Targeting the Assembly of Bacterial Cell Division Protein FtsZ with Small Molecules

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Ⓢ *Supporting Information*

ABSTRACT: FtsZ is the key protein of bacterial cell division and an emergent target for new antibiotics. It is a filament-forming GTPase and a structural homologue of eukaryotic tubulin. A number of FtsZ-interacting compounds have been reported, some of which have powerful antibacterial activity. Here we review recent advances and new approaches in modulating FtsZ assembly with small molecules. This includes analyzing their chemical features, binding sites, mechanisms of action, the methods employed, and computational insights, aimed at a better understanding of their molecular recognition by FtsZ and at rational antibiotic design.
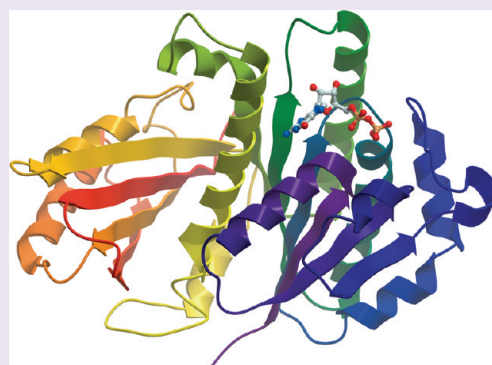
Cell division protein FtsZ is employed by most bacteria to divide. It forms the cytokinetic Z-ring at the division site[1] (Figure 1), is tethered to the inner face of the plasma membrane by FtsA and ZipA, and recruits other accessory proteins of the cell division machinery (divisome), several of which are essential for remodeling the cell wall peptidoglycan at the septum.[2,3] The assembly of the Z-ring is coordinated with DNA segregation and cell growth through many regulatory proteins in different bacteria.[2] The bacterial division proteins differ from the proteins of eukaryotic cytokinesis. Thus, bacterial cytoskeleton and cell division have been recognized as attractive targets for seeking new antibiotics[4,5] with which to fight the widespread emergence of pathogens resistant to current antibiotics.[6] FtsZ has been validated as a target for antibacterial intervention with a synthetic compound active on an *in vivo* model of infection.[7] Very recently, it has been found that antibiotic acyldepsipeptides activate bacterial ClpP peptidase to degrade FtsZ.[8] Here we review small molecule approaches for targeting FtsZ assembly.

FtsZ is a cytoskeletal assembling GTPase that shares the structural fold of eukaryotic tubulin, consisting of an N-terminal nucleotide binding domain and a GTPase activation domain, but with different C-terminal extensions.[9–11] FtsZ monomers assemble forming polar filaments in which the GTP binding site is at the axial association interface between consecutive subunits (Figure 1). Upon filament formation the GTPase-activating domain of one subunit contacts the nucleotide binding site of the subunit below, providing the co-catalytic aspartate residue that completes the active site.[12] Following nucleotide hydrolysis to GDP, FtsZ filaments bend and disassemble.[13,14] Free GDP-

bound subunits exchange their nucleotide and recycle into the FtsZ polymers[15] (Figure 1). Thus, the assembly of purified FtsZ[16] and of FtsZ within the Z-ring in bacterial cells is dynamic, with a subunit half-life on the order of 10 s.[17]

Members of the distinct tubulin/FtsZ superfamily of GTPases[10] assemble forming different functional polymers made of tubulin-like protofilaments.[12] They are thought to have evolved from a primitive GTP-binding protein that formed curving filaments that exerted intracellular forces.[18] Insight into the elusive structure of the bacterial divisome has been provided by cryo-electron tomography of a particular bacterium, *Caulobacter crescentus*, which revealed a putative FtsZ ring consisting of several short (∼100 nm long) and apparently single-stranded FtsZ filaments, attached to the plasma membrane near the division site.[19] Super-resolution light microscopy results suggest that the Z-ring of *Escherichia coli* is composed of a loose bundle of FtsZ protofilaments[20,21] possibly cross-linked by several accessory divisomal proteins. Filaments assembled from purified FtsZ can also coalesce into pairs, bundles, sheets, and other condensates.[22–24] It has been suggested that FtsZ polymers generate the membrane constriction force through iterative cycles of GTP hydrolysis, depolymerization, and repolymerization.[19] Several theoretical models have proposed how FtsZ filament condensation and bending coupled to peptidoglycan septum growth can drive cell division.[25–31] Contractile FtsZ rings have been reconstituted
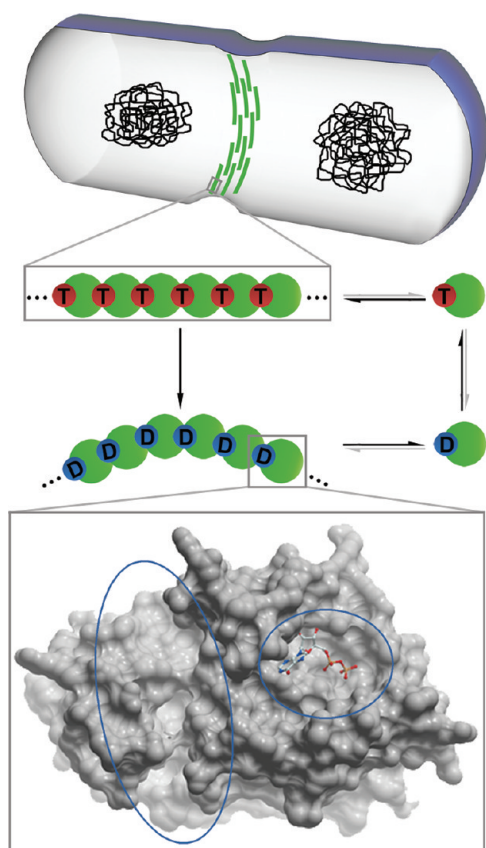
Figure 1. Simplified overview of FtsZ and its role in bacterial cell division. Top: scheme of the localization of the constricting Z-ring made of FtsZ laterally associated filaments (green) at the site of cell division between two daughter bacterial cells that have already separated their DNA (black), in an inside view of a half cell. Middle: architecture and dynamics of an FtsZ filament. Each bead represents an FtsZ molecule, T stands for bound GTP and D for GDP (see text). Bottom: a surface representation of an FtsZ molecule (gray) in a rotated view showing bound GDP at its binding site (encircled, GDP is shown in colored balls and sticks) and the long cleft (oval on the left) between the nucleotide binding and the GTPase activating domains.

that generate constriction force on membranes, and their dynamics has been shown to depend on GTP hydrolysis,[32−34] supporting the idea that FtsZ is capable of generating force for bacterial division. Thus, disturbance of FtsZ assembly dynamics can lead to blocking bacterial cell division.

These characteristics underscore the key role of the nucleotide binding site in regulating FtsZ's function. In addition, there is evidence for a structural activation switch built into FtsZ monomers, which is induced by the assembly contacts and explains the cooperative behavior of single-stranded FtsZ filament polymerization.[22,35−37] In this view, GTP hydrolysis fine-tunes the assembly switch by increasing the unfavorable free energy difference between the inactive and active FtsZ conformations.[22] This contrasts with the classical GTPase switches in which the GTP/GDP binding induces an activating structural change in the unassociated protein.[38] Mapping the flexibility of the FtsZ molecule with computational methods and mutations[39] has predicted a movement of the C-terminal domain onto the N-terminal domain, which closes the cleft between them and is required for switching between the curved and straight association modes of FtsZ.

Tryptophan mutants and fluorescence quenching detect conformational changes compatible with an interdomain movement.[40] The long cleft between the two FtsZ domains (marked with an oval in Figure 1) has been known for a decade[9] and is a potential binding site for small molecules. Recently, compounds have been identified that target the interdomain cleft,[7] suggesting the possibility of modulating FtsZ function by targeting its assembly switch. Interestingly, this cleft structurally overlaps the taxol binding site of tubulin, a well-known target of interfacial antitumor drugs that themselves helped to unravel microtubule structure and assembly. Note that in quite general terms, if a ligand binds more to the protein monomers than to polymers it will inhibit assembly, whereas if it binds more to the polymers it will enhance their assembly,[41] even though its binding site and mechanism may be unknown. A number of small molecules are known to impair bacterial cell division or FtsZ assembly to different extents (previous reviews, refs 4, 5, 42−44). A recent review has pointed out that several of these cytoskeletal inhibitors may be employed as chemical tools to study the subcellular organization of FtsZ.[45] This review specifically focuses on current approaches to directly target FtsZ using small ligands with promising antibacterial activity. In the following sections we will comprehensively address small molecules reported to affect FtsZ function, experimental approaches to study their mechanisms of action, and how to target the nucleotide site or the interdomain cleft with new compounds modulating FtsZ assembly.
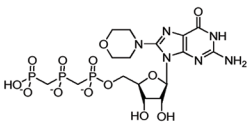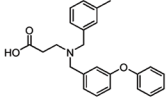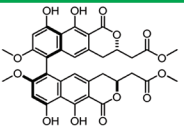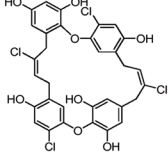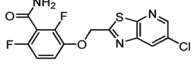
■ SMALL MOLECULE FTSZ INHIBITORS AND METHODS

Since the discovery of its essential role in bacterial cell division, a number of small molecules have been described as inhibiting FtsZ. The reported compounds have different origins: high-throughput screening (HTS) of chemical libraries, natural products discovery, previously known antibacterial compounds, compounds from the tubulin field, and synthetic compounds specifically developed to inhibit FtsZ. A comprehensive list of 40 FtsZ inhibiting compounds of diverse potency and specificity reported to date is provided in Supplementary Table S1, classified into six chemical groups: guanine derivatives, carboxylic acids, phenols and polyphenols, benzam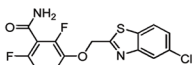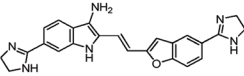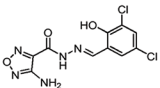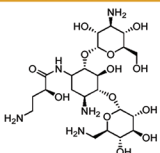ide derivatives, N-heterocycles, and others. Table 1 presents, due to space limitations, several selected examples of these small molecules. Notably, only the guanine nucleotide derivatives have been obtained by a rational design based on the atomic structure of FtsZ.

A number of closely related FtsZ crystal structures are currently available for drug design, including FtsZ from archaea, Gram-positive, and Gram-negative bacteria (though not from *E. coli*). However, the only structure of a FtsZ-inhibitor complex reported at the time of submitting this review is that of FtsZ from *Aquifex aeolicus* with bound 8-morpholino-GTP.[46] Computational docking approaches and *in silico* virtual screening of large chemical libraries for FtsZ inhibitors have met scarce experimental confirmation so far.[47]

Diverse methods have been employed to study the interactions of ligands with FtsZ and bacterial cell division (Supplementary Table S1). A scheme of the workflow in characterizing a FtsZ inhibitor is shown in Figure 2. Biochemical approaches include assembly methods, GTPase, and binding assays. These should be performed at physiological pH and ionic strength, with K^+ and Mg^{2+} cation activities resembling as much as possible the intracellular conditions of

Table 1. Small-Molecule Inhibitors of FtsZ[a]

Chemical group Name (Refs)	Structure	Effects on purified FtsZ	Binding site (Docking Score)[b]	Effects on bacterial cell division / Z-ring	Antibacterial activity MIC (µM)	Cross-reactions with tubulin and eukaryotic cells
Guanine nucleotides 8–morpholino-GTP (46, 47, 79)	(structure)	Inhibits assembly and GTPase in the 100-500 µM range (Ec and Bs) Binds FtsZ with a K_d = 0.4 µM (Mj)	GTP site: crystal structure in complex with FtsZ determined. Competes with a fluorescent nucleotide (Medium)	nd	Ineffective (71)	C8-GTP analogs support microtubule assembly
Carboxylic Acids PC58538 (66)	(structure)	Inhibits assembly and GTPase in the 100 µM range (Bs)	Resistant mutants map around the GTP site (Bs) (Medium)	Filamentation (Bs) and disturbance of Z-ring at 341 µM	Weak. e.g. Bs, 341	nd
Phenols and polyphenols Viriditoxin (61, 80)	(structure)	Inhibits assembly and GTPase in the 10 µM range (Ec)	unknown (High)	Filamentation in *sulA* deleted Ec at 19 µM	Gram + species MRSA, 12	Non-toxic to HeLa cells at 100 µm
Chrysophaentin A (59)	(structure)	Inhibits assembly and GTPase in the 10-50 µM range and binds FtsZ (Ec)	GTP site: GTPγS inhibits binding in STD-NMR. Modelled into the GTP site	nd	MRSA MIC_{50}, 2 Vancomycin resistant *Enterococcus faecium* MIC_{50}, 4	Does not affect tubulin assembly up to 150 µM. Non-toxic to HCT-116 and P388 cells at 74 µM, and to BSC-1 cells at 147 µM
Benzamide derivatives PC190723 (7, 50, 76, 81)	(structure)	Induces assembly and condensation in the µM range (Bs and Sa) and modulates GTPase. Binds FtsZ with a K_d ~ 10 µM (Bs)	Resistant mutants and molecular modelling at the interdomain cleft (Bs) (High docking score in the cleft)	Filamentation and disturbance of Z-ring (Bs) or ballooning (Sa) at 5.6 µM	Bs, 2.8 MRSA, 2.8 Protects mice from a lethal dose of Sa ATCC 19636 (ED_{50}=3.1 mg/kg)	No effect on tubulin at 20 µM. Non-toxic to HepG2 cell at 180 µM. ADME results better than analog 8j
8j (76, 77)	(structure)	Induces assembly and condensation in the µM range and inhibits GTPase in the 10 µM range (Bs)	Possibly the interdomain cleft	Filamentation (Bs) and disturbance of the Z-ring at 0.6 µM. Helical division at 0.06 µM	Sa, ~ 1 Protects mice from a lethal dose of Sa Smith (ED_{50}=41 mg/kg)	Significant plasma protein binding and clearance from hepatocytes in ADME tests
N-Heterocycles Zantrin Z5 (62)	(structure)	Weakly increases assembly and inhibits GTPase in the 5-10 µM range (Ec)	unknown	Filamentation and disturbance of the Z-ring (Ec) in the 40-80 Ec. µM range	Bs JH–642, 40	nd
4-Aminofurazan derivative A189 (68)	(structure)	Inhibits assembly and GTPase in the 100 µM range (Ec)	unknown, GTP site suggested by authors (Medium)	Filamentation and disturbance of Z-ring in Ec M101 at 200 µM	Ec NIHJ JC-2, 405 Sa KMP9, 51	nd
Others Amikacin (82)	(structure)	nd	unknown (High)	Filamentation and disturbance of Z-ring in Ec at 6.8 µM	Ec, lethal at 55 µM	nd

[a]Examples of each chemical group are provided. For a comprehensive list of compounds inhibiting FtsZ and detailed data see Supplementary Table S1. Abbreviations employed for bacterial species: For effects on purified FtsZ, abbreviations refer to the species of origin of FtsZ. Bs, *B. subtilis*; Ec, *E. coli*; Sa, *S. aureus*; Mj, *M. jannaschii*, and Mtb, *M. tuberculosis*. For effects on bacterial cells: Bs, *B. subtilis* 168; Sa, *S. aureus* ATCC 29213; MRSA, methycillin-resistant *S. aureus*; and Mtb, *M. tuberculosis* H37Rv. The *E. coli* strain employed is cited if specified. When a different strain has been used, it is specified. Abbreviations of experimental procedures: ADME, absorption, distribution, metabolism, and excretion; ED_{50}, effective dose 50%; K_d, dissociation constant; MIC, minimum inhibitory concentration; nd, not done; STD-NMR, saturation transfer difference nuclear magnetic resonance. [b]Docking scores indicated only when High or Medium for binding to the nucleotide site. Rating criteria are described in Supplementary Table S2.

bacterial cells,[30] avoiding unnecessary additives such as calcium or polycations. Right angle light scattering is quite popular to monitor the effects of compounds on FtsZ assembly, but reports frequently lack the requisite controls of compound solubility and possible inner filter effects, negative controls with GDP instead of GTP, and disassembly upon GTP consumption. Linear dichroism can also be employed to monitor FtsZ assembly.[48] Often, assembly assays are complemented with electron micrographs, which can give qualitative information of the effect of a ligand on polymer assembly and structure. Small sample ultracentrifugation permits the quantification of steady state FtsZ polymers and the effects of ligands on their assembly. Fluorescence resonance energy transfer[16,40] and anisotropy methods with modified FtsZ[49] can also be employed to measure assembly. FtsZ's GTPase activity is induced by polymerization, and therefore changes induced by
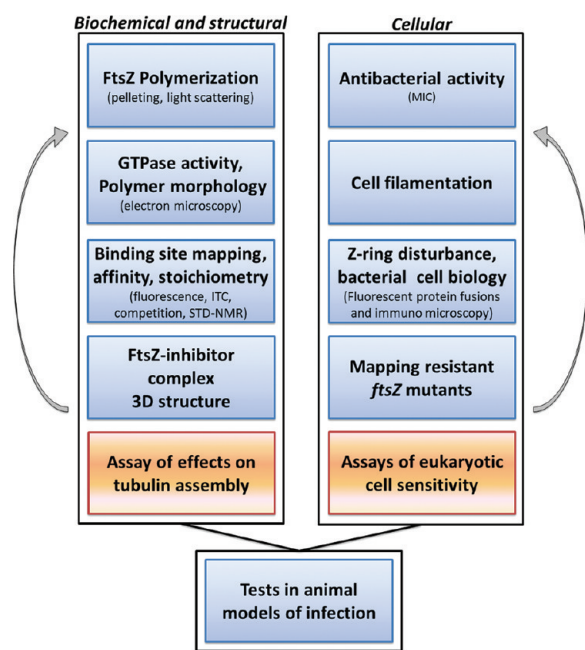
Figure 2. Scheme of the methods that may be employed in going from a hit compound with some activity on FtsZ or bacterial cell (top) toward a lead inhibitor of FtsZ (at the bottom) with characterized mechanisms of action and antibiotic activity (see text). One or several steps of synthetic modification between assays (curved arrows) may be intercalated at some point between the biochemical and cellular assays, resulting in an iterative optimization process.

ligands may simply reflect changes in the extent of polymer formation[46] or be due to modulation of the GTPase activity of the FtsZ polymers.[50] Binding of compounds to FtsZ can be measured employing changes in fluorescence of the compounds themselves,[51−53] of a sensitive tryptophan residue introduced into FtsZ from *E. coli*,[54] or of the fluorescent probe anilino-naphtalenesulfonate (ANS) bound to an unknown site in FtsZ.[55,56] Competition assays to measure ligands binding to the GTP site will be addressed in the nucleotide site section below. Direct ligand binding methods employed include isothermal titration calorimetry (ITC)[57,58] and ultracentrifugation followed by HPLC.[50] Saturation transfer difference nuclear magnetic resonance (STD-NMR) has been employed to map ligand protons in closer contact with FtsZ.[58,59] Biochemical assays have also been employed for the initial discovery of some FtsZ inhibitors. For example, a HTS with a filter assay of fluorescent-FtsZ polymerization[60] was employed to identify viriditoxin from a company library of $>10^5$ extracts.[61] The zantrins were identified by HTS of libraries of $\sim10^4$ small molecules with an enzyme coupled GTPase assay.[62] Interestingly, testing only $\sim10^2$ compounds (29 different scaffolds) with an FtsZ sedimentation assay led to identify one active compound, 3-[5-({4-oxo-2-thioxo-3-[3-(trifluoromethyl)-phenyl]-1,3-thiazolidin-5-ylidene}methyl)-2-furyl]benzoic acid (OTBA).[54]

Several microbiological methods are available to study the effects of inhibitors on FtsZ *in vivo* (Figure 2). The antibacterial spectrum and potency of a compound are commonly evaluated by determining its minimal inhibitory concentration (MIC) in standard assays against a panel of representative bacterial species, which may include pathogenic and antibiotic-resistant strains. Another frequently used method is cell filamentation, a phenomenon that led to the original identification of the *ftsZ*

gene[63,64] and gave the "Fts" (from Filament temperature sensitive) proteins their name. The functional inhibition of FtsZ by a chemical compound in bacterial cells results in impaired division, causing filamentation in bacilli or ballooning in cocci,[7] eventually followed by cell lysis. Antibacterial compounds that do not induce filamentation have been considered off-target regarding cell division.[7,65] In a more specific approach, whether the assembly of the Z-ring is being affected is typically determined by fluorescence microscopy with FtsZ-fluorescent protein fusions or by indirect immuno-fluorescence. However, the Z-ring assembly may be affected by several additional mechanisms, including inhibition of other divisomal components or DNA damaging blocking cell division, by the SOS response or other mechanisms.[45] Therefore, in a further step, whether FtsZ is being targeted directly can be ascertained by generating bacterial mutants resistant to the compound and identifying the mutations in *ftsZ*. In addition, mapping the mutated residues in the FtsZ structure may delineate the putative binding site of the compound.[7,66] Regarding cell-based HTS, since at least *E. coli* and *Bacillus subtilis* apparently lack cell division check points that alter gene expression, a cell-based screen for FtsZ inhibition based on the activation of the σ_F transcription factor during sporulation of *B. subtilis* was designed that allowed the identification of the cell division inhibitor PC58538 from a library of 105,000 synthetic compounds.[66] A screening system (anucleate cell blue assay) to identify inhibitors of chromosome partitioning in *E. coli* was employed to identify the MreB inhibitor A22[67] and also the FtsZ inhibitor A189 from a library of 95,000 compounds.[68]

Standard criteria and methods to validate FtsZ inhibitors should be employed. In our opinion, for a small molecule to be a promising hit, it should specifically interact with purified FtsZ and inhibit bacterial cell division. Specific FtsZ ligands that are ineffective on bacterial division (typically because they do not pass the bacterial envelope) can also provide useful information for the design of more effective compounds or be used as biochemical probes for FtsZ. On the other hand, any effective bacterial cell division inhibitor that does not bind FtsZ may be a valuable antibacterial compound, whose action on other cell components will require further investigation. Testing FtsZ inhibitors for possible cross-effects on microtubule assembly and cytotoxicity on human cell lines is desirable. Optimization of a FtsZ hit into a lead compound demands in-depth investigation of its biochemical and structural mechanisms of action, its effects on the divisome assembly and cell division, and if possible the antibacterial efficiency in an animal model of infection.

TARGETING FTSZ'S NUCLEOTIDE BINDING SITE

An obvious target for the design of small molecule FtsZ inhibitors is the binding site of its natural regulators, GTP and GDP, at the main assembly interface (Figure 1), which is conserved among FtsZs from different organisms.[69] There was a risk that any compounds replacing GTP from FtsZ would also poison assembly of its eukaryotic homologue tubulin. However, probing FtsZ and tubulin with C8-substituted GTP analogues has revealed differences in their nucleotide interfaces.[46] Several of these GTP analogues inhibit FtsZ polymerization but support microtubule assembly, including chloro-, bromo-, iodo-, and methoxy-GTP as well as to a lesser extent pyrrolidino-, morpholino-, and *tert*-butyl-GTP. They constitute a proof of concept that it is possible to selectively inhibit FtsZ[46] (Table 1). The crystal structure of FtsZ in complex with 8-

morpholino-GTP revealed a binding mode similar to that of GDP, consistent with a competitive action on GTP-driven polymerization.[46] However, as the morpholine substituent fits without steric clashes into the active site in the currently available structures of the FtsZ intersubunit interface,[12,69] the structural mechanism of inhibition of FtsZ assembly by these analogues remains to be explained. The differences in the GTP binding sites of FtsZ and tubulin should be exploitable to specifically target the bacterial Z-ring.[70] However, the C8 nucleotide analogues themselves lacked antibacterial activity (presumably due to ineffective uptake of these charged molecules across the bacterial envelope), and C8-substituted cycloSal-GMP prodrugs did not yield satisfactory results.[71]

A further logical step is to find chemically different compounds that can replace the nucleotide in its binding site. There have been several approaches in this direction. The resistance mutations to the synthetic bacterial division inhibitor PC58538 map around the GTP site, and its more potent analogue PC170942 inhibits FtsZ GTPase activity with competitive kinetics.[66] To our knowledge definitive experimental confirmation of whether these compounds bind to the GTP site has not been reported. Crysophaentin A, a natural polyphenolic macrocycle isolated from a marine alga, has a potent antibacterial activity and inhibits FtsZ GTPase.[59] This molecule binds to FtsZ's nucleotide site, according to STD-NMR competition experiments with a GTP analogue.[59]

Given the scarcity of experimental data on GTP mimics for FtsZ, we have performed a docking simulation exercise with most of the compounds reported to interact with FtsZ, in order to gain computational insight into their potential recognition by the FtsZ nucleotide site (Supporting Information). The results were quite consistent with the two state of the art docking methods employed, ICM[72] and Glide.[73] Very similar scoring values were obtained using the atomic structure of FtsZ from either *B. subtilis* or *Methanococcus jannaschii* (Supplementary Table S2). This consistency is likely to reflect the 3D regularity and evolutionary preservation of this well-defined binding site. Several compounds reported to interact with FtsZ have medium to high docking scores into the GTP site, including, in addition to nucleotides, PC58538, the polyphenolic compounds viriditoxin, 2‴-hydroxy-5″-benzylisouvarinol-B, curcumin, caffeic and chlorogenic acids, as well as bis-ANS, the aminofurazan derivative A189, amikacin, DAPI, and edeine B1, though their potential binding to the GTP site has hardly been investigated (Supplementary Tables S1 and S2). However, there are inconsistencies; for example, the crysophaentin A docking scores were unexpectedly low. As examples, A189 and amikacin (Table 1) dock overlapping the guanine nucleotide (Figure 3A,B). Reported FtsZ inhibitors should be experimentally tested for binding to the nucleotide site.

In order to detect the binding of any molecules to the GTP site and measure their affinities within a wide range, a fluorescence assay, which measures the anisotropy change of *mant*-GTP upon binding to nucleotide-free FtsZ monomers, has been developed recently and validated with ³H-GTP competitive binding measurements.[47] As an example of use, the binding affinities of the C8-substituted GTP analogues determined with this method (Figure 3C) correlate with their inhibitory potency on FtsZ polymerization.[46,47] This method has been employed to determine a dominant contribution of the β-phosphate to the free energy change of nucleotide binding.[47] Molecular dynamics and free energy calculations have predicted that hydrolysis of the regulatory γ-phosphate
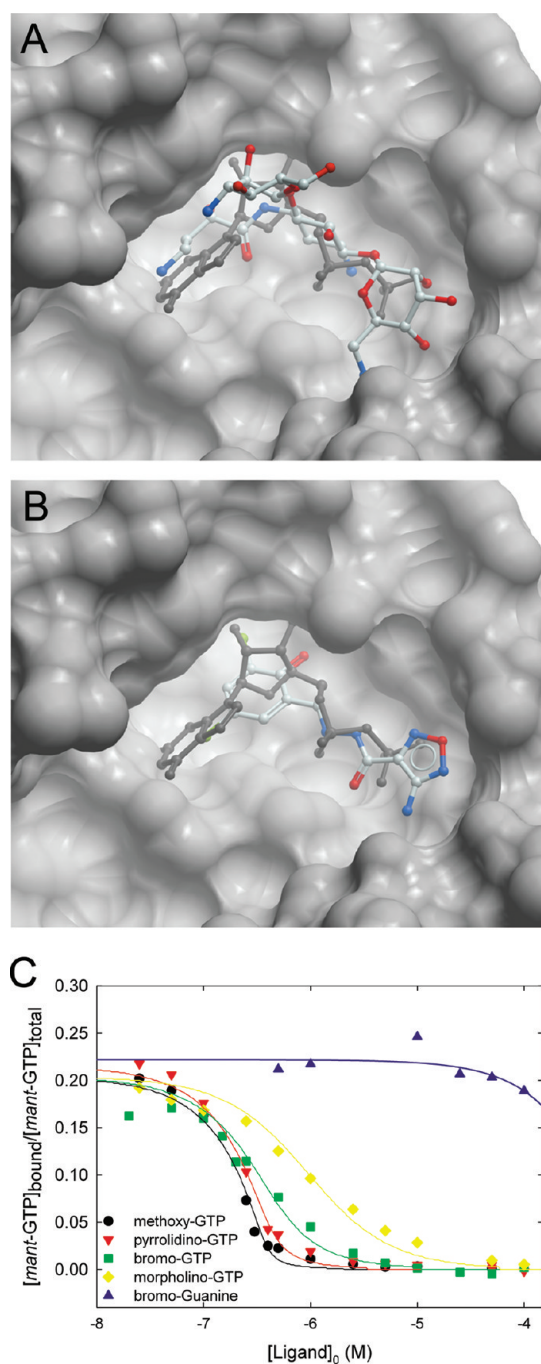

Figure 3. (A, B) Compounds reported to inhibit FtsZ docked into its nucleotide binding site. Two illustrative cases are shown. The binding poses of amikacin (panel A) and 4-aminofuzaran derivative A189 (panel B) predicted by docking on FtsZ (PDB entry 2VXY) are superimposed with a crystallographic GDP molecule (gray sticks, from PDB entry 1OFU). The poses correspond to highest docking scores obtained with ICM (see details in Supporting Information) using the atomic structure of FtsZ from *B. subtilis*. Notice the excellent fit of both ligand structures into the well-defined nucleotide cavity. Amikacin entirely occupies the phosphate and nucleobase binding regions (panel A), whereas A189 mainly targets the phosphate interaction site (panel B). (C) A homogeneous competition assay, based on the anisotropy change of *mant*-GTP, to detect molecules replacing the nucleotide from FtsZ (see text and ref 47). The method is exemplified here with C8-substituted GTP analogues.[46]

and dissociation from the flexible nucleotide binding site results in an accommodation of GDP, which has not been observed in the initial crystal structures.[47] These insights into the FtsZ-nucleotide interactions could be useful in the design of nucleotide mimetics.

◼ TARGETING THE INTERDOMAIN CLEFT AND THE FTSZ ASSEMBLY SWITCH

The long interdomain cleft of FtsZ is a candidate for the binding of ligands that could modulate its flexibility and assembly, according to the structural and biochemical features of this protein. The cleft may close during the functional cycle of FtsZ.[39] This cavity is relatively variable among different FtsZs. Interestingly, in the distant homologue TubZ the N-terminal helix H0 wedges into the cleft.[74] Starting from the very weak effect of 3-methoxybenzamide on *B. subtilis* cell filamentation,[75] researchers at Prolysis (Biota Europe) conducted a fragment-based synthetic program guided by bioactivity. They developed a series of difluoro-benzamide derivatives substituted at position 3 with several orders of magnitude improved cell division and bacterial growth inhibitory properties[7,65,76,77] (Supplementary Table S1). Among these, PC190723[7] (Table 1) was the first FtsZ inhibitor shown to be efficacious in an *in vivo* model of infection, protecting mice from a lethal dose of *Staphylococcus aureus*. This compound is a potent selective bactericide, including against methicillin-resistant *S. aureus* (MRSA). Mutants resistant to this molecule contain aminoacid changes in FtsZ that map its putative binding site to the interdomain cleft.[7] Several FtsZ assembly switch "curved" mutants described overlap these residues,[39] indicating that PC190723 binding could reduce the interdomain flexibility required for FtsZ dynamics stabilizing the active conformation.

The interdomain cleft is extended and variable, unlike the localized and conserved nucleotide binding cavity. Although we have focused our docking simulations only in the taxol-like region of the cleft where resistant mutants were reported, the consistency of results among different methods and crystallographic structures is no longer maintained as it was with the nucleotide site. In fact, PC190723 is the only compound with regular high docking scores in all situations (Table 1, Supplementary Table S2). The phenolic compounds 2‴-hydroxy-5″-benzylisouvarinol-B, curcumin, and chlorogenic acid, as well as DAPI, amikacin, and edeine B1, showed promiscuity, yielding medium docking scores, as did natural nucleotides GTP and GDP. Several of these compounds are likely to give false positives for both sites. Moreover, even with PC190723 we found several alternative high score docking solutions. For example, in the best docking pose found by ICM[72] the benzamide part of the molecule binds into a deep cavity between residues V307 and R191 of *B. subtilis* FtsZ, whereas the thiazolopyridine part is more exposed, making contact with loop residues N33 and N301 (Figure 4A), but very close score solutions were found flipping the positions of these two parts of the molecule. A docking image has been previously presented where benzamide was also bound to the cavity but the thiazolopyridine part was predicted to bind to hydrophobic residues close to I230.[7] Furthermore, with Glide[73] we also found more superficial docking poses that simply block the entrance of the deep cleft (see Figure 4B). This docking ambiguity illustrates the complexity of the rational design in this site. Finding a wedge compound for this long interdomain cleft to specifically affect the conformational dynamics of FtsZ
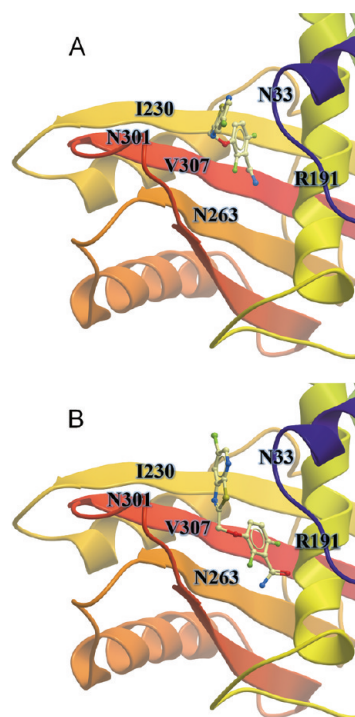

Figure 4. Predicted binding modes of PC190723 at the interdomain cleft of FtsZ. The N-terminal domain is colored blue, core helix H7 (in vertical position in this view) is yellow, and the C-terminal domain is yellow-red. The ligand poses correspond to highest docking scores using the *B. subtilis* atomic structure obtained with ICM (panel A) and Glide (panel B). Key residues are labeled as in ref 7 to highlight the interaction sites. These two different high score docking alternatives illustrate the complexity of the docking at this zone. Given the extension of the cleft and the structural variability that can be found among different species, the ambiguity on the docking results could be even larger (see text for details).

and block its assembly appears computationally very challenging.

PC190723 is a stabilizer of FtsZ polymers from susceptible species, whose binding induces filament assembly and polymorphic condensates depending on solution conditions. The biochemical action of this compound on FtsZ is analogous to that of taxol on tubulin, since both stabilize polymers against disassembly.[50] Related observations and a qualitative increase in FtsZ filament curvature by the close analogue 8j[76] have been reported.[77] Thus, these two benzamide derivatives probably bind to the interdomain cleft in FtsZ, modifying its assembly switch and enhancing polymer formation. It is possible that they create small distortions that propagate to form the abnormal assemblies of FtsZ observed in cells. The challenges are the determination of the structure of a FtsZ-PC190723 complex and the discovery of other compounds that bind to the interdomain cleft. Treatment of *B. subtilis* with PC190723 prevented correct assembly of the Z-ring, causing mislocalization of FtsZ into discrete foci through the elongated cells.[7] A recent study of the cellular effects of compound 8j[77] has indicated that although cell division is blocked, the abnormal FtsZ foci have moderately reduced dynamics and can recruit several septosomal proteins. Subinhibitory concentrations of 8j (or correspondingly higher concentrations of 3-methoxybenzamide) distorted the Z-ring into spirals and generated striking helical cell division events.[77] It is tempting to speculate whether

these drug-induced FtsZ spirals may relate to helical precursor structures of the Z-ring.[21,78]

SUMMARY AND OUTLOOK

Considerable progress has been made recently in the field of antibacterial small molecule FtsZ inhibitors. In addition to a growing list of compounds, their biochemistry and bacterial cell biology is becoming better known. The best characterized ligands bind to the two main cavities in FtsZ, at the interfacial GTP site and at the interdomain cleft. However, there are several important challenges to be addressed in this field. Atomic structures of FtsZ-inhibitor complexes, other than nucleotides, are sorely needed to provide insight for rational drug design. To this end, FtsZ crystallography is quite well developed, whereas conventional protein NMR of FtsZ, potentially another way to determine the 3D structure of these complexes, is hampered by FtsZ self-association in concentrated solution. Finding nucleotide mimetic compounds that specifically inhibit FtsZ and bacterial cell division without poisoning tubulin in human cells is a very interesting challenge. Developing PC190723 into an antibiotic and discovering other compounds that bind to the same site also appears to be of prime interest. Other potential druggable sites in FtsZ, if any, remain to be explored. There is a lack of follow-up on a number of inhibitors initially reported on the basis of their cellular or biochemical effects on FtsZ, with no apparent attempt to characterize their binding sites and mechanism of action or to optimize them. Useful hints for newer FtsZ inhibitors could be provided by a better characterization of several of the existing FtsZ inhibitors. Targeting FtsZ-interacting proteins and FtsZ degradation are also promising antibacterial strategies.

There are a number of approaches that have been employed to discover potential FtsZ inhibitors, with varying results. Past HTS efforts have provided thus far only a few hits whose development has not been reported. The generally applicable cell-based screening methods available are filamentation and Z-ring disturbance, which could be adapted to high throughput if the microscopy and pattern recognition were automated. Among biochemical approaches, homogeneous fluorescence methods to monitor ligand binding or polymerization are susceptible to high-throughput applications. NMR methods to study ligand recognition by FtsZ may be more extensively applied, as well as fragment screening by NMR. Advanced *in silico* screening approaches to drug discovery will need to be better adapted to the FtsZ binding sites, especially for the interdomain cleft. Finally, an eventual crystallographic determination of the structure of FtsZ filaments or accurate all-atom molecular dynamics simulations would provide input for the design of new interfacial drugs inhibiting FtsZ assembly and bacterial cell division. FtsZ has proven to be a good target for potential antibiotics, and it is to be hoped that a more systematic exploration of its binding sites with small molecules will lead to greater success in the near future.

ASSOCIATED CONTENT

ⓢ Supporting Information

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KEYWORDS

Divisome: The bacterial cell division machinery, made up of a number of interacting proteins that localize to the division site to perform their functions; Filamentation: Impairment of cell division in bacilli giving rise to continuous filaments of unseparated cells without septa between them; FtsZ: The protein coded by the ftsZ bacterial cell division gene; the name fts comes from "filament temperature sensitive" cell division mutants. FtsZ is an essential cell division protein in most bacteria; Z-ring: The assembly of FtsZ encircling the inner side of the plasma membrane at the division site, which recruits other cell division proteins

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