SCO4506- and SCO4327-disruptants as converters (fig. S12).

In the next step, we isolated an intermediate from the culture broth of the SCO4326 mutant, which was purified as above and its structure determined as cyclic DHFL (Fig. 1) (figs. S13 to S17).

As a small amount of DHFL accumulated in the culture broth of the SCO4550-disruptant, we predicted that it catalyzed the reaction adjacent to SCO4327. An in vitro enzyme assay was prepared using the recombinant TTHA1092, which contained an ortholog of SCO4550 in *T. thermophilus* HB8. However, we did not detect the formation of cyclic DHFL. Additional enzymes and/or cofactors, and optimized assay conditions, may be necessary.

Recombinant TTHA1568 was prepared to provide an ortholog of SCO4326 in *T. thermophilus* HB8 to convert cyclic DHFL into the next intermediate. A product was obtained and was confirmed to be 1,4-dihydroxy-6-naphthoate by LC-MS (fig. S18); this was previously demonstrated to be an intermediate in the alternative pathway (*13*).

Because the alternative pathway appeared to branch at chorismate with the SCO4506 enzyme, and an examination of the structure of futalosine indicates that the nucleoside moiety is derived from inosine, plus the fact that a tracer experiment (*13*) has implicated a C2 unit from pyruvate or phosphoenolpyruvate between chorismate and inosine forming the C6' and C7' positions of futalosine, we tested the ability of the recombinant enzyme TTHA0803 (an ortholog of SCO4506 in *T. thermophilus* HB8) to catalyze the formation of futalosine. However, we did not detect futalosine, although 3-(1-carboxyvinyloxy) benzoate and m-hydroxybenzoate were formed from chorismate in the absence and presence of flavin mononucleotide (FMN), respectively.

We have succeeded in outlining an alternative pathway for the biosynthesis of MK in microorganisms by a combination of bioinformatics and biochemical experiments. We confirmed the occurrence of the alternative pathway among bacteria and found it (see SOM Text) in several Gram negatives and Archaea, notably in chlamydia and spirochetes. The alternative pathway was distributed only in prokaryotes and was absent in eukaryotes, including lower ones such as fungi, yeasts, and protists. Because humans and commensals, such as lactobacilli, lack this alternative pathway, it is an attractive target for the development of chemotherapeutics. We also searched microorganisms that have both the alternative pathway and the classical pathway or that have both the ubiquinone pathway and the alternative pathway. However, we were not able to find such bacteria among microorganisms whose genome analysis has been completed.

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An Inhibitor of FtsZ with Potent and Selective Anti-Staphylococcal Activity

David J. Haydon,[1] Neil R. Stokes,[1]* Rebecca Ure,[1] Greta Galbraith,[1] James M. Bennett,[1] David R. Brown,[1] Patrick J. Baker,[2] Vladimir V. Barynin,[2] David W. Rice,[2] Sveta E. Sedelnikova,[2] Jonathan R. Heal,[3] Joseph M. Sheridan,[3] Sachin T. Aiwale,[4] Pramod K. Chauhan,[4] Anil Srivastava,[4] Amit Taneja,[4] Ian Collins,[1] Jeff Errington,[1,5] Lloyd G. Czaplewski[1]

FtsZ is an essential bacterial guanosine triphosphatase and homolog of mammalian β-tubulin that polymerizes and assembles into a ring to initiate cell division. We have created a class of small synthetic antibacterials, exemplified by PC190723, which inhibits FtsZ and prevents cell division. PC190723 has potent and selective in vitro bactericidal activity against staphylococci, including methicillin- and multi-drug–resistant *Staphylococcus aureus*. The putative inhibitor-binding site of PC190723 was mapped to a region of FtsZ that is analogous to the Taxol-binding site of tubulin. PC190723 was efficacious in an in vivo model of infection, curing mice infected with a lethal dose of *S. aureus*. The data validate FtsZ as a target for antibacterial intervention and identify PC190723 as suitable for optimization into a new anti-staphylococcal therapy.

Bacterial cell-division proteins, in particular FtsZ, offer unexploited and attractive targets for antibacterial drug discovery because they are essential and widely conserved throughout bacteria (*1*, *2*). During cell division, FtsZ undergoes guanosine 5′-triphosphate (GTP)–dependent polymerization to form the Z ring at the mid-cell. FtsZ recruits other proteins that together drive cell division and the formation of new cell poles (*3*). FtsZ is a distant structural and functional relative of mammalian β-tubulin (*4*–*6*), which is a well-exploited target for anticancer therapy (*6*). This suggests that the protein superfamily is amenable to inhibitor development and may provide structural insights to assist the rational design of FtsZ ligands. Several compounds that block bacterial cell division and/or inhibit the biochemical activity of the FtsZ protein in vitro (*7*–*13*) have been reported; however, so far none has demonstrated efficacy in models of infection or has entered clinical evaluation or use.

3-Methoxybenzamide (3-MBA) ($C_8H_9NO_2$, relative molecular mass of 151.16) (fig. S1) is a small ligand that has been reported to target FtsZ and inhibit cell division in the Gram-positive bacterium *Bacillus subtilis*, resulting in a filamentous morphology (*7*). Although 3-MBA has

[1]Prolysis, Begbroke Science Park, Oxfordshire OX5 1PF, UK. [2]Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, UK. [3]Prosarix, Newton Hall, Cambridge CB22 7ZE, UK. [4]Jubilant Chemsys, B-34, Sector-58, Noida 201301, India. [5]Institute for Cell and Molecular Biosciences, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne NE2 4HH, UK.

*To whom correspondence should be addressed. E-mail: neil.stokes@prolysis.com

a weak antibacterial potency, it is a small and efficient ligand with on-target activity and is able to penetrate the bacterial cell. It therefore provided a good starting point for a fragment-based approach to antibacterial drug discovery. A medicinal chemistry program was undertaken, first to determine the structure-activity relationships of the benzamide and then to explore and extend the methoxy group. The antibacterial activity of, and the microscopic cell morphology caused by, each derivative was measured to direct the iterative rounds of synthetic chemistry (*14*). Analysis of more than 500 analogs yielded a compound, PC190723 ($C_{14}H_8ClF_2N_3O_2S$, relative molecular mass of 355.75) (Fig. 1A), that inhibited cell division and had a vastly improved potency. PC190723 was constructed from substituted benzamide and thiazolopyridine moieties joined by an ether linkage.

PC190723 had potent antibacterial activity against *B. subtilis* as well as all strains and species of staphylococci that were tested [minimal inhibitory concentration (MIC) in the range of 0.5 to 1.0 μg/ml], including a methicillin-resistant *Staphylococcus aureus* (MRSA) strain and a multi-drug–resistant *S. aureus* (MDRSA) isolate that is resistant to many of the major classes of antibiotics (Table 1). The potency of PC190723 was more than 2000 times greater than that of 3-MBA against *S. aureus* (table S1). PC190723 was inactive against a range of other Gram-positive and Gram-negative pathogenic bacteria and did not inhibit the growth of yeast or human hepatocytes (Table 1). In a bacterial survival assay, concentrations of PC190723 equivalent to or greater than the MIC caused a reduction in viable *S. aureus* cell numbers of more than three logarithms within 24 hours (fig. S2), which was consistent with a bactericidal mode of action.

The in vivo efficacy of PC190723 was demonstrated in a murine septicaemia model of staphylococcal infection. A single subcutaneous (SC) or intravenous (IV) administration of PC190723 at 30 mg/kg resulted in a 100% survival of mice inoculated intraperitoneally with a potentially lethal dose of *S. aureus*, as compared with a 0% survival for the group receiving no compound (Fig. 1B). For both routes of administration, dose-dependent efficacy was observed, and the 50%-effective doses (ED_{50}) were 7.3 and 10.2 mg/kg for SC and IV administration, respectively. PC190723 was also efficacious when administered intraperitoneally (ED_{50} of 3.1 mg/kg) (fig. S3).

To verify the mode of action of the compound, *S. aureus* FtsZ was cloned, overexpressed, and purified, and an in vitro assay was developed to demonstrate that PC190723 directly inhibited the GTPase activity of FtsZ in a concentration-dependent manner with an inhibitory concentration (IC_{50}) of 55 ng/ml (Fig. 2A). A green fluorescent protein (GFP)–FtsZ construct (*11*) in *B. subtilis* was used to show that, after PC190723 exposure, FtsZ was distributed as discrete foci throughout the elongated cell, indicating mislocalization (Fig. 2B). This contrasts with other inhibitors of FtsZ, which cause the dissipation of FtsZ assembly (*9*, *11*). Treating rod-shaped *B. subtilis* or spherical *S. aureus* with PC190723 caused the elongation or enlargement, respectively, of the cells (Fig. 2, C and D). In a time-course experiment, PC190723 caused the maximal enlargement of *S. aureus* cells within 2 hours of exposure, which preceded the reduction in cell viability observed in the time-kill study (fig. S4).

Spontaneous PC190723-resistant *S. aureus* American Type Culture Collection (ATCC) 29213 mutants were isolated at compound concentrations equivalent to eight times the agar MIC or higher at a frequency of 2×10^{-8}, which is consistent with a single-gene–product target for PC190723 (*15*), and without further optimization would be suitable for use as a component in a combination therapy. The *ftsZ* gene of the mutants was sequenced, and each was found to contain an amino acid change in FtsZ at one of six residues: R191, G193, G196, V214, N263, and G266, most frequently at G196. It is possible that the resistance to PC190723 by the spontaneously isolated mutants is due to a secondary mutation(s) in proteins other than FtsZ. To test this, an engineered site-directed mutant of *B. subtilis* carrying only a mutation in FtsZ at G196 was created, which reduced its susceptibility to PC190723 (table S1). Mutations at R191, G193, and G266 conferred PC190723 dependency on *S. aureus*, in that the strains failed to grow in the absence of compound, suggesting that these mutant forms of FtsZ cannot function without having compound bound and providing further evidence of a direct interaction in vivo (fig. S5). Spontaneous mutations in residue A47, which was identified in 3-MBA–resistant *B. subtilis* mutants (*7*), were not isolated. A site-directed mutant of *B. subtilis* carrying the A47P mutation was created and, although it showed reduced susceptibility to 3-MBA, remained susceptible to PC190723 (table S1). Taken together, the biochemical, cytological, and genetic data confirm that PC190723 directly blocks FtsZ activity to prevent septum formation and cell division.

An alignment of FtsZ and tubulin protein sequences revealed that several of the PC190723-resistant mutations in FtsZ coincided with the residues of tubulin that form the binding site for the taxanes (*4*–*6*) (fig. S6). This suggests a common mode of ligand-target interaction between

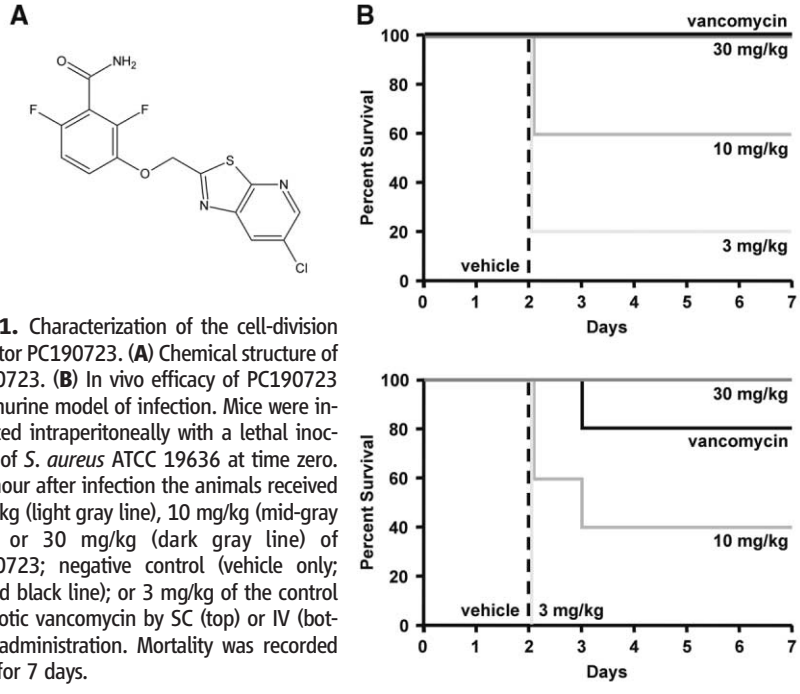

Fig. 1. Characterization of the cell-division inhibitor PC190723. (**A**) Chemical structure of PC190723. (**B**) In vivo efficacy of PC190723 in a murine model of infection. Mice were inoculated intraperitoneally with a lethal inoculum of *S. aureus* ATCC 19636 at time zero. One hour after infection the animals received 3 mg/kg (light gray line), 10 mg/kg (mid-gray line), or 30 mg/kg (dark gray line) of PC190723; negative control (vehicle only; dashed black line); or 3 mg/kg of the control antibiotic vancomycin by SC (top) or IV (bottom) administration. Mortality was recorded daily for 7 days.

Table 1. Microbiological profile of PC190723.

Organism and genotype	MIC (μg/ml)
B. subtilis	1
S. aureus	1
S. aureus (MRSA)	1
S. aureus (MDRSA)*	1
S. epidermidis	1
S. haemolyticus	0.5
S. hominis	1
S. lugdunensis	1
S. saprophyticus	1
S. warneri	1
Enterococcus faecalis	>64
Escherichia coli	>64
Haemophilus influenzae	>64
Pseudomonas aeruginosa	>64
Streptococcus pneumoniae	>64
Saccharomyces cerevisiae	>64
HepG2 (IC_{50})	>64

*Resistant to methicillin, oxacillin, ampicillin, gentamicin, erythromycin, penicillin, azithromycin, amikacin, amoxicillin/clavulanic acid, cephalothin, clindamycin, ceftriaxone, imipenem, lincomycin, streptomycin, perfloxacin, rifampin, and neomycin.

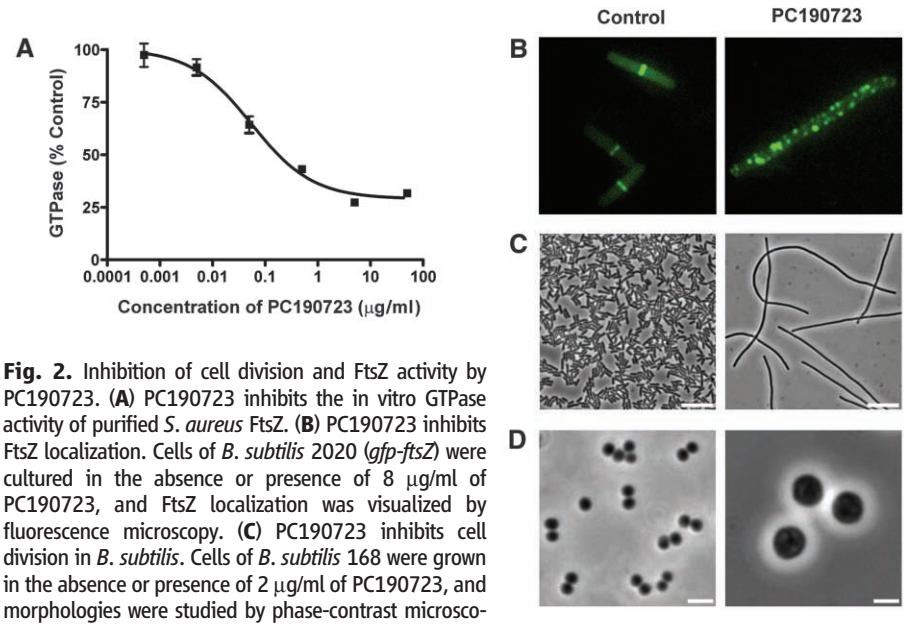

Fig. 2. Inhibition of cell division and FtsZ activity by PC190723. (**A**) PC190723 inhibits the in vitro GTPase activity of purified *S. aureus* FtsZ. (**B**) PC190723 inhibits FtsZ localization. Cells of *B. subtilis* 2020 (*gfp-ftsZ*) were cultured in the absence or presence of 8 μg/ml of PC190723, and FtsZ localization was visualized by fluorescence microscopy. (**C**) PC190723 inhibits cell division in *B. subtilis*. Cells of *B. subtilis* 168 were grown in the absence or presence of 2 μg/ml of PC190723, and morphologies were studied by phase-contrast microscopy. Scale bars, 10 μm. (**D**) PC190723 inhibits cell division in *S. aureus*. Cells of *S. aureus* ATCC 29213 were grown in the absence or presence of 2 μg/ml of PC190723, and morphologies were studied by phase-contrast microscopy. Scale bars, 2 μm.

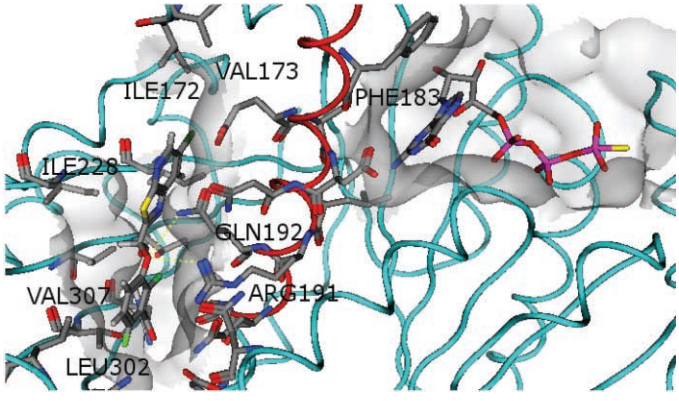

Fig. 3. Interaction of PC190723 with FtsZ. Model of a section of the FtsZ protein showing the position of the proposed binding site of PC190723 (left) relative to the nucleotide-binding site (right). The side chains of residues forming the putative binding pocket are labeled and are shown in ball-and-stick representation. The protein backbone is shown as a light blue ribbon. H7 is highlighted in red. In this model, hydrogen bonds are formed between the phenoxy ether of PC190723 and R191 and Q192 (dashed yellow lines). Blue, nitrogen; yellow, sulphur; gray, carbon; red, oxygen.

the evolutionarily related cytoskeletal elements of prokaryotes and eukaryotes. However, unlike Taxol, PC190723 did not enhance or inhibit tubulin polymerization in vitro (fig. S7). Further, Taxol did not inhibit cell division in bacteria (fig. S8).

A 1.7 Å crystal structure of *B. subtilis* FtsZ apo-form was used to identify a potential binding site(s) for 3-MBA and PC190723. The highest docking score positioned the ligands in a cleft (Fig. 3 and fig. S9) between helix seven (H7) and the C-terminal domain (*16*). The benzamide is predicted to bind in the cleft adjacent to amino acid residues R191, Q192, N263, V307, and T309. The thiazolopyridine portion of PC190723 is predicted to bind in a hydrophobic channel formed by amino acid residues I172, E185, N188, I228, and I230. All of the PC190723-resistant mutations (fig. S6) are in or adjacent to the residues comprising the docking site proposed by the modeling. This region is not part of the target's nucleotide-binding site. The core helix (H7) forms part of the Taxol-binding site in tubulin and has been proposed to act as a lever that can alter the interdomain orientations in tubulin and FtsZ (*4*, *5*, *17*). Interaction of PC190723 with H7 could contribute to the observed inhibition of GTPase activity and FtsZ polymerization. The docking model could also explain the species specificity of these benzamide compounds. Susceptible organisms have a valine at position 307. In nonsusceptible species, an arginine or histidine is present at this position (fig. S6). These larger polar residues partially occlude the cleft. In support of this hypothesis, substituting V307 for an arginine or histidine reduced the susceptibility of *B. subtilis* to 3-MBA and PC190723 (table S1).

PC190723 is representative of a new class of potent small-molecule antibacterial compounds that kill bacterial cells by inhibiting the essential protein FtsZ. In addition to their therapeutic potential, PC190723 and analogs may also be useful reagents for further studies on the biology of FtsZ and bacterial cell division. The potency of PC190723 against drug-resistant *S. aureus*, its efficacy in in vivo models of infection, and its structural and physicochemical properties (table S3) (*18*) make it an excellent candidate for optimization into a therapy to treat staphylococcal infection.

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Supporting Online Material

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