Supplementary data

Supplementary Methods

Murine E. coli Pneumonia Model and Treatment with SCH79797

E. coli cultures were grown overnight in LB broth for 16-17 h, washed with PBS, and then resuspended in PBS to achieve an optical density of 1.0 at 600 nM (Beckman Spectrophotometer; OD of 1 = 4 x 10^8 cfu/mL). Bacteria were then diluted to a concentration of 1 million cfu per 25 µL of PBS prior to instillation into mice.

Intratracheal (IT) instillation was performed for induction of pneumonia. To briefly summarize, mice were first anesthetized with ketamine and xylazine (90 mg/kg and 10 mg/kg intraperitoneally, respectively) and placed on an intubating stand (Braintree Scientific). Then using a fiberoptic light, the oropharynx and vocal cords were visualized, and the *E. coli* solution was injected into the trachea using an insulin syringe attached to a PE-10 catheter. Mice were recovered for up to an hour to ensure they regained normal activity post-procedure. 6 h after infection, mice were reanesthetized with ketamine and xylazine at approximately half the initial dosing, and subsequently SCH79797 or PBS control was administered IT. SCH79797 was resuspended in DMSO to an initial concentration of 50 mM and then diluted in PBS to 10 and 100 µM. A total volume of 30 µl was delivered IT and an equivalent volume of PBS was used as the control for the *in vivo* studies. Since only 1 dose of SCH79797 was given in these studies, short-term survival over the initial 48 h post-infection was the primary outcome for the *in vivo* studies. Non-survival *in vivo* experiments used SCH79797 at a dose of 10 µM since this dose was determined to be the most efficacious.

BAL and Lung Injury Analyses

BAL cell counts were measured using a cell counter (Invitrogen), and a differential was determined using a cytospin to pellet cells on a slide, which were fixed and then stained with hematoxylin and eosin. The supernatant from the BAL fluid was collected and analyzed for total protein using the BCA method (Pierce) as well as for cytokines using ELISAs (R & D). The bacterial burden in the lavage fluid was assessed by diluting the samples in PBS and then plating on LB agar plates. Plates were kept at 37^{o}C overnight, and the following day the colonies were counted.

For histological analysis, mice were euthanized, a luer adapter was placed into the trachea, and 1.5 mL of a 10% zinc formalin solution was instilled into the lungs for fixation and inflation. The trachea was tied off with a suture, and the lungs were removed from the thoracic cavity and immersed in 10% zinc formalin in a vial. The lungs were then processed and sectioned in a standard manner and stained with hematoxylin and eosin. Lungs from a mouse were scored by assessing the degree of hemorrhage and edema in six sections from the lower lobes (3 sections from each lung). The following scoring system was used as has been previously published: no injury, 0; 25% of the field affected, 1; 50% of the field affected, 2; 75% of the field affected, 3; diffuse injury, 4.

Mouse neutrophil isolation

Whole bone marrow was isolated from the femurs and tibias of mice, the red blood cell fraction was lysed with hypotonic saline, and then the remaining cells were sterile filtered with a 70 µM cell strainer (BD Falcon). A 62.5% Percoll density gradient was used to separate the cell fractions, and the neutrophil portion was preserved and washed. Neutrophils were resuspended in the appropriate solution for further studies (usually HBSS with calcium and magnesium), and counted with a hemocytometer.

Mouse neutrophil qRT-PCR and Western blotting for PAR1 expression

For RNA isolation, mouse neutrophils were isolated as outlined above and using the Qiagen Mini Plus RNA kit, cells were lysed (approximately 5 million per condition), genomic DNA removed, and RNA eluted from a spin column. The quantity of RNA was calculated using a NanoDrop system, and converted into cDNA (Applied Biosystems, High-Capacity cDNA Reverse Transcription Kit). Quantitative real-time PCR was then done to detect expression of PAR1, and GAPDH was used as the housekeeping gene (mouse PAR1, GAPDH qPCR primers, Origene; qPCR SYBR-green master mix, Applied Biosystems). Samples were run on a qPCR analyzer (Bio-Rad) and the average Cq was calculated.

Protein was isolated from mouse neutrophils for Western blot analysis using RIPA buffer with protease inhibitor (ThermoFisher, Pierce). The protein fraction was then processed, quantified (BCA method, ThermoFisher) and run on 4-12% Bis-Tris Gel (NuPage, Invitrogen) in a reduced, denatured state. Bone marrow derived mesenchymal stem cells were used as a positive control, as they express PAR1.[22] The Western blot was carried out using standard techniques (primary PAR1 antibody, rabbit polyclonal, Abbiotec; primary β-actin antibody, mouse monoclonal, Neomarkers; secondary antibodies, LiCor) and developed using a LiCor detection system.

Neutrophil bacterial killing and ROS assays

The neutrophil killing protocol utilized is as follows: (a) neutrophils were isolated and seeded at a density of 400,000 cells per 150 µL of HBSS in a 48 well plate; (b) 20 µL of 10x SCH79797 (100 µM) or 20 µL of HBSS was added to appropriate wells; (c) 24 µL of HBSS was then added to each well for final volume of 194 µL and plates were incubated at 37^{o}C; (d) after 2.5 h, 6 µL of DNAse or HBSS was added to appropriate wells; (e) after an additional 30 min, 100,000 cfu of *E. coli* in 50 µL of HBSS was then added to each well; (f) plates were centrifuged at 1600 rpm for 5 min; (g) plates were then incubated at 37^{o}C for 30 min; (h) 25 µL from each well was then aliquoted, diluted and then plated on LB agar plates; plates were incubated at 37^{o}C overnight and colonies were counted the next day. Cell free controls were included as before to calculate neutrophil killing efficiency.

In order to measure ROS production, mouse neutrophils were isolated as outlined above and resuspended in HBSS at a concentration of 2 million cells/mL. Then neutrophils were incubated with DCFH-DA at a concentration of 20 µM, and the suspension was placed on a rotator at 37^{o}C for 20 minutes. Neutrophils were then washed with HBSS once, resuspended in HBSS with calcium and magnesium, and then plated in a 96 well plate at a density of 200,000 cells/100 µL per well. Cells were stimulated with SCH79797 10 µM, phorbol myristate acetate (PMA) 25 nM, and vehicle (control) for 45

min, and then fluorescence was measured on a plate reader with excitation wavelength of 485 nm and emission of 535 nm. Each condition was run in triplicate.

Mouse neutrophil NET visualization and quantification

Neutrophils were isolated and seeded at a density of 1 million per 225 µL of HBSS in a 24 well plate. Go-6976, a protein kinase C (PKC) inhibitor, was added at 10x concentration (100 µM) in 25 µl of HBSS to relevant wells for 30-45 min. PKCs are involved in NET formation, and therefore conditions with a PKC inhibitor were included to determine whether this pathway is required for NET formation using the stimuli tested.[23,24] PMA 25 nM, SCH79797 10 µM, SCH530348 10 µM, or vehicle was then added in 50 µL volume (6x concentration for final volume of 300 µL per well) to appropriate wells and then the plate was incubated at 37^{o}C for 3.5 h. Cells were washed once with HBSS, fixed with 4% formaldehyde for 20 min, washed again with HBSS, and stained with SYTOX green (2 µM, ThermoFisher) for 15 min. After the staining was complete, cells were washed with HBSS and kept hydrated with HBSS prior to visualization. The published images were obtained using a Zeiss AxioObserver D1 microscope with 20x objective in the FITC channel.

NET quantification was done by seeding 200,000 neutrophils in 100 µl of HBSS in a 96 well plate. 20 µL of 6x Go-6976 or HBSS was added to appropriate wells for 30-45 min. 80 µL of 2.5x SCH79797, SCH530348, or PMA or 80 µL of HBSS was then added to appropriate wells for approximately 3 h, and the plate was kept at 37^{o}C. After the incubation period, 50 µL of a micrococcal nuclease solution (500 mU/mL) was added to each well and the plate was incubated at 37^{o}C for 10 min. 2.5 µL of 500 mM EDTA was added to each well to stop the micrococcal nuclease reaction, and the plate was spun at 200 g for 8 min. Then, 100 µL of the supernatant from each well was transferred to a new 96 well assay plate, and 100 µL of PicoGreen solution (Quant-IT PicoGreen dsDNA Assay kit, Life Technologies) was subsequently added to each well. The plate was incubated at room temperature for 5 min and NET formation was quantified using a fluorescent plate reader (excitation: 480 nm; emission: 520 nm).

Assessment of direct antibacterial effect of SCH79797

We carried out two different analyses to ascertain the effect of SCH79797 on *E. coli* growth *in vitro*. The first was a continuous assessment of *E. coli* growth kinetics in the presence of different concentrations of SCH79797 using a Bioscreen assay. *E. coli* K1 was grown overnight in LB broth for approximately 16-17 h, washed and then resuspended in PBS to achieve an OD = 1 at 600 nm. 200,000 cfu of *E. coli* was seeded in the presence or absence of a range of SCH79797 concentrations in a total volume of 200 µL per well in a 100 well honeycomb plate. The bacteria were cultured in 10% LB broth in RPMI-1640 media, and each condition was done in triplicate. *E. coli* growth was assessed every 30 min for 24 h under intermittent shaking conditions by measuring the OD at 600nm (Bioscreen C MBR machine, Growth Curves, USA).

We also carried out a separate set of experiments in which *E. coli* was grown in the presence of varying concentrations of SCH79797 for 6 h in 24 well plates. 500,000 cfu of *E. coli* was seeded in the presence or absence of SCH79797 in a total volume of 500 µl. SCH530348 was also tested to determine if the inhibition of bacterial growth seen with SCH79797 is a general class effect of PAR1 inhibitors. Each condition was done in triplicate, and the media used was 10% LB broth in RPMI-1640. After 6 h, an aliquot

from each well then diluted and plated on LB agar plates. The plates were incubated at 37°C overnight and colonies counted the next day. The data are presented as fold change versus initial inoculum of *E. coli* seeded. Similar studies were done to test the antibacterial activity of SCH79797 against Gram-positive bacteria by utilizing the MRSA strain, TCH 1516. The incubation media used was RPMI-1640 + 5% THB growth media, and aliquots were plated on THB agar plates.

Determination of mechanism of SCH79797 antibacterial action

Minimum inhibitory concentrations (MICs) of SCH79797 in *B. subtilis* and *E. coli* were determined by the broth microdilution method. Overnight pre-inoculum cultures were started by resuspending a single colony in 4 mL of LB medium, and incubated at 22°C (*B. subtilis*) or 30°C (*E. coli*). Inoculum cultures were started by diluting overnight cultures 1:200 in LB medium, and growing at 30°C to an OD600 of 0.15 (*B. subtilis*) or 0.2 (*E. coli*). The cultures were then diluted 1:100 (*B. subtilis*) or 1:400 (*E. coli*) into LB medium containing variable concentrations of SCH79797 in a 96-well plate and grown at 30°C for 24 h. MICs of SCH79797 in *B. subtilis* and *E. coli* were determined to be the lowest concentration of SCH79797 that prevented visible growth after 24 h.

Bacterial cytological profiling was utilized to determine the antibiotic mechanism of action of SCH79797.[25,26] Overnight pre-inoculum and inoculum cultures were prepared as described above. When inoculum cultures reached an OD600 of 0.15 (*B. subtilis*) or 0.2 (*E. coli*), 200 μL of culture was added to 5 mL plastic tubes containing either DMSO or SCH79797 at 0.5x, 1x, 2x, and 5x times the MIC. Cultures were rolled at 30°C. After 30 min and 2 h, 6 μL (*B. subtilis*) or 20 μL (*E. coli*) of cells were stained with 1.5 μL (*B. subtilis*) or 1 μL (*E. coli*) of dye mix. For *B. subtilis*, 100 μL of dye mix contained 3 μL of 1 mg/mL FM4-64, 1 μL of 0.25mM SYTOX Green, and 2.5 μL of 200 μg/mL DAPI in 93.5 μL of 1x T-base. For *E. coli*, 100 μL of dye mix contained 2 μL of 1 mg/mL FM4-64, 4 μL of 0.25 mM SYTOX Green, and 2 μL of 200 μg/mL DAPI in 92 μL of 1x T-base. 7 μL of the stained samples were added to microscope pads containing 1% agarose in 20% LB, and imaged using fluorescence microscopy. Experiments in high-sucrose media were conducted identically, except with growth media and agarose pads containing 0.5 M sucrose (*E. coli*).

Figure S1. Effects of sucrose on *E. coli* morphology in response to the cell wall biosynthesis inhibitor D-cycloserine or the membrane active compound Polymyxin B. Cells were treated with 21.8 µg/mL (1x MIC) D-cycloserine in LB medium (**A, C**), 43.6 µg/mL D-cycloserine in LB medium with 0.5 M sucrose (**E**), 1.1 µg/mL (5x MIC) Polymyxin B in LB medium (**B, D**) or 2.2 µg/mL Polymyxin B in LB medium with 0.5 M sucrose (**F**). Compound concentrations were doubled in the samples containing 0.5 M sucrose as the addition of sucrose reduces the efficacy of many antibiotics, and higher concentrations of D-cycloserine and Polymyxin B were required to affect cells. D-cycloserine experiments were conducted at 1x and 2x MIC as opposed to 5x MIC as only cell debris was visible at the 5x concentration. This figure appears in color in the online version of *JAC* and in black and white in the printed version of *JAC.* (Scale bars: 2 µm)

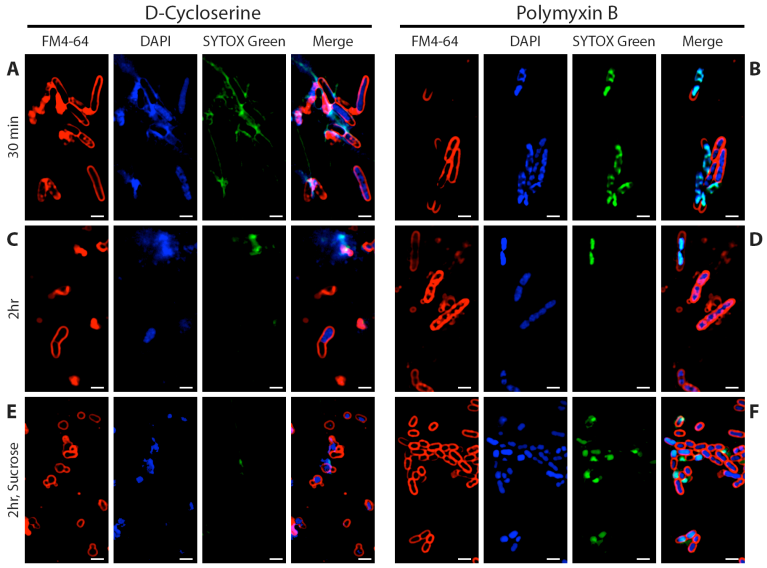

Figure S2. SCH79797 disrupts the membrane of the Gram-positive bacteria, *B. subtilis*. 5x MIC of SCH79797 demonstrates rapid and significant membrane disruption of *B. subtilis* starting at 30 min post-incubation (**A – D**). Cell membranes are stained with FM4-64 (red). DNA is stained with the membrane-permeable dye DAPI (blue) and the membrane-impermeable dye SYTOX Green (green). The presence of SYTOX Green indicates cells with compromised membranes; thus, SYTOX Green is normally absent from untreated cells. This figure appears in color in the online version of *JAC* and in black and white in the printed version of *JAC.* (Scale bars: 2 μm)

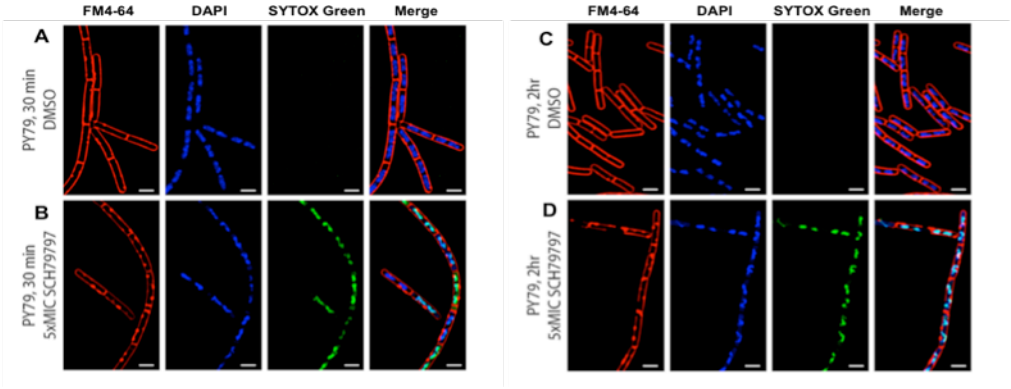

Figure S3. SCH79797 exerts significant bactericidal activity against MRSA. After 6h of incubation, SCH79797 significantly reduced bacterial growth of MRSA (TCH 1516) at a concentration of 1 μM while resulting in substantial bactericidal activity at 10 μM (**p < 0.01 versus TCH only group; ***p < 0.001 versus TCH only group; n = 6 per group; data as mean ± SD).