Inhibition of *Salmonella enterica* Biofilm Formation Using Small-Molecule Adenosine Mimetics

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Biofilms have been widely implicated in chronic infections and environmental persistence of *Salmonella enterica*, facilitating enhanced colonization of surfaces and increasing the ability of the bacteria to be transmitted to new hosts. *Salmonella enterica* serovar Typhi biofilm formation on gallstones from humans and mice enhances gallbladder colonization and bacterial shedding, while *Salmonella enterica* serovar Typhimurium biofilms facilitate long-term persistence in a number of environments important to food, medical, and farming industries. *Salmonella* regulates expression of many virulence- and biofilm-related processes using kinase-driven pathways. Kinases play pivotal roles in phosphorylation and energy transfer in cellular processes and possess an ATP-binding pocket required for their functions. Many other cellular proteins also require ATP for their activity. Here we test the hypothesis that pharmacological interference with ATP-requiring enzymes utilizing adenosine mimetic compounds would decrease or inhibit bacterial biofilm formation. Through the screening of a 3,000-member ATP mimetic library, we identified a single compound (compound 7955004) capable of significantly reducing biofilm formation by *S. Typhimurium* and *S. Typhi*. The compound was not bactericidal or bacteriostatic toward *S. Typhimurium* or cytotoxic to mammalian cells. An ATP-Sepharose affinity matrix technique was used to discover potential protein-binding targets of the compound and identified GroEL and DeoD. Compound 7955004 was screened against other known biofilm-forming bacterial species and was found to potently inhibit biofilms of *Acinetobacter baumannii* as well. The identification of a lead compound with biofilm-inhibiting capabilities toward *Salmonella* provides a potential new avenue of therapeutic intervention against *Salmonella* biofilm formation, with applicability to biofilms of other bacterial pathogens.

Salmonella enterica serovar Typhi and *Salmonella enterica* serovar Typhimurium are among the most commonly encountered foodborne and waterborne pathogens worldwide. Although their respective disease states differ, both organisms are capable of forming bacterial biofilms in mammalian and/or environmental niches. Biofilms are aggregated mixtures of sessile bacteria that are implicated in many chronic infections and are known to facilitate bacterial persistence by increasing antimicrobial resistance and interfering with the host immune response (1, 2). Biofilms are encased within a mixture of secreted and cell wall-associated polysaccharides, glycoproteins, and glycolipids, as well as extracellular DNA, known collectively as extracellular polymeric substances (EPS) (1, 3). This process of surface adherence, self-aggregation, and matrix envelopment serves a critical step in the continued cycle of infection for both *S. Typhi* and *S. Typhimurium*, by increasing the duration of bacterial persistence and shedding, providing resistance to host and environmental stressors, and ultimately enhancing the likelihood of colonizing a new host.

S. Typhi is a human-specific pathogen and the primary etiological agent of typhoid fever (4). Although different levels of availability and efficacy of diagnostic tests in regions in which the disease is endemic can complicate estimations of disease incidence (5), current data indicate that there are over 20 million new cases of typhoid fever each year, resulting in over 200,000 deaths worldwide (6). Following ingestion of viable bacteria, acute infection with *S. Typhi* is initiated by bacterial translocation across the intestinal epithelium and subsequent uptake by local phagocytic cells, permitting systemic dissemination associated with clinical manifestations of acute disease (7). Mortality rates range from 10 to 15% without treatment, due to fatal complications from dissemination to the lung, liver, spleen, and central nervous system

(8), compared to <1% with appropriate treatment (9). Regardless of treatment, 3 to 5% of patients infected with *S. Typhi* recover from the stage of acute disease without fully eliminating the bacteria; instead, they become asymptomatic chronic carriers (10). Chronic carriage is characterized by colonization of the human gallbladder, a unique niche for microbial infection (11), from which bacteria can spread by means of fecal shedding (12). We previously described a mechanism of chronic carriage involving bacterial biofilm formation on cholesterol gallstones present in the gallbladder (13). To date, cholecystectomy in tandem with antibiotic treatment is the primary effective treatment option for chronic infection (14); however, such invasive treatment is poorly suited to the patient care settings in many regions in which typhoid is endemic.

Surface adherence, bacterial aggregation, and biofilm formation are similarly important in the persistence and dissemination of gastroenteritis-causing nontyphoidal *Salmonella* serovars. Biofilm aggregations of *S. Typhimurium* are involved in persistent

Received 23 May 2014 Returned for modification 27 June 2014

Accepted 6 October 2014

Accepted manuscript posted online 13 October 2014

Citation Koopman JA, Marshall JM, Bhatiya A, Eguale T, Kwiek JJ, Gunn JS. 2015. Inhibition of *Salmonella enterica* biofilm formation using small-molecule adenosine mimetics. *Antimicrob Agents Chemother* 59:76–84. doi:10.1128/AAC.03407-14.

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doi:10.1128/AAC.03407-14

colonization of swine and poultry as well as cross-contamination in food preparation and farm environments (15, 16). In spite of efforts to improve detection and prevention, the incidence of food-borne salmonellosis in the United States has changed little in recent years, remaining a major economic and public health burden. In light of the importance of biofilm formation in continued disease spread, the identification of novel compounds that interfere with this process would greatly increase our ability to combat biofilm-mediated infections.

Bacterial kinases participate in the regulation of cellular processes known to be important for biofilm formation, such as quorum sensing (17, 18) and production of extracellular matrix components (19, 20). In recent years, kinases have been assigned a high priority as candidate drug targets due to their endogenous small-molecule-binding motifs and their importance in cellular functions (21). Due to the reliance of several biofilm-associated processes on bacterial kinases and other ATP-requiring proteins, we sought to pursue drug development through interference with ATP-dependent pathways using small-molecule ATP mimetic compounds. Screening of 3,000 putative ATP mimetic compounds for potential biofilm-inhibiting effects resulted in the identification of a promising lead compound (compound 7955004), which was able to inhibit biofilm formation by both *Salmonella* and *Acinetobacter* species in a manner that did not alter bacterial growth or viability. Subsequent testing of compound 7955004 revealed no evidence of mammalian cellular cytotoxicity, even at high concentrations (6-fold greater than the 50% effective concentration [EC_{50}]). Taken together, these results indicate that compound 7955004 is a promising candidate drug for further optimization as a potential antibiofilm therapeutic agent.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and chemical inhibitors. The *Salmonella* strains used in this study were wild-type *S. Typhimurium* 14028s (strain JSG210) and *S. Typhi* TY2 (strain JSG624). Bacteria were stored at -80°C in Luria-Bertani (LB) broth containing 20% glycerol. Overnight cultures were grown in LB broth, tryptic soy broth (TSB), or superoptimal broth (SOB) at 37°C , with aeration. Growth for a 24-h biofilm assay involved normalizing cultures to an optical density at 600 nm (OD_{600}) of 0.8, diluting the cultures 1:100 in TSB diluted 1:20 or in undiluted SOB for *S. Typhi*, and growing the cells at 30°C under static conditions. *Acinetobacter baumannii* 19606 (strain WLS2401), *Staphylococcus aureus* (strain USA300), and *Pseudomonas aeruginosa* (strain PAO1) 24-h biofilm assays were carried out in a similar manner using TSB.

Compound 7955004 [3-(2-furylmethyl)-2-[[5-hydroxy-1H-pyrazol-3-yl)methyl]thio]-3,5,6,7-tetrahydro-4H-cyclopenta[4,5]thieno[2,3-d]pyrimidin-4-on] was purchased from ChemBridge (San Diego, CA). Compounds were prepared at 5 mM concentrations in dimethyl sulfoxide (DMSO) and were stored in the dark at 4°C . The original high-throughput screen was conducted using 5 μM drug concentrations and a Biomek 2000 robot, with dilutions of the drug being made in $1\times$ phosphate-buffered saline (PBS). Controls were treated with 0.1% DMSO (final concentration) and PBS alone. Polystyrene 96-well plates were made by Corning Inc. (Corning, NY). Each plate contained 80 drugs, leaving 16 wells for controls. Cultures and drugs were added simultaneously, and the Biomek 2000 system carried out the assay in a manner similar to that for the rapid attachment biofilm assay described below.

Rapid attachment biofilm assay, EC_{50} determination, and biofilm dispersion assay. To determine biofilm inhibition by the compounds, overnight cultures of the desired bacterial strains were grown in TSB at 37°C on a roller drum or shaker. Cultures were normalized to an OD_{600} of 0.8, and 1:100 dilutions of the normalized cultures in TSB (diluted 1:20) were incubated at 30°C under static conditions, to promote biofilm for-

mation. The OD_{600} was read at 24 h, to observe the growth of planktonic bacteria. Plates were washed in double-distilled water and heat-fixed at 60°C for 1 h. Biofilms were stained for visualization with 33% gentian violet solution (10 ml gentian violet, 18 ml $1\times$ PBS, 1 ml methanol, and 1 ml isopropanol) for 5 min, followed by release of the stain with 33% acetic acid (33 ml glacial acetic acid and 67 ml $1\times$ PBS). The released dye was measured at OD_{570} . The half-maximal effective concentration (EC_{50}) of each compound was determined by finding the concentration corresponding to 50% of the maximum biofilm inhibition achieved using various concentrations of compound 7955004 (0.125 μM to 50 μM). Biofilm dispersion assays were carried out in a similar manner, except that 30 μM compound 7955004 was added after a 24-h biofilm was established and the cells were incubated at 30°C for another 24 h, under static conditions. Biofilms were quantified by gentian violet staining and dye release.

Determination of bacterial viability following drug exposure. In order to establish the potential bactericidal or bacteriostatic effects of compound 7955004, cultures were grown in the presence or absence of the drug and plated for viability. Overnight cultures were grown as described above, back-diluted 1:100, and incubated in TSB for 24 h at 37°C , with aeration, in the presence or absence of 30 μM compound 7955004. Following 0 h, 2 h, 4 h, 8 h, 16 h, and 24 h of incubation, 10- μl aliquots were removed, serially diluted, and plated for viability.

Eukaryotic cellular cytotoxicity indicated by lactate dehydrogenase release. Effects on eukaryotic cells were quantitatively assessed by 12-h and 24-h lactate dehydrogenase (LDH) release experiments using an LDH cytotoxicity detection kit (Clontech, Mountain View, CA). The HepG2 hepatocyte cell line was grown in modified essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine and was incubated in a humidified 5% CO_2 incubator at 37°C . Cells were seeded at 1×10^4 cells ml^{-1} in a polystyrene 96-well tissue culture plate and were allowed to adhere overnight. Adherent HepG2 cells were subjected to 12 or 24 h of drug exposure (at 30 μM or 50 μM) in triplicate. Positive and negative controls consisted of equal volumes of 0.1% DMSO (final concentration) and 2% Triton X-100, respectively. LDH release was measured by a colorimetric assay at 490 nm.

ATP-binding proteins captured by ATP-Sepharose affinity matrix and visualized by silver staining. In order to determine potential ATP-binding protein targets of compound 7955004, bacterial lysates were prepared from wild-type *S. Typhimurium* cultures grown under biofilm-inducing conditions (3 ml of TSB diluted 1:20, with growth at 30°C with aeration). Cells were centrifuged, resuspended in 1 ml of lysis buffer (50 mM HEPES [pH 7.4], 120 mM NaCl, 20 mM MgCl_2 , 1 mM dithiothreitol [DTT], 0.1% Triton X-100, and $1\times$ complete protease inhibitors), and prepared with a FastPrep system and bacterial lysis column B, containing 0.1-mm silica spheres, for extraction of DNA, RNA, and proteins. The lysate was run through ATP-Sepharose resin (22, 23) (graciously provided by Timothy A. J. Haystead, Duke University) and allowed to bind for 1 h. Low-salt (120 mM, 2 times), high-salt (300 mM, 2 times), and low-salt (120 mM, 2 times) rinses were used to wash off nonspecifically binding proteins. Washes were optimized such that no proteins were present in the eluent when analyzed on silver-stained gels. The column was subsequently washed with a solution of 500 μM compound 7955004 to compete protein targets off the column, followed by a wash with 10 μM ATP to clear the column of remaining ATP-bound proteins. Proteins were electrophoresed on a 10% SDS-PAGE gel and stained with silver nitrate (24) to determine the optimal number of wash steps and to identify protein bands of interest. Column elution fractions following the wash with compound 7955004 were subsequently electrophoresed (10% SDS-PAGE) and stained with Sypro Red dye. These protein bands were excised and submitted to the Ohio State University Mass Spectrometry and Proteomics Facility. Identified peptides were analyzed using Mascot Deamon v2.2.1 (Boston, MA) and compared with the NIH/NCBI database.

Data analysis. All experiments were conducted in triplicate, and data represent a minimum of 3 biological replicates. All data analysis, statistical

significance testing, and EC₅₀ calculations were performed using Graph-Pad Prism 6 software. Results from treated and control samples were analyzed for significance using Student's *t* tests or one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison posttest. The EC₅₀ values, defined here as half-maximal observed inhibitory concentrations, were calculated by plotting the percent biomass in treated wells relative to that in untreated control wells. Data were plotted as a function of the log₁₀ compound concentration, and standard sigmoidal dose-response curves were fit to the data; 95% confidence intervals (CIs) are reported to indicate the error of each EC₅₀ determination. *P* values of <0.05 were considered significant.

RESULTS

A candidate adenosine mimetic compound is identified in a high-throughput assay of *S. Typhimurium* biofilm inhibition. A high-throughput screening assay was developed to identify compounds that would inhibit biofilm formation by wild-type *S. Typhimurium* (strain JSG210). A library of 3,000 putative kinase inhibitors was assembled by ChemBridge Corporation from a 450,000-compound library based on the predicted probability of binding to the ATP-binding region of proteins and allosteric sites of kinase targets. ChemBridge selected compounds to include in the library based on the statistical significance of chemical similarity to ATP, on the basis of Tanimoto scores (25). Low-energy conformations of 5'-O-methyladenosine were used to query library compounds for structures mimicking the adenosine portions of ATP, without providing energy for cellular processes. Biofilm formation by wild-type *S. Typhimurium* 14028s was quantified using a rapid attachment biofilm assay adapted from previously described methods (26), with an automated Biomek robot platform. Bacteria and drug (5 μM) were added together at the beginning of the experiment. Biofilm formation was quantitated following 24 h of growth, and candidate drugs were selected based on demonstration of ≥30% reduction in the overall biomass, relative to untreated controls. Initial automated screening resulted in identification of 34 compounds exhibiting ≥30% reduction in biofilm formation, 5 compounds exhibiting ≥40% reduction in biofilm formation, and 3 compounds exhibiting ≥50% reduction in biofilm formation. Manual testing of these compounds using the rapid attachment biofilm assay in 3 separate experiments eliminated all except one compound, i.e., compound 7955004 [3-(2-furylmethyl)-2-[[[(5-hydroxy-1*H*-pyrazol-3-yl)methyl]thio]-3,5,6,7-tetrahydro-4*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidin-4-on] (Fig. 1), which reliably demonstrated ~55% reduction of *S. Typhimurium* biofilm formation, relative to untreated controls, at a concentration of 30 μM.

Compound 7955004 inhibits *S. Typhimurium* biofilm formation in a dose-dependent manner, which is not due to bactericidal or bacteriostatic activity. Biofilms were grown as described above, in the presence of various concentrations of compound 7955004 (0.625 μM to 50 μM). Biofilm formation was inhibited in a dose-dependent manner, reaching a maximum of approximately 55% inhibition at a concentration of 30 μM (*P* < 0.0001) (Fig. 2A), which corresponded to visual observations. Prior to crystal violet quantitation of the biofilms, plates were read at OD₆₀₀ to monitor planktonic growth (Fig. 2B), which showed no significant difference in mean OD₆₀₀ values in the presence versus the absence of compound 7955004 (*P* = 0.8241). We further verified the lack of bactericidal/bacteriostatic activity through bacterial CFU enumeration in the presence or absence of 30 μM drug (Fig. 2C), and no significant differences between the regres-

FIG 1 High-throughput screen of the adenosine mimetic library against *S. Typhimurium* biofilm formation. Compounds that inhibited biofilm formation were identified with their putative interacting proteins by using ATP-Sepharose resin and were correlated with their phenotypic effects. The target protein interactome will be analyzed and derivatization of the lead compounds will be carried out in order to optimize inhibitory effects. The initial library was composed of 3,000 putative ATP mimetic compounds. Preliminary high-throughput screening was used to select compounds of interest based on observation of ≥30% biofilm inhibition in the presence of the drugs. Compound 7955004 showed ≥40% biofilm inhibition in our secondary screen and was prioritized as the lead compound of interest. The chemical structure of the adenosine mimetic compound 7955004 is shown.

sion curves fit to treated versus untreated control data points were found (*P* = 0.3525). The dose-response curve was used to plot the observed activity of compound 7955004, such that 0% corresponds to the minimal observed biofilm inhibition by compound 7955004 and 100% corresponds to the greatest inhibition, permitting calculation of an EC₅₀ of 7.27 μM (95% confidence interval [CI], 5.46 μM to 9.68 μM) (Fig. 2D).

Compound 7955004 was subsequently tested for its ability to inhibit biofilm formation by *S. Typhi*. Although *S. Typhi* forms robust biofilms *in vivo*, biofilm formation *in vitro* is more variable and progresses more slowly than for *S. Typhimurium*. The rapid attachment assay was adapted to promote *S. Typhi* biofilm formation by growth in SOB for an extended period (60 h), followed by biofilm quantitation and data analysis as described above. The results indicated that biofilm inhibition by *S. Typhi* was more modest than that observed for *S. Typhimurium*, reaching a maximum of 20% reduction in the overall biomass, relative to untreated controls, which was achieved with 50 μM drug (*P* < 0.0001) (data not shown). Compound 7955004 was similarly tested for its ability to disperse preexisting 24-h biofilms. Biofilms of *S. Typhimurium* and *S. Typhi* were grown as described, and 30 μM drug was added at 24 h. After 48 h, biofilms were quantified and compared to a biofilm grown without drug. No significant decrease in biofilm presence was observed (*P* = 0.8988) (data not shown).

FIG 2 Compound 7955004 inhibits *S. Typhimurium* biofilm formation in a dose-dependent manner, which is not due to bacterial killing. (A) Quantitation of *S. Typhimurium* biofilm formation via crystal violet staining of the bacterial biomass. Biofilm formation was quantified in the presence of various concentrations of compound 7955004, demonstrating a dose-dependent response reaching a maximum of approximately 55% inhibition at a concentration of 30 μ M, compared to biofilms formed in the presence of solvent alone. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$. (B) Densitometric quantitation of 24-h bacterial growth in the presence of various concentrations of compound 7955004, demonstrating no significant difference among mean OD₆₀₀ values ($P = 0.824$). (C) Bacterial viability determined in the presence or absence of 30 μ M compound 7955004 at various time points (0, 2, 4, 8, 16, and 24 h) by serial dilution and enumeration to calculate CFU/ml, confirming that growth of planktonic bacteria remains unaffected by the presence of the compound ($P = 0.8241$). (D) EC₅₀ (7.27 μ M [95% CI, 5.46 μ M to 9.68 μ M]) calculated by plotting percent biofilm inhibition, relative to wild-type values, as a function of log₁₀ compound concentrations and fitting the data using GraphPad Prism 6.

Compound 7955004 inhibits *Acinetobacter baumannii* biofilm formation in a dose-dependent manner, which is not due to bacterial killing. To determine whether the biofilm-inhibiting effects of compound 7955004 extended beyond *Salmonella* spp., the compound was tested against 3 other biofilm-forming microbial pathogens, i.e., *Staphylococcus aureus* (strain USA300), *Pseudomonas aeruginosa* (strain PAO1), and *Acinetobacter baumannii* (strain 19606). No effect on biofilm formation was observed with *P. aeruginosa*, and only limited effects were detected with *S. aureus* biofilms (data not shown). However, the testing of compound 7955004 against *A. baumannii* resulted in a dose-response curve similar to that observed for *S. Typhimurium*, with the exception that the reduction in the overall biomass, relative to untreated controls, was greater for *A. baumannii* than for *S. Typhimurium*,

with up to 80% biofilm inhibition in the presence of drug concentrations of 30 μ M to 50 μ M ($>5 \mu$ M; $P < 0.0001$) (Fig. 3A). As with *S. Typhimurium*, no difference in *A. baumannii* bacterial growth (OD₆₀₀) following 24 h of incubation in the presence of the drug was detected, implying that the inhibition of biofilm formation was not due to bactericidal or bacteriostatic effects ($P = 0.8478$) (Fig. 3B). An EC₅₀ of 6.05 μ M (95% CI, 3.36 μ M to 10.86 μ M) was determined as described above.

Compound 7955004 does not exhibit host cell toxicity. ATP-binding proteins are common in eukaryotic cells, which raises concerns about potential off-target effects when ATP mimetics are used to treat human disease. To determine the potential cytotoxic effects of compound 7955004, lactate dehydrogenase (LDH) assays were performed with HepG2 cells that had been grown in the

FIG 3 Compound 7955004 inhibits *A. baumannii* biofilm formation in a dose-dependent manner, which is not due to bacterial killing. (A) Quantitation of *A. baumannii* biofilm formation via crystal violet staining of bacterial biomass. Biofilm formation was quantified in the presence of various concentrations of compound 7955004, demonstrating a dose-dependent response reaching a maximum of approximately 80% inhibition at a concentration of 30 μM, compared to biofilms formed in the presence of solvent alone. *, $P < 0.05$; **, $P < 0.01$; ****, $P \leq 0.0001$. The EC_{50} is 6.05 μM (95% CI, 3.36 μM to 10.86 μM). (B) Evidence that planktonic growth is unaffected by the compound at 24 h (OD_{600}) ($P = 0.8478$).

presence of 50 μM, 30 μM, or 0 μM compound 7955004 or Triton X-100 (positive control) for 12 h or 24 h. Following treatment, LDH release was quantified, revealing that no significant difference in mean LDH release was detected for HepG2 cells treated with various concentrations of compound 7955004 versus untreated controls ($P > 0.55$), although all were significantly different from the Triton X-100-treated cells ($P < 0.001$), indicating no acute cellular cytotoxicity at the tested concentrations (Fig. 4A and B). This result was confirmed with a trypan blue exclusion assay (Fig. 4C). Thus, compound 7955004 showed no evidence of acute toxicity for human hepatocytes at a concentration of 50 μM, which was over 6-fold greater than the established EC_{50} .

Putative 7955004 protein targets are identified through ATP-Sepharose affinity chromatography. To determine potential in-

teracting partners for compound 7955004 in the bacterial proteome, wild-type *S. Typhimurium* was grown overnight and bacterial lysates were prepared and run through an ATP-Sepharose displacement affinity matrix (Fig. 5A). In this assay, ATP-binding proteins are immobilized on matrix beads and retained on the column until they are displaced by a binding partner with greater or similar affinity or through allosteric interactions (22). Following exhaustive washes with salt buffer to remove nonspecifically bound proteins, compound 7955004 was added to the column and eluate fractions were collected and electrophoresed on an SDS-PAGE gel. ATP was added after these fractions were collected, to release all remaining bound proteins. After 3 biological replicates of this procedure, two bands (with molecular masses of 60 kDa and 25 kDa) were consistently eluted by com-

FIG 4 Eukaryotic Hep-G2 cells do not exhibit evidence of acute cytotoxicity in the presence of compound 7955004. (A and B) Twelve-hour (A) and 24-h (B) lactate dehydrogenase (LDH) release assays were performed to evaluate the potential cytotoxicity of various concentrations of compound 7955004 on HepG2 hepatocytes. LDH release was compared among adherent HepG2 cells exposed to 2% Triton X-100 (complete lysis), tissue culture medium alone, or 30 μM or 50 μM drug. Colorimetric quantitation of LDH release into the culture medium was conducted, demonstrating that even high concentrations of compound 7955004, at the upper boundary of the experimental dosage, do not exhibit LDH release above levels detected in untreated cells. ($P < 0.001$ relative to Triton X-100 versus $P = 0.55$ relative to untreated controls). (C) Images of HepG2 cells were obtained with an inverted microscope after 24-h drug treatments as described in panel A. Images demonstrate exclusion of trypan blue from the cytosol of cells treated with 0 μM, 30 μM, or 50 μM drug, in contrast to Triton X-100-treated cells, indicating that the tested concentrations of compound 7955004 do not result in compromise of cellular membrane integrity.

FIG 5 ATP-Sepharose resin was utilized to determine candidate targets of compound 7955004. (A) Lysates from *S. Typhimurium* grown under biofilm-inducing conditions were added to ATP-Sepharose resin and incubated on ice for 1 h to permit the interaction of bacterial ATP-binding proteins with the resin. Columns were then washed extensively to remove nonspecifically bound proteins and incubated with compound 7955004 (500 μ M), followed by an ATP wash (10 μ M) to compete off proteins bound to the column. (B) Eluted proteins were electrophoresed on a 10% SDS-PAGE gel and visualized via silver staining to determine potential target proteins. Lane MW, protein molecular weight markers, with values indicated in thousands; lane L, whole-cell lysate flowthrough; lane D, 500 μ M 7955004; lane A, 10 μ M ATP. Protein bands identified in lane D were subsequently submitted for mass spectrometry and were identified as GroEL (upper band, with molecular weight of 60,000; Hsp60) and DeoD (lower band, with molecular weight of 25,000; purine nucleoside phosphorylase).

pound 7955004. These protein bands were cut from the gels, submitted for mass spectrometry, and identified as *S. Typhimurium* GroEL (Hsp60) and purine nucleoside phosphorylase (PNP) (DeoD), respectively (Fig. 5B).

DISCUSSION

Bacterial biofilms were first reported in 1978, when they were observed both in the environment and under conditions associated with chronic human infections (27). Decades of research have now demonstrated that these multicellular community structures can greatly enhance bacterial resistance to antimicrobials and host immune molecules (27, 28). Biofilms are a unique and dynamic bacterial growth environment associated with altered gene expression profiles and increased horizontal transmission of resistance elements, facilitating adaptation to hostile environments. Such functions enhance bacterial growth in difficult host niches and aid the establishment of chronic infections. Numerous studies have demonstrated connections between biofilm formation by nontyphoidal *Salmonella* strains, antimicrobial resistance, and persistence in food animals and throughout the food chain. Our laboratory has demonstrated that biofilm formation in the gallbladder facilitates chronic carriage of *S. Typhi* (13, 29). In light of the importance of these multicellular structures in the infectious cycles of *S. Typhimurium* and *S. Typhi*, we sought to disrupt the formation of bacterial biofilms using small-molecule kinase

inhibitors. It is thought that therapies that function through bactericidal activity present selective pressures for the development of antibiotic resistance (30); therefore, we focused on biofilm formation, a nonessential bacterial process known to facilitate persistent infections.

Reports of multidrug-resistant *Salmonella* strains are now common, and drug discovery is a high-priority research area in an effort to find novel ways to fight infections and to cure disease (21, 31). Small-molecule inhibitors are of great interest and are being explored for their ability to interrupt intracellular signal transduction and protein-protein interactions, as a method to disrupt processes necessary for virulence (32). Recently, small-molecule inhibition of *toxT* expression has been described for *Vibrio cholerae*, and Geske et al. showed that nonnative *N*-acyl homoserine lactone (AHL) signal molecules are able to inhibit quorum sensing and biofilm formation in *P. aeruginosa* (33, 34). Our screen targeted the complex but highly conserved cellular process of biofilm formation, employing an automated, high-throughput, preliminary screening method to assess potential drug targets. Previous research clearly demonstrated a plethora of biofilm-associated proteins and regulatory systems affecting biofilm formation (35); therefore, we employed a top-down approach whereby we began our screen by looking for the desired outcome (significant inhibition of biofilm formation), rather than targeting a particular system. Subsequent efforts were directed at identifying the drug target, in order to begin to investigate the mechanism by which the compound inhibited biofilm formation. Our preliminary screen included 3,000 compounds, from which we identified a single promising compound for further study, validation, and derivatization. Employing an automated preliminary screen permitted us to narrow the candidate compounds to 43, a number that could be tested manually in subsequent assays. The preliminary screen was conducted only once; therefore, it is certainly possible that further replicates of this process or changes in the designated 30% inhibitory threshold would yield other promising candidates from this library.

Our results demonstrate that targeting bacterial kinases/ATP-utilizing enzymes to inhibit biofilm formation could be a promising therapeutic approach for the prevention of *Salmonella enterica* biofilm formation. Compound 7955004 exhibits a dose-dependent response and demonstrates efficacy against biofilm formation at concentrations ranging from 0.625 μ M to 50 μ M. Inhibition of biofilm formation by compound 7955004 is not a result of bactericidal or bacteriostatic activity, as bacterial viability is unaltered after drug exposure. Compound 7955004 did not disperse existing biofilms, suggesting specific effects on early events in the process of attachment or biofilm microcolony formation. It was demonstrated previously that the process of biofilm formation results in increased antibiotic tolerance, which is not exhibited by genetically identical cells in the planktonic phase of growth (28, 36, 37). Therefore, the possibility that compound 7955004 may act by shifting cells out of microcolonies and into the planktonic phase, or maintaining them in a planktonic phase, is of great interest with regard to future studies investigating the potential for use of this compound in tandem with antibiotic treatment.

The core genomes of *S. Typhi* and *S. Typhimurium* share >98% sequence identity (38); however, *S. Typhimurium* is more amenable to laboratory study, genetic manipulation, and *in vivo* animal modeling (13). *S. Typhimurium* forms biofilms in numerous *in vitro* settings with greater speed and less variability than *S.*

Typhi, perhaps due to the former's ability to thrive in diverse host and environmental settings, compared with the latter's stringent host restrictions. Preliminary testing was conducted in *S. Typhimurium* in order to enable subsequent studies of identified lead compounds using available libraries of defined genetic mutations and murine modeling of chronic carriage. However, in spite of the more robust biofilm formation observed in *S. Typhimurium* and the similarity of the genomes of the two organisms, the observed biofilm-inhibiting effect of compound 7955004 was more modest with *S. Typhi* (20% inhibition) than with *S. Typhimurium* (55% inhibition). This could be due to the differing assay conditions required for measurable *in vitro* biofilm formation by *S. Typhi*. Additionally, studies aimed at identifying the interacting partners of compound 7955004 indicated a member of the RpoS regulon, DeoD, as a possible target. DeoD is positively regulated by RpoS (39, 40). Although it is widely used as a laboratory reference strain, *S. Typhi* Ty2 produces a nonfunctional RpoS protein (41), which potentially could diminish the effects of a compound targeting positively regulated components of the RpoS regulon. In addition to further lead compound optimization, additional testing will be conducted using clinical isolates carrying wild-type *rpoS*.

Although functional processes related to biofilm formation may be conserved widely among bacterial species, our laboratory and others have demonstrated that gene expression in *Salmonella* biofilms can be quite variable. Biofilm genes and surface antigens may be heterogeneously expressed in response to different substrata or growth conditions and even in different regions of an individual bacterial community (13, 42–44). As this work involved biofilms grown on a polystyrene surface, further studies will need to be conducted to investigate the potential of this compound to reduce biofilm formation on additional substrata and in different media. *In vivo* studies employing the murine model of chronic gallbladder carriage of *Salmonella* will be of particular interest (12).

Compound 7955004 showed cross-genus activity against *A. baumannii* biofilm formation. *A. baumannii* is a Gram-negative opportunistic pathogen that is frequently reported as a cause of nosocomial infections. Several genes and cellular processes involved in attachment and extracellular matrix production are shared among biofilm-forming bacterial pathogens (45); among these are the production of cellulose, type IV pili, and biofilm-associated protein (Bap) (35). Nonmicrobicidal approaches for *A. baumannii* have shown interference in biofilm formation with the addition of compounds with binding capacities in many domains of response regulators (46). Two-component systems have become a therapeutic target in recent years, due to their tight regulation of many virulence genes in *A. baumannii* and other pathogenic bacteria (47, 48). *Salmonella* and *Acinetobacter* are both able to form biofilms on biotic and abiotic surfaces, and they share biofilm regulatory genes such as *envZ/ompR* and *clpX* (4, 12, 49–52), supporting further investigation of the use of kinase inhibitors against biofilm formation by both genera.

Protein targets elucidated from the ATP-Sepharose studies included GroEL (Hsp60) and a purine nucleoside phosphorylase (DeoD). GroEL is a chaperone protein capable of interacting with and assisting in the proper folding of many proteins in eukaryotes, bacteria, and archaea (53, 54). In *Salmonella*, functional GroEL activity requires interactions with the cochaperonin protein GroES (55). Both GroEL and GroES rely on ATP binding and hydrolysis for their joint functions in cells. GroEL was found to be

essential for biofilm formation in *Mycobacterium smegmatis* and more recently was identified in the extracellular matrix of non-typeable *Haemophilus influenzae* (56, 57). GroEL has also been implicated in bacterial adherence to eukaryotic cells, including intestinal epithelial cells with *Clostridium*, *Lactobacillus*, and *S. Typhi* (58–60). Although mutants of GroEL have been found to be lethal in *Escherichia coli*, previously described mechanisms of GroEL/GroES function reveal that ATP binding occurs at multiple steps, including substrate binding, GroEL/GroES interaction, and enzymatic functionality. Therefore, it is possible that the effects of compound 7955004 reduced the functionality of GroEL but less completely than genetic deletion of *groEL*.

The second major protein target eluted from the ATP-Sepharose column was a purine nucleoside phosphorylase (PNP), DeoD. DeoD is responsible for scavenging and breaking down nucleotides, producing free purine bases and a free sugar that can be utilized as a carbon source. Bacterial DeoD has been greatly studied as a potential drug target because of marked differences in subunit and active site interactions between human and bacterial PNP protein structures (61, 62). DeoD also has been previously implicated in bacterial multicellular behavior, having been found to be significantly increased in biofilms of *E. coli* lacking the biofilm-repressing *ycfR* (63). Additionally development of rugose colony morphology in *Vibrio* (64) and early attachment and biofilm formation in *Streptococcus* have been shown to be dependent on DeoD activity (65). Subsequent studies will focus on employing heterologous deletions in these putative target proteins to establish whether they are important in *Salmonella* biofilm formation and to better understand how compound 7955004 is working in the early stages of biofilm formation.

The data presented demonstrate the promise of synthetic small molecules to generally prevent *Salmonella* biofilm formation or to improve treatment methods for chronic *Salmonella* infections in mammalian hosts. Future work will involve optimization of compound 7955004 with the goals of increasing its potency and broadening its efficacy to additional biofilm-forming pathogens. Efficient targeting of these infectious foci has great potential to facilitate eradication of chronic infections, resulting in decreased global disease burdens.

ACKNOWLEDGMENTS

This work was supported by NIH/NIAID grants AI066208 (to J.S.G.), AI090644 (to J.J.K.), and 1-T32-AI065411 (an NRSA training grant administered by the Center for Microbial Interface Biology at The Ohio State University).

We thank members of Microbial Infection and Immunity, The Ohio State University, for assistance and scientific feedback, P. Hill and D. J. Wozniak for non-*Salmonella* bacterial strains and technical assistance with biofilm assays, C. A. Rappleye for provision of the HepG2 cells, M. E. Drew for Biomek 2000 technical assistance, and K. A. Werbovetz for assistance in obtaining the ATP mimetic library. We also thank the Ohio State Mass Spectrometry and Proteomics Facility for assistance in protein identification and data interpretation (NIH grant P30-CA016058).

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