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GroEL/ES inhibitors as potential antibiotics

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ABSTRACT

We recently reported results from a high-throughput screening effort that identified 235 inhibitors of the *Escherichia coli* GroEL/ES chaperonin system [*Bioorg. Med. Chem. Lett.* **2014**, *24*, 786]. As the GroEL/ES chaperonin system is essential for growth under all conditions, we reasoned that targeting GroEL/ES with small molecule inhibitors could be a viable antibacterial strategy. Extending from our initial screen, we report here the antibacterial activities of 22 GroEL/ES inhibitors against a panel of Gram-positive and Gram-negative bacteria, including *E. coli*, *Bacillus subtilis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*. GroEL/ES inhibitors were more effective at blocking the proliferation of Gram-positive bacteria, in particular *S. aureus*, where lead compounds exhibited antibiotic effects from the low-μM to mid-nM range. While several compounds inhibited the human HSP60/10 refolding cycle, some were able to selectively target the bacterial GroEL/ES system. Despite inhibiting HSP60/10, many compounds exhibited low to no cytotoxicity against human liver and kidney cell lines. Two lead candidates emerged from the panel, compounds **8** and **18**, that exhibit >50-fold selectivity for inhibiting *S. aureus* growth compared to liver or kidney cell cytotoxicity. Compounds **8** and **18** inhibited drug-sensitive and methicillin-resistant *S. aureus* strains with potencies comparable to vancomycin, daptomycin, and streptomycin, and are promising candidates to explore for validating the GroEL/ES chaperonin system as a viable antibiotic target.

The number of lives saved by antibiotics is a hallmark of the success of this class of drugs. However, resistant bacterial strains have been identified for every class of antibiotic, usually within a few years of general therapeutic use.[1–3] The threat of antibiotic resistance is epitomized by the emergence of six multi-drug resistant bacteria referred to as the *ESKAPE* pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species.[4–8] The Centers for Disease Control (CDC) and Prevention Antibiotic Resistance Threat Report lists these bacteria as serious threats (level 4 out of 5) requiring prompt and sustained action.[9] Most alarming is that antibiotic resistance has mounted to the point where therapeutics are severely limited or ineffective for once easily treated infections. For example, ~10,000 people per year are estimated to die from methicillin-resistant *S. aureus* (MRSA) infections in the United States.[10] Moreover, the CDC estimates the direct medical cost of treating antibiotic resistant bacterial infections in the US is more than $20 billion per year.[9] Clearly, the rise of resistant bacterial strains requires enhanced research efforts to ensure an ongoing antibiotic pipeline.

Current antibiotics primarily function by blocking cell wall construction, structure and function of the cell membrane, protein synthesis, DNA structure and function, or folic acid synthesis.[11] Recently developed therapeutics for infections caused by drug-resistant bacteria include the injectable carbapenem beta-lactam, doripenem, which targets penicillin-binding proteins and inhibits

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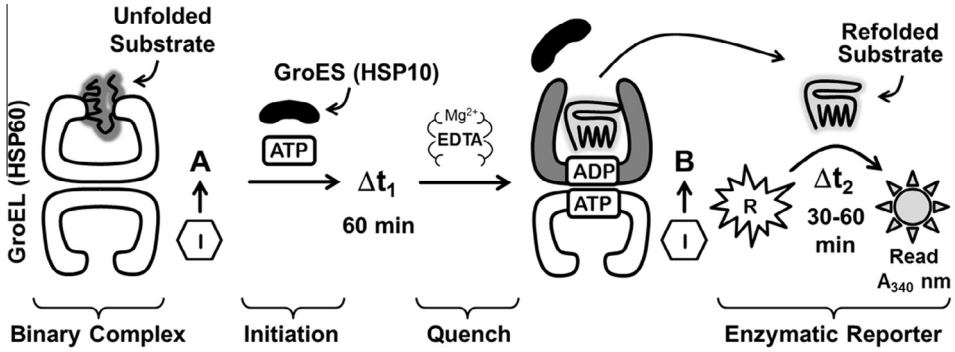

Figure 1. General protocol for chaperonin-mediated biochemical assays. Compounds (**I**) are added at point A to a solution containing GroEL (or HSP60) with bound substrate protein (e.g., malate dehydrogenase, MDH). Addition of GroES (or HSP10) and ATP initiates the refolding cycle, which is quenched with EDTA after a 60 min incubation. Substrates (R) for the refolded reporter enzyme are added and after another 30–60 min incubation (until the DMSO control wells have reached ~90% consumption of NADH), absorbance is measured to evaluate the amount of refolded enzyme present, and by association the extent of chaperonin inhibition. Alternatively, addition of compounds at point B enables determination of off-target inhibition of the reporter enzyme (i.e., native MDH enzyme activity). Chaperonin-mediated ATP hydrolysis is also evaluated using a malachite green assay. Biochemical assays employing Rhodanese (Rho) are performed similarly (refer to Supporting information for detailed protocols).

cell wall synthesis;[12] the cyclic lipopeptide, daptomycin, which inserts into the bacterial membrane and leads to pore formation;[13] quinupristin/dalfopristin, which bind to two different sites on the 50S ribosomal subunit and interfere with protein synthesis;[14] the oxazolidinone, linezolid, which also binds the 50S ribosomal subunit;[15] the tetracycline derivative, tigecycline, which targets protein synthesis via the 30S ribosomal subunit;[16] and the lipoglycopeptide, dalbavancin, which has the same mode of action as vancomycin, binding to the D-Ala-D-Ala motif in the cell wall.[17]

As these examples illustrate, most new antibiotics are derivatives of existing drugs that also target the aforementioned pathways. Unfortunately, bacterial resistance to these drugs is quick to develop. These data argue for the continued pursuit of antibiotics with entirely new modes of action, which may better avoid mechanisms of resistance and have longer effective life times.

An attractive strategy for the development of novel antibiotics is to target bacterial protein homeostasis (proteostasis) mechanisms, in particular molecular chaperones. Molecular chaperones

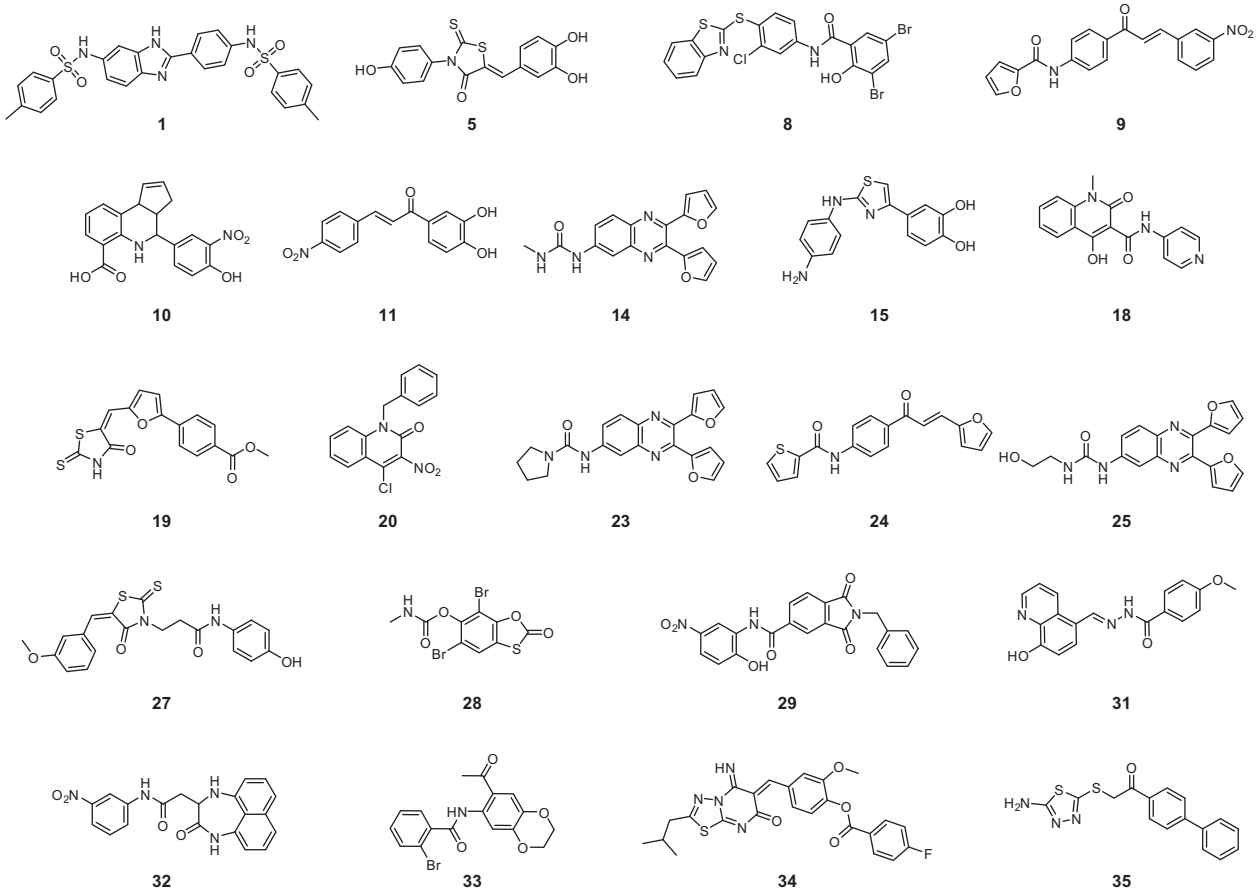

Figure 2. Structures of the 22 compounds under evaluation. For ease of comparison, compound numbering from **1** to **36** was maintained as presented in our previous high-throughput screening study.[37] Compounds **2**–**4**, **6**, **7**, **12**, **13**, **16**, **17**, **21**, **22**, **26**, **30**, and **36** were omitted from evaluation as they were either not commercially available, or purchased compounds were not readily identified by LC–MS and/or did not have acceptable purities confirmed by HPLC.

Table 1
Biochemical IC_{50} results for *E. coli* GroEL/ES inhibitors

#	Biochemical assay IC_{50} values (μM)					
	Native Rho reporter activity	Native MDH reporter activity	GroEL/ES-dRho refolding		GroEL/ES-dMDH refolding	GroEL/ES-dMDH ATPase
1	>100	>62.5	30	*	7.5	119
5	14	>62.5	0.58		0.69	>250
8	>100	7.1	1.4		1.4	>250
9	>100	>62.5	1.4		0.93	80
10	0.25	54	0.47		0.80	174
11	12	>62.5	0.83		1.2	216
14	2.5	>62.5	2.5		3.0	>250
15	23	>62.5	1.7		2.7	>250
18	>100	>62.5	6.8		5.7	>250
19	8.1	>62.5	3.0		4.8	>250
20	>100	>62.5	22	*	5.4	>250
23	2.1	>62.5	2.4		4.7	>250
24	>100	>62.5	2.3		3.6	>250
25	1.4	>62.5	2.6		6.5	>250
27	12	>62.5	9.6	*	4.7	>250
28	5.3	>62.5	0.89		2.6	>250
29	>100	>62.5	28		24	187
31	52	>62.5	18		31	>250
32	10	>62.5	11	*	42	217
33	>100	>62.5	>250		>100	>250
34	>100	>62.5	25		24	>250
35	>100	>62.5	79	#	>100	107

Statistical analyses (two-tailed *t*-tests) were performed for compound log(IC_{50}) values determined from the GroEL/ES-dRho and GroEL/ES-dMDH refolding assays. Compounds for which there is a statistically significant difference between inhibition results have been marked with a '*' between the two assay results being compared (p <0.05). *P*-Values could not be calculated for compounds marked with a '#' as one IC_{50} is greater than the maximum compound concentration tested. For most compounds, IC_{50} values are not statistically different (17/22 compounds), suggesting they are 'on-target' for inhibiting the refolding of the dRho and dMDH substrates. IC_{50} correlations are represented graphically in Figure 3.
IC_{50} = inhibitor concentration resulting in 50% reduction of biochemical activity.

are a specialized class of proteins that help other proteins to properly fold to their native states. Among the molecular chaperones in *E. coli*, the GroEL/ES chaperonin system is the only one essential for growth under all conditions.[18,19] GroEL is a central processing machine that maintains the structural and functional integrity of many other proteins (Fig. 1); for a review, see Refs. 19,20.[19–22] Thus, targeting this one functional node results in a cascading effect that leads to the dysfunction of numerous key cellular pathways, which is lethal to bacteria.[18] Because no other drugs function by targeting chaperonin systems, this strategy should be effective against bacteria that are resistant to current antibiotics.

The central tenet of this antibiotic strategy raises the question of whether bacterial GroEL/ES can be targeted specifically without interfering with the metazoan counterpart in the mitochondria, HSP60/10, whose partial deficiency in humans leads to disease.[23] Human HSP60 shares 48% sequence identity with *E. coli* GroEL, and thus there is the possibility of inhibitor cross-talk between the two chaperonins. However, structural and functional differences between the two systems suggest that it should be possible to develop inhibitors that selectively target bacterial GroEL/ES over human HSP60/10. GroEL is a homo-tetradecameric protein consisting of two, seven-membered rings that stack back-to-back.[24–26] Through a series of events driven by ATP binding and hydrolysis, unfolded substrate proteins are bound within the central cavity of one GroEL ring and encapsulated by the heptameric GroES co-chaperonin lid structure, triggering protein folding in a sequestered chamber.[25–31] GroEL/ES works as an allosterically-controlled, double-ring system, which is regulated through positive intra-ring ATP-binding and negative inter-ring binding. In contrast, studies have indicated that human HSP60/10 operates as a single-ring species, through at least part of the cycle, removing many of the intermediate states associated with the GroEL/ES refolding cycle.[32–34] Thus, it should be possible to develop inhibitors that selectively target the double-ring GroEL/ES cycle with its additional allosteric signals and conformational intermediates. Furthermore, HSP60/10 is in the mitochondrial matrix, which is often protected from small molecule penetration; thus, even if compounds are found that inhibit HSP60/10 in biochemical assays,

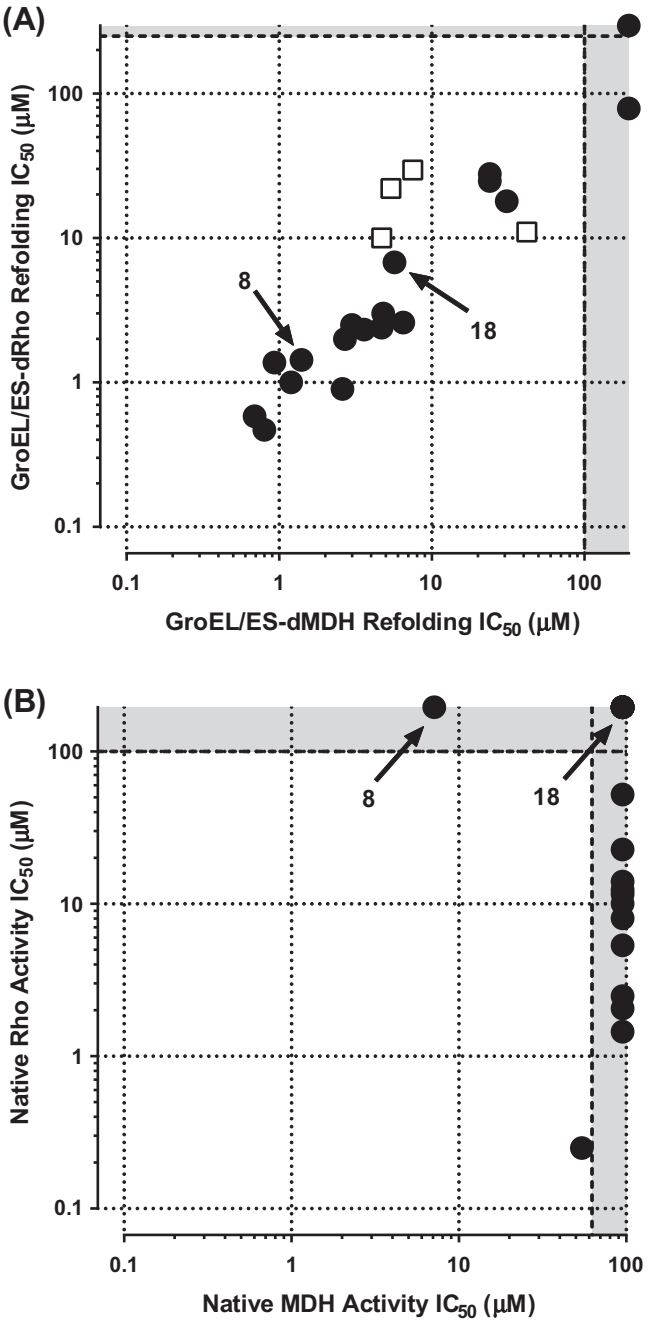

Figure 3. (A) Compounds inhibit nearly equipotently in the *E. coli* GroEL/ES-dMDH and the GroEL/ES-dRho refolding assays. Data plotted in the grey zones represent results beyond the assay detection limits (i.e., >100 μM for the dMDH refolding assay, and >250 μM for the dRho refolding assay). Compounds indicated by the white squares are those with statistically significant differences between their IC_{50} values (p <0.05). (B) While some compounds inhibit either native MDH or Rho individually, only compound **10** inhibits in both counter-screens, and only to a minor extent against native MDH. Data plotted in the grey zones represent results beyond the assay detection limits (i.e., >62.5 μM for the native MDH enzymatic reporter assay, and >100 μM for the native Rho enzymatic reporter assay).

Table 2
E. coli and *B. subtilis* bacterial proliferation EC_{50} results for GroEL/ES inhibitors

#	Bacterial proliferation EC_{50} values (μM)			
	DH5α *E. coli*	MC4100 *ΔacrB E. coli*	SM101 *E. coli*	*B. subtilis*
1	>100	>100	>100	>100
5	>100	92	27	25
8	>100	2.3	0.33	0.10
9	>100	>100	76	>100
10	>100	>100	>100	>100
11	>100	>100	48	>100
14	>100	>100	>100	>100
15	>100	>100	84	>100
18	>100	21	3.3	0.47
19	>100	>100	7.6	16
20	>100	>100	>100	2.8
23	>100	>100	>100	>100
24	>100	>100	>100	>100
25	>100	>100	>100	>100
27	>100	>100	>100	>100
28	>100	>100	>100	43
29	>100	>100	>100	>100
31	>100	>100	19	83
32	>100	>100	68	72
33	>100	>100	>100	>100
34	>100	>100	25	13
35	>100	>100	>100	>100

EC_{50} = effective concentration of compound resulting in 50% reduction of bacterial proliferation.

they may not display cytotoxicity if they failed to reach the mitochondrial matrix.

We previously developed a series of compounds that bind to the ATP sites of *E. coli* GroEL and inhibit the chaperonin refolding cycle.[35,36] We also conducted a high-throughput screen to discover inhibitors of the *E. coli* GroEL/ES chaperonin system that target sites other than the ATP pockets, as we had concerns that ATP-competitive inhibitors may have off-target effects against other ATP-dependent proteins in cells.[37] We chose the *E. coli* GroEL/ES homolog for screening because it is the best characterized chaperonin and has been a model system for studying this class of proteins.[30,31,38–40] It shares high homology with GroEL/ES systems from other bacteria, with 56–97% identical amino acids for GroEL and 44–94% identical amino acids for GroES among the *ESKAPE* pathogens (refer to Table S1 in Supporting information). Thus, *E. coli* GroEL/ES serves as an excellent surrogate to discover chaperonin inhibitors to treat bacterial infections. The assays we developed analyzed the full refolding cycle and used the substrate reporter enzymes β-arylsulfotransferase IV (AST-IV) and malate dehydrogenase (MDH), which require GroEL, GroES, and ATP in order to return to their native, active states (refer to Fig. 1 for a general overview of the assay protocols). From ~700,000 molecules, our high-throughput screening narrowed the number of GroEL/ES inhibitors to 235. We investigated a subset of these hits in greater detail to identify IC_{50} values for inhibiting GroEL/ES-mediated substrate refolding and ATPase activity. While only a few compounds inhibited GroEL/ES-mediated ATPase activity, most were potent inhibitors of the refolding cycle and exhibited minimal to no off-target effects against the reporter enzyme.[37]

Extending from our high-throughput screening studies, we have been investigating the antibiotic potential of 22 of our initial GroEL/ES inhibitor hits (Fig. 2). We first tested the GroEL/ES inhibitors in two additional biochemical counter-screens to further support that they are acting 'on-target' and are not simply artifacts or false-positives. The first counter-screen evaluates for inhibition of GroEL/ES-mediated refolding of a third stringent substrate, Rhodanese (Rho). The second counter-screen evaluates for inhibition of the native Rho enzymatic reporter reaction. Detailed protocols for these two assays are presented in Supporting information).[41–44]

For most of the compounds, we found a direct correlation between inhibition of both the GroEL/ES-mediated dMDH and dRho refolding reactions (Table 1 and Fig. 3A). While some compounds inhibited either the native MDH or Rho reporter reactions, only compound

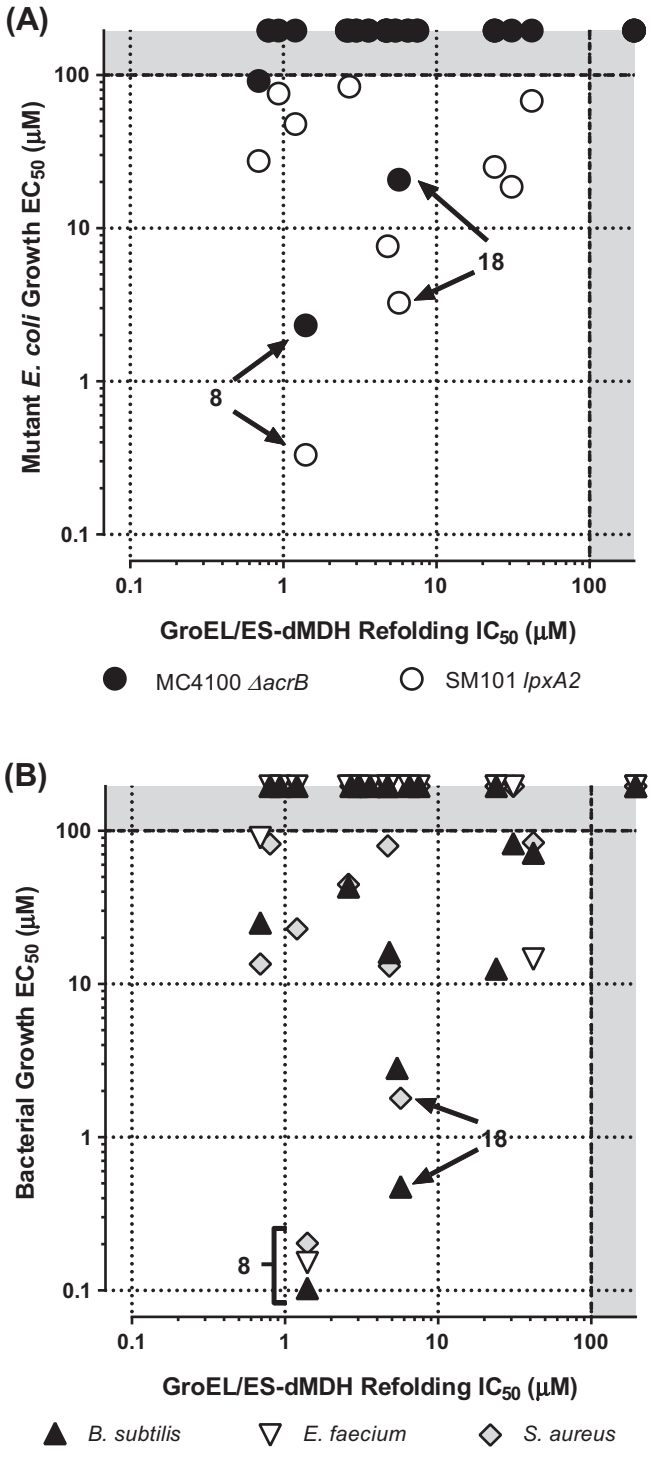

Figure 4. (A) Inhibitors of the *E. coli* GroEL/ES-dMDH refolding cycle are inactive against parent *E. coli* bacteria; however, several exhibit antibacterial effects against mutant *E. coli* strains with compromised efflux pumps (MC4100 Δ*acrB*) and lipopolysaccharide (LPS) outer membranes (SM101 *lpxA2*). (B) Parent *B. subtilis*, *E. faecium*, and *S. aureus* Gram-positive bacteria are more susceptible to the antibiotic effects of GroEL/ES inhibitors. Compound **8** potently inhibits the growth of all three strains, while compound **18** is potent against *B. subtilis* and *S. aureus*, but inactive against *E. faecium*. Data plotted in the grey zones represent results beyond the assay detection limits (i.e., >100 μM).

Table 3
EC_{50} results for GroEL/ES inhibitors against the *ESKAPE* pathogens

	Bacterial proliferation EC_{50} values (μM)						
#	*E. faecium*	*S. aureus*	*MRSA*	*K. pneumoniae*	*A. baumannii*	*P. aeruginosa*	*E. cloacae*
1	>100	>100	>100	>100	>100	>100	>100
5	90	14	9.1	>100	58	>100	>100
8	0.15	0.20	0.13	>100	30	>100	>100
9	>100	>100	>100	>100	>100	>100	>100
10	>100	82	>100	>100	>100	>100	>100
11	>100	23	40	>100	>100	>100	>100
14	>100	>100	94	>100	>100	>100	>100
15	>100	>100	>100	>100	81	>100	>100
18	>100	1.8	1.3	>100	>100	>100	>100
19	>100	13	1.5	>100	>100	>100	>100
20	>100	>100	21	>100	>100	>100	>100
23	>100	>100	>100	>100	>100	>100	>100
24	>100	>100	61	>100	>100	>100	>100
25	>100	>100	>100	>100	>100	>100	>100
27	>100	80	>100	>100	>100	>100	>100
28	>100	45	74	>100	>100	>100	>100
29	>100	>100	15	>100	>100	85	>100
31	>100	>100	56	95	32	>100	>100
32	15	84	54	>100	>100	>100	>100
33	>100	>100	>100	>100	>100	>100	>100
34	>100	>100	>100	>100	>100	>100	>100
35	>100	>100	>100	>100	>100	>100	>100
Ampicillin	0.63	0.059	76	89	2.5	27	>100
Minocycline	<0.05	<0.05	0.35	0.70	<0.05	2.4	1.5
Rifampicin	1.2	<0.05	0.15	5.4	0.47	5.2	6.2
Chloramphenicol	2.1	2.2	2.3	1.4	65	16	2.2
Kanamycin	>100	8.2	>100	45	16	25	37
Streptomycin	50	3.6	>100	3.4	>100	2.9	>100
Vancomycin	0.30	0.20	0.17	>100	17	74	>100
Daptomycin	33	5.7	5.7	>100	>100	>100	>100

EC_{50} = effective concentration of compound resulting in 50% reduction of bacterial proliferation.

10 inhibited in both counter-screens, and only to a minor extent against native MDH (Table 1 and Fig. 3B). Thus, we are confident that compounds are on-target inhibitors of dMDH and dRho refolding. We next evaluated the 22 compounds for their antibiotic effects on *E. coli* cells. A general bacterial proliferation assay (see Supporting information for a detailed protocol) was employed in liquid media using DH5α *E. coli* cells as the initial test strain (EC_{50} values are summarized in Table 2 and graphically in Fig. 4A). Unfortunately, no significant inhibition of bacterial growth was observed for any of the compounds up to 100 μM. Reasoning this

Table 4
Human HSP60/10 biochemical IC_{50} and liver and kidney cytotoxicity EC_{50} results for the GroEL/ES inhibitors

	Biochemical assay IC_{50} values (μM)				Human cell viability EC_{50} values (μM)	
#	GroEL/ES-dMDH refolding		HSP60/10-dMDH refolding	HSP60/10-dMDH ATPase	THLE-3 (liver)	HEK 293 (kidney)
1	7.5	*	89	106	29	34
5	0.69	*	4.9	>250	16	23
8	1.4	*	5.5	3.6	12	78
9	0.93		1.6	>250	>100	>100
10	0.80	*	3.3	140	>100	>100
11	1.2	*	5.2	>250	9.9	12
14	3.0		7.4	>250	>100	>100
15	2.7		10	>250	>100	98
18	5.7	#	>100	>250	>100	>100
19	4.8		16	>250	41	44
20	5.4		2.8	>250	3.6	17
23	4.7		13	>250	>100	>100
24	3.6		8.2	>250	34	55
25	6.5		15	>250	>100	>100
27	4.7	*	13	>250	>100	>100
28	2.6		1.8	>250	>100	>100
29	24	*	38	>250	>100	>100
31	31	#	>100	>250	15	7.7
32	42	#	>100	>250	61	62
33	>100		>100	>250	>100	>100
34	24		26	>250	>100	>100
35	>100		>100	>250	>100	>100

Statistical analyses (two-tailed t-tests) were performed for compound log(IC_{50}) values determined from the GroEL/ES-dMDH and HSP60/10-dMDH refolding assays. Compounds for which there is a statistically significant difference between inhibition results have been marked with a '*' between the two assay results being compared (p <0.05). *P*-Values could not be calculated for compounds marked with a '#' as one IC_{50} is greater than the maximum compound concentration tested. IC_{50} correlations are represented graphically in Figure 5A.
IC_{50} = inhibitor concentration resulting in 50% reduction of biochemical activity.
EC_{50} = effective concentration of compound resulting in 50% reduction in cell viability.

might be due to efflux of the molecules, we tested against an MC4100 Δ*acrB E. coli* strain, which has one of the central components of the AcrA/AcrB/TolC efflux pump removed.[45,46] Compounds **8** and **18** were the most potent inhibitors of this efflux-compromised *E. coli* strain (EC_{50} = 2.3 and 21 μM, respectively), with the remainder of the compounds being inactive.

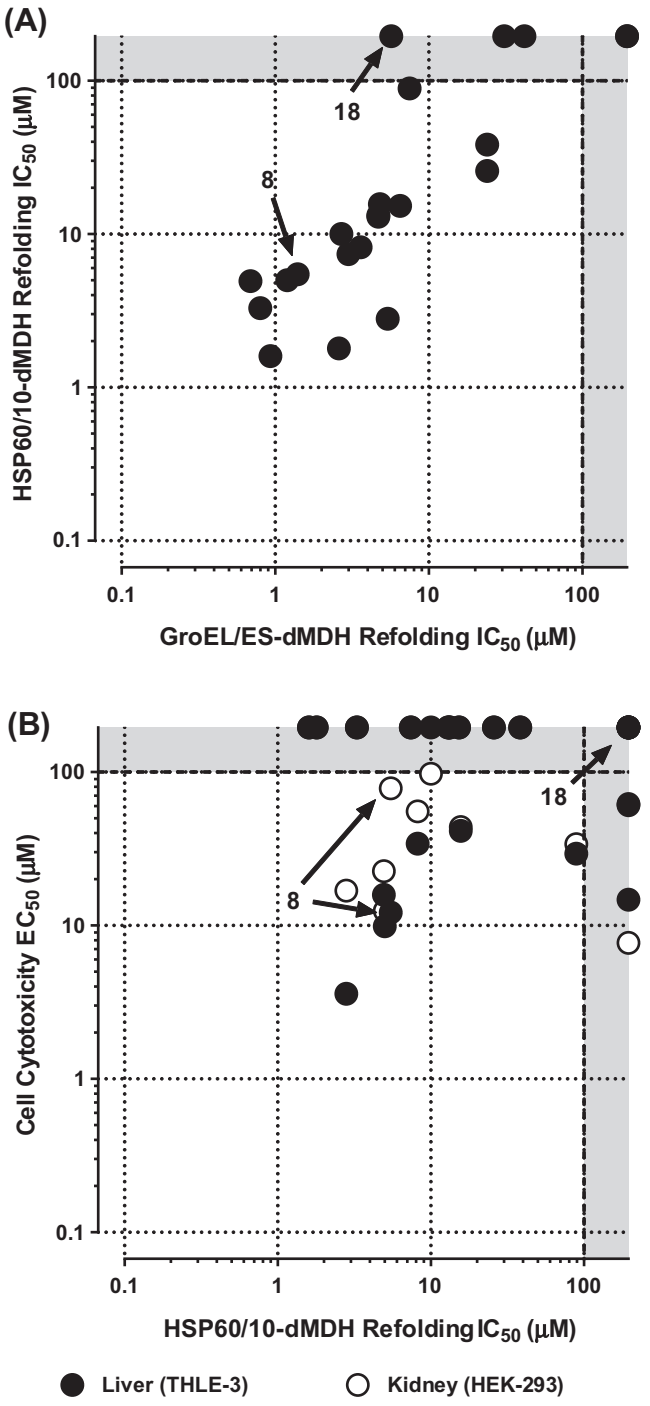

Figure 5. (A) Compounds inhibit both *E. coli* GroEL/ES and human HSP60/10 chaperonin systems, but are generally more selective for the bacterial homolog. Compound **18** is inactive against human HSP60/10, whereas compound **8** exhibits low selectivity for GroEL/ES. (B) Even though compounds can inhibit HSP60/10 biochemical function, many exhibit low or no cytotoxicity to human liver and kidney cells. Compound **18** exhibits no cytotoxicity, whereas compound **8** exhibits moderate or low toxicity. Data plotted in the grey zones represent results beyond the assay detection limits (i.e., >100 μM).

That many of the most potent GroEL/ES biochemical inhibitors were ineffective against the MC4100 Δ*acrB E. coli* cells could be due to the presence of other efflux pumps that were still functional, as it is known that *E. coli* contains several classes of efflux pumps.[47–50] However, another possibility is that the molecules were not able to traverse the highly impermeable lipopolysaccharide (LPS) outer membrane characteristic of Gram-negative bacteria. To probe this, we tested the compounds against a mutant SM101 *lpxA2 E. coli* strain, which has a temperature sensitive *lpxA* allele leading to compromised LPS biosynthesis at non-permissive temperatures, and consequently a greater permeability to molecules.[51,52] We found that 10 compounds inhibited the growth of this *E. coli* strain, with compounds **8** and **18** still proving to be the most effective (EC_{50} values of 0.33 and 3.3 μM, respectively). These results were further supported by the ability of many compounds to inhibit the growth of a Gram-positive bacterium, *Bacillus subtilis* (Table 2 and Fig. 4B), which does not contain an LPS outer membrane. That more compounds failed to inhibit either the SM101 *lpxA2 E. coli* or *B. subtilis* bacteria is putatively because of the presence of efflux pumps that were still intact for these strains, and/or the continued impermeability of compounds across the cell membranes.

While *E. coli* and *B. subtilis* were good model systems for initial proof-of-principle studies, we wanted to elucidate the antibiotic potential of our GroEL/ES inhibitors against a panel of more clinically relevant bacteria, in particular the *ESKAPE* pathogens. We adapted the general bacterial proliferation assay to test molecules against *E. faecium*, *S. aureus* (plus an MRSA strain), *K. pneumoniae*, *A. baumannii*, *P. aeruginosa,* and *E. cloacae* (see Supporting information for detailed protocols). A summary of the EC_{50} values for compounds against these bacteria is presented in Table 3 (and graphically in Fig. 4B), with a comparison against several common antibiotics. As four of the *ESKAPE* pathogens are Gram-negative (*K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. cloacae*), it is not surprising that the GroEL/ES inhibitors were largely ineffective against them. The remaining two *ESKAPE* pathogens, *E. faecium* and *S. aureus*, are Gram-positive bacteria. Compound **8** was very potent at inhibiting *E. faecium* growth (EC_{50} = 0.15 μM), and compound **32** was moderately potent (EC_{50} = 15 μM). Somewhat surprisingly, compound **18**, which emerged as a lead inhibitor against the *E. coli* and *B. subtilis* cells, was inactive against *E. faecium*. The remaining compounds were ineffective against *E. faecium*, which again supports the notion that the presence of an LPS membrane is not the sole determinant leading to compound inactivity. While compounds **8** and **18** emerged as the lead inhibitors of *S. aureus* growth (EC_{50} = 0.20 μM and 1.8 μM, respectively), four other compounds were also moderately effective with EC_{50} values between 10 and 50 μM (**5**, **11**, **19**, and **28**). To determine if they were bactericidal or bacteriostatic, we analyzed lead compounds **8** and **18** against the methicillin susceptible *S. aureus* strain (ATCC 25923) and found that both were acting as bactericidal inhibitors (refer to Fig. S1 in Supporting information). We further tested compounds against an MRSA strain (ATCC #BAA-44: HPV107 strain, SCCmec Type I, Iberian PFGE Type) and found a correlation with the methicillin susceptible *S. aureus* strain (Table 3).

While we have identified numerous inhibitors of GroEL/ES biochemical function, several of which we now know inhibit the growth of pathogenic bacteria (in particular *S. aureus* and MRSA), there remained the possibility that these compounds could be toxic to host cells owing to targeting of HSP60/10. To account for this possibility, in vitro counter-screens were carried out in analogous chaperonin-mediated dMDH refolding and ATPase biochemical assays employing HSP60/10 (Table 4). As indicated in Figure 5A, there was a correlation observed for inhibiting both *E. coli* GroEL/ES and human HSP60/10; however, compounds were generally more potent at inhibiting *E. coli* GroEL/ES. Notably, compounds **1** and

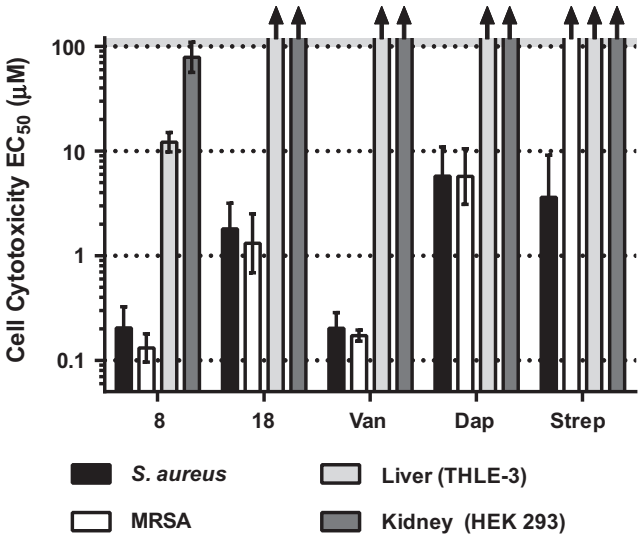

Figure 6. Compounds **8** and **18** are potent antibacterials against *S. aureus* & MRSA, with moderate/low to no toxicity to human liver and kidney cells. Results for Vancomycin (Van), Daptomycin (Dap), and Streptomycin (Strep) are shown for comparison. Arrows (↑) indicate EC_{50} results are >100 μM (the maximum concentrations tested).

18 displayed 12-fold and >17-fold selectivities, respectively, for inhibiting *E. coli* GroEL/ES over human HSP60/10. Unfortunately, the other lead inhibitor against *S. aureus* and MRSA bacteria, compound **8**, was only 4-fold selective. Thus, we were concerned about the cytotoxicity against human cells of **8** and other compounds that inhibited the HSP60/10 refolding cycle.

To gauge for potential cytotoxicity of chaperonin inhibitors to host tissues, we next evaluated compounds in viability assays using cultured human liver (THLE-3) and kidney (HEK 293) cells. These two stable cell lines were chosen because they are derived from the two main organs responsible for drug metabolism and excretion. An Alamar Blue-based cell viability assay was employed to probe for cytotoxicity.[53,54] We observed that inhibition of HSP60/10 biochemical activity did not directly translate into cell toxicity and that many compounds were only moderately toxic or non-toxic to both cell lines (Table 4 and Fig. 5B). This could be due to the fact that the inner mitochondrial membrane is highly impermeable to compounds, and thus inhibitors may not be able to penetrate to the mitochondrial matrix to reach HSP60/10. Compounds **8** and **18** were the most potent inhibitors of *S. aureus* proliferation, with the greatest therapeutic windows compared to liver and kidney cell cytotoxicity (Fig. 6). In particular, compound **8**, which exhibits an EC_{50} of 0.20 μM against *S. aureus* proliferation, has a therapeutic window of 60-fold with THLE-3 cells, and 390-fold with HEK 293 cells. Compound **18**, which exhibits an EC_{50} of 1.8 μM against *S. aureus* proliferation, is non-toxic to the liver and kidney cells up to the maximum concentrations tested (EC_{50} >100 μM; therapeutic window >55-fold).

In conclusion, we have investigated a subset of our previously identified inhibitors of the *E. coli* GroEL/ES chaperonin system for their antibiotic potential against a panel of bacteria including *E. coli* (3 strains), *B. subtilis*, *E. faecium*, *S. aureus* (including an MRSA strain), *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. cloacae*. The reported GroEL/ES inhibitors were largely ineffective at preventing the proliferation of the Gram-negative bacteria. While bacterial LPS outer membranes certainly play a significant role in preventing inhibitors from penetrating these bacteria, our studies with the mutant MC4100 Δ*acrB E. coli* strain indicate that drug efflux is another contributing factor to inhibitor inactivation. Compounds **8** and **18** emerged as the lead candidate GroEL/ES inhibitors that exhibit >50-fold selectivity for blocking the growth of *S. aureus* bacteria versus human liver and kidney cytotoxicities. Their antibiotic efficacies against *S. aureus* are comparable to vancomycin, daptomycin, and streptomycin (Fig. 6). Furthermore, they are effective against the MRSA strain evaluated here. We are pursuing further medicinal chemistry derivatization of these GroEL/ES inhibitors to develop lead analogs with more potent antibiotic effects against *S. aureus* (and ideally other bacteria) that remain non-toxic to mammalian cells. Since these inhibitors were discovered in a targeted GroEL/ES screen, we consider this the putative target, but we cannot rule out the possibility of off-target effects contributing to antibacterial potency. We are designing experiments to delineate the mechanism of action at the protein level and the mode of action at the whole cell level for these GroEL/ES inhibitors. The results presented here are encouraging, leading us to believe we can selectively target bacterial GroEL/ES chaperonin systems as an antibiotic strategy.

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Supplementary data

Supplementary data (comparison of ESKAPE pathogen GroEL and GroES sequence homology; tabulations of log(IC_{50}) and log (EC_{50}) results with standard deviations; bacterial inhibition curves to determine bacteriostatic vs. bactericidal mechanisms of action; experimental protocols for biochemical and cell-based assays; synthetic protocols and characterization data for compounds **10**, **15**, **23**, **24**, and **25**; and HPLC purity and MS characterization of test compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.04.089.

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