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Sulfonamido-2-arylbenzoxazole GroEL/ES inhibitors are potent antibacterials against methicillin-resistant *Staphylococcus aureus* (MRSA)

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Abstract

Extending from a study we recently published examining the anti-trypanosomal effects of a series of GroEL/ES inhibitors based on a pseudo-symmetrical bis-sulfonamido-2-phenylbenzoxazole scaffold, here, we report the antibiotic effects of asymmetric analogs of this scaffold against a panel of bacteria known as the **ESKAPE** pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species). While GroEL/ES inhibitors were largely ineffective against *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. cloacae* (Gram-negative bacteria), many analogs were potent inhibitors of *E. faecium* and *S. aureus* proliferation (Gram-positive bacteria – EC₅₀ values of the most potent analogs were in the 1–2 μ M range). Furthermore, even though some compounds

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Supporting Information.

Supporting information associated with this article can be found in the online version, which includes tabulation of all biochemical IC₅₀, bacterial proliferation EC₅₀, and human cell viability CC₅₀ results; log(IC₅₀), log(EC₅₀), and log(CC₅₀) results with standard deviations; plots of statistical differences between the R- and L-series analogs in the various assays; synthetic protocols and characterization data for test compounds (HPLC purity, MS, and ¹H-NMR); experimental protocols for protein synthesis and purification, and biochemical, bacterial proliferation, and human cell viability assays; and SMILES strings of compound structures.

inhibit human HSP60/10 biochemical functions *in vitro* (IC₅₀ values in the 1–10 μ M range), many of these exhibited moderate to low cytotoxicity to human liver and kidney cells (CC₅₀ values >20 μ M).

Graphical Abstract

Keywords

GroEL; GroES; HSP60; HSP10; molecular chaperone; chaperonin; proteostasis; small molecule inhibitors; *ESKAPE* pathogens; MRSA; antibiotics

INTRODUCTION:

The persistence of antibiotic resistant pathogens is a significant health and economic burden worldwide. Six of the most problematic drug resistant Gram-positive (Gr+) and Gramnegative (Gr-) bacteria are commonly referred to as the *ESKAPE* pathogens. This panel of drug resistant bacteria includes *Enterococcus faecium* (Gr+), *Staphylococcus aureus* (Gr+), *Klebsiella pneumoniae* (Gr-), *Acinetobacter baumannii* (Gr-), *Pseudomonas aeruginosa* (Gr-), and *Enterobacter* species (Gr-).^{1–5} Of particular significance, the CDC recently estimated that ~80,000 individuals are infected with MRSA in the US annually, with ~11,000 deaths – the highest mortality rate amongst all the antibiotic resistant bacteria. *S. aureus*-associated infections range from mild skin infections to life-threatening endocarditis, osteomyelitis, or bacteremia, which are usually treated with penicillinase-resistant beta-lactams or vancomycin. For more resistant strains, newer classes of antibiotics can be prescribed, such as linezolid, daptomycin, dalbavancin, telavancin, tigecycline, ceftaroline, oritavancin, dalbavancin, or tedizolid.^{6–8} Unfortunately, despite the availability of several antimicrobials, treating MRSA infections remains a significant challenge owing to off-target toxicities of some antibiotics, lack of efficacy in life-threatening clinical infections such as bacteraemia, endocarditis, etc., and the emergence of pan-drug resistant strains.⁷ Thus, there is an urgent need for new antibacterial drugs that function against previously unexploited targets and pathways to circumvent predisposed resistance mechanisms.

Towards developing mechanistically unique antibacterial candidates, we have focused on exploiting bacterial protein homeostasis pathways. A network of molecular chaperones and proteases collectively functions to maintain protein homeostasis by assisting proteins to fold to their native, functional states, or ensuring their proper degradation and recycling.^{9, 10} Since such quality control mechanisms are vital to cell survival, targeting them with small molecule inhibitors should be an effective antibacterial strategy. While recent studies have investigated targeting DnaK (a molecular chaperone belonging to the HSP70 family) and

Clp proteases, targeting of the bacterial GroEL/GroES chaperonin system has gone largely unexplored.^{11–14} *E. coli* GroEL, which is the prototypical member of the HSP60 chaperonin family of molecular chaperones, is a homo-tetradecameric protein that forms two, seven-subunit rings that stack back-to-back with one another.^{15–17} Through a series of events driven by ATP binding and hydrolysis, unfolded substrate polypeptides are bound within the central cavity of a GroEL ring and encapsulated by the GroES co-chaperonin “lid”, allowing protein folding within the sequestered chamber.^{17–21} Since the GroEL/ES chaperonin system is essential for *E. coli* survival under all growth conditions, as it likely is for other bacteria, it represents an excellent target for antibacterial development.^{22, 23} Furthermore, GroEL is highly conserved in prokaryotes (generally >50% sequence identity between bacterial species, whether Gram-positive or Gram-negative), thus, targeting this molecular machinery has the potential for broad spectrum applicability. However, since human HSP60 is also highly conserved (48% identity with *E. coli* GroEL), we also need to consider whether GroEL inhibitors have off-target effects against HSP60 in human cells.

Towards our goal of exploiting the GroEL/ES chaperonin system as an antibacterial strategy, we previously reported a high-throughput screen that identified 235 small molecule inhibitors of the *E. coli* GroEL/ES chaperonin system.²⁴ In a subsequent study, we evaluated 22 of these GroEL/ES inhibitor hits for their antibacterial properties against the *ESKAPE* pathogens.²⁵ In another study, we evaluated a series of compound **1**-based GroEL/ES inhibitors for their antibiotic effects against *Trypanosoma brucei* parasites – the causative agents of African sleeping sickness (Figure 1A).²⁶ As an extension of those two studies, herein, we have explored additional compound **1** analogs for their ability to selectively inhibit the prototypical *E. coli* GroEL/ES chaperonin system and growth of the *ESKAPE* pathogens over human HSP60/10 and liver and kidney cells. Overall, this study enabled us to better understand the bioactivity profiles of this molecular scaffold, which provided invaluable SAR to guide future studies to more rationally optimize the pharmacological properties of these antibacterial GroEL/ES chaperonin system inhibitors.

RESULTS AND DISCUSSION

Identifying preliminary SAR of previously developed pseudo-symmetrical compound **1** analogs for antibacterial effects against the *ESKAPE* pathogens.

From our previous study exploring the antibacterial effects of our different GroEL/ES inhibitor scaffolds, we found that the parent hit, compound **1** (based on the benzimidazole core, Figure 1A), exhibited no antibacterial effects against any of the *ESKAPE* pathogens.²⁵ Therefore, before embarking on synthesis of new compound **1** analogs, we tested our previously developed benzoxazole-based GroEL/ES inhibitors (compounds **2–14**), which have antitrypanosomal activities, for their antibacterial properties.²⁶ We expected that this initial evaluation would tell us whether or not this scaffold would be worthwhile to pursue for antibacterial development and, if so, allow us to identify which substituents, substitution patterns, and aryl substructures would provide the most potent antibacterial effects. We employed a standard antibacterial efficacy assay in liquid culture as previously reported, with one modification: the media was cation-adjusted by addition of 25 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+} to better mimic the concentrations of these divalent cations *in vivo*, which can

alter the antibacterial effects of some compounds (e.g. daptomycin is a more potent inhibitor when media is supplemented with additional Ca^{2+} and Mg^{2+} ions).²⁷ Briefly, bacteria were grown at 37°C without shaking (stagnant assay) in media stamped with test compounds. Compounds were first tested at single concentrations of 100 μM , then in dose-response for those that exhibited >50% inhibition of bacterial proliferation (refer to Tables S1A and S2A in the Supporting Information for a tabulation of all EC_{50} results). After 6–8 h (*S. aureus*, MRSA, *K. pneumonia*, *E. cloacae*, and *P. aeruginosa*) or 24 h (*E. faecium* and *A. baumannii*), the absorbance of each well was read at 600 nm to monitor turbidity from bacterial growth, from which EC_{50} results were obtained by plotting the dose-response measurements and fitting data with non-linear regression. A detailed protocol for this assay is listed in the Supporting Information.

Preliminary results from these proliferation assays indicated that all but one compound (**2h-p**) were inactive against the four Gram-negative *KAPE* bacterial species, and only three were able to inhibit *E. faecium* (the *ortho*-, *meta*-, and *para*-substituted analogs of hydroxylated compound **2h**); however, several compounds were able to inhibit *S. aureus* and MRSA proliferation (Figure 1B, and Tables S1A and S2A in the Supporting Information). Furthermore, these compounds were reasonably selective at killing MRSA in relation to their cytotoxicity CC_{50} values obtained from liver (THLE 3) and kidney (HEK 293) cell viability assays, which were determined in the previous study targeting *T. brucei* parasites.²⁶ Preliminary SAR revealed that the sulfonamide end-capping thiophene and *para*-chlorophenyl groups gave the most potent and selective aryl and substituent/substitution patterns, respectively (Figure 1B). Based on these initial results, we decided to combine these two moieties, reasoning that inhibition properties of each could be additive, and developed two parallel series of 2-chlorothiophene-based asymmetrical analogs (Figure 1C, analogs **15–34**) containing variable substructures at the **R**² positions.

Synthesis of the two parallel series of 2-chlorothiophene-based analogs.

We rationalized that the previously developed pseudo-symmetrical compound **1** analogs (i.e. with the same sulfonamide end-capping substructures on either side of the molecules) may not be truly optimized for binding to and inhibiting the chaperonin system. Thus, to better complement the envisioned asymmetric binding sites, we designed two parallel series of analogs with a variety of alkyl and aryl substructures at the **R**² positions on the **Right** (**R**-series) and **Left** (**L**-series) sides of the 2-phenylbenzoxazole scaffold, while keeping the 2-chlorothiophenesulfonamide moiety on the opposite sides (Figure 1C). The same **R**²-groups were used to create the panel of 19 matched-pairs for the different **R**- and **L**-series analogs, with an additional pseudo-symmetrical analog (**15**) that contained the 2-chlorothiophenesulfonamide on both sides (thus, a total of 39 new compounds were synthesized for evaluation). We expected that these matched pairs of analogs would help us to identify whether or not the directionality of the 2-phenylbenzoxazole core would significantly impact inhibitor effects in the various biochemical and cell-based assays they would be tested in. The general syntheses of these analogs are shown in Scheme 1, with detailed procedures and compound characterizations presented in the Experimental section and Supporting Information. Each of the **R**- and **L**-series of analogs were synthesized through 5-step linear protocols employing facile reactions. All final test compounds were

characterized by $^1\text{H-NMR}$ and LC-MS analyses for structural confirmation, and by two independent sets of HPLC conditions for purity identification (all were >95% pure under both conditions).

For synthesizing the **R**-series analogs, 2-chlorothiophenesulfonyl chloride was first coupled to 4-amino-2-nitrophenol in dichloromethane using pyridine as base, giving **35**.^{26, 28, 29} Reduction of the nitro group was accomplished by reacting with tin powder in a 1:10 v/v mixture of hydrochloric acid in glacial acetic acid.^{28–30} Next, an intermediate Schiff base was formed by refluxing **36** and *para*-nitrobenzaldehyde with sodium bicarbonate in anhydrous THF, which was then cooled and cyclized to benzoxazole **37** by addition of DDQ.³¹ The nitro group was again reduced using tin powder in HCl/AcOH, affording intermediate **38**, from which the final bisulfonamido analogs (**15** and **16R-34R**) could be rapidly generated by coupling with a variety of differentially-substituted sulfonyl chlorides (**R**²-SO₂Cl).

For synthesizing the **L**-series analogs, 2-chlorothiophenesulfonyl chloride was first coupled to methyl-4-aminobenzoate in dichloromethane using pyridine as base, giving **39**. The ester was then hydrolyzed with LiOH in a mixture of H₂O/MeOH/THF, affording acid **40**.^{28, 30, 32} The acid was then converted to the acyl chloride, concentrated, and coupled to 2-amino-4-nitrophenol in dichloromethane with pyridine as base, affording the intermediate amide, which was then cyclodehydrated to **41** by refluxing with toluenesulfonic acid in xylenes.^{29, 33} The nitro group was subsequently reduced with tin powder in HCl/AcOH, affording intermediate **42**, from which the final bis-sulfonamido analogs (**16L-34L**) could be rapidly generated by coupling with a variety of differentially-substituted sulfonyl chlorides (**R**²-SO₂Cl).

Evaluating compounds for antibiotic effects against the *ESKAPE* bacteria and inhibition of GroEL/ES-mediated folding of substrate proteins.

Before proceeding with extensive biochemical and biophysical characterization of the new 2-chlorothiophene-based analogs, we first tested for their antibiotic effects on the *ESKAPE* bacteria using the proliferation assay described above (refer to Tables S3A and S4A in the Supporting Information for tabulations of all EC₅₀ results). Much like the initial testing with the pseudo-symmetrical analogs, all but one of the 2-chlorothiophene analogs were inactive against the four Gram-negative *KAPE* bacterial species (**27R** had an EC₅₀ of 37 μM against *A. baumannii*). However, 14 of the 39 new analogs (36%) exhibited antibacterial effects against *E. faecium*, compared to 3 out of 50 (6%) of the pseudo-symmetrical compounds. What was particularly impressive was that 27 out of the 39 new analogs (69%) inhibited *S. aureus* and MRSA, with greater overall potency, compared to the 20 out of 50 (40%) of the pseudosymmetrical compounds (Figure 2A presents a correlation plot of EC₅₀ values for each compound determined in these two assays). To compare assay results between the **R**- and **L**series analogs, we analyzed the log(EC₅₀) values of each data set using two-tailed, paired t-tests (95% confidence level) and looked at differences between paired values (results are plotted in Figure S1 in the Supporting Information). For the *E. faecium* EC₅₀ results, we did not see a statistical difference between results from the **R**- and **L**-series analogs (Figure S1A). However, there was a statistically significant difference between

results of the two series for inhibiting *S. aureus* and MRSA proliferation, with the **L**-series generally providing more potent inhibitors of bacterial proliferation for each strain (Figures S1B and S1C). Although exceptions are evident for individual compounds, there was no statistical difference between inhibiting the drug susceptible *S. aureus* and MRSA strains when the data sets were analyzed for all 89 compounds as a whole (Figure S5A).

Having ascertained that the majority of the **R**- and **L**-series analogs were potent inhibitors of *S. aureus* and MRSA proliferation, we next evaluated their abilities to inhibit the biochemical function of the GroEL/ES chaperonin system. For this, we employed our previously reported assays that monitor GroEL/ES-mediated refolding of two denatured substrate enzymes, malate dehydrogenase (dMDH) and rhodanese (dRho).²⁵ Since these were coupled assays that monitor chaperonin-mediated refolding of substrates by virtue of the enzymatic activities of the refolded substrates, we further counter-screened against the native MDH and Rho enzymes to ensure that compounds were not simply false-positives of the reporter reactions. Detailed protocols for these assays and tabulation of all IC₅₀ results are presented in the Supporting Information.

Because the previously developed pseudo-symmetrical analogs (**1-14**) were not tested in the GroEL/ES-dRho refolding assay, nor the native rhodanese enzymatic reporter reaction counter screen, we also evaluated those compounds in these two assays. When looking at all 89 compounds as a whole, although a correlation is evident, we noticed a statistically significant difference between the GroEL/ES-dMDH and GroEL/ES-dRho refolding IC₅₀ results (Figures 2B and S5B), where compounds were slightly more potent at inhibiting GroEL/ES-mediated refolding of denatured MDH. This difference does not appear to be a result of compounds preferentially inhibiting the native MDH reporter enzyme over rhodanese – while eleven analogs inhibited the native MDH enzymatic reporter reaction, five inhibited native rhodanese, and only to a minor extent. Since only one compound inhibits both native malate dehydrogenase and native rhodanese (**28R**), and only to a minor extent, this supports that compounds are on-target for inhibiting the GroEL/ES-mediated folding cycle.

For the purposes of categorizing inhibitor potencies in the GroEL/ES-mediated refolding assays, we consider compounds with IC₅₀ values >100 μ M to be inactive, 30–100 μ M to be weak inhibitors, 10–30 μ M moderate inhibitors, 1–10 μ M potent inhibitors, and <1 μ M very potent and acting near stoichiometrically since the concentration of GroEL tetradecamer is 50 nM during the refolding cycle (i.e. 700 nM of GroEL subunits). Upon further dissection of the GroEL/ES-dMDH refolding assay IC₅₀ results, we observed that the asymmetric **R**- and **L**-series analogs are generally more potent than the previously developed pseudo-symmetric analogs: 28 out of 38 (74%) of the asymmetric **R**- and **L**-series analogs (**16-34**) have IC₅₀ values less than 10 μ M, whereas only 16 out of 51 (31%) of the pseudo-symmetric analogs (**1-15**) have IC₅₀ values less than 10 μ M. These results are not entirely surprising since the **R**- and **L**-series analogs were hypothesized to better complement the envisioned asymmetric binding sites than the pseudosymmetric compounds. However, we caution on over-interpreting these results since the analog groupings did contain different alkyl and aryl substructures on the sulfonamide end-caps. When comparing the **R**- and **L**-series analogs with each other, though, we do not see any statistically significant differences in either the

GroEL/ES-dMDH or GroEL/ES-dRho refolding assay results (Figure S2A and S2B), suggesting that the orientation of the 2-phenylbenzoxazole core scaffold does not play a significant factor in these compounds binding to and inhibiting the GroEL/ES chaperonin system.

When comparing IC₅₀ results for compounds tested in the biochemical GroEL/ES-dMDH refolding assay with EC₅₀ results for testing in the *S. aureus* proliferation assay (Figure 2C and Tables S3A-B and S4A-B), we note that no compounds are active against bacteria unless they are able to inhibit GroEL. In general, the more potent compounds are at inhibiting the GroEL/ES chaperonin system, the more potent their antibacterial effects, in particular for the **L**-series inhibitors. However, several exceptions are evident where potent GroEL/ES inhibitors are poorly effective, or ineffective, against bacteria, which may owe to poor cell wall permeability and/or efflux, as we had previously observed for other GroEL/ES inhibitor scaffolds.²⁵ Also, while correlations between biochemical IC₅₀ and cell-based EC₅₀ are only suggestive of mechanisms of action in cells, they are not confirmatory. Thus, whether compounds are “ontarget” for GroEL within bacteria is still unknown and warrants further investigation. These studies are ongoing and will be reported in the future.

Characterizing GroEL-inhibitor binding interactions using Isothermal Titration Calorimetry.

To further interrogate inhibitor mechanisms of action against GroEL, we used isothermal titration calorimetry (ITC) to identify the thermodynamic parameters, binding affinities, and binding stoichiometries of compounds **20R** and **20L**. While these two compounds were only moderately active at inhibiting *S. aureus* and MRSA proliferation, they were strong inhibitors of GroEL/ES-mediated folding functions, and thus likely had high binding affinities. Furthermore, because they bore primary amines that would be charged under physiological conditions, they were much more soluble than other inhibitors and thus more amenable to ITC analysis, which requires high concentrations of both protein and ligands in matched aqueous buffers. We performed ITC analyses by titrating 400 μ M GroEL (monomer concentration) into solutions containing either 50 μ M **20R** or **20L** (detailed protocols are presented in the Experimental section). Two representative isotherms for the binding of **20R** and **20L** to GroEL are presented in Figure 3. After subtraction of background heats of mixing and dilution, plots of the integrated heats from compound binding fit well to a single-site binding model.³⁴ Averaged results for the various binding parameters (K_d , n , H , S , and G) obtained from replicate analyses (six replicates for **20R**, and five replicates for **20L**) are presented in Table 1.

From the averaged results, we found that **20R** had a K_d of 36 nM for binding to *E. coli* GroEL, while the K_d of **20L** was about four-fold higher (150 nM), which corresponds reasonably well with the relative IC₅₀ values for inhibiting GroEL/ES-mediated refolding of dMDH and dRho – the IC₅₀ values for **20L** were typically two- to four-fold higher than for **20R**. For both compounds, binding was predominantly enthalpically driven, with minor entropic contributions to affinity. What was particularly interesting from these analyses was the stoichiometry of binding of each compound to GroEL. Since GroEL consists of 14 identical subunits, we anticipated that compounds could bind with a stoichiometry of 14, or potentially 7 if there is negative cooperativity between the two GroEL rings (also assuming

that for inhibitor binding there is no negative cooperativity between subunits within a ring). Since IC₅₀ values were previously found to correlate between GroEL/ES-mediated refolding and ATPase assays for this inhibitor scaffold, the most likely binding sites may be the ATP pockets, of which there are 1 per GroEL subunit (14 per oligomer).²⁶ However, **20R** bound with a stoichiometry of roughly 18 molecules per GroEL tetradecamer, and **20L** with 23 molecules, which could indicate more than one potential binding site per GroEL subunit, and potentially an unknown site outside of the ATP pockets. We are currently pursuing X-ray crystallographic studies to identify specific inhibitor binding sites, which we will report on in future studies.

Counter-screening compounds for inhibition of HSP60/10-mediated refolding of denatured MDH and for cytotoxicity in human cell viability assays.

Having established that the **R**- and **L**-series analogs were potent GroEL/ES chaperonin system inhibitors with anti-staphylococcal properties, we next investigated their selectivity profiles compared to the human HSP60/10 chaperonin system. For this, we evaluated compounds in our previously reported HSP60/10-dMDH refolding assay, which is analogous to that used for determining inhibition of bacterial GroEL/ES, but alternatively employs the human chaperonin system. A detailed protocol and tabulation of all IC₅₀ results for this assay are presented in the Supporting Information. We previously reported that the pseudo-symmetrical analogs (**1-14**) were highly selective for inhibiting *E. coli* GroEL/ES over human HSP60/10 (Figure 3A and S3A).²⁶ However, for the asymmetric compounds developed in this study, selectivity for inhibiting GroEL/ES over HSP60/10 was statistically significant for the **R**-series analogs (Figure S3C), but was lost for the **L**-series analogs (Figure S3B). Correspondingly, differences in IC₅₀ values between the **R**- and **L**-series inhibitors in the HSP60/10-dMDH refolding assay were statistically significant (Figure S4A), which suggests that directionality of the 2-phenylbenzoxazole core does have an effect on compounds binding to and inhibiting the human chaperonin system, unlike the situation for *E. coli* GroEL/ES.

We next evaluated the cytotoxicity of our compounds to human liver (THLE 3) and kidney (HEK 293) cell lines in a well-established, 48 h, Alamar Blue-based cell viability assay. Detailed protocols and tabulation of all cytotoxicity CC₅₀ results for these assays are presented in the Supporting Information. Intriguingly, even though the **L**-series analogs are more potent HSP60/10 inhibitors than the **R**-series, we observed no statistically significant differences between the **L**- and **R**-series CC₅₀ values in the HEK 293 kidney cell viability assay (Figure S4B). Furthermore, we observed an opposing trend where the **L**-series was less cytotoxic to the THLE 3 liver cells compared to the **R**-series analogs, although the differences were small (Figure S4B & C). These results suggest that the compounds may be unable to penetrate the mitochondrial matrix to engage with the HSP60/10 chaperonin system, and instead may be interacting with other targets in the cytosol or other sub-cellular compartments; however, we have yet to confirm this hypothesis. What is particularly promising with these results is that the cytotoxicity of lead analogs has not concomitantly increased as with antibacterial potency. Compared to the previously developed pseudo-symmetrical analogs, several lead asymmetrical analogs have appreciably higher Selectivity Indices (SI) for inhibiting MRSA proliferation over cytotoxicity in the liver cell viability

assay (Figure 4C). The biochemical and cell-based IC₅₀, EC₅₀, and CC₅₀ results for the top four lead inhibitors of the pseudo-symmetrical, **R**-series, and **L**-series analogs are presented in Table 2, ranked based on their selectivity indices. Notably, compounds **24L** and **25L** have selectivity indices of 31 and 46, respectively.

Investigating the potential for MRSA to gain resistance to lead inhibitors.

While selectivity indices (and therapeutic windows with regards to *in vivo* evaluation) are important criteria to consider for antibacterial development, it is also important to determine whether or not bacteria will be able to rapidly develop resistance to lead candidates, especially if they function through new mechanisms of action. To gauge the efficacy of our GroEL/ES inhibitors for eluding acute antibacterial resistance mechanisms, we performed a step-wise, liquid culture resistance assay by consecutively passaging the MRSA strain (ATCC BAA-44) for 12 cycles over 12 consecutive days in the presence of serially-diluted inhibitors **20R**, **28R**, and vancomycin for comparison (a detailed protocol is presented in the Experimental section).^{35, 36} At the end of each cycle (i.e. each 24 h passage), EC₅₀ values were determined for the test compounds, with the assumption that EC₅₀ values would increase over time if MRSA was able to generate acute resistance. A plot of test compound EC₅₀ values over time is presented in Figure 5A. Despite the fact that **28R** was a potent MRSA growth inhibitor (initial EC₅₀ value of 1.7 μ M), the bacteria rapidly developed resistance to compound concentrations in excess of 100 μ M. However, after culturing in the absence of inhibitors for two days (24 h on agar, and another 24 h in liquid media), bacteria regained sensitivity to **28R** (Figure 5B), suggesting resistance is reversible, potentially from up-regulation of efflux pumps. Most importantly, we found that the parent MRSA strain was unable to generate resistance against **20R** over the 12 day passage, even though this analog was only a moderate inhibitor of MRSA proliferation (EC₅₀ = 19 μ M). These results suggest that pan-resistance to these GroEL inhibitor analogs may be difficult to develop, which supports the continued optimization of lead antibacterial candidates based on this molecular scaffold.

CONCLUSIONS.

From exploratory screening of the antibacterial effects of our previously reported pseudosymmetrical compound **1** analogs,²⁶ we were able to develop two parallel **R**- and **L**-series of 2chlorothiophene-based asymmetrical analogs with significantly improved antibacterial efficacy profiles against MRSA. While there were no statistical differences between either the **R**- or **L**series analogs for inhibiting in the GroEL/ES-mediated refolding assays, the **L**-series analogs were found to be more potent at inhibiting *S. aureus* and MRSA proliferation – i.e. preferred from an antibiotic development perspective (Table 3). While a general trend is observed between inhibitor potencies in the biochemical GroEL/ES-dMDH refolding assay and the MRSA proliferation assay (in particular for the **L**-series analogs), further studies are needed to conclusively determine whether or not inhibitors are on-target in bacteria. Using ITC to characterize the binding interactions between GroEL and inhibitors **20R** and **20L**, we found that inhibitor binding was predominantly enthalpically driven. Furthermore, we observed that >14 molecules were bound per GroEL tetradecamer (i.e. more than one molecule bound per GroEL subunit), suggesting that inhibitors could be

binding to unknown sites outside of the ATP pockets. Current studies are underway to identify and characterize these putative binding sites so that more rigorous structure-based optimization of lead candidates can be conducted. Even though the **R**-series analogs were overall less potent at inhibiting HSP60/10, the **L**-series was less cytotoxic against the human liver cells tested, and is likely preferential for further antibacterial optimization going forward. However, before pursuing *in vivo* development of this series, further medicinal chemistry optimization is warranted to increase the selectivity indices for killing MRSA over cytotoxicity to human cells.

EXPERIMENTAL.

General Synthetic Methods.

Unless otherwise stated, all chemicals were purchased from commercial suppliers and used without further purification. Reaction progress was monitored by thin-layer chromatography on silica gel 60 F254 coated glass plates (EM Sciences). Flash chromatography was performed using a Biotage Isolera One flash chromatography system and eluting through Biotage KP-Sil Zip or Snap silica gel columns for normal-phase separations (hexanes:EtOAc gradients), or Snap KP-C18-HS columns for reverse-phase separations (H₂O:MeOH gradients). Reverse-phase high-performance liquid chromatography (RP-HPLC) was performed using a Waters 1525 binary pump, 2489 tunable UV/Vis detector (254 and 280 nm detection), and 2707 autosampler. For preparatory HPLC purification, samples were chromatographically separated using a Waters XSelect CSH C18 OBD prep column (part number 186005422, 130 Å pore size, 5 µm particle size, 19×150 mm), eluting with a H₂O:CH₃CN gradient solvent system. Linear gradients were run from either 100:0, 80:20, or 60:40 A:B to 0:100 A:B (A = 95:5 H₂O:CH₃CN, 0.05% TFA; B = 5:95 H₂O:CH₃CN, 0.05% TFA). Products from normal-phase separations were concentrated directly, and reverse-phase separations were concentrated, diluted with H₂O, frozen, and lyophilized. For primary compound purity analyses (HPLC-1), samples were chromatographically separated using a Waters XSelect CSH C18 column (part number 186005282, 130 Å pore size, 5 µm particle size, 3.0×150 mm), eluting with the above H₂O:CH₃CN gradient solvent systems. For secondary purity analyses (HPLC-2) of final test compounds, samples were chromatographically separated using a Waters XBridge C18 column (either part number 186003027, 130 Å pore size, 3.5 µm particle size, 3.0×100 mm, or part number 186003132, 130 Å pore size, 5.0 µm particle size, 3.0×100 mm), eluting with a H₂O:MeOH gradient solvent system. Linear gradients were run from either 100:0, 80:20, 60:40, or 20:80 A:B to 0:100 A:B (A = 95:5 H₂O:MeOH, 0.05% TFA; B = 5:95 H₂O:MeOH, 0.05% TFA). Test compounds were found to be >95% in purity from both RP-HPLC analyses. Mass spectrometry data were collected using either an Agilent analytical LC-MS at the IU Chemical Genomics Core Facility (CGCF), or a Thermo-Finnigan LTQ LC-MS in-lab. ¹H-NMR spectra were recorded on either Bruker 300 MHz or 500 MHz spectrometers. Chemical shifts are reported in parts per million and calibrated to the *d*₆-DMSO solvent peaks at 2.50 ppm. We previously synthesized compounds **1–14** (including **2a-m-o/m/p**)²⁶ and re-synthesized where necessary due to stock depletion. Synthesis and characterization of intermediates **35–42** are presented below. General sulfonamide coupling steps are presented

for analogs **15** and **16R-34R** and **16L-34L** below, with compound characterizations for each analog presented in the Supporting Information.

35: 5-Chloro-*N*-(4-hydroxy-3-nitrophenyl) thiophene-2-sulfonamide.

To a stirring mixture of 4-amino-2-nitrophenol (5.34 g, 24.6 mmol) in anhydrous CH₂Cl₂ (50 mL) was added 5-chlorothiophene-2-sulfonyl chloride (4.21 g, 27.3 mmol) and pyridine (2.40 mL, 29.4 mmol). The reaction was allowed to stir at room temperature for 18 h and was then diluted with hexanes and the precipitate was filtered, rinsed with 1 M HCl and water, collected, and dried to afford **35** as a reddish-brown solid (7.70 g, 94% yield). ¹H-NMR (300 MHz, *d*₆-DMSO) δ 10.98 (br s, 1H), 10.62 (br s, 1H), 7.60 (d, *J* = 2.7 Hz, 1H), 7.39 (d, *J* = 4.1 Hz, 1H), 7.30 (dd, *J* = 8.9, 2.7 Hz, 1H), 7.22 (d, *J* = 4.1 Hz, 1H), 7.10 (d, *J* = 8.9 Hz, 1H); MS (ESI) C₁₀H₆ClN₂O₅S₂ [M-H]⁻ *m/z* expected = 332.9, observed = 332.8; HPLC-1 = >99% (RT = 8.1 min).

36: *N*-(3-Amino-4-hydroxyphenyl)-5-chlorothiophene-2-sulfonamide.

Tin powder (8.73 g, 73.6 mmol) was added slowly to a stirring mixture of **35** (8.11 g, 24.2 mmol) in a 1:10 mixture of HCl:AcOH (24 mL). The reaction was allowed to stir at R.T. for 18 h, then diluted with EtOAc and H₂O, neutralized with NaHCO₃, and filtered. The filtrate was extracted with EtOAc and the organics dried over Na₂SO₄, filtered, and concentrated. The residue was diluted in a 50% mixture of DCM in hexanes and the precipitate was filtered, rinsed with hexanes, collected, and dried to afford **36** as a brown powder (6.08 g, 82% yield). ¹H-NMR (300 MHz, *d*₆-DMSO) δ 9.81 (br s, 1H), 8.99 (br s, 1H), 7.28 (d, *J* = 4.1 Hz, 1H), 7.18 (d, *J* = 4.1 Hz, 1H), 6.49 (d, *J* = 8.3 Hz, 1H), 6.41 (d, *J* = 2.5 Hz, 1H), 6.13 (dd, *J* = 8.3, 2.6 Hz, 1H), 4.64 (br s, 2H); MS (ESI) C₁₀H₁₀ClN₂O₃S₂ [M+H]⁺ *m/z* expected = 305.0, observed = 304.9; HPLC-1 = 97% (RT = 6.0 min).

37: 5-Chloro-*N*-(2-(4-nitrophenyl)benzo[d]oxazol-5-yl)thiophene-2-sulfonamide.

Compound **36** (2.94 g, 9.65 mmol), 4-nitrobenzaldehyde (2.03 g, 13.4 mmol), NaHCO₃ (2.08 g, 24.8 mmol), and Na₂SO₄ (3.35 g) were stirred in THF (40 mL) for 4 h at reflux (under Ar), then cooled to R.T. DDQ (2.85 g, 12.6 mmol) was then added portion-wise and the reaction was left to stir for 2 h, then filtered. The filtrate was extracted into EtOAc, rinsed with saturated NaHCO₃, 1 M HCl, and brine. The organics layer was dried over Na₂SO₄, filtered, and concentrated. The residue was diluted in a 25% mixture of DCM in hexanes and the precipitate was filtered, rinsed with hexanes, collected, and dried to afford **37** as a brown powder (4.01 g, 95% yield). ¹H-NMR (300 MHz, *d*₆-DMSO) δ 10.74 (s, 1H), 8.37–8.48 (m, 4H), 7.82 (d, *J* = 8.8 Hz, 1H), 7.60 (d, *J* = 1.9 Hz, 1H), 7.43 (d, *J* = 4.0 Hz, 1H), 7.25 (dd, *J* = 8.8, 2.1 Hz, 1H), 7.19 (d, *J* = 4.1 Hz, 1H); MS (ESI) C₁₇H₉ClN₃O₅S₂ [M-H]⁻ *m/z* expected = 434.0, observed = 433.8; HPLC-1 = 91% (RT = 7.7 min).

38: *N*-(2-(4-Aminophenyl)benzo[d]oxazol-5-yl)-5-chlorothiophene-2-sulfonamide.

Tin powder (2.74 g, mmol) was added slowly to a stirring mixture of **37** (2.43 g, 5.58 mmol) in a 1:10 mixture of HCl:AcOH (15 mL). The reaction was allowed to stir at R.T. for 18 h, then diluted with EtOAc and H₂O, neutralized with NaHCO₃, and filtered. The filtrate was extracted with EtOAc and the organics dried over Na₂SO₄, filtered, and concentrated. The

residue was chromatographed over silica (hexanes:EtOAc gradient) and concentrated to afford **38** as an orange solid (936 mg, 41% yield). ¹H-NMR (300 MHz, *d*₆-DMSO) δ 10.54 (s, 1H), 7.78–7.86 (m, 2H), 7.60 (d, *J* = 8.7 Hz, 1H), 7.35–7.40 (m, 2H), 7.18 (d, *J* = 4.1 Hz, 1H), 7.04 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.64–6.71 (m, 2H), 6.04 (br s, 2H); MS (ESI) C₁₇H₁₃ClN₃O₃S₂ [M+H]⁺ *m/z* expected = 406.0, observed = 405.9; HPLC-1 = 97% (RT = 7.6 min).

General sulfonamidation procedure for the synthesis of 16R-34R.

To a stirring mixture of compound **38** (1 eq.) in anhydrous CH₂Cl₂ (5 mL) was added the respective **R**² sulfonyl chloride (1.2 eq.) followed by anhydrous pyridine (1.2 eq.). The reaction was allowed to stir at room temperature for 18 h and was then chromatographed over silica (hexanes:EtOAc gradient) and concentrated. If necessary, the product was further purified by preparatory RP-HPLC (H₂O:CH₃CN gradient), concentrated, and lyophilized. Refer to the Supporting Information for individual compound characterization data.

39: Methyl 4-((5-chlorothiophene)-2-sulfonamido)benzoate.

To a stirring mixture of methyl-4-aminobenzoate (2.76 g, 18.3 mmol) in anhydrous CH₂Cl₂ (50 mL) was added 5-chlorothiophene-2-sulfonyl chloride (4.78 g, 22.0 mmol) and pyridine (1.80 mL, 22.1 mmol). The reaction was allowed to stir at room temperature for 18 h and was then diluted with hexanes and acidified with 1 M HCl. The precipitate was then filtered, rinsed with 1 M HCl and water, collected, and dried to afford **39** as a pinkish-orange solid (5.94 g, 98% yield). ¹H-NMR (300 MHz, *d*₆-DMSO) δ 11.16 (br s, 1H), 7.86–7.93 (m, 2H), 7.56 (d, *J* = 4.1 Hz, 1H), 7.25–7.31 (m, 2H), 7.22 (d, *J* = 4.1 Hz, 1H), 3.80 (s, 3H); HPLC-1 = 99% (RT = 6.2 min).

40: 4-((5-Chlorothiophene)-2-sulfonamido)benzoic acid.

LiOH•H₂O (7.02 g, 167 mmol) was added to a stirring mixture of **39** (5.67 g, 17.1 mmol) in THF (20 mL), MeOH (20 mL), and H₂O (20 mL). The reaction was allowed to stir at room temperature for 2 days and was then diluted with 1M HCl. The precipitate was filtered, washed with H₂O, collected, and dried to afford **40** as an off-white solid (5.20 g, 96% yield). ¹H-NMR (300 MHz, *d*₆-DMSO) δ 12.85 (br s, 1H), 11.09 (br s, 1H), 7.83–7.90 (m, 2H), 7.54 (d, *J* = 4.1 Hz, 1H), 7.19–7.28 (m, 3H); HPLC-1 = >99% (RT = 6.7 min).

41: 5-Chloro-*N*-(4-(5-nitrobenzo[d]oxazol-2-yl)phenyl)thiophene-2-sulfonamide.

Compound **40** (2.96 g, 9.31 mmol) was stirred in SOCl₂ (10 mL) for 6 h at 60°C, then was concentrated to a solid. This was refluxed with 2-amino-4-nitrophenol (2.96 g, 19.2 mmol) and pyridine (1.50 mL, 18.4 mmol) in anhydrous CH₂Cl₂ (50 mL) for 6 h, then stirred at R.T. for 3 days. The reaction was then extracted into EtOAc and rinsed with 1 M HCl and brine, dried over Na₂SO₄, filtered, and concentrated. This amide intermediate was then refluxed with TsOH•H₂O (3.61 g, 19.0 mmol) in xylenes (50 mL) using a Dean-Stark apparatus to remove the residual H₂O. After 18 h, the reaction was cooled, the xylenes decanted off, the sludge extracted with EtOAc, and the combined organics dried over Na₂SO₄, filtered, and concentrated. Flash chromatographic purification over silica (hexanes:EtOAc gradient) afforded **41** as a peach solid (1.81 g, 45% yield). ¹H-NMR (300

MHz, d_6 -DMSO) δ 11.25 (br s, 1H), 8.64 (br s, 1H), 8.32 (d, J = 8.8 Hz, 1H), 8.18 (d, J = 8.1 Hz, 2H), 8.01 (d, J = 8.7 Hz, 1H), 7.60 (br s, 1H), 7.41 (d, J = 8.2 Hz, 2H), 7.24 (br s, 1H); MS (ESI) $C_{17}H_{11}ClN_3O_5S_2$ $[M+H]^+$ m/z expected = 436.0, observed = 436.1; HPLC-1 = 88% (RT = 5.5 min).

42: *N*-(4-(5-Aminobenzo[d]oxazol-2-yl)phenyl)-5-chlorothiophene-2-sulfonamide.

Tin powder (1.53 g, 12.9 mmol) was added slowly to a stirring mixture of **41** (1.78 g, 4.08 mmol) in a 1:7 mixture of HCl:AcOH (8 mL). The reaction was stirred and heated at 60°C for 3 h, then cooled to R.T., diluted with EtOAc and H₂O, neutralized with NaHCO₃, and filtered. The filtrate was extracted with EtOAc and the organics dried over Na₂SO₄, filtered, and concentrated. The residue was diluted in a 10% mixture of DCM in hexanes and the precipitate was filtered, rinsed with hexanes, collected, and dried to afford **42** as a pale-yellow powder (1.59 g, 96% yield). ¹H-NMR (300 MHz, d_6 -DMSO) δ 8.01–8.09 (m, 2H), 7.55 (d, J = 4.1 Hz, 1H), 7.30–7.41 (m, 3H), 7.22 (d, J = 4.1 Hz, 1H), 6.83 (d, J = 2.0 Hz, 1H), 6.64 (dd, J = 8.7, 2.2 Hz, 1H); MS (ESI) $C_{17}H_{13}ClN_3O_3S_2$ $[M+H]^+$ m/z expected = 406.0, observed = 406.1; HPLC-1 = 86% (RT = 6.8 min).

General sulfonamidation procedure for the synthesis of 16L-34L.

To a stirring mixture of compound **42** (1 eq.) in anhydrous CH₂Cl₂ (5 mL) was added the respective **R**² sulfonyl chloride (1.2 eq.) followed by anhydrous pyridine (1.2 eq.). The reaction was allowed to stir at room temperature for 18 h and was then chromatographed over silica (hexanes:EtOAc gradient) and concentrated. If necessary, the product was further purified by preparatory RP-HPLC (H₂O:CH₃CN gradient), concentrated, and lyophilized. Refer to the Supporting Information for individual compound synthesis and characterization data.

Cell information for compound evaluation.

The *ESKAPE* bacteria were purchased from the American Type Culture Collection (ATCC): *E. faecium* (Orla-Jensen) Schleifer and Kilpper-Balz strain NCTC 7171 (ATCC 19434); *S. aureus* subsp. *aureus* Rosenbach strain Seattle 1945 (ATCC 25923); Multi-drug resistant *S. aureus* (MRSA) subsp. *aureus* Rosenbach strain HPV107 (ATCC BAA-44); *K. pneumonia*, subsp. *pneumoniae* (Schroeter) Trevisan strain NCTC 9633 (ATCC 13883); *A. baumannii* Bouvet and Grimont strain 2208 (ATCC 19606); *P. aeruginosa* (Schroeter) Migula strain NCTC 10332 (ATCC 10145); *E. cloacae*, subsp. *cloacae* (Jordan) Hormaeche and Edwards strain CDC 442–68 (ATCC 13047). HEK 293 kidney cells (ATCC CRL-1573) and THLE-3 liver cells (ATCC CRL-11233) were used for compound toxicity assays.

Evaluation of compounds for inhibition of bacterial cell proliferation.

All compounds were evaluated for inhibiting the proliferation of each of the *ESKAPE* bacteria as per previously reported procedures with one minor modification: Liquid growth media was cation-adjusted by addition of 25 mg/L Ca²⁺ and 12.5 mg/L Mg²⁺ to reflect the free concentrations of these divalent cations *in vivo*.²⁷ Detailed protocols for bacterial growth assays are presented in the Supporting Information.

Protein Expression and purification.

E. coli GroEL and GroES, and human HSP60 and HSP10, were expressed and purified as previously reported.²⁵ Detailed protocols for these protein purifications are presented in the Supporting Information.

Evaluation of compounds in GroEL/ES and HSP60/10-mediated dMDH and dRho refolding assays.

All compounds were evaluated for inhibiting *E. coli* GroEL/ES and human HSP60/10-mediated refolding of the denatured MDH and denatured Rho reporter enzymes as per previously reported procedures.²⁶ Detailed protocols for these assays are presented in the Supporting Information.

Counter-screening compounds for inhibition of native MDH and Rho enzymatic activity.

All compounds were counter-screened for inhibiting the enzymatic activity of the native MDH and native Rho reporter enzymes as per previously reported procedures.^{25, 26} Detailed protocols for the assays are presented in the Supporting Information.

Evaluation of inhibitors 20R and 20L binding to GroEL using Isothermal Titration Calorimetry (ITC).

All ITC experiments were performed on a MicroCal VP-ITC system. To minimize background heats of dilution, matched solutions of GroEL and compounds **20R** or **20L** were prepared in buffer containing 20 mM HEPES, pH 7.4, 50 mM KCl, 10 mM MgCl₂, and 1% DMSO. All buffers were freshly prepared from stock solutions on the day of use and degassed under vacuum. For each analytical run, a solution of 400 μ M GroEL (monomer concentration) in the syringe was titrated into 50 μ M solutions of either compound **20R** or **20L** in the sample cell (equilibrated at 20°C for 300 seconds): the first injection was 0.4 μ L (discarded during analysis) and subsequent injections (2–20) were each 2.0 μ L. Each injection was made over 4 s durations with 3-min intervals between subsequent injections. The reference power of the experiment was set to 6 μ cal/s and a 5 s filter period. Sample stirring was set at 600 rpm for all measurements. Six independent replicates were conducted to analyze compound **20R** binding, and five replicates for **20L**. Raw thermograms from each replicate were analyzed independently using the ORIGIN instrument software (MicroCal Inc., version 7.0). In each experiment, the upper baseline was collected after the binding reaction was saturated, and the terminal 7–8 points in the linear region were fit to a straight line and subtracted from the entire data set to remove contributions from background heats of mixing and dilution. The ΔH was obtained by non-linear least-squares fitting of the plot of $-\Delta H$ (mol of injectant)⁻¹ versus molar ratio using a single site binding model.³⁴ The ΔH , S , K_d ($K_A = 1/K_d$), and binding stoichiometries (n) in each titration were obtained, and ΔG was calculated using the Gibbs equation at a temperature of 20°C (293.15 K). Standard deviations were determined based on results from each of the replicate analyses.

Evaluation of compound effects on HEK 293 and THLE-3 cell viability.

Evaluation of compound cytotoxicities to HEK 293 kidney and THLE-3 liver cells were performed using Alamar Blue-based viability assays. HEK 293 cells were maintained in

MEM medium (Corning Cellgro, 10-009 CV) supplemented with 10% FBS (Sigma, F2242). THLE-3 cells were maintained in Clonetics BEBM medium (Lonza, CC-3171) supplemented with the BEGM bullet kit (Lonza, CC-3170) and 10% FBS. All assays were carried out in 384-well plates (BRAND cell culture grade plates, 781980). Cells at 80% confluence were harvested and diluted in growth medium, then 45 μ L of the HEK 293 cells (1,500 cells/well) or THLE-3 cells (1,500 cells/well) were dispensed per well, and plates were sealed with “Breathe Easy” oxygen permeable membranes (Diversified Biotech) and incubated at 37°C, 5% CO₂, for 24 h. The following day, 1 μ L of the compound stocks (10 mM to 4.6 μ M, 3-fold dilutions in DMSO) were pre-diluted by pin-transfer into 25 μ L of the relevant growth mediums. Then, 15 μ L aliquots of the diluted compounds were added to the cell assay plates to give inhibitor concentration ranges of 100 μ M to 46 nM during the assay (final DMSO concentration of 0.1% was maintained during the assay). Plates were sealed with “Breathe Easy” oxygen permeable membranes and incubated for an additional 48 h at 37°C and 5% CO₂. The Alamar Blue reporter reagents were then added to a final concentration of 10%, the plates incubated at 37°C and 5% CO₂, and sample fluorescence (535 nm excitation, 590 nm emission) was read using a Molecular Devices FlexStation II 384-well plate reader (readings taken between 4–24 h of incubation so as to achieve signals in the 30–60% range for conversion of resazurin to resorufin). Cell viability was calculated as per vendor instructions (Thermo Fisher - Alamar Blue cell viability assay manual). Cytotoxicity CC₅₀ values for the test compounds were obtained by plotting the % resazurin reduction results in GraphPad Prism 6 and analyzing by non-linear regression using the log(inhibitor) vs. response (variable slope) equation. Results presented represent the averages of CC₅₀ values obtained from at least triplicate experiments.

Evaluation of MRSA resistance generation against lead inhibitors.

To identify potential resistance toward inhibitors, a liquid culture, 12-day serial passage assay was employed as per procedures reported by Kim. S *et al.*, using the MRSA strain (ATCC BAA-44).^{35, 36} Briefly, MRSA bacteria were streaked onto a Tryptic Soy agar plate and grown overnight at 37°C. A fresh aliquot of Tryptic Soy Broth (TSB) was inoculated with a single bacterial colony and the cultures were grown overnight at 37°C with shaking (250 rpm). The overnight culture was then sub-cultured (1:5 dilution) into a fresh aliquot of media and grown at 37°C for 1 h with shaking, then diluted into fresh media to achieve a final OD₆₀₀ reading of 0.01. Aliquots of the diluted culture (200 μ L) were dispensed to 96 well plates along with addition of 2 μ L of test compounds in DMSO (**20R**, **28R**, and vancomycin as a control). The inhibitor concentration range during the resistance assay was 100 μ M to 48.8 nM (2-fold dilution series). Plates were sealed with “Breathe Easy” oxygen permeable membranes (Diversified Biotech) and left to incubate at 37°C without shaking (stagnant assay). OD₆₀₀ readings were taken at the 24 h time point to monitor for bacterial growth. A second set of baseline control plates were prepared analogously, without any bacteria added, to correct for possible compound absorbance and/or precipitation, as well as plate and media baseline effects. For inoculations on subsequent days, bacteria from the wells with the highest drug concentration where the OD₆₀₀ was >0.2 were diluted with fresh media to OD₆₀₀ of 0.01 and dispensed into a new 96-well plate. Test compounds were added, and the bacteria propagated again as described above. This procedure was repeated each day for a total of 12 days to observe changes in EC₅₀ values over each passage. EC₅₀

values for the test compounds were obtained by plotting the OD₆₀₀ results from each passage in GraphPad Prism 6 and analyzing by non-linear regression using the log(inhibitor) vs. response (variable slope) equation. Results presented represent the averages of EC₅₀ values obtained from at triplicate experiments.

Calculation of IC₅₀ / EC₅₀ / CC₅₀ values and statistical considerations.

All IC₅₀ / EC₅₀ / CC₅₀ results reported are averages of values determined from individual dose-response curves in replicate assays as follows: 1) Individual I/E/CC₅₀ values from replicate assays were first log-transformed and the average log(I/E/CC₅₀) values and standard deviations (SD) calculated; 2) Replicate log(I/E/CC₅₀) values were evaluated for outliers using the ROUT method in GraphPad Prism 6 (Q of 10%); and 3) Average I/E/CC₅₀ values were then backcalculated from the average log(IC₅₀) values. To compare statistical differences between log(I/E/CC₅₀) values between the matched **R**- and **L**-series of analogs, two-tailed, paired t-tests were performed using GraphPad Prism 6 (95% confidence level) and looking at differences between paired values (results are plotted in Figures S1-S5 in the Supporting Information). For compounds where log(I/E/CC₅₀) values were greater than the maximum compound concentrations tested (i.e. >1.8 and >2.0, or >63 and >100 μ M, respectively), results were represented as 0.1 log units higher than the maximum concentrations tested (i.e. 1.9 and 2.1, or 79 and 126 μ M, respectively), so as not to overly bias comparisons because of the unavailability of definitive values for these inactive compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS.

MDH	malate dehydrogenase
Rho	rhodanese
HPLC	high-performance liquid chromatography
¹H-NMR	Proton nuclear magnetic resonance
MS	Mass spectrometry
EtOH	ethanol

AcOH	acetic acid
EtOAc	ethyl acetate
MeOH	methanol
DMSO	dimethyl sulfoxide
IC₅₀	Inhibitory concentration for half-maximal signal in biochemical assay
EC₅₀	effective concentration for half-maximal signal in bacterial proliferation assays
CC₅₀	cytotoxicity concentration for half-maximal signal in human cell viability assays
ITC	isothermal titration calorimetry

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Figure 1.

Progression of hit-to-lead compound **1** analogs. **A.** Compound **1** was an initial hit that emerged from our high-throughput screening for GroEL/ES inhibitors.²⁴ We recently reported on a series of pseudo-symmetrical compound **1** analogs (**2–14** – R^1 represents a variety of alkyl, aryl, and substituted phenyl substructures) that have antibiotic properties against *Trypanosoma brucei* parasites.²⁶ **B.** In the present study, we initially screened the **1–14** analogs for their ability to inhibit a panel of Gram-positive and Gram-negative bacteria, and found that the thiophene (**6**) and *para*-chloro-substituted (**2c-p**) analogs generated the

most potent and selective inhibitors of MRSA proliferation. Data plotted in the gray zones represent EC_{50} and CC_{50} results beyond the assay detection limits (i.e., $>100\ \mu\text{M}$). **C.** Based on initial SAR, compound **15** was developed (bearing the 2-chlorothiophene group on both sides) along with two parallel series of asymmetrical 2-chlorothiophene-based analogs (**16–34**, **Right** and **Left**) containing variable alkyl and aryl substructures at the R^2 positions were developed in an attempt to improve their selectivity indices between efficacy against bacterial proliferation and cytotoxicity in human cell viability assays (structures of the different R^2 groups are presented in Tables S3A and S4A in the Supporting Information).

Figure 2.

Correlation plots of IC_{50} and EC_{50} values for compounds tested in the respective GroEL/ES-dMDH or dRho refolding assays and bacterial proliferation assays. Each data point represents results for individual compounds tested in the respective assays (plotted from results presented in Tables S1-S4 in the Supporting Information). Data plotted in the gray zones represent results beyond the assay detection limits (i.e., $>100 \mu\text{M}$). **A.** Compounds inhibit the proliferation of *S. aureus* (drug susceptible) and MRSA (drug resistant) bacteria nearly equipotently. The **L**- and **R**-series asymmetric inhibitors are generally more potent than the previously developed pseudo-symmetrical inhibitors (**1-14**). **B.** Compounds are on-target for inhibiting chaperonin-mediated substrate refolding as a correlation is evident between IC_{50} values obtained from the GroEL/ES-dMDH and -dRho refolding assays, and compounds do not inhibit in both the native MDH and Rho enzymatic activity counter-screens. **C.** A general trend is observed between inhibiting the GroEL/ES chaperonin system and MRSA proliferation, supporting on-target effects in bacteria.

Figure 3.

Representative analyses of the binding of **20R** (Panel **A**) and **20L** (Panel **B**) to GroEL measured by Isothermal Titration Calorimetry (ITC). Top panels show binding isotherms obtained by titrating GroEL (400 μM monomer concentration) into solutions of either **20R** or **20L** (50 μM) in the ITC cell. Lower panels show the integrated data (solid squares) fit to a single-site binding model (solid lines). The molar ratio refers to the binding stoichiometry of monomeric GroEL to molecules **20R** or **20L**. Average results for the various binding parameters (K_d , n , ΔH , ΔS , and ΔG) obtained from replicate analyses are presented in Table 1.

Figure 4.

Correlation plots of IC_{50} , EC_{50} , and CC_{50} values for compounds tested in the respective GroEL/ES-dMDH and HSP60/10-dMDH refolding assays, liver cell cytotoxicity assay, and MRSA proliferation assay. Each data point represents results for individual compounds tested in the respective assays (plotted from results presented in Tables S1-S4 in the Supporting Information). Data plotted in the gray zones represent results beyond the assay detection limits (i.e., $>100 \mu M$). **A.** The R-series and previously developed analogs selectively inhibit the *E. coli* GroEL/ES over the human HSP60/10 chaperonin system, but the L-series analogs are not selective. **B.** No correlation is evident between cytotoxicity of compounds in the liver cell viability assay and the HSP60/10-dMDH refolding assay, suggesting cytotoxicity may be from off-target effects in cells. **C.** Many compounds are able to selectively inhibit MRSA proliferation with moderate to low cytotoxicity to human liver cells. Results are similar for compounds tested in the kidney cell viability assay.

Figure 5.

Exploring adaptive tolerance by MRSA bacteria to analogs **20R**, **28R**, and vancomycin. **A.** MRSA was serially passaged for 12 days in fresh liquid media supplemented with the selected compounds. Average EC_{50} values of compounds tested after each 24 h passage are plotted from triplicate analyses. MRSA rapidly evolved resistance to **28R**, but retained sensitivity to **20R** and vancomycin throughout 12 day experiment. Data plotted in the gray zones represent EC_{50} results beyond the assay detection limits (i.e., >100 μM). **B.** After re-culturing the **28R**-resistant MRSA strain for two days in the absence of inhibitors (24 h on agar, then another 24 h in liquid medium), the “resistant” MRSA strain regained sensitivity to **28R** (average % growth inhibition results ($\pm$ SD) are plotted from 4 replicate analyses).

^a Reagents and conditions: **A.** (a) CH_2Cl_2 , rt (94%); (b) tin powder, 10% HCl in AcOH, rt (82%); (c) 4-nitrobenzaldehyde, NaHCO_3 , Na_2SO_4 , THF, reflux to rt, then DDQ added (95%); (d) tin powder, 10% HCl in AcOH, rt (41%); (e) $\text{R}^2\text{-SO}_2\text{Cl}$, pyridine, CH_2Cl_2 , rt. **B.** (f) CH_2Cl_2 , rt (98%); (g) $\text{LiOH}\cdot\text{H}_2\text{O}$, 1:1:1 THF:MeOH:H₂O, rt (96%); (h) SOCl_2 , 60°C for 1 h, concentrated, then 2-amino-4-nitrophenol and pyridine in CH_2Cl_2 , reflux to rt, then $\text{TsOH}\cdot\text{H}_2\text{O}$, xylenes, reflux (45%); (i) tin powder, 10% HCl in AcOH, rt (96%); (j) $\text{R}^2\text{-SO}_2\text{Cl}$, pyridine, CH_2Cl_2 , rt.

Scheme 1^a

Table 1.

Isothermal Titration Calorimetry (ITC) results for compounds **20R** and **20L**. Results are averaged from six replicate analyses for **20R**, and five replicate analyses for **20L**.

		20R	20L
Assay IC ₅₀ (μ M)	GroEL/ES-dMDH Refolding	1.3	3.1
	GroEL/ES-dRho Refolding	3.2	11
Stoichiometry n	GroEL _{monomer} : Molecule	0.77 ± 0.16	0.6 ± 0.14
	Molecule : GroEL _{monomer}	1.30 ± 0.27	1.67 ± 0.39
	Molecule : GroEL _{oligomer}	18.2 ± 3.8	23.3 ± 5.4
Log(K _d /nM)		1.56 ± 0.20	2.18 ± 0.03
K _d (nM)		36	150
H (kcal/mol)		-9.78 ± 0.80	-8.30 ± 0.52
S (kcal/molK)		0.001 ± 0.003	0.003 ± 0.002
T S (kcal/mol)		0.20 ± 0.82	0.84 ± 0.55
G (kcal/mol)		-9.98 ± 0.26	-9.14 ± 0.06

Table 2.

Biochemical and cell-based IC_{50} , EC_{50} , and CC_{50} results for the top four lead inhibitors based on Selectivity Index (SI) for inhibiting MRSA proliferation over cytotoxicity in the liver cell viability assay.

Compound Structure & Number	Cell-Based Assays			Biochemical Assay IC_{50} (μM)			
	MRSA Proliferation EC_{50} (μM)	Liver Cell Viability Index (SI) CC_{50} (μM)	Selectivity Index (SI) (CC_{50} / EC_{50})	GroEL/ES refolding of:			
				Native Rho Reporter	Native MDH Reporter	dRho	dMDH
L-Series Analogs 26L 26L 27L 28L	0.53	24	46	>100	>63	6.4	5.1
	1.1	36	31	>100	43	11	6.7
	2.2	27	12	>100	40	11	5.2
	0.97	11	12	>100	37	1.0	1.1
R-Series Analogs 26R 26R 27R 28R	4.5	69	15	72	>63	19	10.0
	2.3	27	12	>100	>63	4.5	2.2
	2.0	22	11	>100	>63	7.3	2.6
	1.7	17	10	53	34	2.0	1.5
Pseudo-symmetrical Analogs 29a 30 29b	2.0	24	12	>100	>63	33	11
	4.6	49	11	>100	52	5.6	2.4
	7.3	47	6.4	>100	>63	22	24

Compound Structure & Number	Cell-Based Assays			Biochemical Assay IC ₅₀ (μM)				
	MRSA Proliferation EC ₅₀ (μM)	Liver Cell Viability Index (SI) CC ₅₀ (μM)	Selectivity Index (SI) (CC ₅₀ / EC ₅₀)	GroEL/ES refolding of:				
				Native Rho Reporter	Native MDH Reporter	dRho	dMDH	HSP60/10-dMDH Refolding
2b-9	3.5	18	5.2	>100	>63	29	15	96

Table 3.

Comparison of the **L**- and **R**-series analogs for which overall scaffolds exhibited preferred inhibition profiles in the various biochemical and cell-based assays. Lead antibacterial candidates are preferred to potently inhibit GroEL/ES and bacteria, and not inhibit human HSP60/10 and liver/kidney cell viability. The log(IC₅₀), log(EC₅₀), or log(CC₅₀) values for the 20 matched **L**- and **R**-series analogs were analyzed by two-tailed, paired t-tests with 95% confidence intervals. Comparative plots are presented in Figures S1-S5 in the Supporting Information.

Assay Inhibition	L-Series	R-Series
<i>S. aureus</i> growth	Preferred	
MRSA growth	Preferred	
GroEL/ES-dMDH refolding	No statistical difference	
GroEL/ES-dRho refolding	No statistical difference	
HSP60/10-dMDH refolding		Preferred
Liver cell viability	Preferred	
Kidney cell viability	No statistical difference	