Published in final edited form as:

Bioorg Med Chem. 2012 August 1; 20(15): 4653–4660. doi:10.1016/j.bmc.2012.06.018.

Development of novel antibacterial agents against methicillin-resistant *Staphylococcus aureus*

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a serious threat to public health because of its resistance to multiple antibiotics most commonly used to treat infection. In this study, we report the unique ability of the cyclooxygenase-2 (COX-2) inhibitor celecoxib to kill *Staphylococcus aureus* and MRSA with modest potency. We hypothesize that the anti-*Staphylococcus* activity of celecoxib could be pharmacologically exploited to develop novel anti-MRSA agents with a distinct mechanism. Examination of an in-house, celecoxib-based focused compound library in conjunction with structural modifications led to the identification of compound **46** as the lead agent with high antibacterial potency against a panel of *Staphylococcus* pathogens and different strains of MRSA. Moreover, this killing effect is bacteria-specific, as human cancer cells are resistant to **46**. In addition, a single intraperitoneal administration of compound **46** at 30 mg/kg improved the survival of MRSA-infected C57BL/6 mice. In light of its high potency in eradicating MRSA *in vitro* and its *in vivo* activity, compound **46** and its analogues warrant continued preclinical development as a potential therapeutic intervention against MRSA.

1. Introduction

Staphylococcus aureus, a gram-positive bacterium, is one of the leading causes of hospital- and community-acquired infections in developed countries.¹ It is estimated that *S. aureus* is commensally found on nasal passages, skin and mucous membranes of 20 - 30% of the human population.^{2, 3} *S. aureus* can cause infection of the bloodstream, lower respiratory track, skin and soft tissue, leading to bacteremia, pneumonia, endocarditis and osteomyelitis.^{1, 4} Initially, *S. aureus* infections could be successfully treated with β -lactam antibiotics, like penicillin and methicillin. However, since the mid-1900s, the emergence of resistant strains of *S. aureus* has been reported,^{5, 6} including methicillin-resistant *S. aureus* (MRSA), which has become endemic in many hospitals worldwide. In addition to β -lactam

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antibiotics, *S. aureus* has also developed resistance to several other classes of antibiotics, including aminoglycosides, macrolides, lincosamides, chloramphenicol, sulfonamides, streptomycin and tetracycline.^{4, 7} The capability of *S. aureus* to resist multiple antibiotics has rendered its treatment difficult, leading to a higher mortality in patients. Thus, development of new antibacterial agents against *S. aureus*, especially strains resistant to multiple antibiotics, has become an urgent public health issue.

Previously, we reported that the cyclooxygenase-2 (COX-2) inhibitor celecoxib and its derivatives exhibited unique antimicrobial activities against various pathogenic bacteria *in vitro*, including *Salmonella* and *Francisella*.⁸⁻¹⁰ This anti-bacterial effect, however, was not noted with rofecoxib, a more potent COX-2 inhibitor, suggesting the dissociation of these two pharmacological activities. In addition, celecoxib was recently reported to inhibit multidrug resistance in pathogenic bacteria,¹¹ and has been used to develop a new class of efflux pump inhibitors.¹² In this study, we further demonstrated the unique ability of celecoxib to directly suppress, though with modest potency, the growth of *S. aureus*, *S. epidermidis* and MRSA. As celecoxib has been shown to suppress cancer cell proliferation, in part, by competing ATP binding of certain signaling kinases, such as phosphoinositide-dependent kinase-1 (PDK-1)¹³ and cyclin-dependent kinases (CDKs),¹⁴ and endoplasmic reticulum Ca²⁺-ATPases,¹⁵ we hypothesize that celecoxib mediates the bacterial killing by blocking ATP-dependent enzymes or transporters that are crucial to cell survival. Pursuant to this premise, we conducted a screening of an in-house celecoxib-based focused compound library, followed by structural optimization, to identify novel agents that exhibit high anti-MRSA potencies without acute cytotoxicity against human cells.

2. Results and discussion

In this study, we screened a celecoxib-based focused compound library to identify candidate anti-*Staphylococcus* agents for lead optimization (Figure 1A). Previously, during the course of our lead optimization of celecoxib to develop novel PDK-1 inhibitors,^{13, 16} we generated a series of derivatives with varying degrees of antiproliferative potency against cancer cells. Of these derivatives, we chose 40 representative celecoxib derivatives for screening against *S. aureus* and *S. epidermidis* (**1 – 40**; Fig. 2), which are noteworthy because they represent the most common causes of medical device-associated infections.¹⁷ This screening netted compound **36** as the lead anti-*Staphylococcus* agent, followed by compound **9**. Further structural modifications of **36** by substituting the phenanthrene ring with various aromatic structures yielded **41 – 47**, of which compound **46** was identified as the optimal agent. General procedures for the synthesis of compounds **1 – 47** are depicted in Fig. 1B.

2.1. Suppressive effect of celecoxib on the growth of *Staphylococcus* bacteria

Pursuant to our previous finding that celecoxib inhibited the proliferation of *Salmonella* and *Francisella* directly in culture medium,⁸⁻¹⁰ we examined its suppressive effect on the growth of *S. aureus* (ATCC 29213), *S. epidermidis* (ATCC 35984), and two different strains of MRSA (ATCC 33592 and SCCmec V_T). Celecoxib exhibited a clear, though modest, activity against these staphylococcal bacteria with the minimum inhibitory concentrations (MIC) of 32 µg/mL for *S. aureus* and both strains of MRSA, and 16 µg/mL for *S. epidermidis*. Exposure of *S. aureus* to celecoxib at the MIC of 32 µg/mL after 24 h resulted in a 6-log decrease in CFU relative to that of control (data not shown). In light of the absence of COX-2-like gene in bacteria,¹¹ this finding suggests that celecoxib's anti-*Staphylococcus* activity was dissociated from its effects on COX-2.

2.2. Identification of novel anti-*Staphylococcus* agents

The dissociation of these two pharmacological activities (antibacterial versus anti-COX-2) provided a molecular basis for the pharmacological exploitation of celecoxib to develop novel anti-*Staphylococcus* agents. As the target for celecoxib's anti-*Staphylococcus* activity remained unknown, we used an in-house, celecoxib-based focused compound library consisting of 40 derivatives with modifications to the terminal aromatic group (R) and polar side chain (Fig. 2), which were screened for growth inhibitory activities against *S. aureus* (ATCC 29213) and *S. epidermidis* (ATCC 35984). Of these derivatives, compounds **1** – **8** and **10** of the carboxamide series, **12** – **15** of the sulfonamide series, **16** – **20** of the amine series, **21**, **22**, **32**, and **37** – **40** did not exhibit appreciable activity at 64 µg/mL or improved activity relative to celecoxib (data not shown). However, other derivatives exhibited multi-fold increases in anti-*Staphylococcus* potency (Table 1), providing a proof-of-concept of our premise that celecoxib could be structurally modified to enhance its anti-*Staphylococcus* activity. Among these more active derivatives, compound **36**, followed by compound **9**, represented the lead agents with MIC values of 2 µg/mL against both *S. aureus* and *S. epidermidis*.

As celecoxib is cytotoxic to cancer cells,¹⁸⁻²⁰ the growth inhibitory activities of celecoxib and selected derivatives were assessed in HT-29 human colon adenocarcinoma cells after 24-h exposure. As shown in Table 1, most of the active anti-*Staphylococcus* compounds examined suppressed the viability of HT-29 cells with high potency (low IC₅₀ values), resulting in low selectivity ratios, defined as IC₅₀/MIC against *S. aureus*. It is noteworthy that compounds **9** and **36**, the lead anti-*Staphylococcus* derivatives, showed lower cytotoxic activity against HT-29 cells relative to the other compounds resulting in the highest selectivity ratios (6 and 12, respectively), indicating a better selectivity in suppressing the growth of *Staphylococcus* versus HT-29 cells.

The above findings underscore the translational potential of compounds **9** and **36** to develop potent anti-*Staphylococcus* agents. Based on these results, we hypothesized that there existed interplay between the terminal aromatic system and the hydrophilic side chain to mediate the anti-*Staphylococcus* activity through the interactions with target protein(s). In light of the modestly improved antibacterial activity of compound **36** relative to compound **9**, we used the former compound as a scaffold for structural modifications by replacing the 2-phenanthrenyl ring with various aromatic systems, generating compounds **41** – **47** (Fig. 1A). These derivatives were assessed for their antibacterial activities against *S. aureus* (ATCC 29213) and *S. epidermidis* (ATCC 35984), which revealed some degree of flexibility in altering the size of the aromatic ring. For example, the phenanthrene ring could be replaced by biphenyl or substituted biphenyls (**42** – **44**) without compromising the anti-*Staphylococcus* activity. Of particular interest is the substitution with an anthracen-9-yl moiety (compound **46**), which gave rise to a twofold increase in the anti-*Staphylococcus* potency with MIC of 0.5 µg/mL (Table 2). Moreover, compound **46** exhibited lower antiproliferative potency against HT-29 cancer cells (IC₅₀, 20 µg/mL), providing a selectivity ratio of 40 relative to that of 12 for compound **36**. It is noteworthy that compound **46** represents a hybrid of compounds **9** and **36**, underlying a subtle structure-activity relationship in interacting with the bacterial target.

2.3. Antibacterial spectra of compounds **9**, **36**, and **41** – **46** against different *Staphylococcus* species

As different strains/species of *Staphylococcus* might respond differently to the antibacterial effects of these novel agents, we further expanded our investigation to include a panel of representative *Staphylococcus* pathogens, consisting of different strains of *S. aureus*, *S.*

epidermidis and MRSA, as well as *S. haemolyticus*, *S. hominis*, *S. intermedius*, *S. saprophyticus*, and *S. lugdunensis*.

As shown in Table 3, the inhibitory potencies of these test agents against the three strains of MRSA, including the multidrug-resistant community-associated MRSA that carries the novel staphylococcal chromosome cassette mec (SCCmec) subtype V,²¹ T were consistent with those of *S. aureus* and *S. epidermidis*. Among these derivatives, compound **46** represented the optimal anti-MRSA agent with MIC of 0.5 µg/mL, followed by compounds **36** and **42 – 44**, all of which exhibited an MIC value of 1 µg/mL. Moreover, as these MRSA strains have been reported to resist different classes of antibiotics,^{7, 21, 22} this finding suggests that a novel antibacterial target is involved in the mechanism of action of these agents.

Other non-*Staphylococcus aureus* species examined, with the exception of *S. intermedius*, showed a lesser degree of susceptibility to compounds **36** and **46**, with MIC around 2 µg/mL. In contrast, the potency of compound **9** remained relatively unchanged across different *Staphylococcus* species.

2.4. Compounds **36** and **46** are bactericidal against *S. aureus*

An antibacterial agent is defined as bactericidal when it exhibits the distinctive endpoint of causing a 99.9% reduction in bacterial inoculum within a 24-h period of exposure.²³ Otherwise, it is considered bacteriostatic. To category these novel agents, represented by compounds **9**, **36**, and **46**, in this regard, their time-killing kinetics were assessed in *S. aureus* ATCC 29213 over a 24-h treatment period as compared to the known bactericidal and bacteriostatic agents ampicillin and chloramphenicol, respectively. Overnight-grown bacteria were inoculated in cation-adjusted Muller Hinton broth (CAMHB) at a concentration of 5×10^5 CFU/mL followed by exposure to individual compounds at 2-to 8-fold their respective MIC values. As shown in Fig. 3, compounds **9**, **36**, and **46** caused time-dependent killing of *S. aureus*, achieving reductions in CFU of 99.14%, 99.91%, and 99.99% respectively, after 24-h exposure to 8 times the MIC. In comparison to ampicillin and chloramphenicol, the bacterial killing effect of compounds **36** and **46** were characteristic of bactericidal, while the antibacterial effect of compound **9** was not sufficient to be defined as bactericidal.

2.5. Intraperitoneal administration of compound **46** improves the survival of MRSA-infected C57BL/6 mice

To further evaluate the therapeutic potential of compounds **36** and **46** against MRSA, inbred C57BL/6 mice were intraperitoneally injected with a lethal dose (7×10^7 CFU) of MRSA (ATCC 33592), followed by a single, intraperitoneal administration of vehicle control or compound **36** or **46** at 30 mg/kg at 1 h post-infection ($N = 5$ for each group). Mice treated with vehicle or compound **36** rapidly developed signs of severe infection that included weight loss of over 20%, significant decrease in body temperature, and lethargy. Survival time for these mice was no more than 2 days (Fig. 4A). Among the five mice that received a single dose of compound **46** at 30 mg/kg, three mice died during the first day post-infection (Fig. 4A). However, two of the compound **46**-treated mice survived for at least 7 days post-infection, exhibiting decreases of body weight at 1 day post-infection that returned to pre-infection levels after 3 – 6 days post-infection (Fig. 4B). It is noteworthy that, other than body weight loss, no other signs of illness were observed in these two mice. Together, these findings provided a proof-of-concept that compound **46** exhibited anti-*Staphylococcus* activity *in vivo*.

2.6. Discussion

Although hospitals have fought MRSA infections since the late 1960s, the past two decades have witnessed severe community-associated MRSA cases affecting healthy, young individuals with no link to the healthcare system.²⁴ To date, *S. aureus* has developed various mechanisms to evade the inhibitory effect of almost all classes of antibiotics, as well as the third-line agent vancomycin.^{25, 26} Consequently, MRSA represents an impending public health crisis, and there is an urgent need to develop new anti-MRSA agents with distinct modes of antibacterial action to overcome the multi-drug resistance. Here, we report the pharmacological exploitation of the suppressive effect of the COX-2 inhibitor celecoxib on the growth of *Staphylococcus* bacteria to develop a novel class of anti-MRSA agents with high potency.

In this study, we tested three different strains of MRSA, all of which exhibit resistance to multiple antibiotics, including oxacillin, clindamycin, sulfonamides, erythromycin, tetracycline, cotrimoxazole, gentamicin, chloramphenicol, and streptomycin.^{7, 21, 22} Despite this multi-drug resistant phenotype, all of the MRSA strains were as sensitive to the inhibitory effects of compounds **46**, **36**, and **9** as the methicillin-sensitive *S. aureus* strains, with MIC values in the range of 0.5 to 2 $\mu\text{g/mL}$. This lack of cross-resistance suggests that the mode of action of these compounds is different from those of existing antibiotics.

Among the forty-seven derivatives examined, there exists a subtle structure-activity relationship in inhibiting the growth of *Staphylococcus* bacteria. Although the majority of the compounds of the carboxamide (**1** – **8**, **10**), sulfonamide (**12** – **15**), and amine (**16** – **20**) series did not show improved activity over celecoxib (MIC, 32 $\mu\text{g/mL}$), conversion of any of these three functional groups into an aminosulfonamide moiety increased the anti-*Staphylococcus* potency by multifold, i.e., **1** and **16** versus **42**, **4** and **15** versus **43**, **20** versus **44**, and **9** versus **46**.

The evaluation of compounds **36** and **46** in MRSA-infected mice demonstrated that compound **46** possesses promising *in vivo* anti-MRSA activity. Despite infection with a lethal dose of MRSA and treatment with only a single dose of drug, two of five compound **46**-treated mice recovered and survived to the study endpoint, while none of the vehicle-treated control mice survived for more than two days post-infection. Studies to develop an optimal formulation for *in vivo* administration of compound **46**, as well as synthetic strategies aimed at increasing aqueous solubility are currently underway.

To shed light onto the potential antibacterial target of these compounds, we are currently identifying bacterial proteins with homology to mammalian targets of celecoxib using the published proteome of *S. aureus* and *S. epidermidis* at the National Center of Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).²⁷ Previously, we demonstrated that celecoxib mediated the antiproliferative effect in cancer cells by targeting a number of non-COX enzymes, including certain signaling kinases, such as PDK-1¹³ and CDKs,¹⁴ and endoplasmic reticulum Ca^{2+} -ATPases.¹⁵ Through BLASTP analysis, we have identified a number of bacterial proteins of *S. aureus* and *S. epidermidis* with some degree of homology to PDK-1 and endoplasmic reticulum calcium ATPases, including a serine/threonine kinase (NP_764450), the copper transporter ATPase copA, potassium transporter ATPase subunit B, and cadmium transporting ATPase. Evaluation of the involvement of these putative targets in the anti-*Staphylococcus* effects of compounds **9**, **36**, and **46**, in conjunction with genomic analysis of drug-resistant mutants, is currently underway. From a translational perspective, understanding the mode of action of these novel agents will foster new strategies for the treatment of staphylococcal infections.

3. Conclusion

The high potency of compounds **36** and **46** in eradicating MRSA *in vitro* justifies continued preclinical development of these compounds in animal models as potential therapeutic agents against infection with MRSA. In addition, identification of the molecular target by which these agents kill *Staphylococcus* bacteria will help design more potent anti-MRSA agents for clinical use.

4. Material and methods

4.1. Chemistry general methods

Celecoxib was prepared from Celebrex® capsules (Amerisource Health, Malvern, PA) by solvent extraction followed by recrystallization from a mixture of ethyl acetate and hexane.

Unless otherwise indicated, all anhydrous solvents were commercially obtained and stored in Sure-seal bottles under nitrogen. All other reagents and solvents were purchased as the highest grade available and used without further purification. Flash column chromatography was performed with silica gel (Sorbent Technologies, 230-400 mesh). Nuclear magnetic resonance spectra (¹H NMR) were acquired on a Bruker DPX 300 model spectrometer. Chemical shifts (δ) were reported in parts per million (ppm) relative to the TMS peak. Coupling constants (*J*) were reported in Hertz throughout. Electrospray ionization mass spectrometry analyses were performed with a Micromass Q-ToF II high-resolution electrospray mass spectrometer. The purity of all tested compounds was determined to be greater than 95% by elemental analyses, which were performed by Atlantic Microlab, Inc. (Norcross, GA) and were reported within 0.4% of calculated values. All synthesized compounds were soluble in organic solvents, including DMSO.

4.2. General procedure of carboxamide series (**9**) and the aminosulfonamide series (**36** and **41 – 47**)

The sulfonamide series compounds **11 – 15**, the amine series compounds **16 – 20**, and the amino acid series compounds **22 – 35** and **38 – 40** were synthesized as previously described.^{16, 28} Syntheses of the active compounds of the carboxamide series (i.e., **9**) and the aminosulfonamide series (i.e., **36** and **41 – 47**) are illustrated by the syntheses of compounds **9** and **36**, respectively, as examples.

Step a. To a suspension of ethyl trifluoroacetate (850 mg, 6 mmol, 1.2 eq.) and NaH (150 mg, 6.25 mmol, 1.25 eq.) in anhydrous THF, individual ketone substrates (5 mmol, 1 eq.) in anhydrous THF were slowly added at 25 °C. The resulting mixture was stirred for 5 h, concentrated, diluted with ethyl acetate, washed, in tandem, with water, 1N HCl and brine. The organic phase was dried over sodium sulfate, filtered and concentrated. The residue was purified by flash column chromatography (EtOAc-hexane, 1:4) to afford pure 1,3-diketone in fair to good yields.

Step b. A mixture of the 1,3-diketone from step a (3 mmol, 1 eq.), individual hydrazine substrates (3.75 mmol, 1.25 eq.), and concentrated HCl (0.4 mL, 1.5 eq.) in ethyl alcohol was refluxed until the reaction completed (monitored with TLC; EtOAc-hexane, 3:7). The resulting mixture was concentrated, diluted with ethyl acetate, and washed with water and brine. The organic phase was dried over sodium sulfate, filtered and concentrated. The residue was purified by flash column chromatography (EtOAc-hexane, 3:7) to yield pure pyrazole ring derivatives.

Step c. To a solution of 4-(5-anthracen-9-yl-3-trifluoromethyl-pyrazol-1-yl)-benzonitrile generated from step b (206 mg, 0.5 mmol, 1 eq.) in DMSO (1 mL) were added Na₂CO₃ (106

mg, 1 mmol, 2 eq.) and H₂O₂ (30%, 0.2 mL, 3 eq.) at 0 °C. The reaction mixture was stirred at 20 °C for 3 h, and water (3 mL) was added. The white precipitate was filtered, washed with water, and dried over sodium sulfate, and filtered to afford compound **9** (177 mg) as off-white crystal in 82% yield.

Step d. A reaction mixture of various 5-aryl-1-(4-nitrophenyl)-3-(trifluoromethyl)-1*H*-pyrazole derivatives (3 mmol, 1 eq.), generated from step b, platinum oxide (PtO₂, 23 mg, 0.1 mmol, 0.03 eq.) in EtOH was stirred overnight under H₂ atmosphere, filtered, and washed with ethyl acetate. The combined filtrate was concentrated and the residue was purified with flash column chromatography (CH₂Cl₂-NH₄OH, 99.9:0.1) to give the corresponding amines with quantitative yields.

4.2.1. 4-(5-Anthracen-9-yl-3-trifluoromethyl-pyrazol-1-yl)-benzamide (9**)**—¹H NMR (DMSO-d₆) δ 7.16 (s, 1H), 7.19 (s, 1H), 7.34 (br s, 1H), 7.41 (s, 1H), 7.57-7.53 (m, 8H), 7.79 (br s, 1H), 8.17 (m 2H), 8.83 (s, 1H). HRMS exact mass of C₂₅H₁₆F₃N₃O, (M + Na)⁺, 454.1143 amu; found 454.1136 amu. Anal. calcd C 69.60, H 3.74, N 9.74; found C 69.42, H 3.85, N 9.84.

4.2.2. N-[4-(5-Phenanthren-2-yl-3-trifluoromethyl-pyrazol-1-yl)-phenyl]-aminosulfonamide (36**)**—Chlorosulfonyl isocyanate (142 mg, 1 mmol, 1 eq.) was added dropwise to an ice-cold solution of *t*-BuOH (74 mg, 1 mmol, 1 eq.) in CH₂Cl₂, which was then added to a mixture of 4-(5-phenanthren-2-yl-3-trifluoromethyl-pyrazol-1-yl)-phenylamine (403 mg, 1 mmol, 1 eq.), generated from step d, and triethylamine (152 mg, 1.5 mmol, 1.5 eq.) in CH₂Cl₂. The reaction mixture was stirred at 25 °C for 1 h, and concentrated. The residue was treated with 20% trifluoroacetic acid in CH₂Cl₂ for 3 h, washed with 10% NaHCO₃, dried over sodium sulfate, and concentrated. The residue was purified by flash column chromatograph (MeOH-CH₂Cl₂-NH₄OH, 2:97.9:0.1) to give **36** (388 mg) as off-white solid in 78% yield. ¹H NMR (DMSO-d₆) δ 7.35-7.15 (m, 7H), 7.46 (dd, *J* = 1.8, 8.7 Hz, 1H), 7.73-7.65 (m, 2H), 7.88 (dd, *J* = 9, 33.6 Hz, 2H), 8.00-8.05 (m, 1H), 8.09 (d, *J* = 1.8 Hz, 2H), 8.82 (m 2H), 9.89 (br s, 1H). HRMS exact mass of C₂₄H₁₇F₃N₄O₂S, (M+Na)⁺, 505.0922 amu; found: 505.0902 amu. Anal. calcd C 59.75, H 3.55, N 11.61; found C 59.98, H 3.71, N 11.51.

Compound **41-47** were prepared by using the same procedure as **36** with the following yields: **41** (277 mg, 70%); **42** (330 mg, 72%); **43** (354 mg, 75%); **44** (391.5 mg, 73%); **45** (303 mg, 70%); **46** (328 mg, 68%); **47** (343 mg, 71%).

4.2.3. N-[4-(5-p-Tolyl-3-trifluoromethyl-pyrazol-1-yl)-phenyl]-aminosulfonamide (41**)**—Melting point: 199-200 °C. ¹H NMR (CDCl₃) δ 2.30 (s, 3H), 7.27-7.11 (m, 11 H), 9.88 (br s, 1H). ¹³C NMR (CDCl₃) δ 151.5, 143.7, 137.9, 131.4, 129.5 (2 ×), 127.0 (2 ×), 126.7 (2 ×), 126.5 (2 ×), 126.0 (2 ×), 116.3, 102.3, 21.1. HRMS exact mass of C₁₇H₁₅F₃N₄O₂S, (M+Na)⁺, 419.0766 amu; found: 419.0755 amu. Anal. calcd C 51.51, H 3.81, N 14.13; found: C 51.30, H 3.79, N 14.08.

4.2.4. N-[4-(5-Biphenyl-4-yl-3-trifluoromethyl-pyrazol-1-yl)-phenyl]-aminosulfonamide (42**)**—Melting point: 170-171 °C. ¹H NMR (CD₃OD) δ 6.96 (s, 1H), 7.25-7.41 (m, 5H), 7.44-7.51 (m, 4H), 7.60-7.63 (m, 4H). ¹³C NMR (CDCl₃) δ 151.5, 150.8, 143.7, 140.8, 140.3, 132.3, 131.5, 128.7 (3 ×), 127.2 (2 ×), 126.9 (2 ×), 126.5 (2 ×), 126.0 (2 ×), 117.1, 116.1 (2 ×), 102.3. HRMS exact mass of C₂₂H₁₇F₃N₄O₂S, (M+Na)⁺, 481.0922 amu; found: 481.0913 amu. Anal. calcd C 57.64, H 3.74, N 12.22; found: C 57.69, H 3.78, N 12.16.

4.2.5. *N*-[4-[5-(4'-Methylbiphenyl-4-yl)-3-trifluoromethyl-pyrazol-1-yl]-phenyl]-aminosulfonamide (43)—Melting point: 194-195 °C. ¹H NMR (CD₃OD) δ 2.37 (s, 3H), 6.95 (s, 1H), 7.35-7.24 (m, 8H), 7.52 (d, *J* = 7.8 Hz, 2H), 7.61 (d, *J* = 8.1 Hz, 2H). ¹³C NMR (CDCl₃) δ 151.5, 150.8, 143.7, 140.8, 140.3, 137.9, 137.0, 132.0, 131.5, 129.5 (2 ×), 127.0 (2 ×), 126.8 (2 ×), 126.5, 126.0 (2 ×), 117.2, 116.3 (2 ×), 102.3, 21.1. HRMS exact mass of C₂₃H₁₉F₃N₄O₂S, (M+Na)⁺, 495.1079 amu; found: 495.1061 amu. Anal. calcd C 58.47, H 4.05, N 11.86; found: C 58.28, H 4.06, N 11.84.

4.2.6. *N*-[4-[5-(4'-Bromobiphenyl-4-yl)-3-trifluoromethyl-pyrazol-1-yl]-phenyl]-aminosulfonamide (44)—Melting point: 157-158 °C. ¹H NMR (DMSO-*d*₆) δ 7.18-7.49 (m, 11H), 7.68-7.22 (m, 4H), 9.93 (s, 1H). ¹³C NMR (CDCl₃) δ 151.5, 150.8, 143.7, 140.8, 140.3, 137.7, 137.0, 132.0, 129.3 (2 ×), 127.2 (2 ×), 126.5 (2 ×), 126.5, 126.0 (2 ×), 122.2, 117.0, 116.1 (2 ×), 102.3. HRMS exact mass of C₂₂H₁₆BrF₃N₄O₂S, (M+Na)⁺, 559.0027 amu; found: 559.0038 amu. Anal. calcd C 49.17, H 3.00, N 10.43; found: C 49.31, H 3.14, N 10.18.

4.2.7. *N*-[4-(5-Naphthalen-2-yl-3-trifluoromethyl-pyrazol-1-yl)-phenyl]-aminosulfonamide (45)—Melting point: 190-191 °C. ¹H NMR (CDCl₃) δ 5.02 (s, 2H), 6.83 (s, 1H), 7.09-7.18 (m, 4H), 7.26 (d, *J* = 6.6 Hz, 2H), 7.49-7.51 (m, 2H), 7.72-7.78 (m, 4H). ¹³C NMR (DMSO-*d*₆) δ 153.2, 141.4, 140.9, 139.6, 132.4, 130.7 (2 ×), 130.5, 129.6, 126.4 (2 ×), 127.5, 125.7, 124.8, 124.6, 122.2, 117.4, 116.7 (2 ×), 108.7. HRMS exact mass of C₂₀H₁₅F₃N₄O₂S, (M+Na)⁺, 455.0766 amu; found: 455.0753 amu. Anal. calcd C 55.55, H 3.50, N 12.96; found: C 55.34, H 3.52, N 12.69.

4.2.8. *N*-[4-(5-Anthracen-9-yl-3-trifluoromethyl-pyrazol-1-yl)-phenyl]-aminosulfonamide (46)—Melting point: 195-196 °C. ¹H NMR (CD₃OD) δ 6.83 (d, *J* = 8.1 Hz, 2H), 6.99 (s, 1H), 7.06 (d, *J* = 8.4 Hz, 2H), 7.48 (s, 4H), 7.56 (s, 2H), 8.06 (d, *J* = 6.0 Hz, 2H), 8.61 (s, 1H). ¹³C NMR (DMSO-*d*₆) δ 153.0, 141.4, 139.6, 132.4, 130.7 (2 ×), 130.5 (2 ×), 129.6, 128.8 (2 ×), 127.5 (2 ×), 125.7 (2 ×), 124.8 (2 ×), 124.6 (2 ×), 122.2, 117.6, 116.3 (2 ×), 108.7. HRMS exact mass of C₂₄H₁₇F₃N₄O₂S, (M+Na)⁺, 505.0922 amu; found: 505.0905 amu. Anal. calcd C 59.75, H 3.55, N 11.61; found: C 59.89, H 3.66, N 11.52.

4.2.9. *N*-[4-(5-Anthracen-2-yl-3-trifluoromethyl-pyrazol-1-yl)-phenyl]-aminosulfonamide (47)—Melting point: 193-194 °C. ¹H NMR (CD₃OD) δ 7.03 (s, 1H), 7.39-7.16 (m, 8H), 7.48 (s, 2H), 7.98 (s, 2H), 8.38 (d, *J* = 9.6 Hz, 1H). ¹³C NMR (DMSO-*d*₆) δ 153.2, 141.4, 139.2, 132.0, 130.7 (2 ×), 130.3 (2 ×), 129.6, 128.4 (2 ×), 127.5 (2 ×), 125.6 (2 ×), 124.8 (2 ×), 124.6, 123.3, 122.2, 117.6, 116.3 (2 ×), 106.7. HRMS exact mass of C₂₄H₁₇F₃N₄O₂S, (M+Na)⁺, 505.0922 amu; found: 505.0930 amu. Anal. calcd C 59.75, H 3.55, N 11.61; found: C 59.93, H 3.59, N 11.68.

4.3. Bacteria stains

S. aureus strains ATCC 29213, ATCC 12598, MRSA strains ATCC 33592, ATCC 49476, *S. epidermidis* strains ATCC 35984 and ATCC 12228, *S. haemolyticus* strain ATCC 29970, *S. hominis* strain ATCC 27844, *S. intermedius* strain ATCC 29663, and *S. saprophyticus* strain ATCC 15305 were obtained from American Type Culture Collection (Manassas, VA). The clinically isolated *S. lugdunensis* and a MRSA strain carrying SCCmec V_T were obtained from the National Taiwan University Hospital (Taipei, Taiwan).

4.4. Antibacterial assays

The MIC of each agent was determined following the guidelines for the broth microdilution method recommended by the Clinical and Laboratory Standards Institute.²⁹ Briefly, bacteria

grown overnight on Luria Bertani (LB) agar plates were suspended in phosphate-buffered saline (PBS) to an O.D. of 1.0 at 600 nm, which was equivalent to 2×10^9 CFU/mL, and then diluted in CAMHB to a final concentration of 5×10^5 CFU/mL. The bacterial suspensions were exposed to the test agents and chloramphenicol at escalating doses, ranging from 0.25 to 64 $\mu\text{g/mL}$, in triplicate in 96-well plates, and the plates were incubated at 37°C for 24 h. The MIC of each agent was defined as the lowest concentration at which no growth of bacteria was observed. Stock solutions of the test agents were made in DMSO at 100 mg/mL, and diluted in culture medium to a final DMSO concentration of 0.1%.

4.5. Antiproliferative assay

The cytotoxicity of individual test agents in HT-29 human colon adenocarcinoma cells was evaluated by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. Briefly, HT-29 cells were seeded into 96-well plates at 1×10^4 cells/well (with a minimum of 6 wells per test condition) in RPMI 1640 medium supplemented with 10% FBS and 10 $\mu\text{g/mL}$ of gentamicin. After overnight incubation at 37°C under 5% CO₂, the medium from each well was removed and replenished with fresh aliquots of the same medium containing various concentrations of test agents dissolved in DMSO (final concentration, 0.1%). Control cells were treated with DMSO alone at a concentration equal to that in drug-treated cells. After 24 h of drug exposure, the medium was removed and replaced by 100 μl of 0.5 mg/mL MTT in 10% FBS-containing medium, and the cells were incubated in the CO₂ incubator at 37°C for 2 h. Subsequently, medium was removed from each well, and the reduced MTT dye was dissolved with 100 μl of DMSO per well. Absorbance at 570 nm was measured with a plate reader. The 50% inhibitory concentration (IC₅₀) of each drug was determined from dose-response curves by using CalcuSyn software (Biosoft, Cambridge, United Kingdom).

4.6. Time-kill assay

To analyze the kinetics of bacterial cell killing, *S. aureus* ATCC 29213 cells at a density of 5×10^5 CFU/mL were treated with test agents at 2-, 4-, and 8-fold MICs in triplicate in 24-well plates. Bacterial survival in medium containing DMSO at a concentration equal to that used for drug-treated bacteria served as control. At different times after the start of drug exposure, a 100 μl aliquot of the bacterial suspension was taken from each well and serially diluted with PBS. The diluted samples were spread onto LB agar plates followed by incubation at 37°C for 16 h. The bacterial colonies on each plate were enumerated, and the number of viable bacteria in each well was expressed as CFU per milliliter.

4.7. In vivo studies

Female C57BL/6 mice (8 to 10 weeks of age) were purchased from the National Laboratory Animal Center (Taipei, Taiwan), and housed as groups under conditions of constant photoperiod (12 h light, 12 h dark) with *ad libitum* access to sterilized food and water in the Laboratory Animal Center, College of Medicine, National Taiwan University. All experimental procedures with these mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the National Taiwan University. Overnight-grown methicillin-resistant *Staphylococcus aureus* (ATCC 33592) were diluted (1:100) in Luria Bertani broth and grown at 37°C to OD₆₀₀=0.6, harvested by centrifugation, washed and resuspended in sterile PBS. Mice were infected by intraperitoneal administration of MRSA (7×10^7 organisms in 0.3 ml PBS; a 100% lethal dose) through a 1 ml syringe with 25 gauge needle. The same bacterial culture was plated onto LB agar to confirm the number of organisms inoculated. At 1 h post-infection, mice were assigned to treatment groups and then received a single treatment of vehicle (40% PEG-400, 10% 2-hydroxyl- β -dextran, 10% DMSO in normal saline), compound **36** or

compound **46** by intraperitoneal injection. Observations of general health and measurements of body weight were recorded daily. The survival time of each mouse was recorded and was defined as the time in days from the start of the study to when mice were sacrificed upon exhibiting signs of significant morbidity, which included, though were not limited to, weight loss of 20% of initial body weight.

Acknowledgments

This work was supported by Award Number UL1RR025755 from the National Center for Research Resources, funded by the Office of the Director, National Institutes of Health (OD) and supported by the NIH Roadmap for Medical Research to C.S.C and by the grant numbers NSC 99-2320-B-002-081 and NSC 100-2320-B-002-104 from National Science Council, Taiwan to H.C.C. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources, the National Institutes of Health, or the National Science Council, Taiwan.

We also thank Wei-Chun Hung, Hsiao-Jan Chen and the Microarray Core Facility of the National Research Program for Genomic Medicine of the National Science Council in Taiwan for assistance with experiments.

Abbreviation

MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
COX-2	cyclooxygenase-2
LB	Luria Bertani
CAMHB	cation-adjusted Muller Hinton broth
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
MIC	minimum inhibitory concentration
IC₅₀	50% inhibitory concentration
CFU	colony-forming unit
PDK-1	phosphoinositide-dependent kinase-1
CDK	cyclin-dependent kinases
SCCmec	Staphylococcal chromosome cassette mec

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Figure 1.

Chemical structures of celecoxib and compounds 1 – 40 in the celecoxib-based focused compound library.

Figure 2.
Chemical structures of compounds **41** – **47**. (B) General synthetic procedures for compounds **1** – **47**.

Figure 3.

The viability of *S. aureus* ATCC 29213 after exposure to various concentrations (2×, 4×, and 8× MIC) of compound 9 (MIC, 4 µg/mL), 36 (MIC, 1 µg/mL), 46 (MIC, 0.5 µg/mL), ampicillin (MIC, 8 µg/mL), and chloramphenicol (MIC, 8 µg/mL) for 2, 4, 8 and 24 h in CAMHB. Numbers of viable bacteria in the broth after each exposure period were enumerated by CFU assay, and the results expressed as CFU/mL. Points indicate means, and bars indicate SD (n = 3). The dashed line represents 99.9% cell killing. Ctl, control.

Figure 4.

Intraperitoneal administration of compound **46** improves the survival of MRSA-infected mice. (A) Effect of intraperitoneal administration of compound **36** and compound **46** on survival of MRSA-infected mice. Female C57BL/6 mice were inoculated intraperitoneally with 7×10^7 CFU of MRSA (ATCC 33592). At 1 h post-infection, mice were treated intraperitoneally once with vehicle (filled circle), compound **36** (open circle) or compound **46** (filled triangle) at 30 mg/kg. (B) Body weight changes in individual compound **46**-treated MRSA-infected mice. The body weights of two out of five MRSA-infected mice (#2 and #4) that survived to 7 days post-infection were monitored during the experiment. Mouse #2 (filled circle), mouse #4 (open circle).

Table 1Anti-*Staphylococcus* (MIC) versus antiproliferative (IC₅₀) activities of test agents

Compound	MIC (μg/mL)		IC ₅₀ (μg/mL) HT-29 cells	Selectivity ratio ^a
	<i>S. aureus</i> (ATCC 29213)	<i>S. epidermidis</i> (ATCC 35984)		
Celecoxib	32	16	18	0.6
9	2	2	12	6
11	4	8	12.5	3.1
23	4	4	3.6	0.9
24	4	4	6.2	1.6
25	4	4	6.5	1.6
26	4	4	4.2	1.1
27	4	4	6	1.5
28	4	4	7.5	1.9
29	4	4	7.2	1.8
30	4	4	3.2	0.8
31	4	4	17.5	4.4
33	4	4	3.3	0.8
34	4	4	19.5	4.9
35	2	4	5.2	2.6
36	1	2	12	12

^aSelectivity ratio = IC₅₀/MIC against *S. aureus*

Table 2

Antibacterial activities (MIC) of test agents vis-à-vis ampicillin and chloramphenicol against *S. aureus* and *S. epidermidis* and selectivity over the antiproliferative activities against HT-29 human colorectal cancer cells

Compound	MIC (µg/mL)		IC ₅₀ (µg/mL) HT-29 cells	Selectivity ratio ^a
	<i>S. aureus</i> (ATCC 29213)	<i>S. epidermidis</i> (ATCC 35984)		
9	2	2	12	6
36	1	2	12	12
41	4	4	28	7
42	1	2	16	16
43	1	2	9	9
44	1	2	10	10
45	1	2	19	19
46	0.5	1	20	40
47	4	4	11	2.8
Ampicillin	32	64	-	-
Chloramphenicol	8	8	-	-

Antibacterial spectra of compounds **9**, **36**, and **41** – **47** vis-à-vis ampicillin and chloramphenicol against a panel of *Staphylococcus* pathogens

Table 3

<i>Staphylococcus</i> species	MIC (µg/mL)													
	9	36	41	42	43	44	45	46	47	Amp	Cm			
<i>S. aureus</i> (ATCC 12598)	4	1	4	1	1	1	2	0.5	4	0.25	4			
<i>S. epidermidis</i> (ATCC 12228)	4	1	4	2	1	1	2	0.5	2	16	2			
MRSA (ATCC 33592)	4	1	4	1	1	1	2	0.5	2	>64	64			
MRSA (SCCmec V _T)	4	1	4	1	1	1	2	0.5	2	>64	32			
MRSA (ATCC 49476)	4	1	4	1	1	1	2	0.5	2	64	4			
<i>S. haemolyticus</i> (ATCC 29970)	4	2	16	2	2	2	4	2	16	0.25	4			
<i>S. hominis</i> (ATCC 27844)	2	2	8	2	2	2	4	2	2	<0.0625	2			
<i>S. intermedius</i> (ATCC 29663)	4	1	8	2	2	2	2	0.5	4	0.125	4			
<i>S. saprophyticus</i> (ATCC 15305)	4	2	8	2	2	2	4	2	8	0.5	4			
<i>S. lugdunensis</i> (NTUH isolate)	4	2	16	2	2	2	4	2	4	32	2			