Variations in the Binding Pocket of an Inhibitor of the Bacterial Division Protein FtsZ across Genotypes and Species

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Abstract

The recent increase in antibiotic resistance in pathogenic bacteria calls for new approaches to drug-target selection and drug development. Targeting the mechanisms of action of proteins involved in bacterial cell division bypasses problems associated with increasingly ineffective variants of older antibiotics; to this end, the essential bacterial cytoskeletal protein FtsZ is a promising target. Recent work on its allosteric inhibitor, PC190723, revealed \textit{in vitro} activity on \textit{Staphylococcus aureus} FtsZ and \textit{in vivo} antimicrobial activities. However, the mechanism of drug action and its effect on FtsZ in other bacterial species are unclear. Here, we examine the structural environment of the PC190723 binding pocket using PockeTFEATURE, a statistical method that scores the similarity between pairs of small-molecule binding sites based on 3D structure information about the local microenvironment, and molecular dynamics (MD) simulations. We observed that species and nucleotide-binding state have significant impacts on the structural properties of the binding site, with substantially disparate microenvironments for bacterial species not from the \textit{Staphylococcus} genus. Based on PockeTFEATURE analysis of MD simulations of \textit{S. aureus} FtsZ bound to GTP or with mutations that are known to confer PC190723 resistance, we predict that PC190723 strongly prefers to bind \textit{Staphylococcus} FtsZ in the nucleotide-bound state. Furthermore, MD simulations of an FtsZ dimer indicated that polymerization may enhance PC190723 binding. Taken together, our results demonstrate that a drug-binding pocket can vary significantly across species, genetic perturbations, and in different polymerization states, yielding important information for the further development of FtsZ inhibitors.

Author Summary

The rise of antibiotic resistance in microbes that cause dangerous diseases necessitates the development of new drugs with novel mechanisms of antimicrobial activity. The recently identified small molecule PC190723 is a promising therapeutic candidate, as it is the only
drug known to directly inhibit FtsZ, a cytoskeletal protein that is critical for bacterial cell division. However, little is known about the effects of PC190723 in bacterial species other than the pathogen Staphylococcus aureus. Here, we evaluate the PC190723 binding site with currently available crystallographic structures of FtsZ using the structural comparison algorithm PocketFEATURE and all-atom molecular dynamics simulations. Our investigations provide insight into the mechanism of PC190723-based inhibition of FtsZ and its potential use as an inhibitor of as-yet untested bacterial species. We also demonstrate the utility of computational analysis of the drug-binding pockets of related proteins, which we expect to accelerate the screening and development of novel antibiotics to combat human disease.

Introduction

Rises in bacterial antibiotic resistance have motivated the development of new classes of drugs with alternative mechanisms of action [1]. One promising target is the cytoskeletal protein FtsZ, a GTPase and homolog of eukaryotic tubulin that plays a central, conserved role in cell division in both eubacteria and archaea [2]. FtsZ forms protofilaments in vitro [3] and assembles in vivo into a ring-like structure termed the “Z-ring,” which acts as a scaffold to recruit other critical division proteins [4] and constricts the cell as division progresses [5,6]. In vitro studies have shown that FtsZ causes constriction when bound to liposomes, either via a membrane-targeting helix or with its in vivo binding partner FtsA, suggesting that FtsZ can generate mechanical force in addition to its scaffolding function [7–9]. All-atom molecular dynamics (MD) simulations predicted that force generation may result from a dramatic bending in GDP-bound filaments induced by nucleotide hydrolysis [10]; this conformational change was later confirmed by X-ray crystallography [6].

Several compounds have been demonstrated to inhibit FtsZ, primarily through three mechanisms [11]: modulation of FtsZ assembly/disassembly [12,13], GTPase activity [14,15], or degradation [16]. However, many of these compounds have as-yet unknown interactions with FtsZ, large minimal inhibitory concentrations, or are associated with toxic effects that make them unsuitable for therapeutic use [11]. In comparison, the FtsZ inhibitor PC190723 and its derivatives are promising drug candidates. PC190723 inhibited cell division at low minimal inhibitory concentration in many Gram-positive bacteria, such as the model organism Bacillus subtilis and the pathogen Staphylococcus aureus, including the methicillin-resistant S. aureus strain [12]. Encouragingly, PC190723 exhibited antimicrobial properties in vivo in mouse models [12], suggesting that this small molecule selectively targets bacterial cell division without affecting tubulin, the eukaryotic FtsZ homolog. Furthermore, single point mutations conferring resistance to PC190723 in S. aureus were identified within ftsZ, suggesting that PC190723 specifically binds to S. aureus FtsZ (SaFtsZ). This observation was later confirmed via the co-crystallization of an SaFtsZ-PC190723 complex in two independent studies [17,18]; thus, currently PC190723 is the only FtsZ-targeting drug with evidence of direct binding. In both co-crystals, PC190723 binds a pocket beneath the H7 loop close to the C-terminus of FtsZ, and many of the FtsZ residues with identified resistance mutations (for example, G193, G196, and N263) lie within 6 Å of the PC190723 molecule in these structures (Fig. 1). Although the PC190723-binding pocket is quite far from FtsZ’s GTP-binding site, it is situated near the T7 loop, which is thought to contact the GTP-binding pocket of an adjacent FtsZ subunit after polymerization, forming the catalytic active site for GTP hydrolysis [19].
While PC190723 inhibits cell division in a variety of bacterial species such as *S. aureus* and *B. subtilis*, its exact mechanism remains unclear. The initial study of PC190723 showed that addition of PC190723 reduced SaFtsZ GTPase activity *in vitro* [12]; in contrast, a recent study reported that PC190723 actually increased SaFtsZ GTPase activity, and exhibited no effect on the GTPase activity of *B. subtilis* FtsZ (BsFtsZ) [20,21]. *In vitro*, PC190723 induced stable bundling...
of SaFtsZ and BsFtsZ filaments; for FtsZ from *Escherichia coli*, a Gram-negative bacterium whose growth is not inhibited by PC190723 treatment, no such bundling was observed [22]. The diverse behaviors observed after PC190723 treatment in FtsZ from diverse species potentially indicate structural complexity in the drug-binding site that has yet to be explored.

Here, we present a structural analysis of the PC190723-binding pocket of FtsZ from multiple species and across a variety of conformational states. We characterized this binding pocket using PocketFEATURE, a statistical algorithm that performs pairwise comparisons of structural information between small molecule-binding pockets in different proteins [23]. This method was previously used to predict inhibitor binding profiles between kinases [23]. PocketFEATURE divides a ligand-binding pocket into a set of microenvironments based on local structural and chemical information, and scores the similarity between this set of microenvironments with those found in other proteins. Using PocketFEATURE, we compared the PC190723-binding pocket from the SaFtsZ-PC190723 co-crystal with FtsZ crystal structures from multiple bacterial and archaeal species, including the Gram-positive *S. aureus*, *B. subtilis*, and *Mycobacterium tuberculosis*, the Gram-negative *Pseudomonas aeruginosa*, and the archaeon *Methanocaldococcus jannaschii* (Table 1). The resulting PocketFEATURE score is a measure of the similarity of that pocket to that of the SaFtsZ-PC190723 co-crystal. PocketFEATURE scores revealed that the microenvironment of the PC190723-binding pocket is dependent on species and the nucleotide-binding state of the protein, with the best similarity scores belonging to *Staphylococcus* species bound to a GDP nucleotide. Similarity scores computed from the coordinates of all-atom MD simulations preserved the ranking order determined by their static crystal structures counterparts, with PC190723-resistant SaFtsZ mutants harboring pockets that were less similar to the SaFtsZ-PC190723 co-crystal than wild-type SaFtsZ pockets. Furthermore, all FtsZ proteins, except those from *Staphylococcus* species, had pockets that were no more similar to the SaFtsZ-PC190723 co-crystal structure than PC190723-resistant SaFtsZ mutants. Finally, FtsZ polymerization increased the pocket similarity of wild-type SaFtsZ to the SaFtsZ-PC190723 co-crystal, but not that of a PC190723-resistant mutant. Taken together, our results suggest that PC190723 binds best to polymerized, nucleotide-bound FtsZ of *Staphylococcus* species.

**Results**

**PC197023 pocket scores from FtsZ crystal structures are highly species-dependent**

Crystal structures of SaFtsZ bound to PC190723 (PDB ID: 4DXD and 3VOB) [17,18] suggest that PC190723 induces antimicrobial activity in this organism by specifically binding FtsZ, but no evidence of direct binding exists in other bacterial species. To probe how species-specific features of the PC190723-binding pocket’s three-dimensional microenvironment affect the drug’s affinity for FtsZ, we mapped the PC190723-binding pocket onto FtsZ molecules from various species (Fig. 1, S1 Fig, Methods) and computed scores for their similarity to the SaFtsZ-PC190723 co-crystal (Fig. 2A, Table 1). The similarity scores roughly fell into categories of high, medium, and low similarity. GDP-bound structures of SaFtsZ and *Staphylococcus epidermidis* FtsZ (SeFtsZ) were highly similar to the binding pocket in the SaFtsZ-PC190723 co-crystal, consistent with a previous report that both species are sensitive to PC190723 [12]. Interestingly, crystal structures of SaFtsZ had similar pocket scores to each other whether or not PC190723 was bound (Fig. 2A). In contrast, we obtained worse similarity scores for FtsZ from all other species; *B. subtilis* (BsFtsZ) and *M. tuberculosis* (MtFtsZ) structures had only intermediate similarity to the SaFtsZ-PC190723 co-crystal, despite evidence of PC190723 sensitivity in *B. subtilis* [12]. FtsZ structures from all other species were associated with comparatively poor similarity scores (Fig. 2A), suggesting that these pockets are less conducive
Table 1. Description of FtsZ crystal structures for which PocketFEATURE scores were computed.

| Organism                        | PDB  | Ligand(s)                        | Chain | Pocket score | Resolution (Å) |
|---------------------------------|------|----------------------------------|-------|--------------|----------------|
| Aquifex aeolicus                | 2R6R | GDP                              | 1     | -4.124       | 1.7            |
| Aquifex aeolicus                | 2R75 | 8-morpholino-GTP, magnesium      | 1     | -3.755       | 1.4            |
| Bacillus subtilis               | 2RHH | sulfate                          | A     | -6.813       | 2              |
| Bacillus subtilis               | 2RHU | sodium, Glycol, sulfate, acetate | A     | -6.837       | 1.76           |
| Bacillus subtilis               | 2RHL | GDP                              | A     | -7.161       | 2.45           |
| Bacillus subtilis               | 2RHL | GDP                              | B     | -7.124       | 2.45           |
| Bacillus subtilis               | 2RHO | GDP, GTPγS                       | A     | -6.96        | 2.45           |
| Bacillus subtilis               | 2RHO | GDP, GTPγS                       | B     | -6.722       | 2.45           |
| Bacillus subtilis               | 2VAM | sulfate                          | A     | -7.413       | 2.5            |
| Bacillus subtilis               | 2VXY | citrate, potassium               | A     | -6.673       | 1.7            |
| Methanocaldococcus jannaschii   | 2VAP | GDP                              | A     | -4.264       | 1.7            |
| Methanocaldococcus jannaschii   | 1W58 | G2P (ester), magnesium           | A     | -3.29        | 2.5            |
| Methanocaldococcus jannaschii   | 1W59 | sulfate                          | A     | -2.932       | 2.7            |
| Methanocaldococcus jannaschii   | 1W59 | sulfate                          | B     | -2.825       | 2.7            |
| Methanocaldococcus jannaschii   | 1W5A | GTP, magnesium                   | A     | -3.642       | 2.4            |
| Methanocaldococcus jannaschii   | 1W5A | GTP, magnesium                   | B     | -3.147       | 2.4            |
| Methanocaldococcus jannaschii   | 1W5B | GTP                              | A     | -3.324       | 2.2            |
| Methanocaldococcus jannaschii   | 1W5B | GTP                              | B     | -3.582       | 2.2            |
| Methanocaldococcus jannaschii   | 1FSZ | GDP                              | A     | -2.621       | 2.8            |
| Mycobacterium tuberculosis      | 1RLU | GTPγS, glycerol                  | A     | -5.899       | 2.08           |
| Mycobacterium tuberculosis      | 1RLU | GTPγS, glycerol                  | B     | -7.024       | 2.08           |
| Mycobacterium tuberculosis      | 1RQ2 | citrate                          | A     | -7.146       | 1.86           |
| Mycobacterium tuberculosis      | 1RQ2 | citrate                          | B     | -7.051       | 1.86           |
| Mycobacterium tuberculosis      | 2Q1X | citrate                          | A     | -6.672       | 2.35           |
| Mycobacterium tuberculosis      | 2Q1X | citrate                          | B     | -6.098       | 2.35           |
| Mycobacterium tuberculosis      | 1RQ7 | GDP                              | A     | -6.867       | 2.6            |
| Mycobacterium tuberculosis      | 1RQ7 | GDP                              | B     | -7.362       | 2.6            |
| Mycobacterium tuberculosis      | 2Q1Y | GTPγS                            | A     | -6.889       | 2.3            |
| Mycobacterium tuberculosis      | 2Q1Y | GTPγS                            | B     | -7.099       | 2.3            |
| Mycobacterium tuberculosis      | 4KWE | GDP                              | A     | -2.916       | 2.91           |
| Mycobacterium tuberculosis      | 4KWE | GDP                              | B     | -3.656       | 2.91           |
| Mycobacterium tuberculosis      | 4KWE | GDP                              | C     | -2.919       | 2.91           |
| Pseudomonas aeruginosa           | 1OFU | GDP                              | A     | -4.052       | 2.1            |
| Pseudomonas aeruginosa           | 1OFU | GDP                              | B     | -4.154       | 2.1            |
| Pseudomonas aeruginosa           | 2VAW | GDP                              | A     | -4.945       | 2.9            |
| Staphylococcus aureus            | 3VO8 | GDP, Calcium                      | A     | -10.82       | 2.26           |
| Staphylococcus aureus            | 3VO8 | GDP, Calcium                      | B     | -10.754      | 2.26           |
| Staphylococcus aureus            | 3VO9 | Selenomethionine                  | A     | -5.784       | 2.71           |
| Staphylococcus aureus            | 3VO9 | Selenomethionine                  | B     | -4.298       | 2.71           |
| Staphylococcus aureus            | 3VO9 | Selenomethionine                  | C     | -4.243       | 2.71           |
| Staphylococcus aureus            | 3VO9 | Selenomethionine                  | D     | -5.201       | 2.71           |
| Staphylococcus aureus            | 3VOA | GDP, Calcium                      | A     | -10.755      | 1.73           |
| Staphylococcus aureus            | 3VOB | GDP, 9PC                         | A     | -11.201      | 2.7            |
| Staphylococcus aureus            | 3VPA | APO                              | A     | -4.32        | 2.49           |
| Staphylococcus aureus            | 3VPA | APO                              | B     | -4.917       | 2.49           |
| Staphylococcus aureus            | 3VPA | APO                              | C     | -4.909       | 2.49           |
| Staphylococcus aureus            | 3VPA | APO                              | D     | -5.109       | 2.49           |

(Continued)
to PC190723 binding. Notably, binding site microenvironment comparison performed by PocketFEATURE captures details that are not detected solely through structural analysis. Root mean squared deviation (RMSD) of the pockets of the crystal structures to the pocket of the SaFtsZ-PC190723 co-crystal (Fig. 2B) clearly identified that Staphylococcus species have pocket structures that are more similar to that of the SaFtsZ-PC190723 co-crystal, but in contrast to PocketFEATURE, RMSD is unable to distinguish among non-Staphylococcus species.

Table 1. (Continued)

| Organism                  | PDB  | Ligand(s)           | Chain | Pocket score | Resolution (Å) |
|---------------------------|------|---------------------|-------|--------------|----------------|
| Staphylococcus aureus     | 4DXD | GDP, 9PC            | 1     | -12          | 2.01           |
| Staphylococcus epidermidis| 4M8I | GDP                 | A     | -10.001      | 1.43           |
| Thermobifida fusca        | 4E6E | chloride, magnesium, sulfate | A | -3.471       | 2.12           |

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Fig 2. PC197023-binding pocket similarity scores depend on species and nucleotide-binding state. (A) PocketFEATURE scores for FtsZ structures from various species compared to the PC190723-binding pocket of SaFtsZ (PDB ID: 4DXD). GDP-bound Staphylococcus species display more similarity (more negative scores) to the SaFtsZ-PC190723 co-crystal, with APO Staphylococcus structures and structures from other bacterial species showing less similarity to the SaFtsZ-PC190723 co-crystal. (B) RMSD of the 3D coordinates of FtsZ crystal structures from the PC190723 co-crystal coarsely separate the proteins into close and distant relations of GDP-bound Staphylococcus FtsZ, but this measurement does not capture additional features of the drug pockets that distinguish between structures of medium and low similarity to the SaFtsZ-PC190723 co-crystal.

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PC197023 pocket score is dependent on nucleotide state

For SaFtsZ, we also noted a marked decrease in similarity score for the APO state (no nucleotide) relative to GDP-bound structures, with APO scores similar to that of BsFtsZ (Fig. 2A). This observation suggests that conformational changes associated with nucleotide binding may tune the binding affinity of PC190723 for FtsZ; no GTP-bound SaFtsZ structures exist, leaving it unclear whether GTP hydrolysis significantly affects pocket score. Interestingly, there did not appear to be any nucleotide dependence in 8sFtsZ (Fig. 2A), and MfFtsZ displayed the opposite tendency to SaFtsZ (Fig. 2A), with worse similarity to the SaFtsZ-PC190723 co-crystal for GDP-bound structures relative to GTP-bound or APO structures. However, all BfFtsZ and MfFtsZ binding pockets have medium to low similarity compared to GDP-SaFtsZ, and thus may not be optimal for PC190723 binding regardless of nucleotide state. These data suggest that the identity of the nucleotide bound to an FtsZ monomer has variable effects on PC190723 binding across species. In the case of SaFtsZ, pocket scores predict that PC190723 is more likely to bind FtsZ when GDP is also bound to it.

Resistance mutations substantially reduce PC190723 pocket scores

We sought to determine if FtsZ mutations distort the microenvironment of the PC190723 binding site. We therefore compared the pockets of wild-type SaFtsZ with those of the PC190723-resistant mutants G193D, G196C, and N263K [12]. Since crystal structures of these mutants do not exist, we conducted all-atom MD simulations on SaFtsZ GDP-bound wildtype and drug-resistant (mutant) monomers in order to evaluate how the mutations affected the PC190723-binding pocket. All SaFtsZ simulations were initialized from the crystal structure of the GDP-bound SaFtsZ without PC190723 (PDB ID: 3VO8) (Fig. 3A, Methods). We observed an initial, rapid decline in similarity to the static FtsZ-PC190723 co-crystal for all simulated monomers. Close investigation into the individual microenvironments contributing to the pocket similarity score for each frame revealed that while the majority of the 20 residues included in the pocket score computation remained within the binding pocket, some had decreased contribution to the total similarity score. For example, over the simulation trajectory of the wild-type SaFtsZ monomer, an average of 17 out of the 20 residues contributed to the similarity score at any given time point during the simulation, resulting in an overall shift in the PocketFEATURE score to less negative values (decreased similarity). Thus, small fluctuations in the amino acid functional centers within the pocket can perturb the local physiochemical properties calculated for each residue, pointing to the subtle structural balance required for the most optimal drug-binding environment that can be extremely sensitive to thermal fluctuations. Nevertheless, the time-scale and magnitude of this change as the simulations reached a steady state were consistent across all FtsZ monomers (Fig. 3A), indicating that comparisons between simulated protein structures can be established.

To compare pocket scores from MD trajectories across species, we also carried out MD simulations of ScFtsZ and BsFtsZ monomers initialized from their crystal structures (PDB IDs: 2RHL and 4M8I, respectively); we observed similar initial decreases in pocket similarity (Fig. 3A). Despite the general change in pocket score to the SaFtsZ-PC190723 co-crystal in all simulations relative to the pocket score computed from static crystal structures, SaFtsZ and ScFtsZ monomers maintained better similarity to the SaFtsZ-PC190723 co-crystal compared to the BsFtsZ monomer. These results are consistent with the comparisons of pocket score magnitudes determined from static crystal structures (Fig. 2A), indicating that important features of the PC190723-binding pocket are conserved in MD simulations.

All three SaFtsZ mutant monomers had significantly worse pocket scores in comparisons with the SaFtsZ-PC190723 co-crystal than a wild-type monomer, and similar scores to that of
Further examination of the microenvironment pocket scores of the SaFtsZ mutants and BsFtsZ (Fig. 3B) revealed that a subset of residues (203–207 in \(S.\) \textit{aureus}) across all four simulations had average score magnitudes below those of wild-type SaFtsZ. Interestingly, these residues correspond to amino acids within the T7 loop, a highly conserved structural element near the site of monomer association that plays an important role in GTP hydrolysis [19]. In \textit{E. coli}, mutations affecting GTP hydrolysis occur either in the nucleotide pocket or in the T7 loop [2,19]. Truncations of this loop in SaFtsZ disrupt hydrolysis [24]. Taken together, affinity of PC190723 to FtsZ is likely closely coupled to the FtsZ structural transition that occurs during the hydrolysis cycle, and mutations in FtsZ can sufficiently disrupt the PC190723 binding environment.
Absence of nucleotide, but not nucleotide hydrolysis, strongly affects PC190723 pocket score

Given the large difference in pocket scores in comparisons with the SaFtsZ-PC190723 co-crystal between APO and GDP-bound SaFtsZ crystal structures, we wondered whether it was nucleotide binding or hydrolysis that had a greater effect on pocket microenvironment. Since there are no GTP-bound crystal structures of SaFtsZ, we conducted MD simulations of the APO state and GTP-bound SaFtsZ monomers (Fig. 3C) to complement our GDP-bound monomer simulations (Fig. 3A). Due to the potential for small atomic differences between the GTP- and GDP-bound states of FtsZ, we conducted three independent simulations of each monomer (Fig. 3C). For each replicate, we observed that GTP- and GDP-bound SaFtsZ had similar drug-pocket scores, and better similarity scores than the APO state. These results illustrate the sensitivity of the drug pocket to allosteric effects of the nucleotide bound on the opposite side of the protein, and suggest that occupancy of the nucleotide-binding site, rather than the identity of the nucleotide located there, alters the conformation of the pocket to favor PC190723 binding.

To test whether the chemical and physical properties evaluated by PocketFEATURE are captured by traditional structural-only metrics such as RMSD, we calculated the RMSD of the 20 pocket residues aligned to the same 20 residues of the 4DXD SaFtsZ-PC190723 co-crystal structure for each MD trajectory (Fig. 3D). Large-magnitude pocket scores did not correspond with low RMSD from the SaFtsZ-PC190723 co-crystal, with SaFtsZ and ScFtsZ having values intermediate between the low values of APO SaFtsZ and BsFtsZ and the high values of SaFtsZG193D. Taken together, these data indicate that PocketFEATURE provides a distinctly informative metric for comparing similarity between pockets, and that PC190723 binding may be unique to Staphylococcus species.

FtsZ polymerization improves the PC190723 pocket score

FtsZ hydrolysis and polymerization are intrinsically coupled. Key catalytic residues for GTP hydrolysis reside on the T7 loop of the adjacent subunit near the dimer interface [19], and mutations near the active site designed to modulate hydrolysis also affect the dynamics of polymer assembly [25]. Since the PC190723-binding pocket is located close to this interface, we hypothesized that it may be affected by FtsZ polymerization. We conducted MD simulations of GDP-bound dimers of wild-type SaFtsZ (Fig. 4A) and the G193D mutant, and examined the pocket scores of each subunit of the dimers (Fig. 4B). In the subunits of the wild-type dimer, we observed a statistically significant difference (t-test, \( p < 2.2e-16 \)) in pocket scores, with the subunit with the pocket closest to the dimer interface having a better similarity score to the SaFtsZ-PC190723 co-crystal (Fig. 4B). No such difference was evident in the PC190723-resistant G193D mutant dimer; both subunits had pocket scores throughout the trajectory that were similar to those of a wild-type subunit with a pocket away from the dimer interface (Fig. 4B).

At later times in the MD simulation, the PC190723 pocket score of the wild-type dimer subunit with the better pocket score rapidly shifted to a value similar to that of the other subunit with poorer similarity (S2A Fig). To probe the cause of this change, we examined the conformations of the wild-type dimer from 130–180 ns. We observed a conformational shift at the dimer interface that opens the GDP-binding pocket, likely as a prelude to the release of the bound nucleotide (S2B Fig). Given that this biologically relevant release of GDP at the dimer interface has a corresponding drastic affect on the PC190723-binding pocket in our MD simulations (S2A Fig), this further confirms the allosteric coupling between the bound nucleotide and PC190723 binding. Since we were interested in the PC190723-binding pocket in a stable...
GDP-bound dimer, we focused on the pocket similarity score during the first 120 ns of the trajectory (Fig. 4B), which showed that polymerization increases the pocket similarity to the SaFtsZ-PC190723 co-crystal as compared with FtsZ monomers. While these calculations were made over a shorter timescale than that required for the system to reach convergence, our results suggest that polymerization induces pocket deformations with an increased likeness to the co-crystal drug pocket.

Discussion

Our analyses of FtsZ crystal structures and MD trajectories indicate that multiple factors including species, allosteric binding, genetic perturbations, and polymerization state all contribute to the conformations of the PC190723-binding pocket. In particular, polymerization and allosteric binding of a guanosine nucleotide play a pivotal role in stabilizing the PC190723 pocket; the drug pocket is also subject to species-level differences, despite the close structural similarities of FtsZ from all characterized species at the level of entire monomers [26]. Using
all-atom MD simulations to quantify perturbations to the PC190723-binding pocket due to resistance-inducing mutations, we have provided an indirect assessment of PC190723 binding affinity that can be used as a point of comparison for at least two relevant applications. First, our results impart insight into the interpretation of experimental results suggesting different responses of SaFtsZ and BsFtsZ to PC190723 [20]. Second, our analyses illustrate how evaluation of the 3D environment of a drug-binding pocket can illuminate the molecular properties and mechanisms governing drug-protein interactions.

Our prediction that PC190723 has high affinity for its binding pocket in FtsZ structures from the *Staphylococcus* species but not in FtsZ from other species has important implications for its development as a future therapeutic. Previously, experimental evidence of PC190723’s antimicrobial properties in *S. aureus* and *B. subtilis* seemed to agree well with *in vitro* studies indicating that the drug acts through excess polymer stabilization [12,22]; in contrast, another *in vitro* study reported increased enzymatic activity in SaFtsZ and no change in BsFtsZ [20]. Our analysis indicates that the PC190723-binding pocket in BsFtsZ is less similar to the SaFtsZ-PC190723 co-crystal than the non-drug bound SaFtsZ structure, both as a static crystal structure (Fig. 2A) and in our MD trajectories (Fig. 3A). This observation, combined with evidence from our MD simulations that BsFtsZ pocket scores are on par with the scores of point mutations that confer PC190723 resistance in *S. aureus* [Fig. 3A], argues that BsFtsZ is unlikely to strongly bind PC190723 in this particular pocket, and therefore is likely inhibited by PC190723 either via binding to an alternate, unidentified pocket or through an unknown, indirect mechanism. Our analysis of the pocket’s microenvironment indicates that a major reason for the decrease in pocket similarity is the incompatibility of the residues around the catalytically important T7 loop near the polymerization interface (Fig. 3D); in the case of BsFtsZ, our data are more consistent with *in vitro* evidence that BsFtsZ GTPase activity is unaffected by the addition of PC190723 [20]. Taken together, the magnitude of the drop in pocket similarity for all species other than *S. aureus* and *S. epidermidis* indicates that PC190723 may be specifically effective for binding to *Staphylococcus* FtsZ proteins and very close relatives. One limitation of the PocketFEATURE algorithm is that evaluation requires some *a priori* knowledge or estimate of a potential binding site, either from a similar protein structure or through cavity detection software. Our PocketFEATURE analysis specifically evaluated one subset of microenvironments within the pocket identified in the SaFtsZ-PC190723 co-crystal; it is possible that PC190723 may bind to FtsZ from other species either through an alternative pocket or through different contacts within the same pocket. Genetic studies of mutations in FtsZ from other species that provide resistance to PC190723 would clarify whether such binding pockets exist. Evaluating whether PC190723 exerts similar antimicrobial effects on the other species we investigated in this study would yield further insight into the potential efficacy of PC190723 and its derivatives as universal, cross-species bactericides. This determination is particularly important for *M. tuberculosis*, another virulent bacterial species that is resistant to a variety of therapeutic drugs. Although our PocketFEATURE results indicate that the 3D microenvironments of BsFtsZ and MtFtsZ are similar (Fig. 2A), suggesting that PC190723 does not bind to the same pocket in MtFtsZ and SaFtsZ, confirmation of the inhibition of cell division in *M. tuberculosis* would justify the investigation of PC190723 compatibility with other potential binding pockets on the surface of MtFtsZ.

Our predictions that nucleotide binding and polymerization significantly enhance PC190723 binding to SaFtsZ (Fig. 4B) are consistent with experimental evidence that PC190723 stabilizes polymer formation [22]. In current models of Z-ring assembly and function, FtsZ monomers in the cytosol bind GTP, polymerize into filaments either in the cytoplasm or on the membrane, hydrolyze, and then return to the cytosol to further the constriction process [27–29]. Our simulations of APO, GDP-, and GTP-bound SaFtsZ
monomers (Fig. 3B) and a GDP-bound SaFtsZ dimer (Fig. 4B) capture many of the FtsZ structural states at various points of this cycle, and our pocket scores indicate that the optimal PC190723-binding pocket exists when FtsZ is in a nucleotide-bound, polymerized state. Our observations that the PC190723-binding pocket is disrupted as the FtsZ dimer begins to open at the nucleotide-binding site near the dimer interface (S2 Fig) motivate future simulations of FtsZ dimers bound to PC190723 to determine whether the dimer is stabilized. Additionally, further experiments exploring PC190723 binding to an SaFtsZ dimer in various nucleotide states to validate the predictions of our MD simulations of GTP-bound SaFtsZ may shed light onto the mechanism by which PC190723 inhibits cellular division in S. aureus.

Our study also has important implications regarding the strengths and versatility of PocketFEATURE. By evaluating the local physical and chemical properties that dictate drug binding, we can begin to build a mechanistic understanding of the general effects of protein microenvironments. Previous applications of PocketFEATURE demonstrated its ability to distinguish similar binding pockets in static, unrelated proteins [23]. However, the significance of PocketFEATURE scores for binding affinity has only been loosely correlated. Importantly, our study showcases PocketFEATURE’s ability to distinguish pockets in structures with close homology (Fig. 3A,B) that had scores within a smaller range than was previously studied [22]. Our results establish that PocketFEATURE is able to not only distinguish aspects of the drug-binding pocket in FtsZ structures from different species that are not evident from methods such as RMSD, which only considers structural information; the algorithm also can evaluate how protein pockets in molecules from resistant mutants may differ from the optimal binding structure. PocketFEATURE can therefore aid in the design of alternative mutants with equivalent structural and chemical features. Applications of PocketFEATURE are limited in part by the dependence of similarity score evaluation on the set of existing 3D structures of protein-drug co-crystals, a data set that determines the range of pocket variability. Excitingly, our work shows that relevant differences in pocket structure can be detected in dynamic trajectories (Fig. 3A, B and 4B), highlighting the utility of MD simulations for exploring protein-drug interactions, particularly in mutant proteins whose structures have yet to be solved through X-ray crystallography or nuclear magnetic resonance.

**Methods**

**Microenvironment characterization and PocketFEATURE scores**

To compare inhibitor-binding pockets across FtsZ homologs, we aligned the proteins (S1 Fig) to identify the pocket residues and then utilized the PocketFEATURE method [23] in which microenvironments are defined as spherical regions around the functional centers of a residue. Utilizing the FEATURE characterization system [30], we calculated 80 physiochemical properties over six concentric spherical shells within each microenvironment, with a total microenvironment radius of 7.5 Å [30]. Each microenvironment is therefore represented by a feature vector with 480 properties. PocketFEATURE evaluates pocket similarity by combinatorially comparing the microenvironments of the two pockets and calculating a Tanimoto coefficient ($T_c$) for each pair, where $T_c$ is the ratio of the numbers of similar properties and total unique properties. These $T_c$ scores are then evaluated against a set of equivalent microenvironment pairs derived from a dataset of 1116 sites from non-redundant 3D structures in the PDB, fit to a normal distribution, and normalized for background frequency. Microenvironments are aligned to the mutual best scoring microenvironment pairs with a normalized $T_c$ score $<-3$. For example, for two sites A and B, microenvironments A1 and B1 will only be aligned if the scores for all other combinations of microenvironment pairs are smaller in magnitude. The
PocketFEATURE binding site similarity score is the sum of the best-scoring, non-redundant microenvironment pairs. Further details of the method can be found in [23].

In this study, we define the “ideal” PC190723-binding pocket as the microenvironment of the 20 amino acid residues within 6 Å of the PC190723 molecule in an SaFtsZ-PC190723 co-crystal structure (PDB ID: 4DXD) [17]. For comparison with other crystal structures, we defined the PC197023-binding pocket as the equivalent amino acids after a structural alignment (Fig. 1, S1 Fig) with the flexible structure alignment method jFATCAT on RCSB.org [31]. We then used PocketFEATURE to compare the feature lists of each protein pocket to the SaFtsZ-PC190723 binding pocket and output a similarity score. This method was applied to both static FtsZ crystal structures and to dynamic structures of FtsZ simulated via MD.

**Equilibrium MD simulations**

MD simulations were performed on the FtsZ crystal structures of *S. aureus* (PDB ID: 3VO8), *S. epidermidis* (PDB ID: 4M8I), and *B. subtilis* (PDB ID: 2RHL) using NAMD [32] with the CHARMM27 force field [33,34] and CMAP corrections [35]. Simulations were initialized from crystal structures with missing residues at the N- and C-termini, but no missing internal residues. For *S. aureus* FtsZ, residues 12–316 of chain A were present; for *S. epidermidis* FtsZ, residues 11–321; for *B. subtilis* FtsZ, residues 12–316. All water molecules retained in the crystal structure were removed prior to simulation and the structures were then resolvated in a box of water molecules described by the TIP3P model [36] with 14 Å padding and neutralized with NaCl using the solvate and auto-ionize VMD extensions [37]. Single point mutations were introduced into the *S. aureus* crystal structure with the mutate residue VMD modeling extension [37]. *S. aureus* FtsZ crystal structures were initialized from the 3VO8 structure. All structures underwent energy minimization prior to the equilibrium simulations. Long-range electrostatic forces were evaluated via the particle-mesh Ewald summation approach with a grid spacing of <1 Å. An integration time step of 2 fs was used [38]. Bonded terms and short-range, non-bonded terms were evaluated every time step, and long-range electrostatics were evaluated every other time step. Constant temperature (T = 310 K) was maintained using Langevin dynamics [39], with a damping coefficient of 1 ps⁻¹. A constant pressure of 1 atm was enforced using the Langevin piston algorithm [40] with a decay period of 200 fs and a time constant of 50 fs.

For all analyses of MD simulations, amino acid pocket scores were evaluated for the last 30 ns of the trajectories to ensure structural equilibration. Smoothing was carried out with the “geom_smooth” function using a generalized additive model method in the R package ggplot2 [41].

**Supporting Information**

S1 Fig. Sequence alignment of FtsZ proteins from eight prokaryotic species. Blue, strictly conserved amino acids; red, highly conserved amino acids (75% conservation). The secondary structure elements of SaFtsZ appear above the sequence; orange and green bars denote helices and sheets, respectively. (EPS)

S2 Fig. MD simulations predict a relationship between PocketFEATURE score and the conformation of the T7 loop. (A) 180-ns MD trajectories of FtsZ wild-type dimers (red/orange) and mutant dimers (green). A and B refer to the subunits with the PC190723 pocket exposed to the solvent and situated close to the dimer interface, respectively. At ~130 ns, we observed a large decrease in the similarity of the wild-type subunit B pocket to the SaFtsZ-PC190723 co-
crystal. (B) This drop is concurrent with a large change in the protein structure over the next 50 ns. By 180 ns, the GDP molecule (blue) is exposed to the solvent due to a conformational change in the T7 loop.

EPS

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Author Contributions
Conceived and designed the experiments: AM JH TL GT RBA KCH. Performed the experiments: AM JH TL GT. Analyzed the data: AM JH. Contributed reagents/materials/analysis tools: AM JH TL GT. Wrote the paper: AM JH TL GT RBA KCH.

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