 to vertebrate angiogenesis genes, and it was on the basis of these conserved processes that the
251 antifungal compound TBZ was initially suspected, later confirmed, to be an angiogenesis
252 inhibitor²⁰.

253 While TBZ somewhat reduced the abundance of tubulin proteins in human cells²⁰, at angiogenesis-
254 inhibiting doses, the overall morphology of TBZ treated animals was normal, suggesting that only
255 certain cell types, specifically those endothelial cells involved in forming the vasculature, might
256 be uniquely susceptible to TBZ. Here, we find that TBZ does indeed specifically modulate the
257 microtubules in vascular endothelial cells. Several currently identified microtubule targeting drugs
258 have been reported to interfere with polymerization dynamics by binding β -tubulin^{11,16} close to or
259 at the colchicine binding site. Building on previous work^{27,48}, our *in silico* modeling results suggest
260 that TBZ's binding site, while in close proximity to the colchicine binding site, is distinct from it,
261 thereby uncovering a novel β -tubulin effector site likely specific to other benzimidazoles and TBZ
262 analogs.

263 In contrast to β -tubulin anticancer drugs, which have largely shown pan-isotype activity, to our
264 knowledge, this study presents an unusual case of isotype-specific drug targeting in the β -tubulin
265 gene family. Fungal suppressor studies on benzimidazole resistance have repeatedly found
266 resistant mutations in β -tubulin; we found that 8 of 9 human β -tubulins natively harbor the same
267 suppressor mutations and consequently exhibit unfavourable steric clashes interfering with TBZ
268 binding. We demonstrate both *via* human cell culture microtubule assays and humanized yeast
269 drug sensitivity tests that TBZ selectively targets only TUBB8 among the nine human β -tubulins,
270 thus disrupting microtubule dynamics and reducing end-binding protein accumulation at the plus
271 ends of microtubules in susceptible cells.

272 With TUBB8 thus acting as the specific target, it follows that of all human cell types, vascular
273 endothelial cells must in turn be particularly sensitive to inhibition of TUBB8, leading to selective
274 disruption of the vasculature relative to other human tissues. It remains to be seen why TBZ's
275 vascular disrupting activity is restricted to immature or newly forming blood vessels, but we
276 speculate that this subset of the vasculature lacks reinforcing cell-cell contacts typical of larger,
277 more established vasculature, leading to greater sensitivity to TBZ-induced microtubule
278 disruption. As β -tubulin isotypes tend to be broadly expressed and often substitute for one another
279 in microtubule structures^{74,75}, one possibility is that TUBB8 inhibition simply leads to the loss of
280 interactions with endothelial cell-specific components, thus specifically impacting
281 vasculogenesis/angiogenesis. However, gene-gene and gene-drug interactions can often proceed
282 by less obviously direct mechanisms to selectively impact cell types or phenotype penetrance *via*
283 conditional cell-specific or dosage-dependent synthetic interactions^{76,77}. It would thus not be
284 surprising for the consequences of inhibiting TUBB8 in vascular endothelial cells to be similarly
285 indirectly mediated by endothelial cell-specific synthetic interactions. Further experiments
286 characterizing TBZ's selective activity against newly forming/formed vasculature and the
287 vascular-specific roles of TUBB8 in tetrapods could offer valuable insights into the cytoskeletal
288 dynamics underlying vasculogenesis and angiogenesis.

289 Based on chemical properties and signature resistance mutations observed against benzimidazole
290 compounds, we identified a larger class of extensively used fungicides and pesticides that all
291 exhibit vascular disruption activity. While our results suggest possible new clinical applications
292 for these compounds, they also highlight the potential caveats of their use in at-risk populations,
293 especially for the two compounds (albendazole and mebendazole) that are FDA approved for
294 human use. The WHO recommends the use of both albendazole and mebendazole as essential
295 antihelmenthics worldwide for children up to the age of 14 against soil-transmitted helminth
296 infections. Moreover, these compounds are widely used as public health interventions in pregnant
297 women after the first trimester in regions where hookworm and whipworm infections exceed
298 20%^{78,79}. While in the US, the risk of mebendazole use during pregnancy has not been assigned,
299 our data add weight to WHO recommendations that these drugs should not be administered in the
300 first trimester of pregnancy and suggest their use be carefully evaluated in patients in which
301 angiogenesis inhibition might pose risks, including using caution later in pregnancy in light of the
302 evidence that the compounds disrupt immature vasculature and might prove harmful to a

303 developing fetus. Conversely, while efforts in the angiogenesis field have been often motivated
304 towards developing anticancer therapies, the wide use of the compounds discussed here and their
305 FDA-approved status could open alternative paths to treating other angiogenesis and/or vascular
306 related diseases, such as diabetic retinopathy, macular degeneration, and hemangioma. It remains
307 to be seen if other benzimidazoles sharing similar chemical profiles to those tested in our work
308 (such as ciclobendazole, nocodazole, oxibendazole, and oxfendazole) also exhibit vascular
309 disrupting activity.

310 More broadly, our framework of leveraging phenotypic relationships between species and
311 repurposing model organisms to systematically explore drug mechanisms opens new routes for
312 drug repurposing and discovery, and highlights the power of systems biology and evolution-guided
313 approaches in advancing our knowledge of conserved genetic modules and how their disruption
314 manifests in disease. This work also illustrates how duplicated genes diversify their functions and
315 reinforces the therapeutic benefits of finding drugs specific to individual gene family members.
316 As evidenced by the high degree of replaceability of conserved genes from cross-species
317 complementation assays⁸⁰⁻⁸², we anticipate that the combination of humanized yeast and
318 phenolog-based disease modeling can be extended beyond vascular disruption to other conserved
319 processes and therapies targeting them.

320

321 **MATERIALS AND METHODS**

322 **Multiple sequence alignment**

323 Human gene sequences were downloaded from the Uniprot database. The multiple sequence
324 alignment for *S. pombe*, *S. cerevisiae*, and 9 human β -tubulin genes was constructed using MAFFT
325 v7⁸³ and visualized in Geneious v10 (<https://www.geneious.com>).

326 **Molecular modeling of β -tubulins**

327 Homology models of human and fungal β -tubulins were constructed using as a reference structure
328 the previously determined *Ovis aries* β -tubulin crystal structures (PDB: 3UT5 and 3N2G)^{46,47}. The
329 template was prepared using the Molecular Operating Environment (MOE.09.2014) software

330 package from Chemical Computing Group. The structure was inspected for anomalies and
331 protonated/charged with the Protonate3D subroutine (310K, pH 7.4, 0.1 M salt)⁸⁴. The protonated
332 structure was then lightly tethered to reduce significant deviation from the empirically determined
333 coordinates and minimized using the Amber10:EHT forcefield with R-field treatment of
334 electrostatics to an RMS gradient of 0.1 kcal mol⁻¹ Å⁻¹. Homology models of the wild-type fungal
335 β-tubulin were prepared by creating 25 main chain models with 25 sidechain samples at 298K (625
336 total) within MOE. Intermediates were refined to an RMS gradient of 1 kcal mol⁻¹ Å⁻¹, scored with
337 the GB/VI methodology, minimized again to an RMS gradient of 0.5 kcal mol⁻¹ Å⁻¹ and protonated.
338 The final model for each variant was further refined by placing the protein within a 6 Å water
339 sphere and minimizing the solvent enclosed structure to an RMS gradient of 0.001 kcal mol⁻¹ Å⁻¹.
340 Models were evaluated by calculating Phi-Psi angles and superimposed against the reference
341 structure. Homology models for each human β-tubulin were prepared similarly, based on
342 generating a total of 625 models and averaging to make a final model for each β-tubulin isotype.

343 ***In silico* docking of TBZ into β-tubulins**

344 Potential binding sites were evaluated using the Site Finder application and recent computational
345 work on benzimidazole binding to parasitic β-tubulins^{27,85}. Conformational variants of TBZ were
346 created in 3-D within MOE. A database of conformations was then used to dock TBZ to the wild-
347 type homology model using induced fit and template similarity protocols. The placement was
348 scored with Triangle Matcher and rescored with London dG. Poses were refined with the
349 Amber10:EHT forcefield with GVBI/WSA dG scoring. Candidate poses were then identified by
350 inspecting polar contacts. Geometry optimization was carried out with MOPAC 7.0 using AM1.
351 Conformational analysis of the bound structure was evaluated with LowModeMD⁸⁶. 2-D contact
352 maps were created using Ligand Interactions⁸⁷.

353 **Cell culture**

354 HUVEC cells were purchased from Clonetics and were used between passages 4 and 9. HUVECs
355 were cultured on 0.1% gelatin-coated (Sigma) plates in endothelial growth medium-2 (EGM-2;
356 Clonetics) in tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO₂. NIH-3T3 cells
357 were obtained from Vishy Iyer at the University of Texas at Austin and cultured in Dulbecco's
358 Modified Eagle's Medium (DMEM) with 10% bovine calf serum.

359 **Immunohistochemistry**

360 Cell lines were cultured in 6-well plates and treated with thiabendazole dissolved in 1% DMSO.
361 Control cells received 1% DMSO. After 24 h, cells were fixed with methanol at -20 °C for 10 min
362 and subsequently with 4% paraformaldehyde in PBS at room temperature for 10 min. Cell
363 membranes were permeabilized with 0.2% Triton X-100 in PBS, and nonspecific antibody binding
364 sites were blocked with 5% goat serum for 1 h at room temperature. Cells were incubated with
365 primary antibodies to EB1 (BD Bioscience) and α -tubulin (Sigma) at 4 °C overnight. After
366 washing with PBST, primary antibodies were detected by Alexa Fluor-488 or 555 goat anti-rabbit
367 or mouse immunoglobulin (IgG). 4',6-Diamidino-2-phenylindole (DAPI dye, Sigma) was added
368 as needed to visualize nuclei.

369 **Cell transfection and perfusion**

370 EB3-eGFP cDNA obtained from Anna Akhmanova was cloned into the vector CS2+⁸⁸. TUBB4
371 (Origene, RG203945) and TUBB8 (Origene, RG213889) cDNAs were purchased and cloned into
372 the vector CS107-RFP-3Stop. HUVEC cells were transfected by nucleofection (Lonza) according
373 to the manufacturer's instructions. To analyze the effect of TBZ in living cells, we used a closed
374 perfusion system (POC-R2, Pecon) connected to a peristaltic pump (Ismatec). 1% DMSO, 250 μ M
375 TBZ or 1% DMSO diluted in EBM-2 medium was flowed at 100 μ l/min rate for the indicated
376 times.

377 **Western blotting**

378 HUVECs were cultured in 6-well plates and treated with 1% DMSO or 1% DMSO, 250 μ M TBZ
379 for 24 hours. Cells were lysed in cell lysis buffer (Cell Signaling Technology) containing 1 mM
380 PMSF and analyzed by SDS-PAGE and western blotting using anti-EB1 (BD Bioscience) or anti-
381 EB3 (Millipore) or anti-Clip170 (Santa Cruz) antibodies.

382 **Imaging and image analysis**

383 Immunohistochemistry experiments, live HUVECs, and live *KDR:GFP* transgenic *Xenopus laevis*
384 were imaged using an inverted Zeiss LSM5 Pascal and Zeiss LSM700 confocal microscope, and
385 super-resolution structured illumination (SR-SIM) combined with Zeiss LSM710 microscope.

386 Comet lengths were measured using the software Fiji. Confocal images were cropped and
387 enhanced in Adobe Illustrator and Adobe Photoshop for the compilation of figures.

388 **Benzimidazole clustering analysis**

389 81 commercially used benzimidazole compounds spanning a wide range of classes were curated
390 from PubChem⁸⁹. JOELib (<http://joelib.sourceforge.net>), OpenBabel⁹⁰, and Chem Mine features
391 were computed using ChemMine tools⁷³. Heatmaps were visualized using Morpheus
392 (<https://software.broadinstitute.org/morpheus>). Clustergrams were generated by hierarchical
393 clustering on the one minus Pearson correlation coefficient with average linkage.

394 **Humanizing yeast β -tubulin using CRISPR-Cas9**

395 The human TUBB4 and TUBB8 open reading frames were integrated chromosomally (from start
396 to stop codon) into *Saccharomyces cerevisiae* in place of the endogenous *TUB2* open reading
397 frame using CRISPR/Cas9 genome editing as described in Akhmetov *et al.*⁹¹. Two sgRNAs were
398 designed targeting the yeast *TUB2* locus using the Geneious (v10.2.6) CRISPR-Cas9 tools suite,
399 purchased as oligos from IDT, and cloned into yeast CRISPR-K/O vectors using the yeast toolkit
400 (YTK)⁹² to express a synthetic guide RNA sequence, Cas9 nuclease, and a selectable marker
401 (URA3)^{52,93}. Repair templates were constructed by PCR amplification of the human β -tubulin ORF
402 (from the human ORFeome⁹⁴) flanked by 75 bp of target chromosomal boundary at the *TUB2*
403 locus to facilitate recombination *via* homology directed repair. BY4741 (S288C) yeast strains were
404 co-transformed with the CRISPR/Cas9 vector and repair template using Zymo Research Frozen-
405 EZ Yeast Transformation II Kit. Transformants were selected on SC-URA media. Surviving
406 colonies were screened by colony PCR, and Sanger sequenced to confirm replacement.

407 **Humanized yeast growth assays**

408 Assayed benzimidazole compounds were all dissolved in 100% DMSO to prepare stock solutions
409 of 5 or 10 mg/ml based on solubility. Candidate VDA compounds were titrated in ranges of 5-
410 1000 μ g/ml into growth medium depending on solubility (**Fig. S5** lists specific concentrations) for
411 subsequent growth assays. Liquid growth assays were performed in triplicate in 96-well format
412 using a Biotek Synergy HT incubating spectrophotometer. Humanized tubulin strains were pre-

413 cultured to saturation in YPD and diluted into 150 μ L of media to have $0.05-0.1 \times 10^7$ cells/ml.
414 Assays were typically run for 48 hrs with absorbance measured every 15 min.

415 ***Xenopus* embryo manipulations and VDA assays**

416 *Xenopus* embryos were reared in 1/3 \times Marc's modified Ringer's (MMR) solution. Each drug was
417 treated to embryos from stage 31 until stage 38 with 10 μ g/ml or 20 μ g/ml in 1% DMSO diluted
418 in 1/3X MMR. Embryos were fixed at stage 38 with MEMFA, and whole-mount *in situ*
419 hybridization for *erg* was performed as described in Sive *et al.*⁸⁷.

420

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430

431 **AUTHOR CONTRIBUTIONS**

432 Conceptualization and methodology, R.K.G., H.J.C., J.D.G., A.H.K., J.B.W., E.M.M.;
433 Computational analyses, R.K.G., H.J.C., J.D.G.; Investigation, R.K.G., H.J.C, C.L., J.D.G.;
434 Formal analysis and visualization, R.K.G., H.J.C., E.M.M.; Writing – R.K.G., H.J.C, J.B.W.,
435 E.M.M.

436

437 **COMPETING INTERESTS**

438 The authors declare no competing interest.

439 **FIGURE LEGENDS**

440 **Figure 1. Thiabendazole (TBZ) significantly reduces EB1 comet length at microtubule plus** 441 **ends in cultured human cells.**

442 Immunohistochemical analysis of α -tubulin in two human cell lines using confocal microscopy
443 does not show a definite distinction between 1% DMSO-treated control (**A, C**) and 1% DMSO,
444 250 μ M TBZ-treated cell lines (**B, D**), but images from super-resolution microscopy reveal that
445 the accumulation of end-binding (EB) protein 1 at the plus end of microtubules is significantly
446 reduced with TBZ treatment (**B**) compared to the control (**A**) in HUVECs. In NIH-3T3 cells, the
447 reduced EB1 comet length following TBZ treatment (**D**) compared to control (**C**) is not as
448 pronounced as in HUVECs, as quantified by comet length (**E**). Scale bars, 20 μ m in (A) and (C),
449 2 μ m in (A') and (C').

450 **Figure 2. Uncovering the molecular mechanism of thiabendazole.**

451 (**A**) TBZ elicits varying activity across different clades of life being toxic to fungal and nematode
452 clades but behaves as a vascular disrupting agent in tetrapods. (**B**) Of the 9 human β -tubulins, 8
453 have amino acids at positions 167, 198, and 200 that confer TBZ resistance to fungal tubulins (see
454 Table S1), as seen in a multiple sequence alignment of human and *Schizosaccharomyces pombe*
455 β -tubulins; only TUBB8 lacks resistance mutations. (**C**) *In silico* docking of TBZ (orange) into a
456 homology modeled yeast β -tubulin 3D structure (see Methods) indicates TBZ is well-
457 accommodated by a binding pocket in wild-type yeast *NDA3* that abuts the 3 major β -tubulin TBZ
458 resistance mutation sites. In contrast, docking of TBZ into homology models of human TUBB4
459 and TUBB8 indicates the potential for differential binding, with TUBB8 accommodating TBZ
460 whereas, in the case of TUBB4, TBZ is sterically blocked. Polar contacts are illustrated *via* dashed
461 lines, and residues lining the proposed binding pocket are shown in cyan. Intramolecular hydrogen
462 bonding between E198 and Y200 in TUBB4 reorganizes the geometry of the binding pocket.
463 Residues involved in steric clashing are depicted with a partial mesh surface. (Note that due to
464 steric clashes between TBZ and TUBB4 at the proposed binding pocket, TBZ was superimposed
465 from our binding model to measure interactions).

466 **Figure 3. TBZ specifically inhibits the human β -tubulin TUBB8, not TUBB4, in humanized**
467 **yeast and HUVEC cell culture.**

468 (A) Overview. TBZ's isotype specificity was identified in 2 ways. (Left) Recombinant human β -
469 tubulins TUBB4 and TUBB8 were individually overexpressed in HUVEC cell culture to monitor
470 comet lengths in the presence of TBZ. (Right) Using humanized yeast wherein yeast *TUB2* was
471 singly humanized by either of 2 replaceable human β -tubulins TUBB4 or TUBB8 to screen for
472 differential sensitivity towards TBZ. (B) Reduced EB3 comet length after 1% DMSO, 250 μ M
473 TBZ treatment compared to 1% DMSO treated control. A. Comet length is similar in EB3, TUBB8
474 transfected HUVECs compared to EB3 transfected controls expressing native tubulins, but comets
475 are longer in most EB3, TUBB4 transfected cells. B. Comet length is statistically similar between
476 cells treated with 1% DMSO; however, following 30 minutes of 1% DMSO, 250 μ M TBZ
477 treatment *TUBB4* transfected cells have significantly longer EB3 comets than HUVECs with
478 TUBB8 or expressing native tubulins. (C) Growth profiles of humanized yeast strains show TBZ's
479 isotype specificity to TUBB8. When grown in the presence of TBZ, Strains carrying the wild-type
480 *TUB2* (blue) and human TUBB8 (green) genes are sensitive to TBZ while humanized TUBB4
481 strains (orange) are resistant. Mean +/- standard deviation indicated by solid lines and shaded
482 boundaries, respectively.

483 **Figure 4. Global trends in benzimidazole resistance mutations and chemical structural**
484 **similarities suggest numerous potential vascular disrupting agents.**

485 (A) 3 β -tubulin mutations, (F167Y, E198A, F200Y) conferring benzimidazole resistance have
486 been globally observed among parasitic nematode and fungal species. Each icon represents an
487 instance of β -tubulin suppressor mutations occurring in benzimidazole resistant parasitic fungal or
488 nematode species (See **File S1** for list of species showing benzimidazole resistance). (B)
489 Commonly used benzimidazoles hierarchically clustered by their chemical properties suggest new
490 vascular disrupting agents with similar molecular mechanisms to TBZ. (Left) Clustergram of 81
491 widely used benzimidazole compounds spanning a wide range of drug classes grouped by chemical
492 features (See **File S5** for the full list of compounds and features analysed). (Right) Zooms of black
493 boxes indicate 3 clades containing TUBB8 specific VDA candidates (top) and proton-pump
494 inhibitors (bottom).

495 **Figure 5. Commercially used benzimidazole pesticides, antifungals, and antihelmintics are**
496 **also TUBB8-specific and disrupt vasculature.** *In situ* hybridization of blood vessels (using the
497 *erg/flk1* probe¹⁸) in *Xenopus laevis* embryos indicate the disruption of the vasculature caused by
498 the presence of human and animal antihelmintics and broad-spectrum pesticides as compared to
499 the DMSO control. Insets show growth profiles for yeast strains with humanized β -tubulin TUBB4
500 (orange) and TUBB8 (green) compared to wild-type (blue) when grown in the presence of each
501 compound.

502 **Supplementary Figures**

503 **Figure S1. Homology modeling and *in silico* docking studies predict the TBZ binding site in**
504 **the fungal β -tubulin *ND43* structure.** TBZ is well accommodated in wild-type *ND43*'s predicted
505 binding pocket (**A**) as opposed to its F200Y mutant (**B**). 3D structures and 2D contact maps shown
506 on the left and right respectively indicate the steric clashes TBZ faces in the F200Y binding pocket.
507 Cyan meshes (in 3D structures) and red highlights on the ligand (in 2D contact maps) indicate
508 steric clashes in the binding pocket

509 **Figure S2. Only TUBB8 favorably binds TBZ among the 9 human β -tubulins.**

510 (**A**) 2D contact maps highlight ligand interactions between TBZ and TUBB4 (left) or TUBB8
511 (right). TBZ forms polar contacts with residues Q134, E198, F200, and L253 in TUBB8. In
512 TUBB4, reorientation of the proposed binding pocket is observed. Substantial steric clashing is
513 shown in red on TBZ. (**B**) *In silico* docking of TBZ into other human β -tubulin homology models
514 suggests substantial steric clashes due to unfavorable binding pockets among 8/9 human β -tubulin
515 isotypes. Cyan meshes (in 3D structures) and red highlights on the ligand (in 2D contact maps)
516 indicate steric clashes in the binding pocket

517 **Figure S3. Yeast strains with modified β -tubulin are differentially sensitive to TBZ.**

518 (**A**) Deletion of yeast α -tubulin *TUB3* makes *Saccharomyces cerevisiae* (Baker's yeast) sensitive
519 to TBZ. (**B**) Growth profiles of *tub3 Δ* yeast strains with wild-type *TUB2* (blue), humanized β -
520 tubulins TUBB4 (orange), or TUBB8 (green) in increasing concentrations of TBZ show
521 differential sensitivities to the drug.

522 **Figure S4. Growth profiles of benzimidazole treated yeast strains.**

523 Plots depict varying doses of albendazole, benomyl, carbendazim, and colchicine. Our data show
524 that colchicine is a pan-isotype inhibitor whereas albendazole, benomyl, and carbendazim show
525 some degree of specificity for TUBB8.

526 **Figure S5. Proton-pump inhibitors do not elicit growth defects in humanized strains.**

527 (A) Yeast strains harboring the beta-tubulin gene *TUB2* (blue), TUBB4 (orange), and TUBB8
528 (green) are not inhibited by proton pump inhibitors (drug conc. 40 µg/ml). (B) Triclabendazole is
529 a pan-isotype β-tubulin inhibitor inhibiting both wild-type and humanized yeast strains (Left) and
530 lethal to developing *Xenopus laevis* embryos at stage 38. (Right).

531

532 **Supplementary information**

533 **File S1.** Species resistance table curated from literature

534 **File S2.** Yeast site finder statistics

535 **File S3.** Docking free energy scores across β-tubulin isotypes

536 **File S4.** Yeast wt β-tubulin induced fit TBZ docking scores

537 **File S5.** Benzimidazole chemical features

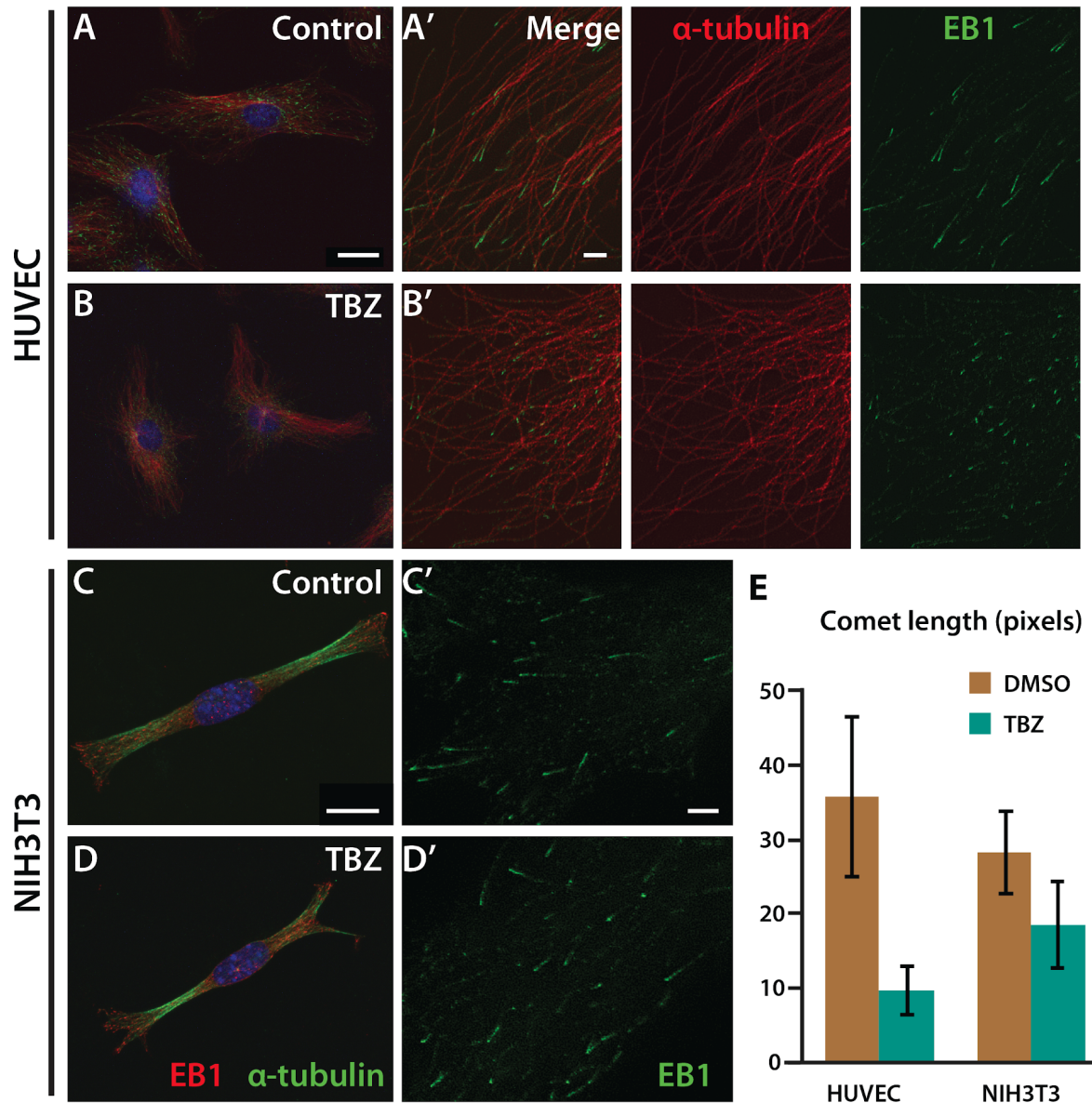


Figure 1. TBZ significantly reduces EB1 comet length at microtubule plus ends in cultured human vascular endothelial cells.

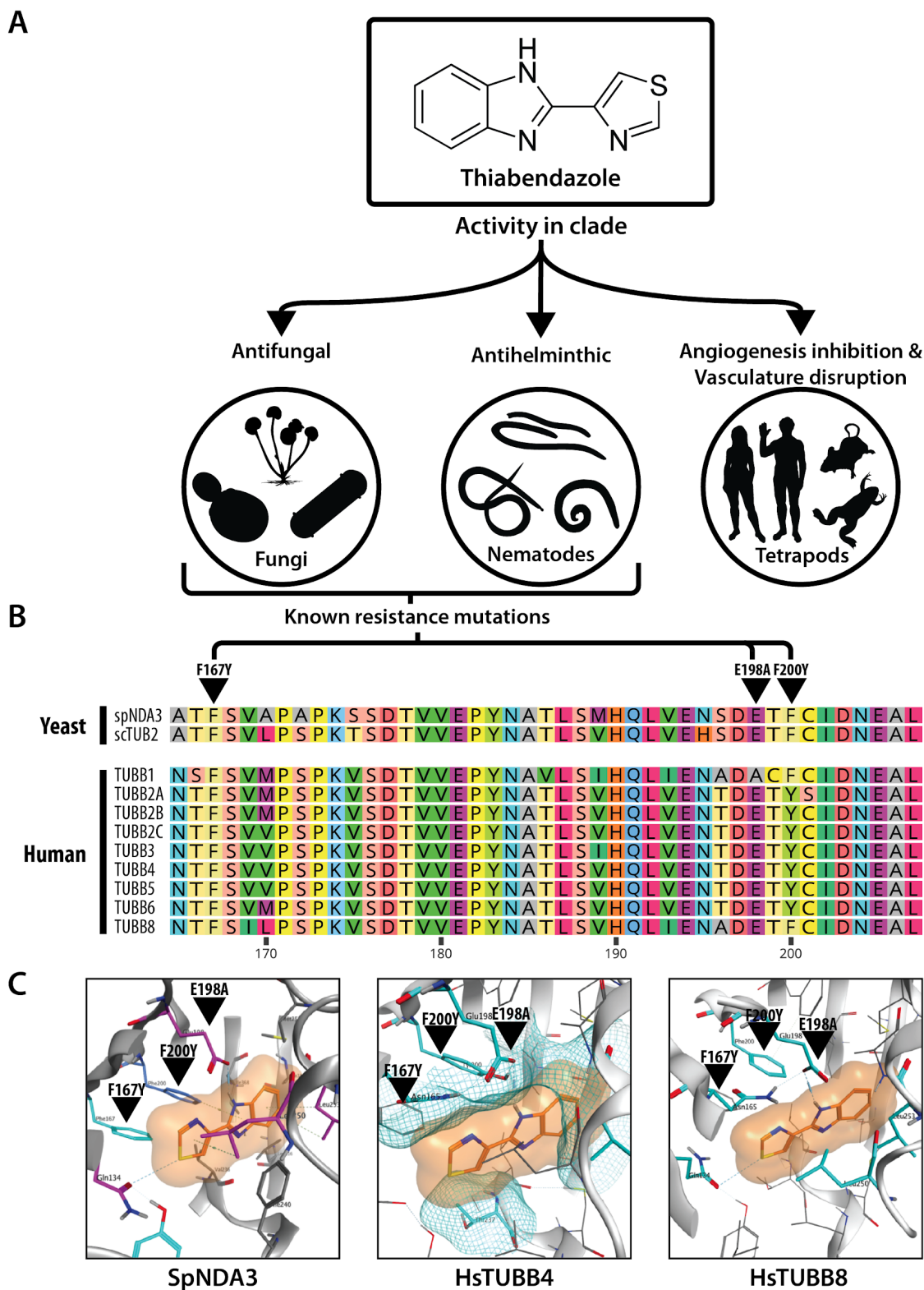


Figure 2. Uncovering the molecular mechanism of thiabendazole.

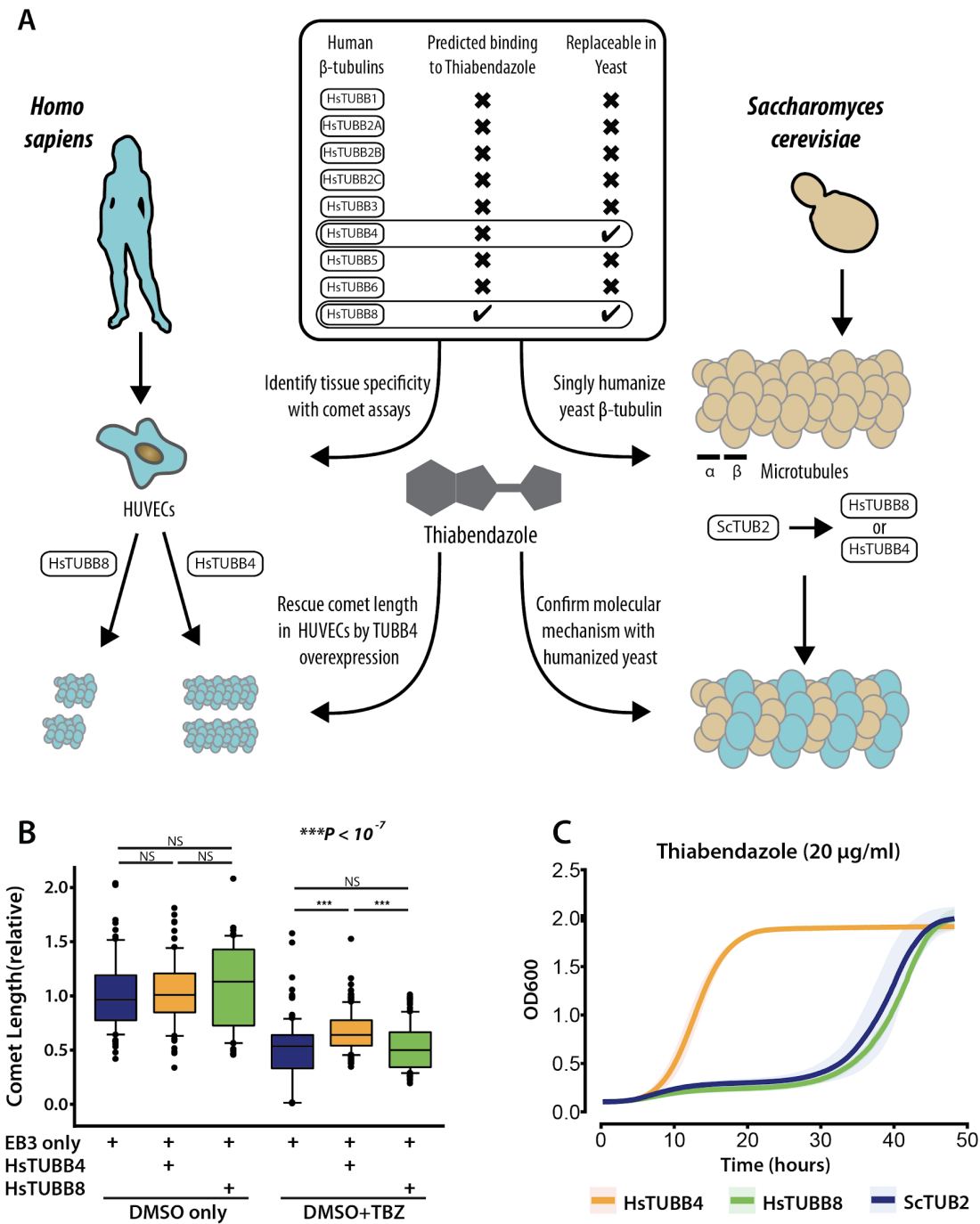


Figure 3. TBZ specifically inhibits the human β -tubulin TUBB8, not TUBB4, in HUVEC cell culture and humanized yeast .

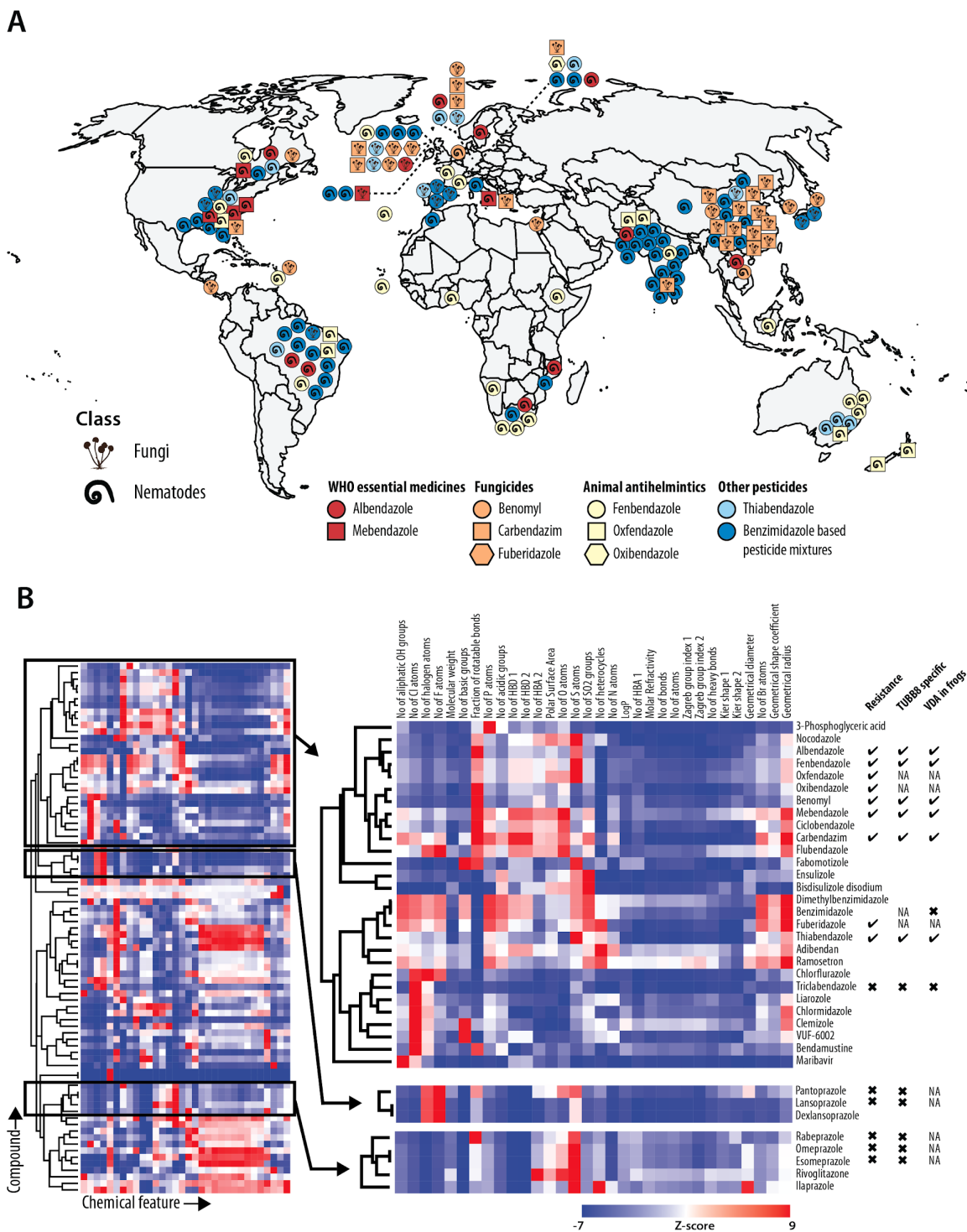


Figure 4. Global trends in benzimidazole resistance mutations and chemical structural similarities suggest numerous potential vascular disrupting agents.

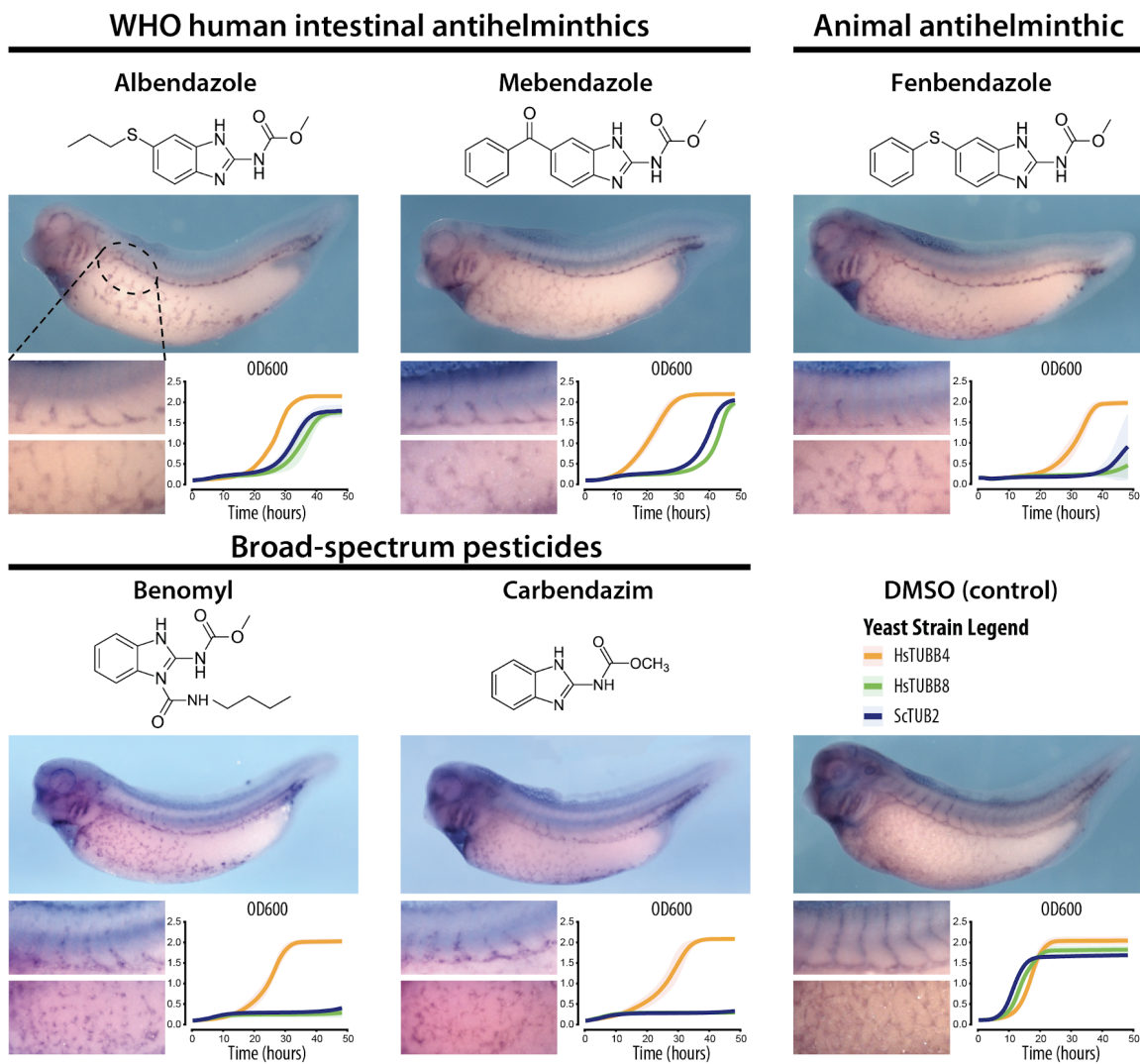


Figure 5. Commercially used benzimidazole pesticides, antifungals, and antihelminthics are also TUBB8-specific and disrupt vasculature.

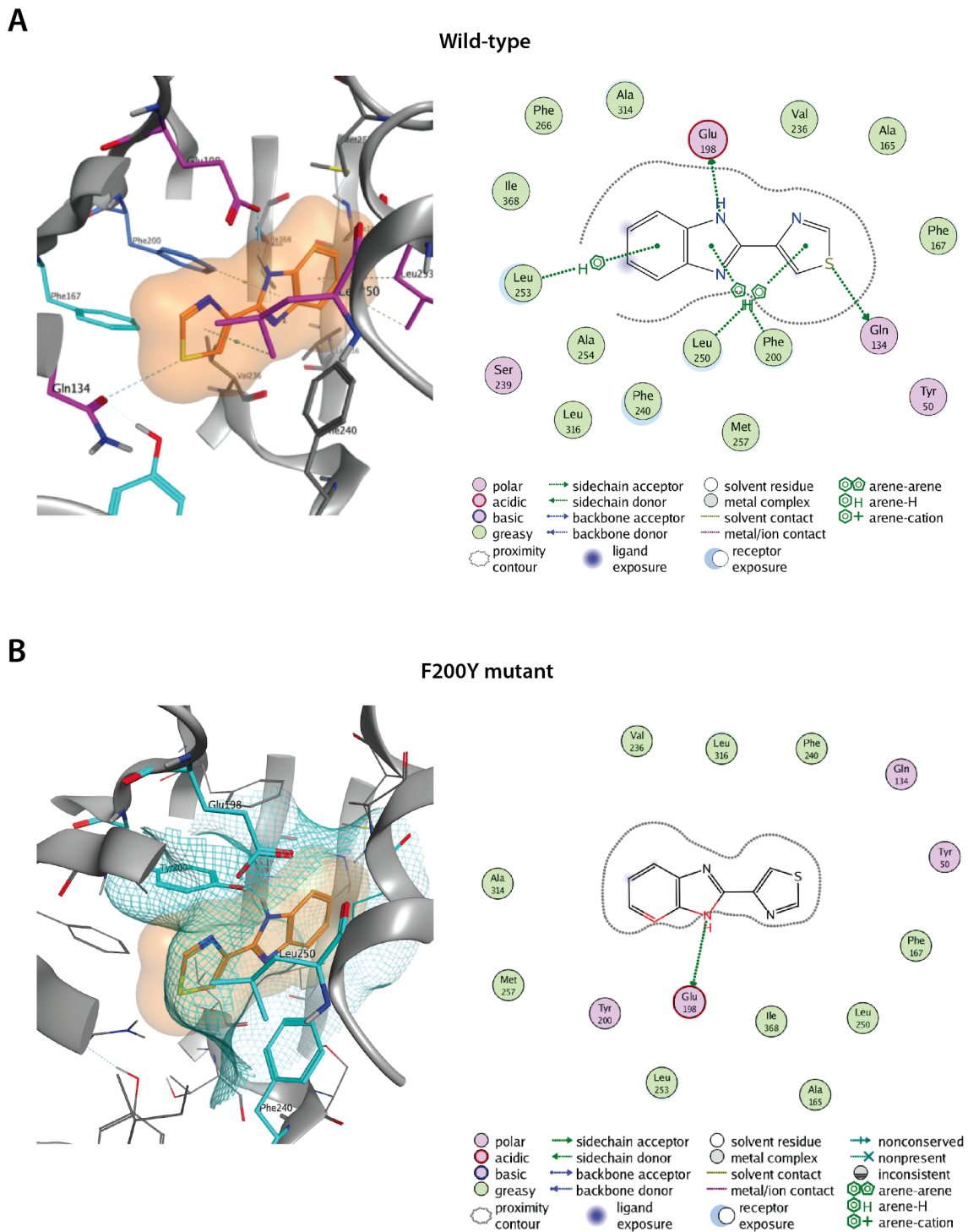
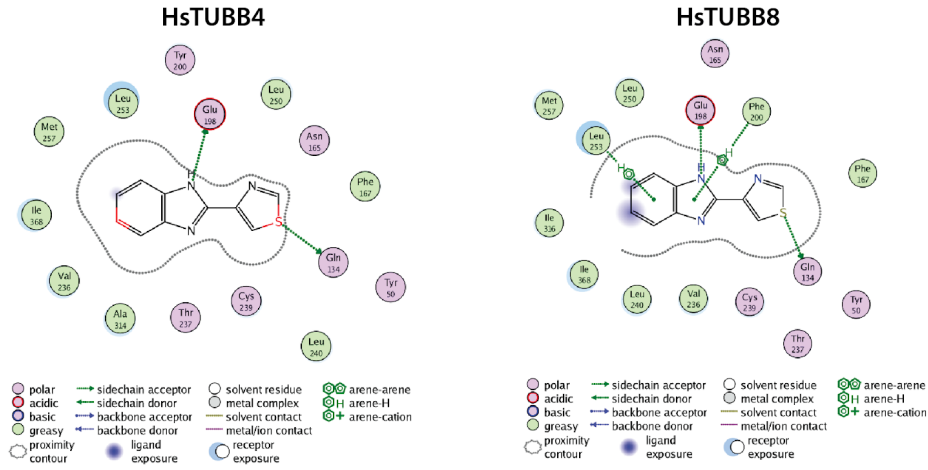


Figure S1. Homology modeling and *in silico* docking studies predict the TBZ binding site in the fungal β -tubulin *NDA3* structure.

A



B

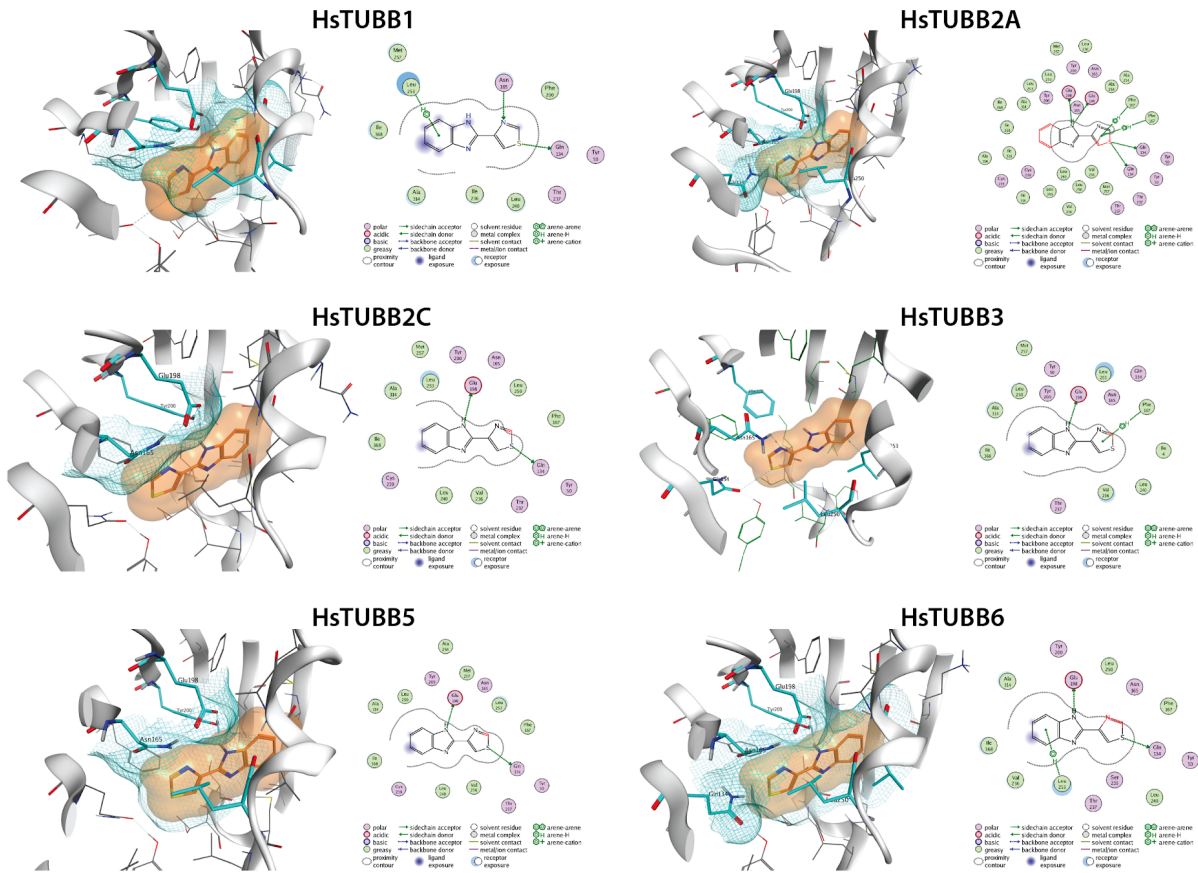
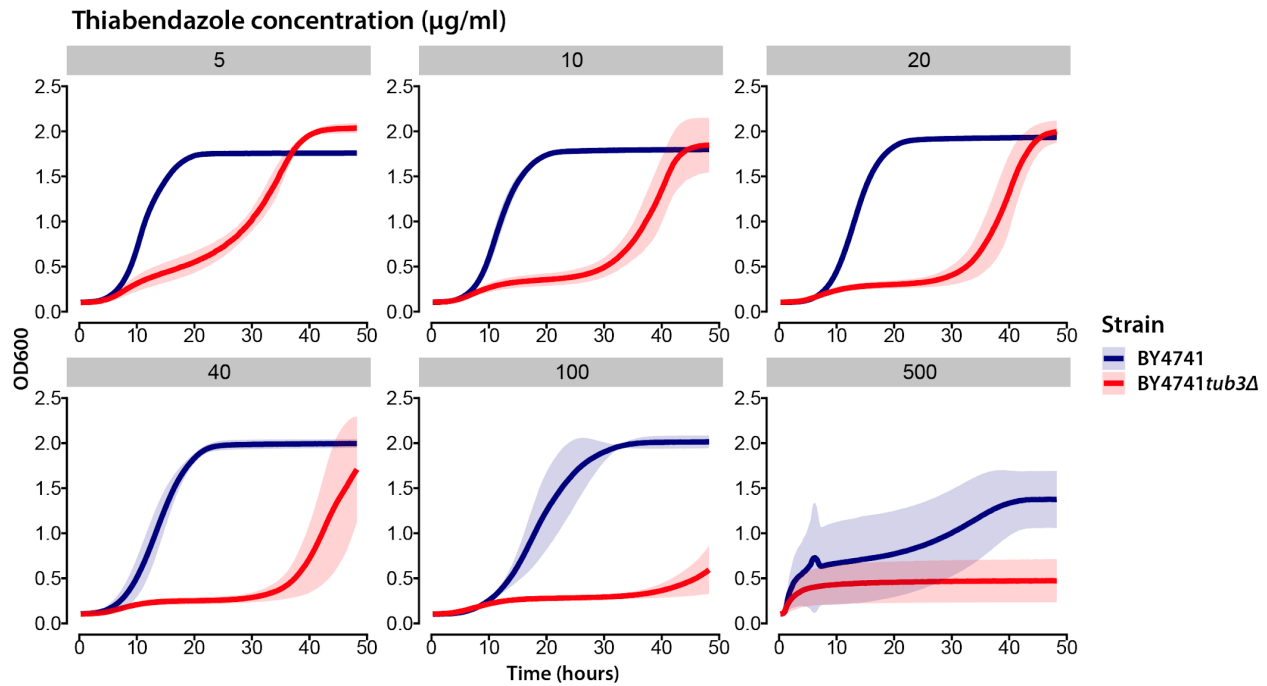


Figure S2. Only TUBB8 favorably binds TBZ among the 9 human β -tubulins.

A



B

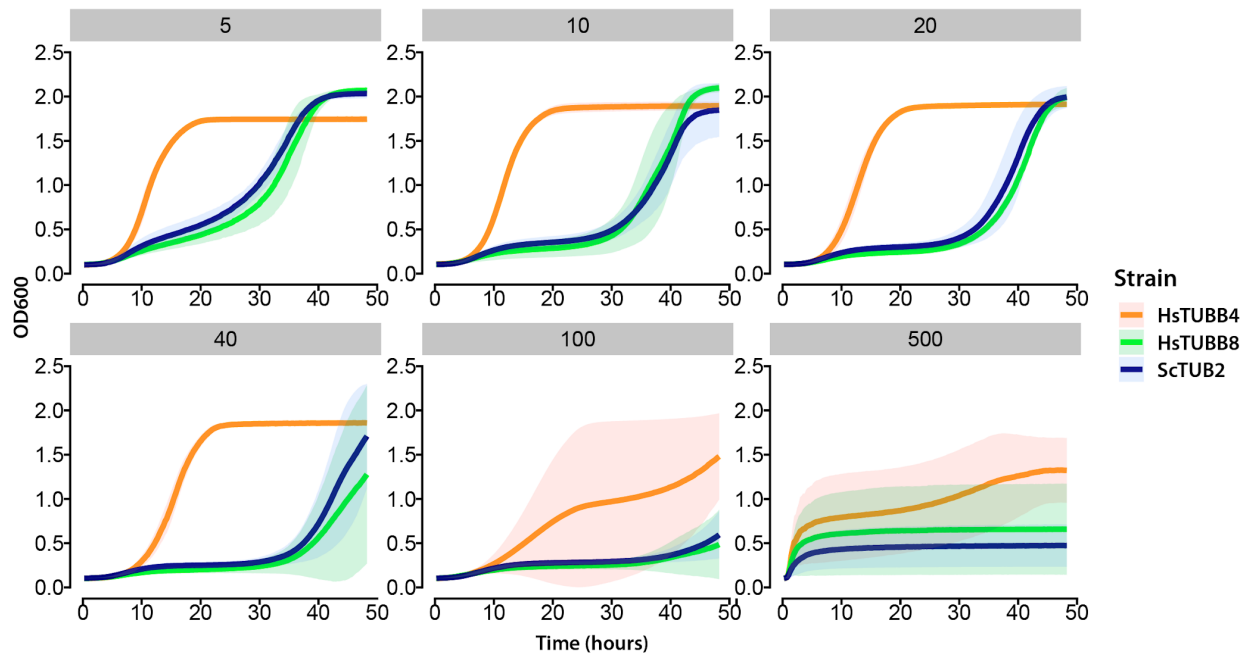


Figure S3. Yeast strains with modified β -tubulin are differentially sensitive to TBZ.

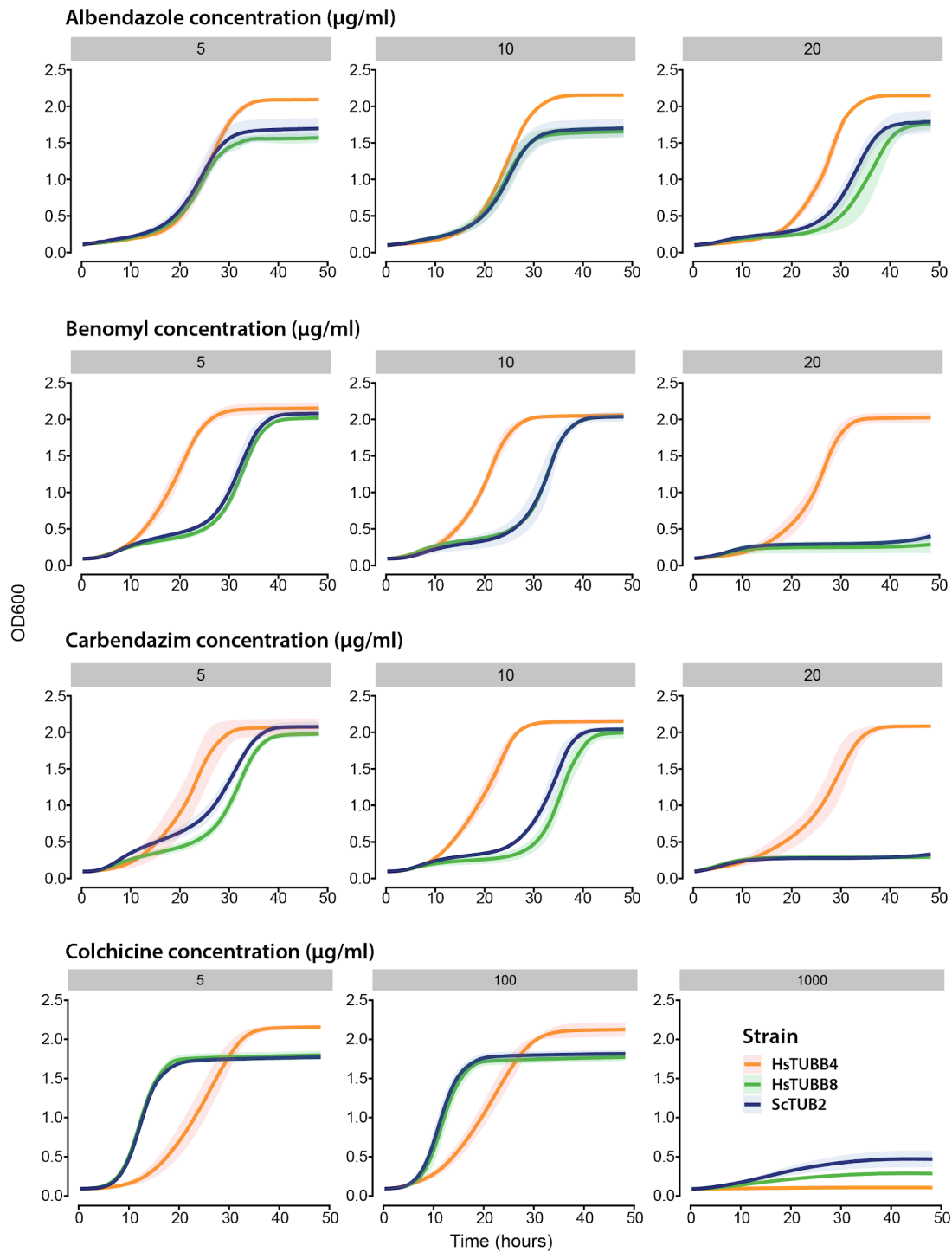
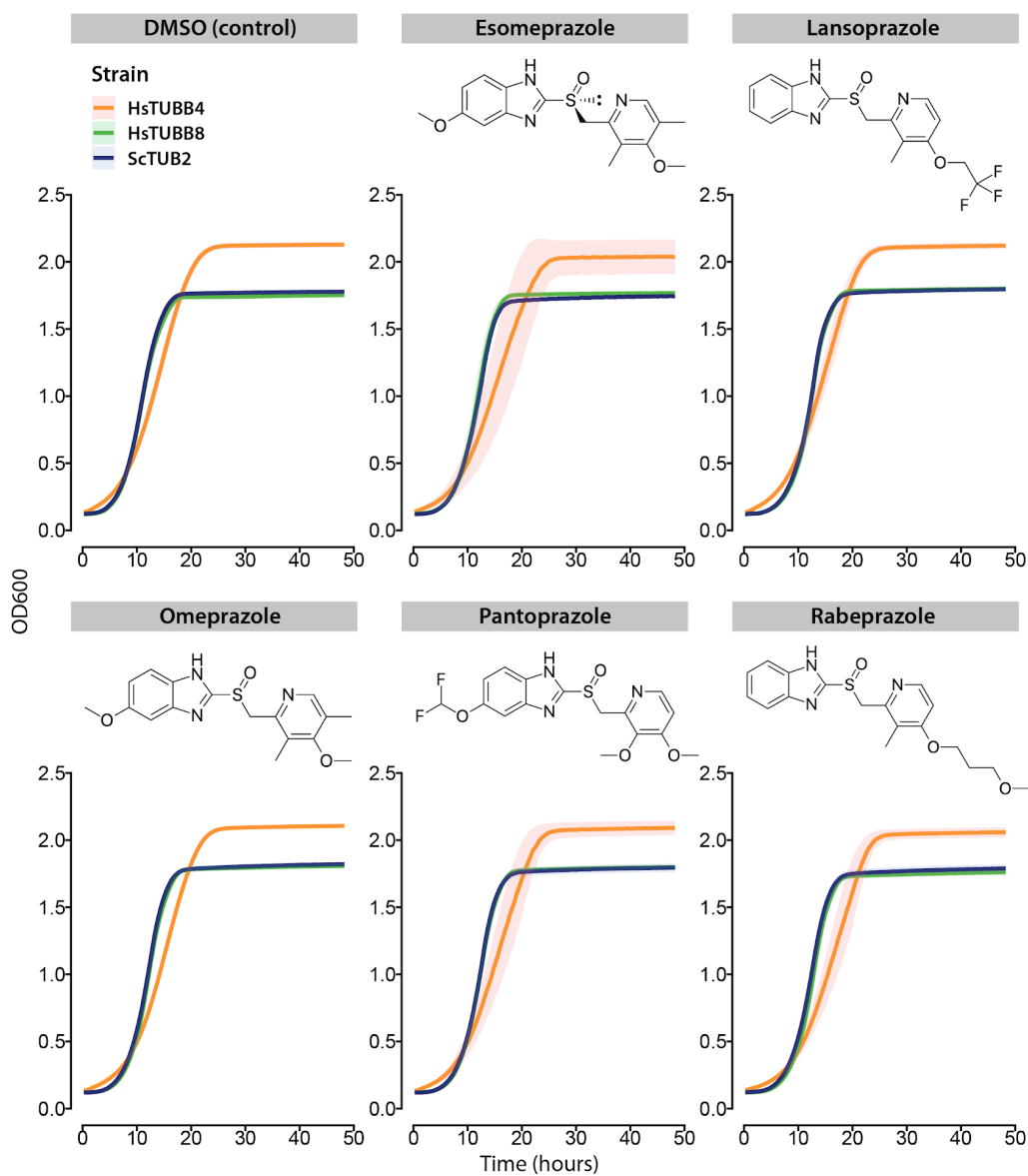


Figure S4. Growth profiles of benzimidazole treated yeast strains.

A



B



Figure S5. Proton-pump inhibitors do not elicit growth defects in humanized strains.

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