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Divin: A Small Molecule Inhibitor of Bacterial Divisome Assembly

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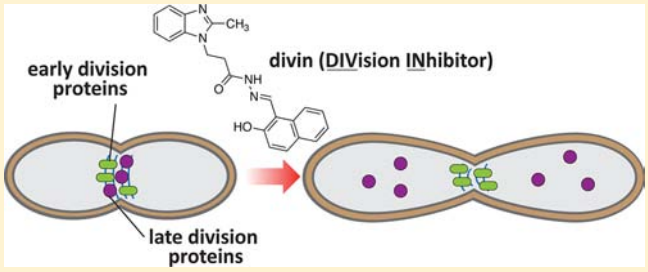
Supporting Information

ABSTRACT: Bacterial cell division involves the dynamic assembly of division proteins and coordinated constriction of the cell envelope. A wide range of factors regulates cell division—including growth and environmental stresses—and the targeting of the division machinery has been a widely discussed approach for antimicrobial therapies. This paper introduces divin, a small molecule inhibitor of bacterial cell division that may facilitate mechanistic studies of this process. Divin disrupts the assembly of late division proteins, reduces peptidoglycan remodeling at the division site, and blocks compartmentalization of the cytoplasm. In contrast to other division inhibitors, divin does not interact with the tubulin homologue FtsZ, affect chromosome segregation, or activate regulatory mechanisms that inhibit cell division indirectly. Our studies of bacterial cell division using divin as a probe suggest that dividing bacteria proceed through several morphological stages of the cell envelope, and FtsZ is required but not sufficient to compartmentalize the cytoplasmic membrane at the division site. Divin is only moderately toxic to mammalian cells at concentrations that inhibit the growth of clinical pathogens. These characteristics make divin a useful probe for studying bacterial cell division and a starting point for the development of new classes of therapeutic agents.



INTRODUCTION

Bacterial cell division was historically considered a 'simple' process in which cytokinesis occurred in the absence of cytoskeletal elements.[1] However, the past two decades of research have transformed this view. During division, more than a dozen different proteins assemble at the division site (Figure 1), including FtsZ, a homologue of eukaryotic tubulin. The resulting complex is referred to as the 'divisome'.[2]

Divisome assembly in *Escherichia coli* is hierarchical and stepwise.[3] FtsZ is the first protein to assemble at the site of cell division and is required for the localization of downstream division proteins.[4] The remaining division proteins arrive in two distinct stages and are categorized as either 'early' or 'late' proteins based on their temporal localization.[2] Several division proteins form subcomplexes in *E. coli* and *Caulobacter crescentus* cells prior to arriving at the division site or within the divisome, including FtsL/B/Q and FtsW/I/N.[3,5]

The dynamics and biochemical mechanisms of division proteins are not well understood,[6] although several biological functions have been proposed. Various early division proteins (e.g., FtsA, ZipA, ZapA, and FzlA) tether FtsZ to the inner membrane or regulate the quaternary structure of the bacterial tubulin.[2] Several late division proteins are involved in chromosome segregation (e.g., FtsK) and peptidoglycan (PG) remodeling (e.g., FtsI and FtsW).[6] Other late division proteins (e.g., FtsQ, FtsL, and FtsB) do not have assigned biochemical

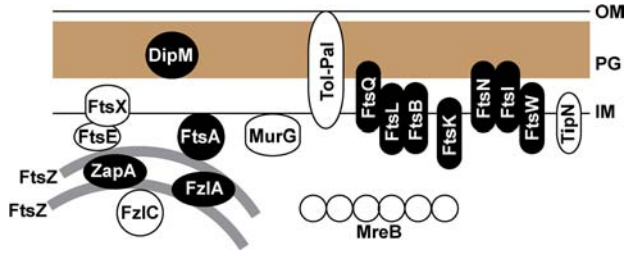


Figure 1. A cartoon illustrating components of the *C. crescentus* divisome. More than one dozen proteins assemble at the division site and span the three layers of the cell envelope. Several proteins associate physically within the divisome. The cartoon illustrates the order of divisome assembly, starting with FtsZ on the left and chronologically progressing to the right. Division proteins investigated in this study are shaded black. Outer membrane, OM; inner membrane, IM; peptidoglycan, PG. Cartoon adapted from Goley et al.[6]

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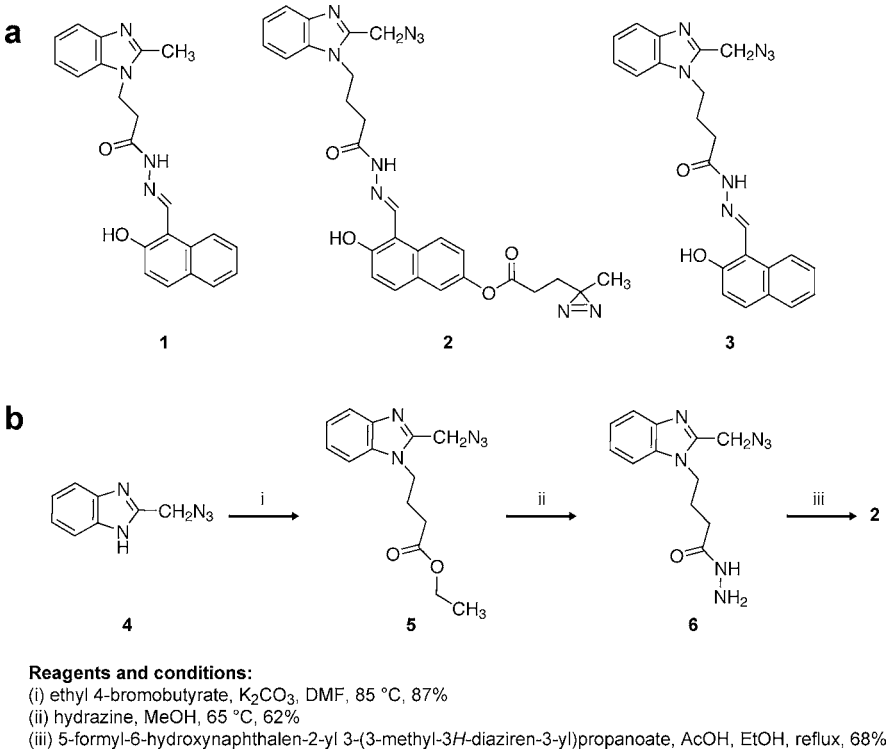

Figure 2. (a) Structures of divin **1**, its photoactivatable analog **2**, and the control probe **3**. (b) Synthetic scheme for the synthesis of **2**.

functions and may play a structural role in maintaining the divisome.[7,8]

Concurrent with divisome assembly, dividing bacterial cells constrict several different layers of the cell envelope.[2] Gram-negative *E. coli* and *C. crescentus* cells constrict the layers of the cell envelope simultaneously at the division site. *C. crescentus* cells finish the constriction of the inner membrane slightly earlier than the other layers of the envelope and compartmentalize the cytoplasm of mother and daughter cells prior to their physical separation.[9]

The physical mechanisms for cell envelope constriction in dividing cells are not well understood, and two models describing this process have been proposed.[10] One hypothesis is that the GTPase activity of FtsZ converts chemical energy into the mechanical energy required to constrict dividing cells.[10] GTP-bound homopolymers of FtsZ form straight protofilaments that become curved upon substrate hydrolysis to GDP.[11] As FtsZ filaments are tethered to the membrane through FtsA/ZapA, changes in the shape of FtsZ filament may deform the membrane at the division site.[12,13] In support of this hypothesis, studies of recombinant FtsZ interacting with liposomes demonstrated that the protein produces enough force to alter membrane shape in vitro when incubated with GTP.[10,14] Furthermore, cells expressing a mutant of FtsZ that has reduced GTPase activity do not constrict at the division plane and become unpinched, elongated filaments, thereby suggesting that FtsZ may create a critical force to deform membrane shape.[15] These studies, however did not investigate whether FtsZ mutants that inhibit division influence the assembly of late division proteins at the divisome in vivo.[15] Based upon these data, it is difficult to rule out a role of other division proteins in providing a mechanical input essential for division.

The second model for constricting the cell envelope proposes that cell wall growth (e.g., PG and β-glucan synthesis) provides force for constricting cells during division and stabilizes the FtsZ-driven constriction.[16−18] Several early and late division proteins (e.g., DipM, FtsI, and FtsW) are involved in PG remodeling, and depletion or deletion of their coding genes produces a smooth or deep constrictions at the site of division.[6] In *C. crescentus* cells lacking the putative endopeptidase DipM, FtsZ is correctly localized at the division site, and yet the cells frequently exhibit constrictions that are inhibited or relaxed.[16] Although it is unclear how this mutation produces various cell morphologies, these observations suggest an essential role of PG remodeling in constricting the division plane in cells.

Cell division requires the coordination of DNA synthesis, divisome assembly, and PG remodeling. To orchestrate these processes and respond to various extracellular stresses, bacteria use several mechanisms that position the division site at the midcell and regulate the progression of division.[19] The majority of these mechanisms block division by targeting FtsZ and altering its properties, including inhibition of its GTPase activity and the bundling of protofilaments.

FtsZ activity is regulated during cell growth to position the divisome at the midcell.[20] Proteins that regulate FtsZ include MipZ in *C. crescentus* and MinC and SlmA in *E. coli*. MipZ and MinC are concentrated at the cell poles where they bind FtsZ directly and stimulate the depolymerization of FtsZ protofilaments. The concentration gradient and activity of MipZ and MinC guide divisome assembly to the midcell.[20,21] The DNA-binding protein SlmA also binds to FtsZ, negatively regulates its activity, and ensures that the position of the division site does not overlap with the chromosome.[22]

In addition to regulating FtsZ during growth, bacteria may terminate division when available energy is reduced or in response to DNA damage.[19] KidO is a homologue of NADH-dependent oxidoreductases in *C. crescentus* and may act as a metabolic sensor to destabilize FtsZ protofilaments when

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energy levels are low.[23] Following DNA damage, *E. coli* cells activate the SOS response, which leads to the downstream sequestration of FtsZ monomers and inhibition of polymerization.[2,24] *C. crescentus* uses a different SOS response mechanism in which SidA binds to the late division protein FtsW, interferes with divisome assembly, and inhibits late stages of cell division.[5]

Several classes of antimicrobial agents inhibit bacterial cell division by binding directly to FtsZ or activating the regulatory mechanisms described above.[25] For example, PC190723 was reported to reduce the GTPase activity of FtsZ, stabilize protofilaments, and inhibit cell division of Gram-positive bacteria.[26,27] The aminocoumarin and quinolone families of antibiotics target DNA gyrase, introduce DNA damage, and disrupt DNA segregation.[28] The β-lactam family of antibiotics blocks PG remodeling at the division site and simultaneously activates the SOS response by stimulating the DpiBA two-component signal transduction system.[29] Overall, these classes of small molecules either target FtsZ directly or trigger regulatory mechanisms that cause downstream changes in the function of division machinery. The introduction of new classes of compounds that directly target the divisome may provide deeper insight into the molecular mechanisms of bacterial division and present new classes of clinical antimicrobial agents.

In this manuscript, we introduce divin (DIVision INhibitor; *N*′-[(*E*)-(2-hydroxynaphthalen-1-yl)methylidene]-3-(2-methyl-1*H*-benzimidazol-1-yl)propanehydrazide), a small molecule that has a unique mechanism of inhibiting bacterial division. We have demonstrated that divin does not bind to FtsZ or activate regulatory mechanisms that inhibit FtsZ. Instead, divin blocks cell division by influencing the spatial and temporal localization of late division proteins. The misplacement of late division proteins reduces PG remodeling at the division site significantly and prevents compartmentalization of the cytoplasm. We conclude that cell division proceeds through several morphological stages of the constricting cell envelope and that compartmentalization of the cytoplasm requires other factors in addition to the activity of FtsZ. Divin is a potent bacteriostatic agent against clinical pathogens and has low toxicity against mammalian cells. In addition to representing a new class of chemical biological probes for studying divisome dynamics, divin may be an attractive starting point for the development of therapeutic agents.

RESULTS AND DISCUSSION

Discovery and Characterization of Divin. We identified divin (**1**, Figure 2) in a high-throughput screen of small molecule libraries to isolate chemical inhibitors of MipZ.[30] In *C. crescentus* cells, MipZ spatially and temporally coordinates cell division, and its ATP hydrolysis activity is critical for polar localization.[11] We identified **1** as a weak inhibitor of the ATPase activity of MipZ in vitro; however, **1** did not perturb the polar localization of MipZ-YFP in vivo (Figure S1). Interestingly, *C. crescentus* and *E. coli* cells treated with **1** had incomplete constrictions at the division site (Figure 3). As division arrest occurred in *E. coli* cells, which lack *mipZ* in their genome, we concluded that the primary biological activity of **1** is not centered upon binding to MipZ.

We observed that treatment with **1** reduces the growth rate (Figure S2) and transmembrane potential ($\Delta\Psi$) of bacterial cells without altering the permeability of their membranes (Figure S3). To investigate whether depolarization of $\Delta\Psi$ is sufficient to produce division defects, we treated *C. crescentus* cells with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) at various concentrations and monitored cell morphology over time. Cells dosed with CCCP did not inhibit division at late stages (data not shown), suggesting that the depolarization effect of **1** is not responsible for inhibition of cell division. As **1** has no obvious physicochemical characteristics that would make it an effective ionophore, it is unclear whether it directly causes the depolarization of $\Delta\Psi$ in vivo.

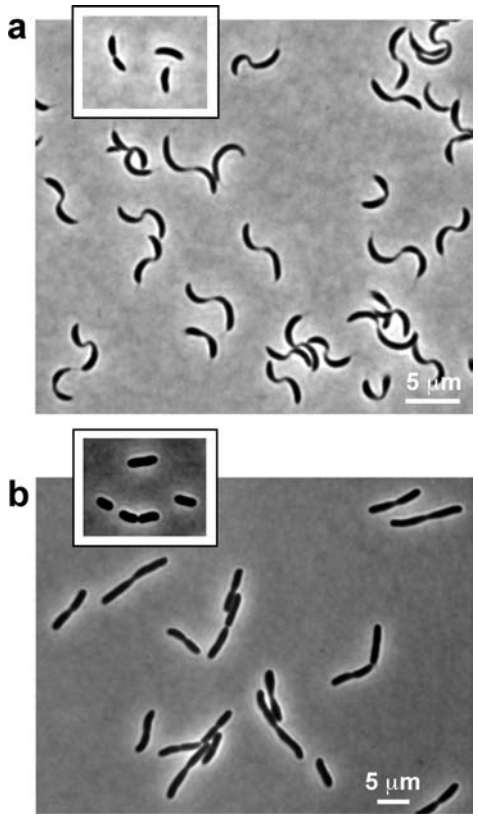

Figure 3. Divin arrests constriction in dividing cells. (a) *C. crescentus* CB15N cells treated with **1** (5 μM) after 11 h of incubation. (b) *E. coli* JW5503 cells treated with **1** (50 μM) after 12 h of incubation. Image inset shows untreated cells.

By measuring the time-dependent viability of bacteria treated with **1**, we determined that divin is a bacteriostatic agent (Figure S4). Consistent with this observation, we found that the inhibition of division was reversible—cells resumed division after **1** was washed out (Figure S5). Monitoring cells after removal of the compound, we observed that the newly formed daughter cells have sharp, pointed poles (Figure S5; 105 and 135 min), in contrast to the oblate shapes that are typical of new poles in *C. crescentus* cells.[31] The shape of the poles of drug-treated *C. crescentus* cells suggested that **1** disturbs the balance between elongation of the midcell and constriction at the division site.

The morphology of cells treated with **1** indicated that it arrests cell division after the initiation of the midcell constriction of the cell envelope. To test whether division is blocked prior to compartmentalization of the cytoplasm, we performed fluorescence recovery after photobleaching (FRAP) experiments with *C. crescentus* strain MT97 cells. We bleached MipZ-YFP molecules at one pole and measured the change of fluorescence at both poles of cells undergoing division. If the cytoplasm of the mother and daughter cells is continuous, the rapid diffusion of MipZ-YFP will produce a recovery of

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fluorescence in the bleached region of cells. After the cytoplasm has become compartmentalized, the exchange of MipZ-YFP between the cells is blocked, and fluorescence would not recover after bleaching. In all of the cells treated with **1**, the bleached pole recovered fluorescence, while the signal at the opposite pole was reduced (Figure 4). Control cells in the

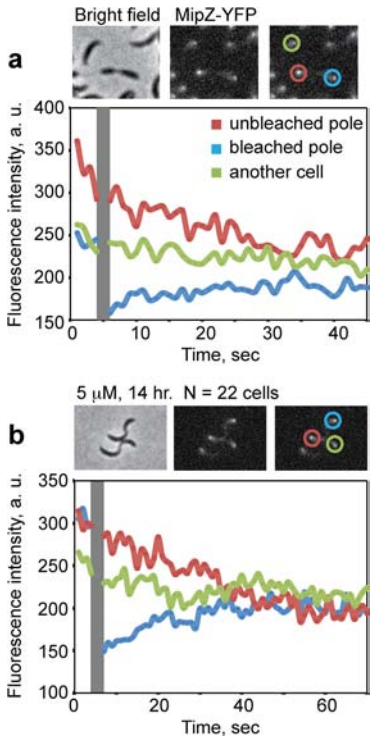

Figure 4. Divin-treated *C. crescentus* MT97 cells share a continuous cytoplasm between the mother and daughter cells. Images in each panel demonstrate bleached cells and unbleached cells in the imaging field. In the fluorescence image, three colored circles represent the regions for which fluorescence intensities were plotted. The gray bar in the fluorescence intensity plots represents the bleaching period. (a) A positive control sample treated with DMSO. (b) Cells treated with 5 μM of **1** for 14 h. For both DMSO and divin-treated samples, one representative data set is shown.

imaging field did not show dramatic changes in fluorescence. These results suggest that the cytoplasm in the mother and daughter cells was continuous and that divin treatment blocks compartmentalization of the cytoplasm.

Inhibition of cell division appeared 6 h after treating *C. crescentus* cells with **1** at its minimum inhibitory concentration (MIC, 5 μM; Table 1). Treating *E. coli* cells with a concentration of **1** that was 2- or 4-fold higher than the MIC (12.5 μM; Table 1) produced a division arrest after 4 h of incubation. We found that **1** is subjected to drug efflux pumps in bacterial cells, as adding a pump inhibitor and knocking out *tolC* improved the MIC of *C. crescentus* and *E. coli* cells, respectively (Table 1). Thus, **1** may accumulate slowly in bacterial cells, and bioavailability may explain the observed delay in causing the formation of division defects.

We confirmed the structure of **1** using NMR spectroscopy (Figure S6) and mass spectrometry (Figure S7), resynthesized it, and found that the synthetic version matched the biological activity of the compound we identified from our screen of commercial small molecule libraries. From the structural analysis, we found that **1** existed as trans$_{C(O)-N}$ and cis$_{C(O)-N}$

Table 1. MIC of Divin

organism	strain	MIC, μM	efflux pump inhibitor[a], μM
Caulobacter crescentus	CB15N	5	none
Caulobacter crescentus	CB15N	3	25
Caulobacter crescentus	CB15N	1.5	50
Escherichia coli	BW25113	>50	none
Escherichia coli	JW5503 (BW25113 Δ*tolC*)	12.5	none
Escherichia coli	YJE24 (BW25113 Δ*tolC*, Δ*recA*)	12.5	none
Vibrio cholerae	clinical isolate	3	none
Shigella boydii	clinical isolate	50	none
Acinetobacter baumannii	clinical isolate	25	none
Bacillus cereus	clinical isolate	50	none

[a]MC-207,110 was used to inhibit efflux pumps.

isomers with 60% of the compound in the trans-isomer at 25 °C in $CDCl_3/CD_3OD$ (95/5%). Due to a spontaneous interconversion and equilibrium between the two rotamers, it was not possible to discern which isomer is active in vivo.

We determined the solubility of **1** to be 0.02 mg/mL in buffer solutions, and it was stable in solution for >24 h (Figure S8, Table S1). The solubility of **1** prevented us from preparing agar plates containing a high concentration of the small molecule and using the solid growth medium to isolate and characterize suppressor mutations that influenced drug resistance (see Supporting Information).

As studies of **1**-resistant strains did not provide useful information on its target, we designed and synthesized a photoactivatable probe and its control (**2** and **3**, respectively; Figures 2 and S9−12) to profile the bacterial proteome for proteins that bind **1**.[32] **2** contains a diazirine group and can be photoactivated to covalently attach it to nearby amino acids in the target protein.[33] **2** and **3** also incorporate an azide to react with a small molecule tag (e.g., fluorescein, rhodamine, or biotin) through click chemistry and serve as a molecular handle to separate and enrich the covalent protein−drug complex.[34] As **3** does not contain a diazirine group, proteins coeluting with this compound will help us identify false-positives.

Several rounds of structure−activity relationship studies on **1**[35] made it possible for us to design analogs with in vivo activity that is virtually identical to **1**. Both **2** and **3** had MICs against *C. crescentus* and *E. coli* cells that were similar to **1** (Table S2) and caused an identical phenotype of late stage division inhibition in *C. crescentus* cells (Figure S13). The determination of the cellular target(s) of divin using **2** and **3** is currently underway.

Divin Does Not Bind FtsZ or Activate Regulatory Mechanisms That Target FtsZ. Several classes of molecules have been reported to bind FtsZ and inhibit division.[25,27] To test the hypothesis that **1** targets FtsZ directly, we measured the enzymatic activity of recombinant *C. crescentus* FtsZ in the presence of **1**. Using radioactive GTPase assays, we determined a Michaelis constant, K_M (80 ± 36 μM) and rate constant, k_{cat} (4 ± 0.5 min^{-1}) of recombinant FtsZ. When testing the potential inhibitory activity of **1**, we set the final concentration of GTP to the K_M to increase the sensitivity of the enzyme assays. We found that **1** does not inhibit *C. crescentus* FtsZ

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despite using a 25-fold higher concentration of the compound to the enzyme in the assay (Figure S14).

Divin may target FtsZ indirectly by triggering the SOS response or inhibiting DNA segregation. We tested these possibilities by performing three experiments: (1) FtsZ localization in divin-treated *C. crescentus* cells; (2) divin sensitivity of *E. coli* and *C. crescentus* cells that lack essential components of the SOS response; and (3) distribution of DNA in *C. crescentus* and *E. coli* cells.

We treated *C. crescentus* MT196 cells expressing FtsZ-YFP with **1** and observed FtsZ localizes at the midcell (Figure S15). In cells with an extended constriction, we occasionally observed more than one foci/band of FtsZ-YFP at the constriction site. These observations indicated that FtsZ protofilaments are assembled and maintained at the division site in the presence of **1**.

Upon detecting DNA damage, *E. coli* cells activate the SOS response to arrest cell division. Knocking out the *recA* gene disables SOS.[36] We constructed *E. coli* strain YJE24 in which *recA* was disrupted by a transposon and tested its sensitivity to **1**. We found that YJE24 cells have an MIC (12.5 μM) that is identical to the parent strain (JW5503) containing an intact copy of *recA* (Table 1). Moreover, **1** blocked the division of YJE24 cells (Figure S16) similar to the parent strain (Figure 3b). These results indicate that **1** does not activate the SOS response to inhibit cell division in *E. coli*. We have also tested the sensitivity of *C. crescentus* Δ*sidA* cells to **1**, and concluded that **1** does not act through the SOS response pathway in *C. crescentus* cells (Figure S17).

Segregation of replicated chromosomes involves structural maintenance of chromosome (SMC) proteins, topoisomerases (e.g., TopoIV), and some components of the divisome (e.g., FtsK) that participate in chromosome partitioning.[37−39] Inhibition of these proteins produces an abnormal orientation and distribution of DNA, blocks chromosome segregation, and halts cell division. By visualizing the orientation and distribution of DNA using microscopy, we tested whether **1** interferes with proteins that interact with DNA.

In *C. crescentus*, the chromosome is oriented with the origin of replication located at the pole containing the stalk or flagellum and the terminus at the other cell pole.[40] The two chromosome termini are located at the midcell during division and are the last regions of DNA separated.[40] We visualized DNA near the termini using a fluorescent repressor operator system (FROS) in *C. crescentus* and observed a normal orientation of termini at the midcell of cells treated with **1** using microscopy (Figure S18a; cells indicated by arrows). Thus, **1** does not inhibit SMC proteins as the inhibition of this family of proteins misorients the termini to the poles, instead of the midcell region.[39]

In addition to visualizing the chromosome termini in *C. crescentus*, we observed the distribution of DNA in *E. coli* cells. We treated cells with **1**, labeled DNA with DAPI, and imaged cells (Figure S18b). Cells treated with **1** typically contained two regions of DNA with a clear physical separation between the regions that corresponded to the site of the partially constricted cell wall. This observation suggested that **1** does not affect TopoIV, as the inhibition of this enzyme produces aggregation of replicated chromosomes.[41] Overall, these data from *C. crescentus* and *E. coli* cells demonstrated that **1** does not affect replication, segregation, and orientation of chromosome.

Divin Inhibits Assembly of a Functional Divisome. As temperature-sensitive mutations in several division proteins produce partial constrictions that are similar to the divin-induced phenotype,[1,6,42,43] we explored whether divisome assembly was altered in cells treated with the small molecule. By expressing translational fusions of fluorescent proteins to 11 different division proteins in *C. crescentus* (Figure 1), we quantified the temporal disassembly of the divisome after treatment with **1** (Table 2 and Figure S19). We found that FtsK and FtsI were the first proteins to delocalize in *C. crescentus* cells (6 h after treatment; concurrent with presentation of the division defect). We did not observe any significant delocalization of the two proteins prior to the 6 h time point.

Table 2. Divin Disassembles Late Division Proteins from the Divisome in *C. crescentus* Cells

	time (h)[b,c]		
division proteins[a]	6	8	12
ZapA	+	+	+
FzlA	+	+	+
DipM	+	+	−
FtsA	+	+	+
FtsN	+	+	+
FtsQ	+	−	−
FtsI	−	−	−
FtsK	−	−	−
FtsL	+	−	−
FtsW	+	−	−
FtsB	+	−	−

[a]Division proteins are listed in their chronological order of assembly; the earliest protein is indicated at the top of the first column, and the last protein at the bottom of the first column.[6] [b]A plus sign (+) indicates protein localization at the constriction site, and a minus sign (−) indicates delocalization. [c]Raw images and results from image analysis are shown in Figure S19.

FtsK plays a role in partitioning replicated chromosomes, and depletion of the C-terminal domain of FtsK in *C. crescentus* cells causes inaccurate partitioning of the chromosome termini.[37] As FtsK delocalized after treatment with **1**, we asked whether the termini are partitioned abnormally in cells treated with **1**. We analyzed the previous FROS data in *C. crescentus* cells and found that the distribution of the termini is normal in cells treated with **1**. Most of compound-treated cells contained two separate termini at the midcell, which had an equal distance to the pole where the origin was anchored (Figure S18a; cells indicated by asterisks). In contrast, some cells displayed a skewed distribution of the termini in which the distance to one pole was larger than to the other pole (Figure S18a; cells indicated by plus signs). This 'mis-segregation' of termini was observed in 20% of cells (n = 111) treated with DMSO and 31% of the cells treated with **1** (n = 133) and was not statistically significant. Thus, the disassembly of FtsK from the divisome in the presence of **1** likely occurs after the completion of chromosome partitioning.

The other protein that delocalized was FtsI, which remodels the PG specifically at the site of cell division. To better understand the effect of FtsI delocalization upon treatment with **1**, we visualized PG remodeling in *C. crescentus* cells using HADA, a fluorescent analog of D-alanine (Figure S20).[44] When incubated for a short time interval (i.e., 2−8% of the time required for cell doubling), HADA specifically labels the active site of PG synthesis and enables visualization of nascent PG in cells.[44] We found that HADA robustly labeled PG at the

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division site of *C. crescentus* cells (Figure S20). Six hours after treating *C. crescentus* cells with **1**, active PG remodeling continued at the division site, despite the delocalization of FtsI from this region of cells (Figures S20, S21). Eight hours after treatment with **1**, HADA labeling of the division site decreased significantly (Figures S20, S21) and coincided with the dissociation of several late division proteins—FtsQ, FtsL, FtsW, and FtsB—from the divisome (Table 2, Figure S19). Of these proteins, FtsW has been hypothesized to participate in PG synthesis by transporting PG precursors into the periplasm.[45] Overall, these results suggest that cells continue to remodel PG at the division site for 2 h after the displacement of FtsI from the divisome and that the dissociation of other late proteins contributes to the significant reduction of PG synthesis at the constriction site.

We observed that the division proteins FtsQ, FtsL, FtsW, and FtsB dissociated from the divisome following the disassembly of FtsK and FtsI after treatment with **1** (Table 2; Figure S19). Most of these proteins normally assemble after the arrival of FtsK and FtsI at the site of cell division, although they do not require FtsK and FtsI for their localization.[6] FtsW, FtsI, and FtsN form a subcomplex within the cytokinetic ring in *C. crescentus*.[5] Thus, the departure of FtsI from the divisome of cells treated with **1** may influence the delocalization of FtsW. We found that FtsN was localized at the division site up to 12 h after treatment with **1**, despite the disassembly of FtsI and FtsW within this time frame.

FtsL, FtsB, and FtsQ delocalized in *C. crescentus* cells after 8 h of treatment with **1**. In *C. crescentus*, FtsB and FtsQ require FtsL for localization to the midcell.[6] In *E. coli*, FtsL, FtsB, and FtsQ form a subcomplex, and their interactions are independent of other division proteins.[46] Thus, their simultaneous departure from the divisome in *C. crescentus* cells treated with **1** is consistent with previous observations.

Six hours after the mislocalization of FtsI and FtsK in *C. crescentus* cells due to **1**, we observed that several early division proteins remained at the constriction site, with the exception of DipM (Table 2, Figure S19). DipM delocalization may be due to departure of a particular divisome protein and/or reduction in PG remodeling at the constriction site (Figure S20), as DipM binds to PG directly[16,47,48] and associates more strongly with septal PG than with PG in other regions in the cell.[16] It is unclear why DipM localization persisted for 4 h after the significant reduction in PG synthesis due to treatment with **1** (Figure S20, Table 2).

Overall, localization studies of 11 division proteins in *C. crescentus* demonstrated that **1** disrupts divisome assembly with a preference for disassembling late division proteins from the complex. The sensitivity of late division proteins to **1** in *C. crescentus* was similar in *E. coli* cells (Figure S22, Table S3). Four division proteins that we tested in *E. coli* dispersed from the division plane in an order comparable to proteins in *C. crescentus*, starting with delocalization of FtsK and followed by FtsL, FtsQ, and FtsN. However, the disassembly of late division proteins after treatment with **1** does not necessarily support a direct, physical interaction of **1** with these proteins in *C. crescentus* and *E. coli* cells.

Divin As a Potential Therapeutic for Infectious Diseases. Our experiments demonstrated that **1** effectively blocks reproduction of model Gram-negative bacteria. We explored whether **1** inhibits the growth of clinically relevant strains of bacteria and found that it has an MIC of 3 μM against the Gram-negative pathogen *Vibrio cholerae* (Table 1). **1** was also toxic to clinical isolates of *Shigella boydii*, *Acinetobacter baumannii*, and *Bacillus cereus* (Table 1).

The antibiotic activity of **1** against bacterial pathogens prompted us to measure its toxicity in mammalian cells. We quantified the hemolysis of red blood cells (RBCs) and the viability of human epithelial kidney (HEK) cells. RBCs treated with **1** did not display a significant increase in hemolysis (Figure 5), although the HEK cells treated with **1** showed a slight decrease in viability (72 ± 5% viable, compared to the DMSO control). Overall, the antibiotic activity of **1** against bacterial pathogens and its mechanism of action make the compound a compelling starting point for developing a new class of clinical therapeutic agents.

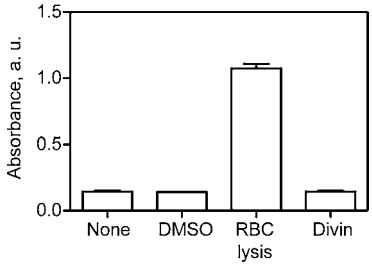

Figure 5. Divin is not toxic to RBCs. Divin (25 μM) was incubated with RBCs for 17 h, and hemolysis of RBCs was quantified by measuring the absorbance of heme at λ = 405 nm. A RBC lysis solution (EpiCentre Biotechnology) was used as a positive control. The error bars represent standard deviations from three independent experiments.

CONCLUSION

We have demonstrated that **1** inhibits cell division by perturbing divisome assembly without affecting FtsZ. Both the activity and localization of FtsZ were normal after treatment with **1**, and proteins that activate the SOS response and block cell division were not required for the **1** sensitivity *E. coli* and *C. crescentus* cells to **1**. **1** did not interfere with replication, partitioning, and orientation of chromosomes, thereby ruling out the possibility that **1** inhibits cell division indirectly. As demonstrated by microscopy experiments, **1** caused dissociation of late division proteins from the divisome.

Although we have not yet established a direct physical interaction between **1** and a division protein, the biological activity of **1** is unique and makes it an important chemical probe for studying the dynamics of bacterial division. Several applications of **1** that do not require information about its target include: (1) characterization of new division proteins, as **1** reduces the association of several late division proteins within the divisome without influencing early proteins. This difference in drug sensitivity between early and late proteins may be useful when characterizing the assembly of members of the divisome; (2) synchronization of *E. coli* cells, as **1** is reversible and bacteriostatic; (3) visualization of cell wall synthesis dynamics at different cell widths; (4) visualization of FtsZ polymer structures at the division site using cryo-electron microscopy, as **1** does not perturb FtsZ activity and localization, and its treatment produces cells with a width ($\leq$500 nm) that is compatible with electron cryomicroscopy; and (5) studies of the relationship between localization and function of division proteins. The localization of some division proteins, such as FtsI to the midcell in *C. crescentus*, is dependent on its

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function,[49] while other proteins (e.g., FtsN) have separate domains for activity and divisome localization.[50] It is possible that treatment with **1** influences the activity of some early division proteins without altering their localization at the divisome. As the biochemical/structural roles of division proteins are elucidated, **1** and other chemical probes may facilitate building the connection between divisome localization and function.

Our investigations demonstrate that **1** arrests division after the initial constriction at the division site and prior to compartmentalization of the cytoplasm. These data have at least two implications. First, they support a model for bacterial division in which multiple, morphological stages of the envelope constriction occur at the midcell. Temperature-sensitive mutants and depletion strains of division proteins produce a range of phenotypes: mutations in *ftsI*, *ftsQ*, and *ftsN* generate shallow constrictions, and mutations in *ftsA* and *ftsK* produce deep constrictions.[6] **1** causes deep, extended constrictions in *C. crescentus* cells and arrests constrictions at different stages in *E. coli* cells. The integration of these results with previous genetic studies produces several insights into bacterial division, including different stages of cell constriction do not follow the order of division protein assembly[42,51] and constrictions must progress through several stages/checkpoints, rather than advancing to completion once initiated. **1** may complement available genetic tools and provide insight into the relationship between divisome assembly and progression of the constricting cell division plane.

A second implication derived from the activity of **1** is that normal FtsZ activity and localization at the division site are insufficient for cytoplasm compartmentalization. Our results support a model in which FtsZ is influenced by other division proteins that collectively constrict the cell envelope during cell division. We found that **1** caused the dissociation of FtsI and FtsW from the divisome and lowered PG remodeling at the constriction site. Using **1** in combination with high-resolution imaging may generate new insights on the mechanisms by which PG remodeling generates force and/or provides feedback that influences the activity and structure of FtsZ to constrict dividing cells.[52]

To further develop **1** as a chemical probe, we are investigating the structure−activity relationship of the compound to improve its solubility and biological activity and are identifying its molecular target. Drawing lessons from eukaryotic cell biology, the development of a collection of chemical probes may have a deep impact on the study of prokaryotic cytoskeletons and their associated proteins.[25,53]

EXPERIMENTAL SECTION

General Methods for Optical Microscopy. For all microscopy experiments, we transferred a 1 μL cell suspension onto 1% agarose pads. For bright field and epifluorescence imaging, we used a Nikon Eclipse TE2000E inverted microscope with an Andor DU-895 EMCCD camera, a Perfect Focus system, and an encoded z-stage for phase contrast and epifluorescence microscopy. For DNA labeling with DAPI, we added the dye at a final concentration of 1 μg/mL and incubated for 10 min at 25 °C prior to imaging. For membrane labeling with FM 4-64, we added the probe at a final concentration of 4 μg/mL, incubated for 15 min at 25 °C, and washed the cells once with growth medium prior to imaging.

FRAP. We used MT97 cells for FRAP experiments. This strain expresses YFP, which absorbs green light (λ_{max} = 514 nm) and fluoresces in the green-yellow region of the electromagnetic spectrum (λ_{max} = 533 nm). We used a 514 nm Argon ion laser (Melles Griot) to excite the fluorophore. Samples were imaged using a Nikon Eclipse Ti inverted microscope equipped with an oil immersion objective (CFI Plan Apo Lambda DM 100× oil, 1.45 NA) and a 1.5× tube lens. The yellow emission was collected using a 560/50 emission filter (Chroma). The laser beam was split into two beams using a beam splitter. A focused beam (0.5 μm full width at half-maximum height (fwhm), 30 kW/cm2 peak intensity, 100 ms in duration) was used to selectively bleach one tip of the cell at t = 0. A broad probe beam (40 μm fwhm, 100 W/cm2) was used to record the fluorescence recovery with time (with 100 ms exposure at 1 Hz frame rate). The two beams were combined into the microscope in epi illumination mode. The beams were synchronized with the camera frames with fast shutters (Uniblitz LS2, Vincent Associates). The bleach beam was controlled with an extra home-built shutter that was only open at t = 0. We used a back-illuminated EMCCD camera with 16 × 16 μm pixels (iXon DV-887, Andor Technology). Each pixel corresponds to 105 × 105 nm^2 at the sample (150× magnification).

During the time series of FRAP experiments, we acquired images of a bleached cell and at least one more cell in a same window to compare the fluorescence intensity changes in the bleached cell with the signals in unbleached cells. In addition, we photobleached swarmer cells (with a single focus of MipZ-YFP per cell) in the absence of **1**, since it is possible for YFP to slowly recover from the dark state at high laser intensities.[54,55] The laser intensity we used for bleaching did not promote reversible photobleaching. Following data acquisition, fluorescence intensities were measured using ImageJ.

Assays for Measuring the Localization of Division Proteins. For experiments with *C. crescentus* cells, we prepared the cells by diluting the overnight culture 10- or 20-fold into fresh PYE medium. We preincubated the diluted culture for 1 h at 200 rpm and 30 °C and added **1** or DMSO as the solvent control. For experiments with *E. coli* cells, we diluted overnight cultures to an optical density of 0.1 in M8 media. We incubated the diluted cultures for 30 min prior to compound treatment (DMSO or **1**).

Since the experiments with *C. crescentus* cells required long periods of incubation (up to 12 h), we found that the fraction of population that exhibits normal localization decreased over time in the DMSO control sample. Thus, we grew the DMSO control cells for the same amount of time as the cells treated with **1** for a more accurate comparison (e.g., 12 h DMSO sample vs 12 h divin sample). Experiments with *E. coli* cells required a shorter incubation (up to 5 h) than *C. crescentus*, and the DMSO control sample was induced immediately after the preincubation, imaged, and analyzed for comparison with cells treated with **1**.

To quantify the fraction of population with a normal localization of protein, we analyzed the images manually. The manual analysis was necessary since cells treated with **1** have an unusual morphology that provides challenges for Matlab-based scripts to segment the cells correctly. In the analysis, we only considered cells with visible constrictions and determined whether fluorescence foci were found at the division constriction in these cells. Cells with visible constrictions were marked on the bright-field image, which was used as a reference for the fluorescence image to count the cells with a midcell localization. Regardless of the intensity of the fluorescence, as long as the fluorescence was discernible at the midcell, we categorized the cell as a wild-type phenotype. We counted at least 40 cells per experimental condition and performed each experiment at least twice to ensure reproducibility of the data. After counting the number of cells displaying a midcell localization out of the total number cells analyzed, we calculated two-sided p-values between the DMSO and samples treated with **1** using Fisher's exact test (Graphpad Prism).

Furthermore, we used two 'quality control tests', as manual data analysis can be subjective. In one test, one person analyzed several data sets on two different occasions that were several weeks apart from each other and determined whether the results from the two occasions were the same. We also had two different individuals analyze the same sets of data to compare the results to each other. In both 'quality control tests', we found that the results were very similar to each other and did not change the p-value of the data sets, indicating that the manual analysis was objective and accurate.

Determination of MICs. We determined the MIC of *E. coli* and *C. crescentus* strains in liquid media using the macrodilution method according to the CLSI guidelines.[56] For clinical pathogens, including *V. cholera*, *S. boydii*, and *A. baumannii* strains, we used 96-well microplates (100 μL/well) and the microdilution method from the CLSI guidelines.[56] We used PYE media for *C. crescentus* and M8 media for all other organisms.

RBC Hemolysis Assay. We used sheep RBCs from Lampire Biological Laboratories. The assay was performed as previously described[30] using 25 μM of **1**. The cells were treated with the compound for 17 h.

Mammalian Cytotoxicity Assay. We measured the cytotoxicity of small molecules on HEK cells using the CellTiterGlo Luminescent Cell Viability Assay (Promega). The assay was performed as previously described[30] using 25 μM of **1**. The cells were treated with the compound for 17 h, and the final concentration of DMSO was 0.082% v/v.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental results, microbiological procedures, compound characterizations, membrane assays, HADA labeling, and GTPase assay with FtsZ. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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