1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

Conformational flexibility Of A Highly Conserved Helix Controls Cryptic Pocket Formation In FtsZ

Aisha Alnami^{1,2}, Raymond S. Norton^{3,4}, Helena Perez Pena¹, Shozeb Haider¹, Frank
Kozielski^{1*}

¹ Department of Pharmaceutical and Biological Chemistry, School of Pharmacy,
University College London, 29-39 Brunswick Square, London WC1N 1AX, UK,

² Department of Pharmaceutical Chemistry, Faculty of Pharmacy, King Abdulaziz
University, 21589, Jeddah, Saudi Arabia,

³ Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, Monash
University, Parkville, Victoria 3052, Australia,

⁴ ARC Centre for Fragment-Based Design, Monash University, Parkville, Victoria
3052, Australia.

20 *Mycobacterium tuberculosis* is responsible for more than 1.6 million deaths per
 21 year. Overcoming failure from established therapies owing to multidrug
 22 resistance requires the identification of novel targets. One potential antibacterial
 23 target is filamentous temperature sensitive protein Z (FtsZ), which is the
 24 bacterial homologue of mammalian tubulin, a validated cancer target. *M.*
 25 *tuberculosis* FtsZ function is essential, with its inhibition leading to arrest of cell
 26 division, elongation of the bacterial cell and eventual cell death. However, the
 27 development of potent inhibitors against FtsZ has been a challenge due to the
 28 lack of structural information. Here we have solved multiple crystal structures of
 29 *M. tuberculosis* FtsZ in complex with coumarin analogues. Coumarins bind
 30 exclusively to two novel cryptic pockets in nucleotide-free FtsZ but not to the
 31 binary FtsZ-GTP or GDP complexes. Our findings provide a detailed
 32 understanding of the molecular basis for cryptic pocket formation, controlled by
 33 the conformational flexibility of the H7 helix, and thus reveal an important
 34 structural and mechanistic rationale for coumarin's antibacterial activity.

35
 36
 37

38 Tuberculosis (TB) is one of the leading causes of morbidity and mortality caused by
39 an infectious agent. The World Health Organization estimates that the latent form of
40 TB has infected nearly one-quarter of the world's population. Ten million people have
41 developed the disease (equivalent to 142 cases per 100,000 population), and about 1.3
42 million died in 2017. Additionally, 558,000 reported cases were classified as
43 multidrug resistant tuberculosis (MDR-TB) patients, and 8.5% of these patients
44 developed extensively drug-resistant tuberculosis (XDR-TB)[1].

45 After a 40-year gap, the US Food and Drug Administration (FDA) approved
46 two new anti-TB agents, Bedaquiline in 2012 [2] and Delamanid in 2014 [3], to be
47 used as adjuncts to current anti-TB agents in treating MDR-TB[4]. However,
48 multidrug-resistant TB infections remain a substantial threat to public health
49 worldwide, creating a pressing need to explore novel anti-TB agents with new
50 mechanisms of action.

51 FtsZ, the bacterial homologue of mammalian tubulin, is highly conserved
52 among all types of bacteria, including Mycobacterium strains [5]. FtsZ is an essential
53 cell division protein, with both GTPase and polymerisation activities. In a GTP-
54 regulated manner, FtsZ subunits polymerise at the centre of the cell into
55 protofilaments to form a dynamic ring-like structure called the Z-ring [6]. The Z-ring
56 functions as a scaffold for the assembly of the divisome, a multiprotein complex that
57 causes contraction of the Z-ring, finally resulting in septum formation and cell
58 division [7, 8]. Abnormalities in polymerization and/or GTPase activity result in
59 inhibition of the Z-ring and septum formation, leading to inhibition of cell division
60 and cell death.

61 In the effort to develop inhibitors of bacterial cell division that target FtsZ,
62 several groups have identified inhibitors based on natural products, synthetic small
63 molecules, and nucleotides. However, many of these hits have been described as
64 “false positives” or possessing “irreproducible activity” [9, 10], and progress of the
65 majority of these projects has been hampered by the lack of available structural data
66 such as crystallographic FtsZ-inhibitor complexes for structure-based drug design. It
67 has been proposed that it may be an “intrinsic challenge” to crystallise FtsZ in
68 complex with inhibitors [11]. Hitherto, only a single binding site for PC190723 and
69 related compounds [12] has been identified in *Staphylococcus aureus* FtsZ, but not in
70 any other bacterial species [13].

71 In this study we successfully identified two novel inhibitor-binding pockets in
72 FtsZ from *M. tuberculosis* in crystal structures with a resolution of 1.7 Å. We show
73 that these pockets are cryptic in nature since they appear in nucleotide-free FtsZ but
74 are absent in the nucleotide-bound form. Furthermore, their formation and dissolution
75 depend on the structural dynamics of H7, a highly conserved helix across all bacterial
76 FtsZ.

77

78 **Results**

79 **Overall description of the binary FtsZ-nucleotide bound structure.** We
80 determined the structure of *M. tuberculosis* FtsZ in the absence and presence of the
81 coumarin analogue 4-hydroxycoumarin (Fig. 1a). Data collection and refinement
82 statistics are summarised in Tables S1 and S2. FtsZ crystallises as a dimer, with one
83 binary FtsZ-nucleotide complex and one molecule of nucleotide-free FtsZ. Since we
84 did not add GTP or GDP to the purification buffer or the crystallisation conditions the
85 nucleotide originates from expression in *E. coli*. FtsZ contains an N-terminal
86 enzymatic domain and a shorter C-terminal domain designated the GTPase-activating
87 domain, which are separated by a long helix named H7 (Fig. 1b) that is structurally
88 conserved across all bacterial species (Fig. S1). The nucleotide-binding pocket is
89 located in the N-terminal domain (helices H1-H6 and strands β 1- β 6), while the C-
90 terminal domain (H8-H10 and β 7- β 10) contains the important T7 loop at the tip of
91 helix H7, responsible for the binding of various auxiliary proteins (Fig. 1b).

92

Fig. 1. Structures of FtsZ-inhibitor complexes. **a**, Chemical structures of coumarin and 4-hydroxycoumarin as known inhibitors of the bacterial cell division protein FtsZ. **b**, Overall structure representing the binary GTP-bound form of *M. tuberculosis* FtsZ. The N-terminal enzymatic domain is coloured in red (helices) and green (β-strands), whereas the C-terminal GTPase-activating domain is coloured in pink and light green for helices and β-strands, respectively. Loop regions in both domains are shown in grey. The central helix H7 separating the two domains is coloured in purple. The bound nucleotide GTP is shown as a liquorice model. **c**, Surface representation of Nucleotide-free FtsZ highlighting the position of binding pockets (BP) 1 and 2. **d**, Interactions of 4-hydroxycoumarin in BP1. Residues surrounding the inhibitor are illustrated in stick representation and 4-hydroxycoumarin is coloured in red. Hydrogen-bond interactions are displayed as dotted lines. **e**, Difference electron density map of the inhibitor contoured at 1σ fully covering the inhibitor in BP1. **f**, Volume of BP1 with bound 4-hydroxycoumarin and a water molecule **g**, Molecular interactions of 4-hydroxycoumarin in BP2. Residues surrounding the inhibitor and 4-hydroxycoumarin is coloured in red. Hydrogen-bond interactions are displayed as dotted lines. **h**, Electron density of the inhibitor contoured at 1σ fully covering the inhibitor in BP2. **i** Volume of BP2 with bound 4-hydroxycoumarin and two water molecules.

114

115 **Molecular interactions of 4-hydroxycoumarin with FtsZ.** 4-hydroxycoumarin
116 binds exclusively to nucleotide-free FtsZ, and not to the nucleotide-FtsZ complex. It
117 binds to two novel inhibitor-binding pockets not previously identified in FtsZ (Fig.
118 1c). Binding pocket 1 (BP1) is formed by secondary structure elements $\beta 2$, $\beta 2'$ and $\beta 3$
119 (Fig. 1d). 4-hydroxycoumarin binds with full occupancy and the electron density fully
120 covers the entire molecule (Fig. 1e; Fig. S2). The benzyl ring is oriented inwards,
121 towards the more hydrophobic part of the inhibitor-binding pocket. The 4-hydroxy
122 group points towards the solvent and does not form any interactions with FtsZ,
123 although it is in close proximity to several water molecules. (Fig. S3). The benzyl
124 group forms hydrophobic interactions with residues Leu47 of $\beta 2'$ and Met49, from
125 the turn between $\beta 2'$ and $\beta 3$ and Lys55 and Asp57 of $\beta 3$, with 4-hydroxycoumarin
126 aligned almost parallel to the elongated side chain of Lys55. 4-hydroxycoumarin
127 forms two hydrogen bond interactions with residues of the inhibitor-binding pocket
128 including the carbonyl oxygen, which interacts with the nitrogen atom of the Lys55
129 side chain (3.66 Å) and the backbone nitrogen of Ser50 (3.01 Å). A third interaction
130 with the side chain nitrogen of Lys55 is mediated via a structural water molecule. The
131 ring oxygen of the pyrone group also interacts with the main chain nitrogen of Ser50
132 (3.04 Å). The volume of BP1 is 104.4 Å³ (Fig. 1f).

133 A second 4-hydroxycoumarin binding pocket (BP2) appeared later during
134 refinement. 4-hydroxycoumarin in BP2 is sandwiched between Glu185, Asn189,
135 Gln192 of helix H7 and Ile225 (strand $\beta 7$), Ala262 (strand $\beta 8$), Glu302 and Arg304
136 (strand $\beta 10$) (Fig. 1g). Similar to BP1, the benzyl group points towards the protein
137 interior, establishing various hydrophobic interactions, whereas the more hydrophilic
138 pyrone ring system points towards the solvent, establishing several water-mediated
139 hydrogen-bond interactions. The electron density around the inhibitor is well defined
140 and 4-hydroxycoumarin binds in the second pocket with full occupancy (Fig. 1h, Fig.
141 S2). There are several short- and long-distance hydrogen-bond interactions between
142 residues of FtsZ and water molecules. Long-distance interactions (3.6 to 4.9 Å) occur
143 between the 4-hydroxy group and the side chain oxygen atoms of Ser227, Glu302 and
144 Arg304, as well as between the pyrone oxygen atoms with main chain carbonyl
145 oxygen and the carboxyl side chain of Glu185. The 4-hydroxy substituent establishes
146 a hydrogen bond interaction with a structural water molecule whereas the two oxygen

147 atom of the pyrone ring show a second interaction with a water molecule (Fig. S3).
 148 The volume of BP2 is 146.6 Å³ (Fig. 1i).
 149
 150 **Structural changes associated with 4-hydroxycoumarin binding.** 4-
 151 hydroxycoumarin binds in two distinct pockets. We describe these as cryptic pockets
 152 because they are present exclusively in the nucleotide-free state but absent in
 153 nucleotide-bound FtsZ. So what is the structural rationale for this selectivity of 4-
 154 hydroxycoumarin for nucleotide-free FtsZ over the nucleotide-bound form?
 155 Overlaying the structure of nucleotide-free FtsZ-coumarin binary complex with that
 156 of the FtsZ-nucleotide complex (Fig. 2) reveals significant structural differences. BP1
 157 for 4-hydroxycoumarin in the nucleotide-free structure is formed by strands $\beta 2$, $\beta 2'$
 158 and $\beta 3$, whereas in the FtsZ-nucleotide complex strand $\beta 2'$ converts into a 2-turn
 159 helix H2 (Fig. 2a). This structural rearrangement from a β -strand to a helix leads to a
 160 shift of Leu47 by 3.15 Å such that its side chain is projected into BP1, thereby
 161 restricting the binding of 4-hydroxycoumarin. Additionally, Ser50, whose main chain
 162 nitrogen forms hydrogen bond interaction with the oxygen atom of the pyrone ring is
 163 shifted by 2.49 Å. Thus, BP1 for 4-hydroxycoumarin in nucleotide-free FtsZ is absent
 164 in the nucleotide-bound form.

165

166

167 **Fig 2.** Structural differences of the 4-hydroxycoumarin binding pockets between the nucleotide-bound
 168 (cyan/yellow) and the nucleotide-free form of FtsZ (orange), 4-hydroxycoumarin (red) is shown as a
 169 liquorice model. **a**, Comparison of the BP1 in nucleotide-free and -bound FtsZ. 4-hydroxycoumarin
 170 would sterically clash with helix H2 in the nucleotide-bound form. **b**, Overlay of BP2 in the nucleotide-
 171 free form and the nucleotide-bound proteins. Arrows represent the reorientation of the side chains
 172 around the binding site, allowing 4-hydroxycoumarin to bind.

173

174 When overlaying the nucleotide-free and nucleotide-bound structures to
 175 compare BP2 (Fig. 2b), the most obvious difference is the side chain conformation of
 176 Arg304. The guanidinium side chain runs parallel to the inhibitor when 4-

hydroxycoumarin is bound, but it can reorient itself to form a new hydrogen bond interaction with Asp296 or towards $\beta 7$ and thus occlude BP2. Also, in the nucleotide-bound form, the side chains of Glu185 and Asn189 are in closer proximity to the putative 4-hydroxycoumarin binding pocket and would have to move away to make space for the inhibitor.

182

Comparison of the 4-hydroxycoumarin binding pockets. An important question is whether the two new cryptic binding pockets are already present in the native nucleotide-free form, or if the inhibitor induces the formation of the binding pockets? To be able to directly compare the 4-hydroxycoumarin binding pockets, we superimposed nucleotide-free FtsZ and the nucleotide-free FtsZ-coumarin complexes (Fig. 1d,g; Table S1). Both native and coumarin-bound complexes crystallise in the same space group with very similar cell parameters displaying the same crystal contacts. In both nucleotide-free structures, the residues of the BP1 are essentially identical. The only noticeable difference is a slight change in conformation of the Leu47 side chain, which slightly rotates to orient itself more favourable with respect to the 4-hydroxycoumarin (Fig. 1d). The native nucleotide-free structure contains four water molecules bound in place of 4-hydroxycoumarin.

The situation is distinct for BP2 since significant differences can be observed: in the native structure the side chains of Ile225 and Arg304 clearly adopt two alternative conformations where one of those extends into the BP2. Two additional side chains (Glu185 and Asn189) also display conformational changes (Fig. 1g).

199

Conformational dynamics of FtsZ states. To provide a structural rationale for the selectivity of 4-hydroxycoumarin towards the nucleotide-free FtsZ over the nucleotide-bound form and understand the differences in the conformational dynamics of the complexes, we carried out long timescale molecular dynamics simulations. Two sets of statistically independent simulations for nucleotide-bound (GTP and GDP) and nucleotide-free states were simulated for 3 μ s each. During the simulations, the nucleotides were retained in the complexes in their bound state. However, the 4-hydroxycoumarin was removed from its corresponding binding pockets in the nucleotide-free state. This was done to study the transient nature of the binding pockets and exclude any influence of the ligand on the formation or disappearance of the cryptic pockets in the simulated system. We analysed the

dynamic interconversion of structural conformations between distinct FtsZ states and identified relevant intermediates, via RMSD based clustering of all MD simulation trajectories using the k-medoids algorithm (Fig. S4) [14].

214

Notably, there was no exclusive unidirectional relationship between nucleotide-free and nucleotide-bound states. The final ensembles of the nucleotide-free form could not be reached from the GTP or GDP-bound states directly. BP1 is fully formed when strand $\beta 2'$ is present (cluster 4), while it is absent in nucleotide-bound states when a complete helix H2 is present (clusters 1 and 6) (Fig. S5). The intermediate clusters highlight varying levels of incomplete helicity of helix H2, multiple conformations of the loop T3 (between $\beta 3$ and helix H3), which interacts with the phosphate groups in the nucleotide-bound state and the loop between $\beta 6$ and H7 helix (Fig. S6). A more detailed analysis of the clusters indicated that the long helix H7 (Residues 176-199) is also highly dynamic and prone to helical bending. In the nucleotide-free state, helix H7 is bent towards the C-terminal domain, away from the nucleotide-binding site, where H7 helix did not show considerable kinking in the nucleotide-bound states. This suggested that helix H2, which forms the boundary of BP1, behaved like a constrained spring that was wound when helix H7 was straight (nucleotide-bound states) and relaxed into the $\beta 2'$ -strand when helix H7 moved away towards the C-terminal domain in the nucleotide-free state. The linked inter-conversion of helix H2 and the $\beta 2'$ strand, concomitant with helical H7, bending resulted in the formation or disappearance of BP1.

233

Helix H7 bending controls the formation of cryptic binding pockets. Flexible helices frequently support conformational transitions between different functional states of proteins [15] and tend to be conserved in evolution, implicating them in functional roles. Of all the amino acids, glycine and proline have the strongest destabilising effect on helices [16, 17]. Glycine in particular disfavours helix folding since the entropic cost of tethering glycine in a helix exceeds the gain of enthalpy upon helix formation [18-20]. H7 helical bending was calculated as a function of the nucleotide-bound/free states from the simulations. Analysis was carried out separately on each simulated state.

243

Fig 3. Dynamics of helical H7 bending. **a**, Heat map of H7 helix bending in the FtsZ-GTP (left), FtsZ-GDP (middle) and nucleotide-free FtsZ (right) states plotted as a function of simulation time. **b**, Structural conservation of helix H7 highlighting the position of Gly193 (red box) in *M. tuberculosis* (current structures), *B. subtilis* (PDB id 2VXY), *P. aeruginosa* (PDB id 2VAW), *M. jannaschii* (PDB entry 1W5E) and *A. aeolicus* (PDB entry 2R75). Conformational snapshot at 3 μs of **c** FtsZ-GDP and **d** the nucleotide-free complex illustrating helical bending. Helix H7 is represented as a cylinder. The colour legend representing the kink is the same as in Fig. 3a.

Helix H7 distortion distribution was then extrapolated as a heat map and was colour coded according to the local angle magnitude (Fig. 3a). The flexibility of helix H7 is greater in the nucleotide-free state, followed by GDP-bound and GTP-bound states. In the GTP-bound state, the C-terminal end of helix H7 is tethered to neighbouring structural elements via Gln192 making hydrogen bond interactions with Gln30 of helix H1 and Arg304 (β10) (Fig. 4a). Mild flexibility is observed between residues 189-195, with an average kink of 12°-18°. Furthermore, the N-terminal region of helix H7 forms part of the nucleotide-binding site. The side chain of Asp184 establishes hydrogen bond interactions with the guanine nucleotide, while Phe180 makes a π-stacking interaction with the imidazole ring of the purine moiety. There are a number of other hydrogen bond interactions, for example between the γ-phosphate and Thr42 (loop between β2 and helix H2) and the backbone nitrogen atom of Ala68 and Ala70 (loop between β3 and helix H3), which constrain the nucleotide within its binding site and indirectly restrict helix H7 bending. It is also noteworthy that Thr42 sits at the edge of helix H2, which in the nucleotide-bound form prevents the formation of BP1.

Fig 4. Tethering of BP1 and BP2 via H7 helix in **a** nucleotide-bound and **b** nucleotide-free FtsZ structures. Helix H7 is coloured purple, GTP is coloured yellow and 4-hydroxycoumarin are coloured red. Orange spheres represent hydrogen bond interactions.

In the GDP-bound state, we observe similar tethering of helix H7 with H1, although interactions with β 10 are not present. Helical bending is observed between residues 190-194 and is most pronounced at Gly193 (Fig. 3a). During sampling of the first 600 ns, additional helical bending is also observed around Ser182. The loss of a γ -phosphate in GDP results in loss of the interaction with Thr42 and Ala68 and the additional volume generated permits enhanced flexibility of helix H7. The greatest variation in helical flexibility is seen in the nucleotide-free state. In addition to Ser182

282 and Gly193, a helical kink is also seen at Val186. Comparing helix H7 flexibility
283 across the three states indicated that bending occurs predominantly at Gly193, a
284 highly conserved residue in H7 helix across the bacterial FtsZ family (Fig. 3b). This
285 suggests that the helical movement of H7 is conserved and possibly common to all
286 bacterial FtsZ proteins. Gly193 is positioned near the C-terminal end of helix H7 and
287 act as a hinge point for kink movements, thereby exerting its effects on the farther N-
288 terminal part of helix H7, adjacent to the nucleotide-binding site. The nucleotides act
289 like a wedge that restricts H7 helical motion. Therefore, in the nucleotide-free state,
290 G193 acts like a primary pivot point of helix H7, localizing on its concave side (Fig.
291 3d). A comparison of the different states, from both crystallographic structures and
292 MD simulation trajectories, suggests that helical bending is heavily influenced by
293 steric hindrance of the nucleotide and affects the collective dynamics of helix H7 at
294 its N-terminal apex.

295 Is the formation of BP2 in the nucleotide-free state also linked with the
296 dynamics of helix H7? In the GTP-bound state, Glu185 makes hydrogen bonds with
297 Asn189, which is stable throughout the course of the simulation. Ile225, whose
298 hydrophobic side chain forms the base of BP2 is positioned opposite Asn189. In
299 addition, the side chain of Arg304, which in the crystal structure points into BP2,
300 makes interactions with Gln192 and Asp296. Gln192 also forms stable hydrogen
301 bond interactions with Gln30. These interactions strongly tether helices H1 and H7
302 and β 9 together. This tethering binds the local structural elements from the N-terminal
303 and C-terminal domains together and prevents the formation of BP2.

304 In the GDP-bound state, tethering is observed between helices H1 and H7
305 through residues Arg26 and Glu185, Gln30 and Gln192, Lys33 and Asp196.
306 However, interactions between helix H7 and Arg304 of β 10 are lost. Even though the
307 volume of BP2 increases slightly from that observed in the GTP-bound state, it is still
308 not large enough to accommodate 4-hydroxycoumarin.

309 In contrast, in the nucleotide-free state, the conformational dynamics of helix
310 H7 prevent any stable interactions or tethering of the local structural elements
311 together at its C-terminal end. As a result, the side chains of Arg304 and Asp189 can
312 adopt multiple conformations. The effects of H7 helical bending on its N-terminal
313 region allow the side chain of Glu185 to rearrange and form the boundary of BP2.
314 The multiple side chain reorganisations impart a defined shape to BP2, making it
315 large enough to accommodate 4-hydroxycoumarin. In summary, the formation of BP1

316 and BP2 are directly linked with the structural dynamics of helix H7 in the
317 nucleotide-free state.

318

319 **4-hydroxycoumarin binding sites are involved in pocket crosstalk.** Small
320 molecule binding sites in proteins have been shown to sometimes contribute to
321 protein allosteric signalling [21, 22]. Since both 4-hydroxycoumarin binding pockets
322 appear together in the nucleotide-free crystal structure and the dynamics of helix H7
323 bending contributes to the formation and dissolution of both BP1 and BP2, we
324 investigated whether there was any communication between the two sites. We used
325 protein conformations extracted from MD trajectories to detect the formation and
326 spatiotemporal evolution of the pockets formed in the nucleotide-free state of FtsZ.
327 Such communication termed as “pocket crosstalk” has been effectively used to study
328 pharmaceutically relevant targets, including the purine nucleoside phosphorylase,
329 adenosine receptor in a membrane environment and the kinase family [23]. A merging
330 and splitting frequency matrix was calculated into a 3D network graph, where nodes
331 represent the pockets and the edges indicate communication between two pockets or
332 how often two pockets exchange atoms. A total of five pockets was identified that
333 existed for more than 24% during the course of the nucleotide-free simulation run.
334 Pocket identifier (pID) 7 (green) corresponds to BP1, pID 4 (blue) is BP2, pID 3 (red)
335 corresponds to the nucleotide binding site and pID 20 (pink) and 14 (orange) are two
336 sub-pockets that form as a result of the fluctuations seen in loop T3 (Fig. 5a). These
337 pockets have average dynamic volumes of 24, 119, 201.1, 55, and 50 Å³, respectively
338 (Fig. S7).

339 The reduced volume of BP1 can be explained by the existence of the nearby
340 nucleotide-binding site, which has a persistence of ~80%. Furthermore, the high
341 fluctuations of loop T3 in the nucleotide-free state allow the formation of two minor
342 pockets (pID 20 and 14) with a persistence of 45 and 55%. These sub-pockets have
343 the ability to merge with the nucleotide-binding pocket, resulting in a single larger
344 binding pocket (Fig. 5a; Fig. S7). BP2 is situated diagonally across helix H7 on the
345 distal side. Since the conformation of H7 controls the volume of both BP2 and the
346 nucleotide-binding site, it was therefore not surprising that the nucleotide-binding site
347 and BP2 displayed a crosstalk pattern during our simulations. This suggests that a
348 possible communication pathway exists between BP2 and BP1 that is mediated via
349 the nucleotide-binding site. Furthermore, this might also explain the formation and

disappearance dependence of BP1 and BP2 on the dynamics of helical bending in the nucleotide-free state. However, in the nucleotide-bound state, the communication between BP2 and BP1 is blocked by the presence of the nucleotide and its strong interactions within its binding site.

354

355

Fig 5. Communication between binding pockets. **a**, Spatial location of 4-hydroxycoumarin binding pocket (BP) 1 (green), 2 (blue), nucleotide-binding pocket (NBP; red) and two additional sub-pockets (salmon and pink) computed for the nucleotide-free FtsZ structure. The pockets are connected via black lines (edges) and exhibit a persistency of at least 24%. Helix H7 is coloured purple. **b**, Dynamic cross-correlation analysis of the time-correlated α motions calculated from FtsZ-GTP (left), FtsZ-GDP (centre) and nucleotide-free FtsZ (right) complex simulations. The cross-correlation between helix H7 (dashed box) and BP1 (*), BP2 (□) and between BP1 and BP2 (□) are illustrated proportional to the shading, as indicated by the scale on the right.

363

364

Dynamic cross-correlation between helix H7 and 4-hydroxycoumarin binding

pockets. To investigate the relative residue movement between the α atoms of BP1, BP2 and helix H7, we generated dynamical cross-correlation (DCC) heat maps (Fig. 5b). The DCC was calculated separately over all 30000 frames for GTP-bound, GDP-bound and nucleotide-free complex. In the nucleotide-bound states, the interactions between BP1 and helix H7 are positively correlated (marked *), whereas the interactions between BP2 and H7 helix (marked □) are anticorrelated. The DCC between BP1 and BP2 (marked □) are also anticorrelated. However, in the nucleotide-

373

free state the DCC between BP1 and helix H7 is anticorrelated, whereas the movement between BP2 and helix H7 is correlated. The interactions between BP1 and BP2 are also partially positively correlated. This is consistent with our pocket crosstalk analysis, which revealed an allosteric communication between BP1 and BP2. Taken together, our analysis confirms that formation of the 4-hydroxycoumarin binding pockets in the nucleotide-free state and its absence in the nucleotide-bound state are a result of cryptic communication in *M. tuberculosis* FtsZ.

381

382 Discussion

383 The antibacterial effects of coumarin and its analogues have been quantified in
384 various bacterial strains including *S. aureus*, *E. coli*, *P. aeruginosa* and *M.*
385 *tuberculosis* *H₃₇Rv* [24, 25]. Duggirala and colleagues [26] were able to prove that the
386 4-hydroxycoumarin inhibited the both the polymerization and GTPase activities of *E.*
387 *coli* FtsZ, providing a direct link between inhibition of FtsZ and its antibacterial
388 activity. In the absence of structural data for the inhibition of FtsZ by coumarins,
389 modeling attempts have been undertaken to predict the coumarin-binding pocket for
390 *M. tuberculosis* FtsZ [26]. These docking studies using the *E. coli* FtsZ structure as a
391 template predicted that coumarin binds to FtsZ at a site distinct from the GTP binding
392 site, in close proximity to the binding pocket identified in *S. aureus* FtsZ (Fig. S8).
393 This predicted binding pocket is part of the T7 loop of *S. aureus* FtsZ. However, the
394 two new coumarin-binding pockets that we report in this work are not located in the
395 predicted location. The reasons for this discrepancy may be that subtle structural
396 differences between *E. coli*, *S. aureus* and *M. tuberculosis* FtsZ structures are more
397 significant than anticipated. Moreover, it is worth emphasizing that all previous
398 attempts to model coumarin binding were done on the GTP-bound structure, where
399 the cryptic pockets that we report are not formed.

400 *S. aureus* FtsZ exists in open and closed conformations [27]. To date, only a
401 single inhibitor-binding pocket has been described by structural methods, namely for
402 *S. aureus* FtsZ [12, 13, 28]. This binding pocket is located in a hydrophobic cleft
403 between the C-terminal subdomain and helix H7 in proximity to the T7 loop and is
404 distinct from the two new pockets identified in our study (Fig. S8). In the *S. aureus*
405 complex (Table S2), the inhibitor PC190723 (or its analogues) is bound to the FtsZ-
406 GDP binary complex in the open conformation. Since there is only one binary FtsZ-
407 GDP complex in the asymmetric unit, it is unclear if this represents a transient

inhibitor-binding pocket or if this inhibitor could also bind to nucleotide-free FtsZ. Superimposition of *M. tuberculosis* and *S. aureus* FtsZ closed conformation structure (PDB entry 3WVGK) reveals a C α RMSD of ~ 0.4 Å (Fig. S9a). This confirms that the coumarin analogues stabilize the closed conformation of *M. tuberculosis* FtsZ, unlike *S. aureus* FtsZ where the inhibitor stabilizes the open conformation. A comparison of *M. tuberculosis* FtsZ with the inhibitor-bound open conformation *S. aureus* FtsZ highlights a significant rotation of the C-terminal domain by $\sim 27^\circ$ (Fig. S9b). In addition to this rotation, the H7 helix in *S. aureus* displays a downward shift by about one helical turn (Fig. S9c). It is interesting to note that none of the *S. aureus* structures (open or closed conformations) displayed H7 helical bending or the formation of equivalent binding sites for coumarin analogues. Nevertheless, we do not exclude the possibility that even more binding pockets will be identified in the future in FtsZ.

Fig 6. Structural comparison of FtsZ-4-hydroxycoumarin and tubulin-inhibitor complexes. The nucleotide-free FtsZ (orange)-4-hydroxycoumarin (red space fill) complex has been superimposed with clinically relevant (a) tubulin-taxol (cyan) complex, (b) tubulin-colchicine (pink) complex and (c) tubulin-vinblastine (yellow) complexes.

427

428 Bacterial FtsZ also shares homology with mammalian tubulin. Interestingly,
429 certain taxol-related analogues that have been shown to be inactive against various
430 tumour cell lines and do not act on tubulin are still potent inhibitors against *M.*
431 *tuberculosis* FtsZ with MIC values of 2.5 μ M [29]. Comparison of the FtsZ-4-
432 hydroxycoumarin binary complexes with medically relevant tubulin-drug complexes
433 (Fig. 6), namely tubulin in complex with the anticancer agents taxol (PDB entry 1JFF,
434 [30]), colchicine (PDB entry 4O2B, [31]) and vinblastine (PDB entry 5J2T, [32]),
435 highlighted that the two transient 4-hydroxycoumarin binding pockets on FtsZ do not
436 overlap with either colchicine (Fig. 6b) or vinblastine (Fig. 6c). In contrast, one of the
437 4-hydroxycoumarin binding pockets overlaps with the taxol-binding pocket on tubulin
438 (Fig. 6a), indicating that FtsZ and mammalian tubulin share not only the same
439 ancestor with respect to their overall folds but also some conservation of their
440 inhibitor-binding pockets.

441 Finally, our results provide novel insights into the structural and dynamic
442 mechanisms into cryptic pocket formation on *M. tuberculosis* FtsZ. We report on the
443 formation and dissolution of two novel pockets on *M. tuberculosis* FtsZ. A
444 combination of crystallographic and computational work has allowed us to understand
445 structural dynamics in a manner that is not evident from static structures alone. The
446 atomistic level details in understanding the cryptic pocket formation not only opens
447 new avenues to study and understand FtsZ function but can also be leveraged to
448 design more potent and selective inhibitors.

449

450 **Methods**

451 **Reagents and Constructs.** 4-Hydroxycoumarin was bought from hit2lead /
452 Chembridge with a purity of > 90%. BL21(D3) pLysS competent cells and Bugbuster
453 protein extraction reagent were purchased from Novagan. LB medium and LB-agar
454 were purchased from MP Biomedicals. TB medium was bought from Fluka.
455 NuPAGE®LDS Sample Buffer (4X), NuPAGE®Sample Reducing Agent (10X),
456 NuPAGE® MES SDS Running Buffer (20X), SeeBlue® Plus2 Pre-stained Protein
457 Standard, SimplyBlue™ SafeStain and NuPAGE®Novex® 4-12% Bis-Tris Protein
458 Gels (1.0 mm 10 well) were purchased from Life Technologies. Tris
459 (hydroxymethyl) aminomethane, Imidazole, distilled water, $MgCl_2$, $MgAc_2$,
460 Glycerol, Dimethyl sulfoxide (DMSO), Ethylene glycol tetraacetic acid (EGTA),

NaCl, Benzonase Nuclease, Chloramphenicol, Ethylenediaminetetraacetic acid (EDTA), N-(2-acetamido)-2 amino ethanesulphonic acid (ACES), KCl, and Isopropyl-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma Aldrich. Ethanol, NaOH, Sodium citrate dihydrate, and Isopropanol were purchased from Fisher. 5 ml HisTrap FF columns, Superose 12 was bought from GE Healthcare. Ampicillin sodium salt was bought from Cayman Chemical. SnakeSkin® Dialysis Tubing was purchased from Thermo Scientific. PEG4000 was purchased from Melford. Ammonium acetate was purchased from Lab Chemie. 24 well linbro plates and coverslips were bought from Hampton Research.

Protein expression and purification of FtsZ from *M. tuberculosis*. Full-length FtsZ from *M. tuberculosis* (residues 1-379) was cloned into the pProEx expression plasmid, yielding a PreScission Protease cleavable N-terminal hexahistidine-tagged protein. The plasmid was transformed into BL21(DE3) pLysS. A single colony was mixed into a 20-mL flask containing TB medium, supplemented with 100 µg/ml ampicillin and 34 µg/mL chloramphenicol. The flask was incubated at 37°C for 8 h. The culture was distributed equally into six 2L flasks containing 1 L TB medium, supplemented with 100 µg/mL ampicillin, and cultured overnight at 37°C. The next day, cells were induced by the addition of 0.5 mM IPTG for 24 h at 20°C. Cells were then harvested by centrifugation at 8000 rpm (Avanti® JE centrifuge and JLA-16.250 rotor from Beckman Coulter) for 10 min at 4°C. The supernatant was discarded, and the pellets were stored at -80°C until used. The harvested cells were resuspended in buffer A (50 mM HEPES pH 7.2, 300 mM NaCl, 5% glycerol and 10 mM imidazole) and lysed on ice by sonication, for ten cycles of 30 sec on/off. The lysate was transferred into precooled 250 mL centrifuge bottles and centrifuged at 20,000 rpm (Avanti® JE centrifuge and JLA-16.250 rotor from Beckman Coulter) for 15 min at 4°C.

The resulting supernatant was loaded into a 5 ml His-Trap FF column, equilibrated with 15 mL (1 mL/min) of buffer A (50 mM HEPES pH 7.2, 150 mM NaCl, 5% glycerol and 10 mM imidazole). The column was washed with 100 mL (1 mL/min) washing buffer B (50 mM HEPES pH 7.2, 150 mM NaCl, 5% glycerol and 50 mM imidazole) and the protein was eluted using buffer C (50 mM HEPES pH 7.2, 150 mM NaCl, 5% glycerol and 250 mM imidazole).

494 The eluted protein was mixed with the appropriate amount of PreScission
495 protease (1 mg of PreScission protease / 50 mg protein), transferred into 10 K
496 SnakeSkin Dialysis Tubing and placed overnight at 4°C into cleavage buffer (50 mM
497 HEPES pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.01% Tween 20 and 1 mM DDT).
498 Any uncleaved His-Tagged protein or PreScission protease was removed by running
499 the protein through a second 5 ml HisTrap FF column. Cleaved FtsZ was dialysed
500 against dialysis buffer (25 mM MES pH 7.2, 0.1 mM EDTA, 10 mM DDT, 50 mM
501 NaCl and 5% glycerol) and concentrated to 18 mg/mL using an Amicon Ultra-15, 10
502 kDa concentrator, frozen in liquid nitrogen and stored at -80°C.

503

504 **Protein crystallisation of native FtsZ and in complex with 4-hydroxycoumarin.**

505 Purified FtsZ (18 mg/mL) was crystallised in Linbro plates using the hanging drop
506 method. 1 µl of protein was mixed with a drop of 1 µl of reservoir solution containing
507 27.5% PEG4000, 0.4 M ammonium acetate and 0.1 M Na citrate pH 5.6 and
508 incubated at 18°C for at least six weeks. For the native *M. tuberculosis* FtsZ structure,
509 crystals were immersed in a cryoprotectant solution composed of reservoir solutions
510 plus 15% glycerol while, for the FtsZ-4-hydroxycoumarin structure, crystals were
511 soaked for 30 min in a cryoprotectant solution composed of liquid mother solution
512 plus 15% DMSO containing 37.5 mM of 4-hydroxyloumarin. Finally, all crystals
513 were flash-frozen in liquid nitrogen and stored until used.

514

515 **Data collection, structure determination and refinement.** Diffraction data for
516 native *M. tuberculosis* FtsZ and the two coumarin analogue complexes were collected
517 at Diamond Light Source, Oxfordshire, at beamline i04. Data were processed using
518 iMosflm [33] and scaled using SCALA [34] to resolutions as shown in Table 1. The
519 structures of native FtsZ and the FtsZ-4-coumarin complexes were solved in space
520 group P₆₅ by molecular replacement (PHASER MR in CCP4 suite [35] using the
521 previously published *M. tuberculosis* FtsZ structure (PDB ID: 1RQ7;[36]) as a search
522 model. All structures were improved using Coot and refined with PHENIX [37]. The
523 calculation of R_{free} used 5% of the data. Crystallographic and refinement statistics are
524 shown in Table 1. Native FtsZ contains residues Asn6 to Thr63 and Gly69 to Phe312
525 in chain A, whereas chain B contains Asn6 to Gly59 and Ala70 to Asp313.

526

Molecular Dynamics (MD) Simulations. The starting structures for molecular dynamics simulations were prepared after the final crystallographic refinement of the resolved coordinates. Three separate systems GTP, GDP and nucleotide-free (NF) were prepared as follows: While preparing the NF state, the bound coumarin ligands in binding pockets 1 and 2 were removed. The protonation state of the complexes was determined using the software PDB2PQR [38]. This was followed by the systems being set up using xleap [39] employing the ff14SB forcefield [40]. The parameters for GTP and GDP were downloaded from the Amber parameter database [41]. The protonated complexes were solvated using TIP3P water [42] and the edge of the box was set to at least 10 Å from the closest solute atom. The system was neutralised using counter ions. The protocol was identical for each system. Each system was minimized and equilibrated under NPT conditions for 5 ns at 1 atm. The temperature was increased gradually to 298 K using a timestep of 4 fs, rigid bonds, a cut-off of 9 Å and particle mesh Ewalds summation switched on for long-range electrostatics. The protein was fixed during equilibration, while the ions and solvent molecules were allowed to move. The heavy atoms of the protein and co-factors were constrained by a spring constant set at 1 kcal mol⁻¹ Å⁻². The production simulations were run using ACEMD molecular dynamics software [43] in the NVT ensemble using a Langevin thermostat with a damping of 0.1 ps⁻¹ and hydrogen mass repartitioning scheme to achieve time steps of 4 fs. Two sets of simulations were run for each system for 3 μs (30000 frames) starting from different velocities to improve statistics. VMD [44] was used to visualise the trajectories and the figures were made using ICM-Pro software (www.molsoft.com).

550

Clustering. Trajectories from all three simulations (sampling 9 μs) were pooled together before clustering was carried out. All frames were aligned and grouped based on the RMSD metric, such that similar ones are in the same cluster while the dissimilar structures are in other clusters. Clustering was done via the *k-medoids* algorithm [14] with a smart initialisation method implemented in the BiKi Lifesciences software suite (www.bikitechnologies.com). The representative conformation called medoids is based on the most centrally located conformation in the cluster and is less sensitive to outliers in comparison with other methods. The size of the circle is proportional to the size of the cluster and the edges denote the number

of inter-conversions between them. The Fruchterman-Reignold layout was used to generate the cluster graph.

562

Helical bending. The dynamics of helix H7 was analysed using Bendix software [45] implemented in the Visual Molecular Dynamics package [44]. The Bendix algorithm is based on calculating a window of four residues to give local helix axes that are joined by a spline [46]. The helical bending is plotted as a heatmap, which is colour coded according to the local angle magnitude, highlighting non-linear helix behaviour. The maximal and local angles were plotted over the course of each simulation set.

570

Pocket crosstalk. Spatio-temporal evolution of the binding pockets was investigated using Pocketron [23] implemented in the BiKi Lifesciences software suite (www.bikitechnologies.com). The algorithm identifies pockets and tracks their evolution over time along an MD trajectory. In brief, residue exchange between adjacent pockets, termed as pocket crosstalk, was monitored. This is another means of identifying communication between different parts of the protein [21, 22]. Alignment-independent identification of pockets on the protein surface was carried out, followed by quantification and visualisation of the volume and surface area of all the pockets found in the protein, for each conformation present in the MD trajectory. Each pocket is then assigned a unique pocket identifier (pID). Communication between pockets, describing the merging and splitting is plotted. More specifically, a “merge” event occurs when atoms belonging to the same pocket in the current frame have belonged to separate pockets in a previous frame. Similarly, a “split” event occurs when atoms of a single pocket separate into two or more distinct pockets. The algorithm translates the merging and splitting frequency matrix into a 3D network graph, where nodes represent geometric center of the pockets, and the edges indicate communication between two pockets. The thickness of the edge is directly proportional to the frequency of the merging and splitting events between two connected pockets. Finally, the signal transmission network between pockets across the protein surface is illustrated as interconnected pocket motions. The entire 3 μ s nucleotide-free trajectory with a stride of 2 (>15000 frames) was used to analyse pocket crosstalk for each system. The minimum persistence time for each pocket was fixed at 24%.

Dynamic cross-correlation maps. The systems were simplified by representing each residue as a single C α node. All 30000 conformations pooled for each system was used to construct a local contact matrix. Two nodes (excluding neighbouring nodes) are in contact if they are within a distance of 6.5 Å. The connectivity between two nodes is termed an edge. The dynamic cross-correlation maps (DCCM) were calculated using the program MD-TASK program [47]. The generated dynamic cross-correlational map has the ability to identify highly correlated or anticorrelated nodes. However, to compute communication pathways, it is useful to construct a matrix (C) where small values indicate highly correlated or anticorrelated motions. The edges between the nodes can be weighted on how often the interactions exist. This can be functionalised by $w_{ij} = -\log(|C_{ij}|)$; where w_{ij} can be thought of as a distance in the functionalised correlation space between node-node pairs i and j .

Data Availability

The structures of native FtsZ in the presence of GDP (PDB ID 6YM1) or GTPyS (PDB ID 6YM9) as well as FtsZ-GDP-coumarin (PDB ID 6Y1U) and FtsZ-GTPyS-coumarin (PDB ID 6Y1V) ternary complexes can be downloaded from the Protein Data Bank. Simulation trajectories and all other data supporting the findings of this study are available from the corresponding author upon reasonable request.

Reporting summary

Further information is available in the Nature Research Reporting Summary linked to this article

References

1. Organization, W.H., *Global tuberculosis report 2013*. 2013: World Health Organization.
2. Osborne, R., *First novel anti-tuberculosis drug in 40 years*. Nature Biotechnology, 2013. **31**: p. 89.
3. Ryan, N.J. and J.H. Lo, *Delamanid: first global approval*. Drugs, 2014. **74**(9): p. 1041-1045.
4. Organization, W.H., *WHO consolidated guidelines on drug-resistant tuberculosis treatment*. 2019: World Health Organization.
5. Harry, L.M.a.E., *The Bacterial Cytoskeleton*. Australian Biochemist, 2009. **40**: p. 4-8.

- 628 6. RayChaudhuri, D. and J.T. Park, *Escherichia coli* cell-division gene *ftsZ*
629 *encodes a novel GTP-binding protein*. Nature, 1992. **359**(6392): p. 251-
630 254.
- 631 7. Stricker, J., et al., *Rapid assembly dynamics of the Escherichia coli FtsZ-ring*
632 *demonstrated by fluorescence recovery after photobleaching*. Proceedings
633 of the National Academy of Sciences, 2002. **99**(5): p. 3171-3175.
- 634 8. Anderson, D.E., F.J. Gueiros-Filho, and H.P. Erickson, *Assembly dynamics of*
635 *FtsZ rings in Bacillus subtilis and Escherichia coli and effects of FtsZ-*
636 *regulating proteins*. Journal of bacteriology, 2004. **186**(17): p. 5775-5781.
- 637 9. White, E.L., et al., *2-Alkoxy carbonylaminopyridines: inhibitors of*
638 *Mycobacterium tuberculosis FtsZ*. Journal of Antimicrobial Chemotherapy,
639 2002. **50**(1): p. 111-114.
- 640 10. Reynolds, R., et al., *A new 2-carbamoyl pteridine that inhibits*
641 *mycobacterial FtsZ*. Bioorganic & medicinal chemistry letters, 2004.
642 **14**(12): p. 3161-3164.
- 643 11. Hurley, K.A., et al., *Targeting the bacterial division protein FtsZ*. Journal of
644 medicinal chemistry, 2016. **59**(15): p. 6975-6998.
- 645 12. Matsui, T., et al., *Structural reorganization of the bacterial cell-division*
646 *protein FtsZ from Staphylococcus aureus*. Acta Crystallographica Section D:
647 Biological Crystallography, 2012. **68**(9): p. 1175-1188.
- 648 13. Fujita, J., et al., *Structural flexibility of an inhibitor overcomes drug*
649 *resistance mutations in Staphylococcus aureus FtsZ*. ACS chemical biology,
650 2017. **12**(7): p. 1947-1955.
- 651 14. Park, H.-S. and C.-H. Jun, *A simple and fast algorithm for K-medoids*
652 *clustering*. Expert systems with applications, 2009. **36**(2): p. 3336-3341.
- 653 15. Högel, P., et al., *Glycine perturbs local and global conformational flexibility*
654 *of a transmembrane helix*. Biochemistry, 2018. **57**(8): p. 1326-1337.
- 655 16. Liu, L.-P. and C.M. Deber, *Uncoupling Hydrophobicity and Helicity in*
656 *Transmembrane Segments α -HELICAL PROPENSITIES OF THE AMINO*
657 *ACIDS IN NON-POLAR ENVIRONMENTS*. Journal of Biological Chemistry,
658 1998. **273**(37): p. 23645-23648.
- 659 17. Li, S.-C. and C.M. Deber, *A measure of helical propensity for amino acids in*
660 *membrane environments*. Nature structural biology, 1994. **1**(6): p. 368-
661 373.
- 662 18. O'Neil, K.T. and W.F. DeGrado, *A thermodynamic scale for the helix-forming*
663 *tendencies of the commonly occurring amino acids*. Science, 1990.
664 **250**(4981): p. 646-651.
- 665 19. Lyu, P.C., et al., *Side chain contributions to the stability of alpha-helical*
666 *structure in peptides*. Science, 1990. **250**(4981): p. 669-673.
- 667 20. Pace, C.N. and J.M. Scholtz, *A helix propensity scale based on experimental*
668 *studies of peptides and proteins*. Biophysical journal, 1998. **75**(1): p. 422-
669 427.
- 670 21. Stank, A., et al., *Protein binding pocket dynamics*. Accounts of chemical
671 research, 2016. **49**(5): p. 809-815.
- 672 22. Nussinov, R., *Introduction to protein ensembles and allostery*. 2016, ACS
673 Publications.
- 674 23. La Sala, G., et al., *Allosteric communication networks in proteins revealed*
675 *through pocket crosstalk analysis*. ACS central science, 2017. **3**(9): p. 949-
676 960.

- 677 24. Chiang, C.C., et al., *A novel dimeric coumarin analog and antimycobacterial*
678 *constituents from Fatoua pilosa*. Chemistry & biodiversity, 2010. **7**(7): p.
679 1728-1736.
- 680 25. de Souza, S.M., F. Delle Monache, and A. Smânia, *Antibacterial activity of*
681 *coumarins*. Zeitschrift fuer Naturforschung C, 2005. **60**(9-10): p. 693-700.
- 682 26. Duggirala, S., et al., *Phytochemicals as inhibitors of bacterial cell division*
683 *protein FtsZ: coumarins are promising candidates*. Applied biochemistry
684 and biotechnology, 2014. **174**(1): p. 283-296.
- 685 27. Wagstaff, J.M., et al., *A polymerization-associated structural switch in FtsZ*
686 *that enables treadmilling of model filaments*. MBio, 2017. **8**(3).
- 687 28. Tan, C.M., et al., *Restoring methicillin-resistant Staphylococcus aureus*
688 *susceptibility to β -lactam antibiotics*. Science translational medicine, 2012.
689 **4**(126): p. 126ra35-126ra35.
- 690 29. Huang, Q., et al., *Targeting FtsZ for antituberculosis drug discovery:*
691 *noncytotoxic taxanes as novel antituberculosis agents*. Journal of medicinal
692 chemistry, 2006. **49**(2): p. 463-466.
- 693 30. Löwe, J., et al., *Refined structure of $\alpha\beta$ -tubulin at 3.5 Å resolution*. Journal of
694 molecular biology, 2001. **313**(5): p. 1045-1057.
- 695 31. Waight, A.B., et al., *Structural basis of microtubule destabilization by potent*
696 *auristatin anti-mitotics*. PLoS One, 2016. **11**(8): p. e0160890.
- 697 32. Prota, A.E., et al., *The novel microtubule-destabilizing drug BAL27862 binds*
698 *to the colchicine site of tubulin with distinct effects on microtubule*
699 *organization*. Journal of Molecular Biology, 2014. **426**(8): p. 1848-1860.
- 700 33. Battye, T.G.G., et al., *iMOSFLM: a new graphical interface for diffraction-*
701 *image processing with MOSFLM*. Acta Crystallographica Section D:
702 Biological Crystallography, 2011. **67**(4): p. 271-281.
- 703 34. Evans, P., *Scaling and assessment of data quality*. Acta Cryst. 2006.
- 704 35. Winn, M.D., et al., *Overview of the CCP4 suite and current developments*.
705 Acta Crystallographica Section D: Biological Crystallography, 2011. **67**(4):
706 p. 235-242.
- 707 36. Leung, A.K., et al., *Structure of Mycobacterium tuberculosis FtsZ reveals*
708 *unexpected, G protein-like conformational switches*. Journal of molecular
709 biology, 2004. **342**(3): p. 953-970.
- 710 37. Adams, P.D., et al., *PHENIX: a comprehensive Python-based system for*
711 *macromolecular structure solution*. Acta Crystallographica Section D:
712 Biological Crystallography, 2010. **66**(2): p. 213-221.
- 713 38. Dolinsky, T.J., et al., *PDB2PQR: an automated pipeline for the setup of*
714 *Poisson-Boltzmann electrostatics calculations*. Nucleic acids research,
715 2004. **32**(suppl_2): p. W665-W667.
- 716 39. Case, D., et al., *AMBER 2015; University of California: San Francisco, CA,*
717 *2015*. There is no corresponding record for this reference.[Google
718 Scholar], 2015.
- 719 40. Hornak, V., et al., *Comparison of multiple Amber force fields and*
720 *development of improved protein backbone parameters*. Proteins:
721 Structure, Function, and Bioinformatics, 2006. **65**(3): p. 712-725.
- 722 41. Meagher, K.L., L.T. Redman, and H.A. Carlson, *Development of*
723 *polyphosphate parameters for use with the AMBER force field*. Journal of
724 computational chemistry, 2003. **24**(9): p. 1016-1025.

- 725 42. Joung, I.S. and T.E. Cheatham III, *Determination of alkali and halide*
726 *monovalent ion parameters for use in explicitly solvated biomolecular*
727 *simulations*. The journal of physical chemistry B, 2008. **112**(30): p. 9020-
728 9041.
- 729 43. Harvey, M.J., G. Giupponi, and G.D. Fabritiis, *ACEMD: accelerating*
730 *biomolecular dynamics in the microsecond time scale*. Journal of chemical
731 theory and computation, 2009. **5**(6): p. 1632-1639.
- 732 44. Humphrey, W., A. Dalke, and K. Schulten, *VMD: visual molecular dynamics*.
733 Journal of molecular graphics, 1996. **14**(1): p. 33-38.
- 734 45. Dahl, A.C.E., M. Chavent, and M.S. Sansom, *Bendix: intuitive helix geometry*
735 *analysis and abstraction*. Bioinformatics, 2012. **28**(16): p. 2193-2194.
- 736 46. Dalton, J.A., I. Michalopoulos, and D.R. Westhead, *Calculation of helix*
737 *packing angles in protein structures*. Bioinformatics, 2003. **19**(10): p.
738 1298-1299.
- 739 47. Brown, D.K., et al., *MD-TASK: a software suite for analyzing molecular*
740 *dynamics trajectories*. Bioinformatics, 2017. **33**(17): p. 2768-2771.

743 Acknowledgements

744 We are grateful for PharmAlliance for initial seed funding and the Faculty of
745 Pharmacy, King Abdul-Aziz University for Aisha Alnami's PhD studentship. We
746 thank Diamond Light Source for providing access to beamlines I04 and I24, which
747 contributed to the results.

749 Author contributions

750 The manuscript was written through contributions of all authors. All authors have
751 approved the final version of the manuscript. This publication is part of the doctoral
752 thesis of Aisha Alnami.

754 Competing interests

755 The authors declare no competing financial interests.

756 Additional information

757 **Supplementary information** is available for this paper.

758 **Correspondence and requests for materials** should be addressed to F.K.

759

760