Raf-kinase inhibitor GW5074 shows antibacterial activity against methicillin-resistant *Staphylococcus aureus* and potentiates the activity of gentamicin

Aim: Increasing antimicrobial resistance has compromised the effectiveness of many antibiotics, including those used to treat staphylococcal infections like methicillin-resistant *Staphylococcus aureus*. The development of combination therapies, where antimicrobial agents are used with compounds that inhibit resistance pathways is a promising strategy. **Results/methodology:** The Raf kinase inhibitor GW5074 exhibited selective *in vitro* activity against Gram-positive bacteria, including clinical isolates of *S. aureus* with a minimum inhibitory concentration (MIC) of 2–8 µg/ml. GW5074 was effective *in vivo* in the *Galleria mellonella* infection model. The compound showed synergy with gentamicin by lowering MIC by fourfold, compared with gentamicin MIC alone. **Conclusion:** This work demonstrates the antimicrobial properties of GW5074 and supports further investigation of the kinase inhibitors as antibiotic adjuvants.

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The clinical lifespan of antibiotics is limited by how quickly targeted pathogens evolve resistance. The finite lifespan of these essential medicines means that there is a pressing need to identify new antimicrobial agents and discover novel antibacterial targets [1,2]. Multidrug-resistant (MDR) bacteria have emerged as a major threat to hospitalized patients as they are significantly more difficult to treat and are associated with higher mortality rates. Worryingly, the challenges posed by the rise of MDR bacteria are being amplified by the withdrawal of big pharma from the arena over the last decade, which has resulted in a paucity of new antibiotics entering the development pipeline [3,4].

Multiple approaches are being explored to control MDR bacteria and their associated bacterial infections. One of these approaches is to expand the use of antibiotics by using them in combination with compounds that inhibit resistance pathways [5]. For example, clavulanic acid, tazobactam and

sulbactam are β -lactamase inhibitors that have been used successfully in combination with β -lactams antibiotics, and they have prolonged and expanded their effectiveness [6–8]. Aminoglycoside, macrolide and lincosamide antibiotics are also susceptible to enzyme-mediated deactivation through phosphorylation by kinases [9–13]. The clinical success of β -lactam/ β -lactamase inhibitor combinations suggests that other similar antibiotic/kinase inhibitor strategies might also be useful.

Aminoglycosides, in particular, are very effective agents against a number of serious bacterial infections and gentamicin is one of the most used aminoglycosides to contribute to the treatment of severe Gram-positive infections, such as staphylococcal and enterococcal endocarditis [14]. However, while very effective in clearing bacterial infections, the use of gentamicin is limited by its dose-dependent nephrotoxicity [15,16]. In this study, we show that the Raf kinase

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inhibitor GW5074 shows direct antibacterial activity against Gram-positive bacteria; rescues *Caenorhabditis elegans* nematodes from an *S. aureus* infection [17]; acts synergistically with gentamicin against methicillin-resistant *Staphylococcus aureus* (MRSA) *in vitro* and clears staphylococcal infections in the *Galleria mellonella* model.

Materials & methods

Bacterial strains & culture conditions

The bacterial strains tested for sensitivity to GW5074 (BML-E1307; Enzo Bio Sciences, NY, USA) are listed in Table 1. Glycerol stocks of bacteria were stored at -80°C until needed. Bacteria were grown on Mueller–Hinton agar (MHA) and in Mueller–Hinton Broth (MHB; CM0405, Oxoid, UK), Brain Heart Infusion medium (BHI; Bacto Brain Heart Infusion, 237200, BD Biosciences, MD, USA) or Bacto Tryptic Soy Broth (TSB; 21825, BD Biosciences). Media were prepared using MilliQ water as described in the manufacturer's instructions. Cultures were grown on MHA or BHI plates at 37°C in an incubator, or shaken in liquid growth medium at 200 rpm and 37°C in a temperature-controlled shaking incubator (NY, USA).

Minimum inhibitory concentration & minimum bactericidal concentration

Minimum inhibitory concentrations (MICs) were determined according to CLSI guidelines using broth microdilution in Mueller–Hinton medium. Bacterial suspensions were prepared from colonies grown overnight on MHA plates and diluted with MHB to a final inoculum of 5×10^5 cfu/ml. After incubation at 37°C

for 20 h, the MIC was determined by observing the lowest concentration of compound that produced no visible bacterial growth. A 10 µl aliquot of culture from the MIC assay was plated on MHA and then colony formation was examined after incubation overnight at 35°C. Minimum bactericidal concentration (MBC) was determined to be the compound concentration that produced a 99.9% reduction in the number of viable cells.

Killing curve assay

Time–kill assays were performed according to the protocol in Pamer *et al.* [19]. In brief, an overnight culture of *S. aureus* MW2 was diluted in fresh MHB to a density of 5×10^5 cfu/ml in 5 ml round-bottom tubes (BD Biosciences). The compound was added to each tube at 0.5× MIC, 1× MIC and 4× MIC and incubated with shaking at 37°C. At periodic intervals – 0, 4, 6 and 24 h of incubation aliquots were taken from the tubes and serially diluted and plated on MHA plates. The plates were incubated overnight at 37°C and colonies were counted to measure viability. All experiments were performed in duplicate.

Resistance selection studies

The single-step resistance assay was performed as previously described [20,21]. Briefly, an overnight MHB culture of *S. aureus* strain MW2 was diluted to an optical density at 600 nm (OD_{600}) to 0.05 and grown at 37°C until mid-log phase (OD_{600} ~0.4–0.5). Cells were collected by centrifugation and resuspended into fresh MHB medium to obtain 10^9 cells per ml. The cells at 10^9 cells per ml were plated onto MHA plates

Table 1. Bacterial strains used in this study.

Name	Strain
<i>Staphylococcus aureus</i>	MW2
<i>Enterococcus faecium</i>	E007
<i>Klebsiella pneumonia</i>	WGLW2
<i>Acinetobacter baumannii</i>	ATCC 17978
<i>Enterobacter aerogenes</i> Hormaeche and Edwards	ATCC 13048
<i>Pseudomonas aeruginosa</i>	PA14
<i>Staphylococcus aureus</i> clinical isolates	BFSA375; BFSA378; BFSA379; BFSA380; BFSA381; BFSA329; BFSA304; BFSA308
<i>Staphylococcus aureus</i>	ATCC 29213
<i>Staphylococcus aureus</i>	USA100
<i>Staphylococcus epidermidis</i>	NIHLM021
<i>Staphylococcus aureus</i>	VRSA [18]
<i>Staphylococcus aureus</i>	MW2 R1; MW2 R2; MW2 R3

BFSA are current *S. aureus* clinical isolates at Rhode Island Hospital (the clinical isolates were collected with the approval of the Rhode Island Hospital Institutional Review Board). Other bacterial strains are from the lab collection.

containing 2×, 4× and 8× MIC GW5074. The plates were incubated for 48 h and checked at 24 h for survived colonies. To determine resistance development by sequential passaging in liquid medium [19], an overnight culture of *S. aureus* MW2 was diluted to OD₆₀₀ of 0.1 in 5 ml of MHB supplemented with 2 µg/ml, 4 µg/ml and 8 µg/ml of GW5074 in triplicate experiments. After incubation for 24 h at 37°C with shaking at 200 rpm, the highest compound concentration that contained visible growth in each experiment was used to determine the concentrations of GW5074 to be tested on the following day. For the next round of selection the culture in the tube with the highest compound concentration containing visible growth was adjusted to an OD₆₀₀ of 0.1 and used as the inoculum for the subsequent resistance selection. The cultures were incubated for 24 h with shaking as described above. The same method of sequential passaging in liquid medium was used to develop *S. aureus* MW2 isolates that are resistant to gentamicin.

Screening of the Nebraska Transposon Mutant Library

The Nebraska Transposon Mutant Library (NTML), containing 1920 transposon mutants, was obtained from the University of Nebraska Medical Center via the Biodefense and Emerging Infections Research Resources Repository [22]. The library was screened against different concentrations of GW5074. In brief, NTML transposon mutants were transferred from 384 plates with a 96-pin multiblot replicator into 384-well plates containing TSB medium and various concentrations of GW5074. The plates were incubated at 37°C for 48 h and observed at 24 and 48 h for bacterial growth.

Transposon library screening

A highly saturated *mariner* insertion library constructed in *S. aureus* HG003 [23] was obtained as a gift from M Gilmore (MGH, Massachusetts Eye and Ear Infirmary). A frozen aliquot of the library was diluted 1:1000 in 40 ml BHI medium and incubated at 37°C overnight with shaking at 200 rpm. The overnight bacterial culture was diluted 1:100 in 100 ml BHI medium containing 1×, 2×, 4× and 8× the MIC of GW5074 and grown for 7 h at 37°C with shaking at 200 rpm before being serially diluted and plated out over the corresponding concentration of GW5074 on BHI plates. The plates were incubated for 24 h to select for survived colonies. If no surviving colonies were observed the plates were incubated for an additional 24 h. The surviving colonies were then grown overnight in 5 ml TSB and their genomic DNA was isolated using a DNeasy Blood and Tissues kit (Qiagen, CA, USA) according

to the manufacturer's protocol. Purified genomic DNA was digested with *AciI* enzyme (New England BioLabs, MA, USA) and then ligated using T4 DNA ligase (New England BioLabs) [24]. The resulting DNA was purified with the QIAquick PCR purification kit (Qiagen). DNA fragments carrying transposon chromosome junction sequences were amplified by PCR with the following primers: Martn-F (TTT ATG GTA CCA TTT CAT TTT CCT GCT TTT TC) and Martn-ermR (AAA CTG ATT TTT AGT AAA CAG TTG ACG ATA TTC), as previously described [24,25]. PCR products were directly sequenced with the Martn-F primer at Genewiz (NJ, USA).

Checkerboard assay

Gentamicin, kanamycin and streptomycin (Sigma Aldrich, G1264; K1377; S6501; MO, USA) were dissolved in double distilled water at 50 mg/ml, and GW5074 (GW5074; Enzo Life Sciences, NY, USA) was dissolved in DMSO at 5 mg/ml. Checkerboard assays were performed as detailed elsewhere [26]. Briefly, 100 µl of MHB medium were added to each well of a 96-well plate. The first compound of the combination was serially diluted along the ordinate and the second along the abscissa. An inoculum corresponding to a 0.5 McFarland turbidity standard was prepared from *S. aureus* MW2 in MHB, and each well of the microplate was inoculated with 100 µl of a bacterial suspension containing 5×10^5 cfu/ml, then the plate was incubated at 37°C for 20 h. The Σ FICs were determined using the following formula: Σ FIC = FIC A + FIC B, where FIC A is the MIC of compound A in the combination/MIC of drug A alone, and FIC B is the MIC of compound B in the combination/MIC of drug B alone. Combinations are considered synergistic when Σ FIC is ≤ 0.5 , indifferent or no interaction when Σ FIC is > 0.5 to < 4 and antagonistic when Σ FIC is > 4 [27].

Mammalian cell lines & culture conditions

Cell culture media and reagents were purchased from Thermo Fisher Scientific and ATCC. HKC-8 cells, the immortalized human renal proximal tubular epithelial cells, were a generous gift from R Gong and L Dworkin, Division of Kidney Diseases and Hypertension, Rhode Island Hospital, RI, USA. The cells were maintained in Dulbecco's Modified Eagle Medium/Nutrient F-12 Ham and supplemented with 10% fetal bovine serum. The HepG2 liver cells were from ATCC (HB-8065) and cells were grown in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum.

Mammalian cells cytotoxicity assay

The cell proliferation reagent WST-1 (Roche, IN, USA) was used to determine the mammalian cell cyto-

toxicity of GW5074. Cells were seeded into 96-well tissue culture plates (Falcon, 353072, NY, USA) at a 70–80% density. The next day the culture medium was replaced with fresh medium containing serially diluted GW5074. After exposure to GW5074, the tetrazolium salt WST-1 reagent was added into the medium and the cells were further incubated for 4 h. The absorbance $A_{450\text{nm}}$ was measured with a reference wavelength of 610 nm using a Bio-Rad Benchmark Plus Spectrophotometer (Bio-Rad Laboratories, CA, USA). Experiments were performed twice with three replicates each. The results were normalized against controls and presented as a percentage of cell viability.

Red blood cell hemolysis assay

Human erythrocytes were purchased from Rockland Immunochemicals (human red blood cells, 10% washed pooled cells, R407–0050). The assay was performed as described in [28]. In brief, red blood cells were diluted to 2% with PBS and 190 μl aliquots were added to a 96-well plate. Then 10 μl of GW5074 in PBS at 20x of the final tested concentrations ranging from 0.125 $\mu\text{g/ml}$ to 64 $\mu\text{g/ml}$ were added to the wells with red blood cells. The 20% Triton X-100 and PBS were included as positive and negative controls, respectively. The plate was incubated at 37°C for 1 h before being centrifuged at 500 $\times$ g for 5 min to pellet the intact erythrocytes, then 100 μl of the supernatant from each well was transferred to a new 96-well plate. The percentage of hemolysis was determined by measuring absorbance at 540 nm (Bio-Rad Benchmark Plus spectrometer, Bio-Rad Laboratories) and then normalizing the mean experimental absorbance value to the mean absorbance of the Triton X-100 treated samples, which represented 100% hemolysis. Each well value was multiplied by 100 to yield the percentage of hemolysis. Experiments were performed in triplicate.

Galleria mellonella survival studies

Galleria mellonella larvae were purchased from Vanderhost Wholesale (OH, USA) and stored in the dark at room temperature until used. The experiments were performed according to previously published protocols with some modifications [29]. Larvae weighing 230–250 mg were selected for the experiments. Each group included 15 larvae, and experiments were repeated twice using larvae from independent batches. There were two negative control groups in each experiment, the first group underwent no manipulation and the second (uninfected control) was injected with PBS only to control for the impact of physical trauma on the larvae.

To prepare the inoculum, an *S. aureus* MW2 overnight culture was diluted 1:100 into TSB medium and was grown until mid-log phase, then harvested by cen-

trifugation at 3000 rpm for 10 min. The cell pellet was washed with PBS and then centrifuged as above. This wash was repeated, then the cell density of the resultant bacteria suspension was determined by measuring optical density at 600 nm (OD 600). The bacterial suspension was diluted with PBS to give the desired cfu/ml. *G. mellonella* larvae were inoculated with 10 μl of bacterial suspension containing 5×10^4 cfu in the last left proleg of each one using a 10 μl Hamilton syringe (20779, Sigma Aldrich, MO, USA). Two hours later the larvae were injected with GW5074 at 6 mg/kg, vancomycin at 30 mg/kg. PBS was used for injections of control group. Later the larvae in Petri dishes were placed into a 37°C incubator and their survival was monitored each 24 h for 5 consecutive days. Larvae were scored for survival by observing for movement after gentle touch.

Results

GW5074 exhibits antistaphylococcal activity *in vitro* & in a whole animal *C. elegans* infection model

The *S. aureus* MW2 (MRSA)–*C. elegans* liquid infection assay [30] was used to screen the Biomol ICCBL Known Bioactivities 2012 library which contains 472 diverse compounds of known biological activity. Hits were defined as compounds that increased survival of the *C. elegans* nematodes infected with MRSA. GW5074, a commercially available Raf kinase inhibitor (Figure 1), that directly inhibited growth of *S. aureus* MW2 during infection and rescued worms, was selected for further study. The rate of *C. elegans* survival ranged from 94.7 to 100% for GW5074-containing wells (Figure 2).

GW5074 shows selective activity against Gram-positive bacteria

GW5074 was examined for direct antibacterial activity against the following staphylococcal strains: USA100; a clinical isolate of *S. aureus* with high-level resistance to vancomycin MIC ≥ 1024 $\mu\text{g/ml}$ that contains a 57.9-kb multiresistant conjugative plasmid with integrated Tn1546 (*vanA*) from *Enterococcus faecalis* [18]; *Staphylococcus epidermidis* strain NIHLM021 and recent clinical isolates of *S. aureus* obtained from our laboratory collection. Activity against a panel of other ESKAPE (an acronym for the *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) pathogens was also examined. These studies showed that GW5074 exhibited selective antimicrobial activity toward Gram-positive micro-organisms with MICs ranging from 2 to 8 $\mu\text{g/ml}$, compared with the >64 $\mu\text{g/ml}$ for all Gram-negative bacteria tested (Tables 2 & 3).

In MBC experiments, 10 μ l of *S. aureus* MW2 bacterial cultures, prepared using the same protocol as to that for the MIC assays, were spotted onto MHA plates to evaluate growth inhibition upon exposure to GW5074 (Figure 3). Based on the MBC data, we found that GW5074 demonstrates bacteriostatic activity toward *S. aureus* MW2, and this finding was further supported by the results from the time–kill assay (Figure 4).

GW5074 rescues *G. mellonella* from staphylococcal infection *in vivo*

In order to further evaluate the antistaphylococcal efficacy of this compound *in vivo*, we used the *G. mellonella* model [29]. First, we tested the toxicity of GW5074 by administering a range of doses and we did not observe any difference in larval survival between groups injected with GW5074 at up to 6 mg/kg and the control groups injected with PBS only. We therefore concluded that GW5074 is nontoxic at these doses, and then we evaluated the efficacy of GW5074 in this model.

Administration of a single dose of GW5074 at 6 mg/kg to larvae that were infected with 5×10^4 cfu of *S. aureus* MW2 resulted in increased larval survival, compared with the PBS-treated control group ($p > 0.0001$) (Figure 5). On day 5 postinfection, the percentages of larvae survival infected with *S. aureus* MW2 from two combined independent experiments was 6.7% for the control group that received PBS, 42.1% for the GW5074 group and 66.7% for the vancomycin group.

Development of resistance to GW5074

Interestingly, we were unable to develop GW5074 resistant isolates. More specifically, during sequential passaging in liquid medium (detailed in the ‘Materials & methods’ section) aimed at resistance development of *S. aureus* MW2 to GW5074, bacterial growth could only be observed at up to $2 \times$ MIC (8 μ g/ml of GW5074). Also, we attempted to select *S. aureus* MW2 that was resistant to GW5074 by other commonly used laboratory techniques, including broth microdilution [31] with which we got the same results as described above, and single-step resistance development with no colonies observed even after 48 h of incubation on plates containing $2 \times$ and $4 \times$ MIC of GW5074.

Finally, we screened the NTML library of *S. aureus* for resistant mutants to GW5074. This library is a collection of approximately 2000 mutants of *S. aureus* USA300 LAC in which the nonessential genes in the genome were disrupted by the insertion of the *mariner* Tn *bursa aurealis* [32]. Upon screening, we did not

Figure 1. Chemical structure of GW5074 compound.

observe any bacterial growth in the wells of the 384-well screening plates, therefore we concluded that the library did not contain mutants resistant to GW5074. Of note is that there are also 32 kinase mutants in the library that were tested separately for their susceptibility to GW5074 to assess whether the compound interferes with kinase activity. We only observed an insignificant decrease in MIC from 4 μ g/ml for wt *S. aureus* USA300 LAC to 2 μ g/ml for the following transpo-

Figure 2. GW5074 rescues *Caenorhabditis elegans* from methicillin-resistant *Staphylococcus aureus* infection. A 384-well assay plate was coinoculated with *C. elegans*, *S. aureus* MW2 and either DMSO (negative control), vancomycin (positive control) or screening compounds. The well containing GW5074 compound is shown. Bright field images of assay wells are shown on the left, on the right are images of dead worms. (A) GW5074; (B) vancomycin; (C) DMSO.

Table 2. Antimicrobial activity of GW5074 compound.	
Bacterial strain	GW5074, MIC µg/ml ug/ml
<i>Staphylococcus aureus</i> MW2	4
<i>S. aureus</i> ATCC 29213	4
USA100	4
<i>Staphylococcus epidermidis</i> NIHLM021	4
VRSA	2
BFSA375	2
BFSA378	2
BFSA379	2
BFSA380	2
BFSA381	2
BFSA329	4
BFSA304	4
BFSA308	4
<i>Enterococcus faecium</i> E007	8
<i>Klebsiella pneumoniae</i> WGLW2	>64
<i>Enterobacter aerogenes</i> 13048	>64
<i>Acinetobacter baumannii</i> 17978	>64
<i>Pseudomonas aeruginosa</i> PA14	>64

son mutants: sensor histidine kinase, sensor histidine kinase Sae S, staphylokinase Sak, acetylglutamate kinase argB and 1–2 µg/ml for hydroxyethylthiazole kinase.

Toxicity studies

GW5074 was tested for the hemolysis of red blood cells and also for cytotoxicity toward mammalian cells. Human erythrocytes tested in the *ex vivo* hemolysis assay with increasing concentrations of GW5074 showed no hemolysis over the concentration range of 0–64 µg/ml (Figure 6). We also used HepG2 and HKC-8 cells to test the cytotoxicity of the GW5074 toward mammalian cells in the WST-1 cell prolifera-

tion assay (Roche, IN, USA). Both cell lines responded similarly upon exposure to increasing concentrations (0–64 µg/ml) of the compound. The drug showed no toxicity at concentrations below 8 and 16 µg/ml against HepG2 and HKC-8 cells, respectively, and it reduced cell viability to ~60% (HepG2) and ~34% (HKC-8) at the highest concentration tested (Figure 7).

Transposon insertion library identifies mutants that show resistance to low concentrations of GW5074

In further efforts to uncover the mechanism of antimicrobial action of GW5074, we screened the highly saturated *mariner* library [23] of *S. aureus* strain HG003

Table 3. Minimum inhibitory concentration values of gentamicin in the absence and presence of GW5074 in <i>S. aureus</i> strains.			
Strain	Antibiotic	MIC alone	Combined MIC
<i>Staphylococcus aureus</i> MW2	Gentamicin	1	0.25
BSFA308	Gentamicin	4	1
BFSA329	Gentamicin	4	1
<i>S. aureus</i> MW2 R1	Gentamicin	4	1
<i>S. aureus</i> MW2 R2	Gentamicin	8	2
<i>S. aureus</i> MW2 R3	Gentamicin	8	2
The isolates of <i>S. aureus</i> MW2 R1, 2 and 3 were selected during resistance development to gentamicin. MIC: Minimum inhibitory concentration.			

for GW5074-resistant mutants. Cultures of pooled library mutants were grown in the presence of increasing concentrations of GW5074 (MIC for transposon insertion library mutants 2 µg/ml). Mutant selection experiments were carried out at 2, 4, 8 and 16 µg/ml of GW5074. After incubation for 7 h at 37°C, we observed visible bacterial growth at only MIC and 2× MIC concentrations. Colonies appeared after 24 h incubation when the serial dilutions of these bacterial cultures were plated over corresponding GW5074 concentrations.

We also screened colonies that grew at MIC and 2× MIC concentrations for transposon insertion sites by inverse PCR, and sequenced them to determine the genes disrupted by transposon insertion. The sequencing data were analyzed using the BLASTN program against the query *S. aureus* NCTC 8325 (CDS: CP000253.1). The first identified resistant mutant carried an insertion in a conserved hypothetical protein (CDS: ABD30536.1). When we assigned GO terms to protein sequences within the BLAST2GO pipeline using BLAST+ against the NCBI nr database (including GenBank, CDS translations, along with PDB, swissProt, PIR) and InterProScan the functional annotation to this sequence was described as encoding the extracellular matrix-binding protein *ebhA*. We identified a second GW5074-resistant mutant that carried an insertion in another hypothetical protein (Anchor: CP000253.1, chromosomal position: 2,722,678...2,723,606) and shared 20% similarity with tributyrin esterase. We found that the third insertion mutant selected at 2× MIC involved an insertion in the gene encoding *fmhA*, a protein that is involved in peptidoglycan biosynthesis [33].

GW5074 potentiates the activity of aminoglycoside antibiotics

We performed checkerboard assays to examine whether, in addition to showing direct action against *S. aureus*, GW5074 could increase *S. aureus* susceptibility when coadministered with clinically used aminoglycoside antibiotics. We found that the MIC of gentamicin necessary against *S. aureus* MW2 alone was 1 µg/ml and in combination with GW5074 it decreased to 0.25 µg/ml (FIC = 0.5). Based on this finding, we screened a collection of clinical isolates to identify the gentamicin-resistant *S. aureus*, and tested these isolates for synergy between GW5074 and gentamicin. The MIC for gentamicin with clinical isolates BFS308, and BFS329 alone was 4 µg/ml, but in combination with GW5074 it decreased to 1 µg/ml (Table 2). We also developed *S. aureus* MW2 gentamicin-resistant mutants using sequential passaging by microdilution in 96-well plates. *S. aureus* MW2 mutants R1, R2 and R3, with elevated resistance to gentamicin of 4 and 8 µg/ml, also showed

Figure 3. GW5074 demonstrates bacteriostatic activity toward *Staphylococcus aureus* MW2. 10 µl of bacterial cultures from MIC assay from each well were spotted onto MHA plates to see the growth inhibition of *S. aureus* MW2 upon exposure to GW5074. MHA: Mueller–Hinton agar; MIC: Minimum inhibitory concentration.

fourfold reduction in MIC value for gentamicin (FICs were 0.375 and 0.5). Interestingly, we did not find synergy between GW5074 and either kanamycin or streptomycin (FIC = 0.625), however the MICs for streptomycin and kanamycin in combination with GW5074 (1 µg/ml) dropped to 4 and 8 µg/ml compared with the MIC for streptomycin, kanamycin and GW5074 alone (8, 16 and 8 µg/ml, respectively).

Discussion

The Raf kinase inhibitor GW5074 (BML-E1307, Enzo Bio Sciences) was initially identified as an antimicrobial compound that can rescue *C. elegans* from staphylococcal infection *in vivo* [17]. When the compound was tested for antimicrobial activity against a panel of ESKAPE pathogens, it showed selective activity against Gram-positive pathogens. We used

Figure 4. Time-kill curve for GW5074 and *Staphylococcus aureus* MW2. At intervals of 0, 4, 6 and 24 h of incubation colony counts were performed and charted on graph with the survivor colony count on the ordinate (logarithmic scale) and time on the abscissa (arithmetic scale).

the *G. mellonella* infection model to confirm the antimicrobial properties of this compound. Interestingly, we found that GW5074 acts synergistically to gentamicin; and that staphylococcal resistance cannot develop easily, suggesting that it might be associated with an essential gene or be multi-factorial.

Figure 5. Survival of *Galleria mellonella* inoculated with 5×10^4 cfu of *Staphylococcus aureus* upon treatment with GW5074 (6 mg/kg) and vancomycin (30 mg/kg). Graph represents combined data from two independent experiments (each group had $n = 15$ larvae, statistical Mantel-Cox test was performed for PBS vs E1307 and PBS vs vancomycin, $p < 0.0001$). Vancomycin was used as positive control.

In our studies, we used two invertebrate models to identify and evaluate the efficacy of this compound. First, we identified the activity of GW5074 using the whole animal *C. elegans*-based assay. *C. elegans*, a soil-dwelling nematode is susceptible to many of the pathogens that infect humans, including *S. aureus* [34]. The innate immune response of this nematode consists of an array of numerous antimicrobial proteins, many of which are conserved in higher organisms [35]. Therefore, *C. elegans* is useful as an alternative host to study innate immunity and to screen for pathogenic factors [36]. Our lab, in collaboration with others, successfully used this nematode in the *C. elegans* infection model to screen and identify new antimicrobial agents and compounds that affect pathogen virulence or modulate the innate immune response of nematodes during pathogen infection [17,37–40].

In our studies, we also took the advantage of the *G. mellonella* infection model to further evaluate the antimicrobial properties of GW5074. Despite the lack of adaptive immune response, this insect model host showed remarkable similarity to vertebrates in innate immune response [41]. The immune responses of *G. mellonella* include phagocytic cells and also antimicrobial peptides [41]. These similarities facilitated the study of staphylococcal pathogenesis using this model as a host [42–44]. The *G. mellonella* infection model is also a quick and inexpensive way to test the efficacy of antimicrobial agents against *S. aureus* *in vivo* [29,45,46]. Altogether, the combination of these two models allowed us to first identify the antimicrobial agents and then evaluate their antimicrobial activity against *S. aureus* without exposing mammalian hosts.

Regarding the potential mode of action for this compound, as noted above, GW5074 is a Raf kinase inhibitor in mammalian cells and its function is well described in the literature [47,48]. Despite the fact that prokaryotic and mammalian kinases share very little sequence homology, the known 3D protein crystal structure revealed an unexpected similarity in kinase function in important binding pockets [11]. Therefore, the possibility that GW5074 exerts antimicrobial activity by inhibiting *S. aureus* kinases was a reasonable first hypothesis. Subsequently, we screened 32 kinase mutants from the Nebraska transposon insertion library. However, we saw neither an increase in resistance nor a significant sensitivity to this compound on tested bacteria. Taken together, these findings suggest that the targeted kinase(s) is/are not represented in the library, and since we were unable to develop the resistant mutant at concentrations higher than $2 \times \text{MIC}$, the targeted kinases are important for bacterial cell viability. However, it is worth mentioning that we observed the slight increase in sensitivity of the hydroxyethylthiazole kinase mutant to GW5074. This

is interesting since this kinase is important for vitamin B1 biosynthesis, a metabolic process that is exclusive to bacteria, plants and lower eukaryotes, but absent in humans. It is notable that the vitamin B1 biosynthesis pathway has been proposed as a promising but 'untapped' antibiotic target [49].

Also, based on the sequencing data from the isolates with a small change in MIC, our studies indicate some additional potential targets. More specifically, the first two insertion mutants, identified using the highly saturated *mariner* insertion library constructed in the *S. aureus* HG003 strain, involved insertions in two hypothetical proteins, one of which has functional annotation as the extracellular matrix-binding protein *ebhA*. *Ebh* is cell wall associated fibronectin-binding protein that binds to Fn (fibronectin) glycoprotein, a major component of the extracellular matrix and a major protein of blood plasma that facilitates *S. aureus* colonization and dissemination [50]. *S. aureus* expresses an array of adhesive proteins that interact with molecules of the host extracellular matrix, and these cell wall associated adhesive proteins have been identified as having the potential to be a crucial element in the development of antistaphylococcal vaccines or for developing new antiadhesive strategies [50].

The second insertion mutant involved a gene encoding the *fmhA* protein that was identified in *S. aureus* as similar to *femAB*-like proteins, which are essential for synthesis of the peptidoglycan pentaglycine interpeptide bridge of the cell wall. Inactivation of *femAB* leads to reduced growth rates and hyper-susceptibility to practically all known antibiotics [51]. However, the inactivation of *fmhA* and *fmbC*, but not *fmbB*, which is involved in the first step of interpeptide formation, had no effect on growth, antibiotic susceptibility or peptidoglycan composition of the cell wall [33]. It is reasonable to hypothesize that *fmhA* function might provide a structural anchor for the cell wall adhesive *ebhA* protein. Developing antimicrobial agents that target structural anchors for adhesive proteins could prevent staphylococcal colonization of the host and decrease the severity of infection and therefore increase the chances for success of antibiotic therapy.

Shakya *et al.* used the screen-well protein kinase inhibitor library from Enzo Life Sciences, which includes the GW5074 compound, in an *in vitro* screening to discover and analyze chemoselective relationships across different antibiotic kinases. In this study, GW5074 demonstrated broad specificity against antibiotic kinases that play an important role in developing resistance in bacteria [52]. We wanted to test this theory and assess the ability of this compound to act synergistically with known antibiotics, like aminoglycosides, in killing *S. aureus*. The checkerboard assays with

Figure 6. GW5074 compound does not cause hemolysis of human red blood cells. Human red blood cells were incubated with serially diluted GW5074 compound (64–0.125 µg/ml). Triton 2% was used as a positive control and DMSO as a negative.

S. aureus MW2 and its gentamicin-resistant mutants, as well as clinical isolates showed the synergy between GW5074 and gentamicin. However, we found that highly gentamicin-resistant *S. aureus* clinical isolates expressed aminoglycoside modifying enzymes, specifically: *aac*(6')-*aph*(2'') FIC = 0.625 and *aac*(6')-*aph*(2'')-*aph*(3')-IIIa FIC = 0.725. And that in both of these cases gentamicin MIC was lower in combination with GW5074 than alone.

Regarding the practical ramifications of this work, gentamicin is highly effective in the treatment of MRSA bacteremia and infective endocarditis, but it is also highly nephrotoxic [15,16,53]. The class of compounds similar to GW5074 may provide the possibility to reduce the initial dose of gentamicin in treatment of MRSA infections and therefore reduce its nephrotoxic effects assuring safer antimicrobial therapy. It should be noted, however, that while GW5074 potentiates the antimicrobial activity of gentamicin, there is also the possibility that it might increase the toxicity of gentamicin *in vivo*.

We also need to note that this compound has some obvious limitations. GW5074 showed toxicity at high concentrations toward mammalian HepG2 and HKC-8 cells as demonstrated in a toxicity assay with the WST-1 reagent, which was not surprising since Raf kinases are candidates for cancer treatment and some are in clinical trials [54].

Conclusion

The compound GW5074 demonstrated antimicrobial activity against MRSA and synergy with gentamicin. Because staphylococcal resistance against this compound cannot develop easily, further work on this type

Figure 7. Dose toxicity studies with GW5074 in mammalian cells. The cell lines HKC-8 and HepG2 were treated with increasing concentrations of GW5074 for 24 h and cell viability was assessed by WST-1 assay. (A) HKC-8 cells; (B) HepG2 cells.

of adjuvant has the potential to identify compounds that decrease the dose-dependent nephrotoxic effects of gentamicin. A functional analysis of this class of chemicals could decrease or eliminate its excessive toxicity toward mammalian cells when in use as antibiotic adjuvant.

Future perspective

Staphylococcus aureus is a leading cause of recurrent diseases that range from soft tissue infections to bacteremia, pneumonia, endocarditis, osteomyelitis, arthritis and toxic shock. The emergence of methicillin- and vancomycin-resistant *S. aureus* strains, combined with the lack of new antimicrobial agents, is a public emergency. Among the potential targets for new antimicrobial agents are protein kinases that are essential for sustaining bacterial growth, expressing virulence and development of antibiotic resistance. In the near future we plan to explore GW5074-like compounds and other kinase inhibitors identified in the *C. elegans*–MRSA screen for their antimicrobial activities and cellular targets. We also plan to perform a structure functional analysis of GW5074 where chemical modifications will be made to the parent molecule in order to determine functional groups.

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Executive summary

- GW5074 is a Raf kinase inhibitor with antimicrobial properties against Gram-positive bacteria and current clinical isolates.
- GW5074 effectively rescues *Caenorhabditis elegans* and *Galleria mellonella* from *Staphylococcus aureus* infection *in vivo*.
- GW5074 acts synergistically with gentamicin *in vitro* and lowers gentamicin MIC by fourfold.
- Compounds with similar chemical structure to GW5074 could potentially be used as antibiotic adjuvants to gentamicin in antistaphylococcal therapy to decrease nephrotoxicity.

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