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Deciphering resistance against multi-targeting antibiotics

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2023

The discovery of antibiotics more than 90 years ago initiated a period of drug innovation and widespread implementation in healthcare and agriculture. Unfortunately, unlike most other drugs, antibiotics tend to lose their efficacy over time due to the emergence and dissemination of antibiotic resistance among bacterial pathogens. The emergence of resistance traits to multiple classes of antibiotics, resulting in strains with multidrug-resistance (MDR) phenotypes, has progressively narrowed the available treatment options for many pathogens. Drug resistant bacterial infections are currently responsible for 700,000 worldwide deaths annually, and this number is predicted to rise up to 10 million by 2050, which would make antibiotic resistant infections the leading cause of death. (O'Neill, 2016)

The emergence and dissemination of antibiotic resistance is driven by evolutionary processes that have provided the bacteria their outstanding ability to adapt to challenging environments for millennia. (Aminov, 2009; D'Costa et al., 2011) Resistance, in general, mainly can be mediated by two mechanisms: chromosomal resistance mutations or horizontal gene transfer via broad host-range plasmids. The relative importance of these mechanisms in resistance evolution is highly variable and depends on the antibiotic and the bacterial pathogens considered. (Hughes & Andersson, 2015) This work is mainly concerned with inhibitors of the Topoisomerase IV - DNA gyrase complex and against those antibiotics the relevance of plasmid-mediated resistance is much lower. Accordingly, we focus on chromosomal resistance mutations.

The emergence of resistance puts antibiotic development efforts in a precarious position. Pharmaceutical research and development costs, which are estimated to be \$400–\$800 million per approved agent, combined with an estimated 95% failure rate, pose a considerable barrier to new drug development in general. (Payne et al., 2007; Spellberg et al., 2004) Additionally, due to the rise of drug-resistant bacteria, the commercial success of antibiotic development is unpredictable. Paradoxically, as the need for new antibiotics is greater than ever, large pharmaceutical companies have mostly abandoned the market, accounting for only 4 of the 42 antibiotics currently under development. (Pew Charitable Trust, 2020; Jackson et al., 2018)

Consequently, the main driving force of current antibiotic development is the notion of finding 'resistance-proof' antibiotics. By principle, an antibiotic could be called such if resistance cannot evolve, because mutations that confer clinically significant resistance are exceedingly rare. (Bell & MacLean, 2018) Antibiotics with multiple independent cellular targets is an emerging

alternative. There are multiple mechanisms by which antimicrobial compounds may inhibit multiple bacterial targets. (Silver, 2007) In the case of hybrid drugs, two antibiotic pharmacophores with dissimilar targets are covalently linked to form one molecule. (Domalaon et al., 2018. Klahn & Brönstrup, 2017) Other antibiotics target two or more non-overlapping regions of a single bacterial protein and furthermore, equipotently inhibit two or more bacterial proteins. (Oldfield & Feng, 2014; Silver, 2007) Although it is a major focus of the pharmaceutical industry, designing multi-targeting antibiotics remains challenging. Until now, only a handful of antibiotic candidates displayed a balanced inhibition of multiple microbial targets. (Ince et al., 2002; Strahilevitz & Hooper, 2005; Tari et al., 2013) Additionally, due to the shortage of in-depth resistance studies, our knowledge on the tempo and mode of resistance development against multi-targeting antibiotics remains limited.

To establish the resistance-proof nature of an antibiotic, it is essential to precisely estimate the rate of resistance evolution at the earliest stages of development. Unfortunately, standard microbial protocols are slow, have a low coverage, and may fail to predict the frequency and molecular mechanisms of antibiotic resistance by genomic mutations. This is especially problematic in examining potential resistance against multi-targeting antibiotics, where a high level of resistance demands the simultaneous acquisition of multiple, rare mutations, many of which seemingly provide little benefit individually.

Directed evolution with random genomic mutations (DIvERGE), a recently published genome engineering method from our laboratory, addresses the aforementioned shortcomings of current *in vivo* mutagenesis techniques. (Nyerges et al., 2018) DIvERGE enables mutagenesis of predefined long genomic regions with broad, controllable mutagenesis spectra for each nucleotide position. It also allows very high mutation rates of the target sequences, enables multiple rounds of mutagenesis and selection, and is applicable to a range of host species without the need for prior genomic modification.

DIvERGE is based on a unique single-strand DNA oligonucleotide design strategy where long, continuous genomic segments are covered by the alignment of partially overlapping mutagenized DNA oligonucleotides. The randomized oligonucleotides fully cover the locus of interest and induce mutagenesis at their target after incorporation. Similarly to prior approaches,

DIvERGE proceeds via cell growth, oligo delivery and incorporation and mutagenesis, leading to a highly elevated genetic diversity of the mutagenized population.

DNA gyrase and Topoisomerase IV protein complexes offer an exceptional target for developing multi-targeting antibiotics, because of the homology of their corresponding subunits and the substantial overlap in their three-dimensional protein structures. (Bisacchi & Manchester, 2015; Collin et al., 2011) Both DNA gyrase and Topoisomerase IV are type II topoisomerases. In prokaryotes they form heterotetramers, with two subunits, GyrA-GyrB and ParC-ParE, respectively. Broadly speaking, the GyrA (ParC) subunit is involved with interactions with DNA, it contains the active-site tyrosine responsible for DNA cleavage, and the B subunit contains the ATPase active site. (Champoux, 2001) DNA gyrase and Topoisomerase IV are involved in breaking and rejoining double-stranded DNA, and thus, they determine changes in DNA topology during replication.

The body of the dissertation is based on two main scientific publications. In both, we focused on resistance against multi-targeting antibiotics, utilizing DIvERGE and traditional microbiological methods. In the first paper, we attempted to describe resistance against a novel topoisomerase inhibitor currently in clinical development, termed gepotidacin. (Szili et al., 2019) Based on the experiences gathered in this work, we later attempted to aid the early development of novel antibacterial compounds with potential multi-targeting activity. (Nyerges et al., 2020)

Gepotidacin (GSK2140944) is an exemplary candidate to study resistance evolution toward multi-targeting antibiotics. (Biedenbach et al., 2016; Flamm et al., 2017) It is a novel triazaacenaphthylene antibiotic candidate currently in phase 3 clinical trials and is expected to enter the clinical practice in the upcoming years. (Scangarella-Oman et al., 2020; Taylor, Morris, et al., 2018) The molecule inhibits bacterial DNA gyrase and Topoisomerase IV with a novel mode of action. Using a standard frequency of resistance test, early studies have failed to identify resistant clones of Neisseria gonorrhoeae and Escherichia coli against this new compound, suggesting that individual mutations cannot provide substantial resistance to gepotidacin. (Farrell et al., 2017; Nyerges et al., 2018) In our work, we demonstrated that contrary to expectations, reduced susceptibility to gepotidacin can evolve rapidly and previous works failed to identify resistance due to the limitations of the methods used.

In the second work, our collaborators have rationally designed a novel series of antibacterial compounds, endeavoring to achieve a balanced and simultaneous inhibitory effect on subunit B of DNA gyrase (GyrB) and subunit E of Topoisomerase IV (ParE). We characterized the biochemical and antibacterial properties, resistance development, as well as the *in vivo* efficacy of two representative compounds, termed ULD1 and ULD2.

Prior studies have demonstrated that gepotidacin selectively inhibits both bacterial DNA gyrase and Topoisomerase IV by a unique mechanism. Therefore, we subjected the four potential target genes to a single round of directed evolution with random genomic mutation (DIvERGE) mutagenesis in clinically relevant pathogen *Klebsiella pneumoniae*. Amino acids D82 within the GyrA subunit and D79 of the ParC subunit are found to be mutated in all the clones isolated, and no further mutations have been found. Subsequent saturation mutagenesis of the two mutational hot spots has proven that the combination of two specific mutations (GyrA D82N and ParC D79N) is responsible for a high-level reduction of susceptibility to gepotidacin. The double mutants were found to display as high as 2,080-fold reductions in gepotidacin susceptibility levels compared to the corresponding wild type strain of K. pneumoniae. In contrast, the single mutants were found to show no considerable changes in gepotidacin susceptibility. These findings are consistent with prior single-step resistance selection studies that failed to recover mutants with significant resistance.

Antibiotic resistance typically induces a fitness cost in the form of reduced bacterial growth rates, and such costs shape the long-term stability of antibiotic-resistant populations. Therefore, we have investigated the fitness effects of target-mediated resistance to gepotidacin in *K. pneumoniae* by pairwise competition experiments between the wild type strain and a specific mutant strain in nutrient-rich antibiotic-free bacterial medium (Mueller-Hinton II broth [MHBII]) at 37°C. We have found that the mutation combination of GyrA D82N and ParC D79N, which confers resistance to gepotidacin, significantly decreases fitness in *K. pneumoniae*. However, the fitness cost of gepotidacin resistance-causing mutations is comparable to the fitness cost of mutations associated with clinically relevant fluoroquinolone resistance. Importantly, the ParC D79N mutation is found to confer no measurable fitness cost individually. We also investigated whether mutants with resistance to gepotidacin display reduced virulence *in vivo* in a murine thigh wound infection

model. No significant decrease in in vivo virulence was observed for any of the mutants compared to the wild type strain.

Several prior works demonstrate that certain resistance mutations may be present in bacterial populations long before being exposed to a given antibiotic. Based on this knowledge, we hypothesized that prolonged use of other antibiotics might select for mutations that serve as stepping stones toward resistance to gepotidacin. The most likely antibiotic family for this purpose would be fluoroquinolones, as they are widely employed in clinical practice, and share a target with gepotidacin in the DNA gyrase – Topoisomerase IV complex. To reinvestigate this issue, we have focused on ciprofloxacin, a widely employed and well-characterized fluoroquinolone drug. Several ciprofloxacin resistance-conferring mutations and mutation combinations in clinical isolates provide no relevant cross-resistance against gepotidacin. On the contrary, we have found that the GyrA D82N single-mutant strain displays an over 16-fold increase in ciprofloxacin resistance level compared to that of the wild-type *K. pneumoniae* ATCC 10031 strain. Remarkably, the same mutation confers only a 2-fold increase in gepotidacin MIC level. As might be expected, the GyrA D82N and ParC D79N double mutants also display significant resistance to ciprofloxacin. This finding raise the possibility that the D82N mutation of GyrA might be present in fluoroquinolone-resistant clinical isolates, increasing the likelihood of the subsequent emergence of double mutants with resistance to gepotidacin. Indeed, our systematic search of online databases proved that that both mutations occur in a wide range of Gram-negative and Gram-positive bacteria, including fluoroquinolone resistant clinical isolates of *E. coli* and other species belonging to the *Salmonella, Mycoplasma, Clostridium, Citrobacter, Streptococcus*, and *Neisseria* genera. *Neisseria* and *Streptococcus* are especially clinically relevant in this context, as infections caused by these bacteria are reported to be the targets of gepotidacin in recent clinical trials.

To establish further that ciprofloxacin promotes reduced susceptibility to gepotidacin, we have performed adaptive laboratory evolution under ciprofloxacin stress with wild type *K. pneumoniae* ATCC 10031, as well as the wild type and *ΔmutS* hypermutator strains of *E. coli* K-12 MG1655. In line with previous clinical and laboratory studies, ciprofloxacin resistance was seen to emerge quickly in our experiments, especially in *ΔmutS* hypermutator lineages. Three randomly selected ciprofloxacin-resistant lineages per strain were selected for gepotidacin MIC measurements. Strikingly, all have been found to display a >64- to 1,058-fold increase in

gepotidacin resistance compared to the corresponding control strains. We also carried out whole genome sequencing of 2 and 3 evolved lineages derived from *E. coli* K-12 MG1655 and *K. pneumoniae* ATCC 10031, respectively. Although these lineages display markedly reduced gepotidacin susceptibilities, they do not carry mutations at GyrA D82 or ParC D79, suggesting that multiple other mutations may also select for reduced susceptibility to gepotidacin. Indeed, whole-genome sequencing has revealed that these ciprofloxacin-resistant lineages carry putative resistance mutations in genes involved in membrane efflux (acrR, soxR, and marR) and the general stress response (cusS and rpoB). Overall, these results strongly suggest that long-term exposure to ciprofloxacin stress promotes reduced susceptibility to gepotidacin.

In the second half of my doctorate program I have attempted to aid the early development of novel antibacterial compounds with potential multi-targeting activity. (Nyerges et al., 2020) Our collaborators (Peterlin lab, University of Ljubljana) have recently discovered a novel class of DNA gyrase inhibitors with a pyrrolamidobenzothiazole scaffold, inspired by the marine natural product oroidin. (Gross et al., 2003; Tomašič et al., 2015) To transform these molecules into broadly effective antibiotics, they have executed modifications at several sites on the pyrrolamidobenzothiazole-6-carboxylic acid scaffold based on the co-crystal structure with subunit B of DNA gyrase and molecular dynamics (MD) simulations. The molecular modifications were aimed at designing novel inhibitors that display equipotent dual-targeting activity towards subunit B of DNA gyrase (GyrB) and subunit E of Topoisomerase IV (ParE). The chemical modifications have yielded 2 especially promising antibiotic leads, ULD1 and ULD2.

When characterizing the bioactivity of these two molecules, first we have determined the minimum inhibitory concentrations (MICs) of ULD1 and ULD2 against a panel of Gram-negative and Gram-positive clinical pathogens. ULD1 and ULD2 were found to display potent antibacterial activity against ESKAPE pathogens (*S. aureus, Enterococcus sp., Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii), Streptococcus sp., and Clostridium difficile.* The MIC values against all studied multidrug-resistant *Staphylococcus*, *Enterococcus*, and *Streptococcus* isolates were below 2 μg/mL. Notably, ULD1 and ULD2 exerted activity against all MRSA, VRSA (MRSA/VRSA: methicillin-resistant/vancomycin-resistant *S. aureus)*, and vancomycin-resistant *Enterococcus* (VRE) isolates, which frequently cause difficult-to-treat skin and soft-tissue infections (SSTIs). (Ramakrishnan et al., 2015) We hypothesize that further

chemical modifications of ULD1/ULD2 could increase the potency of this compound class to inhibit Gram-negative pathogens as well. Encouraged by the excellent bioactivity of the molecules in MRSA and VRSA strains, we have focused on determining the antibacterial activity of ULD1 and ULD2 against a geographically and genetically diverse set of S. aureus clinical isolates, including 56 MRSA and 28 vancomycin-intermediate and vancomycin-resistant strains, inclusive of recent clinical isolates. A large fraction of these isolates were simultaneously resistant to multiple other available antibiotics too. In sharp contrast to other approved antibiotics against staphylococcal infections, both ULD1 and ULD2 were found to exert a potent activity against all tested isolates (MIC ≤ 1 µg/mL).

To explore the potential resistance mechanisms, we have determined the spontaneous frequency-of-resistance against ULD1, ULD2 in *S. aureus*. Novobiocin, an antibiotic formerly used in clinical practice, has served as a reference compound. Novobiocin's main target is the DNA gyrase subunit B, but second-step resistance mutations occasionally occur in Topoisomerase IV. (Fujimoto-Nakamura et al., 2005) Using a standard protocol for spontaneous frequency-of-resistance analysis (Bell & MacLean, 2018; Ling et al., 2015), we have exposed 10^{10} to 10^{12} bacterial cells derived from stationary-phase cultures of *S. aureus* ATCC 700699 (VISA) and *S. aureus* ATCC 43300 (MRSA) to increasing concentrations of ULD1, ULD2, and novobiocin, respectively. We have assessed the frequency-of-resistance and the mutant prevention concentration (MPC) for all 3 compounds. MPC is the drug concentration threshold above which the proliferation of resistant mutants does not occur (i.e., the concentration required to avoid the emergence of all first-step resistant mutants). (Bell & MacLean, 2018) In agreement with prior laboratory studies and clinical observations, the frequency-of-resistance against novobiocin was relatively high (Fig 5), and an up-to 120-fold increment in the MIC of novobiocin in the isolated *S. aureus* mutants have been detected. (Fujimoto-Nakamura et al., 2005; Vickers et al., 2007) By sharp contrast, no resistant variants of *S. aureus* have been detected when the bacterial cells were exposed to ULD1 at concentrations 8-fold the wild-type MIC (Fig 5). Moreover, resistant mutants isolated upon exposure to ULD1 at lower compound concentrations provided only minor changes in ULD1 susceptibility.

To investigate the molecular basis of mild ULD1 resistance, we have collected 400 independently isolated, ULD1 resistant clones from the frequency-of-resistance assay plates and

have sequenced their gyrB and parE genomic regions using Pacific Biosciences single-molecule real-time (SMRT) amplicon sequencing. Sequence analyses have revealed that all ULD1-resistant isolates had missense mutations that mapped to gyrB. Four different positions in the ULD1-binding pocket of GyrB (R144, G85, I175, T173) have mutated repeatedly. All the mutated amino acid residues in *S. aureus* are located in the binding pocket of GyrB and form secondary interactions with ULD1.

As ULD2 has a high affinity towards both of its target proteins and exerts an excellent dual-target enzyme inhibition, we hypothesized that the frequency-of-resistance against ULD2 could be exceptionally low. Notably, no ULD2 resistant mutants have emerged when 4×10^{12} *S. aureus* ATCC 700699 (VISA) cells were exposed to ULD2 at a concentration of only 4 times the wild-type MIC (Fig 5). We estimate that the MPC is as low as 0.16 μg/mL for ULD1 and 0.08 μg/mL for ULD2 in *S. aureus* ATCC 700699 (VISA).

While these results were encouraging, our prior experience with gepotidacin suggests that combination of multiple, specific mutations at all drug targets, in the long run, can eventually render even multi-targeting antibiotics ineffective. (Szili et al., 2019) To test this possibility, we have repeated the frequency-of-resistance assays with 2 ULD1-resistant *S. aureus* VISA laboratory isolates, both of which carried a single mutation at GyrB, the primary target of ULD1/ULD2. These mutations—GyrB R144I and I175T—were relatively frequent in the isolated single-step resistant mutants, and they conferred a moderately decreased susceptibility to ULD1. Populations of these single-mutant strains have been exposed to increasing concentrations of ULD1 and ULD2, separately. Spontaneous resistant mutants have appeared at a frequency of 10^{-8}–10^{-11}. All isolated second-step resistant mutants have displayed a relatively low resistance level, i.e., they could be inhibited by 1 μg/mL of ULD2. Sequence analyses have revealed that all detected second-step mutations are localized at ParE, the other target of ULD1/ULD2, at positions that are homologous to the binding sites in GyrB (Table 3). In sum, the observed mutations provide strong evidence in support of a dual-targeting mechanism of action for ULD1 and ULD2.

We have also investigated whether long-term exposure to ULD1 and ULD2 could select for a high level of resistance. To this aim, we performed adaptive laboratory evolution experiments with VISA under ULD1, ULD2, and novobiocin stresses. To accurately assess potential resistance mechanisms, 10 parallel evolving populations have been exposed to gradually increasing

concentrations of each compound. Following laboratory evolution, a single clone from each population has been isolated and subjected to drug susceptibility tests. In agreement with prior clinical observations and laboratory studies (Bisacchi & Manchester, 2015; Vickers et al., 2007), a high level of novobiocin-resistance has emerged rapidly. In novobiocin-adapted strains, an up-to-320-fold increase in novobiocin MIC (i.e., 16 μg/mL) has been detected, compared with the wild type strain. In contrast, only a relatively modest, 25-fold increase in the MICs of ULD1 and ULD2 have been detected in lineages adapted to ULD1 or ULD2, respectively (1 μg/mL for ULD1 and 0.5 μg/mL for ULD2). In order to elucidate the molecular mechanisms underlying ULD2 resistance, 5 ULD2-adapted strains have been subjected to whole-genome sequencing. We have focused on de novo mutations that have accumulated in several lineages independently during the course of laboratory evolution. Such mutations have been found in the target proteins (GyrB, ParE), as well as in a regulator of purine biosynthesis (PurR), and another enzyme involved in the uridine diphosphate (UDP) biosynthesis pathway (PyrH). The exact roles of these mutations in shaping ULD1/ULD2 susceptibilities remain to be discovered.

To explore the potential costs of resistance, we have investigated the growth phenotype of laboratory evolved, ULD1/ULD2 resistance-conferring *S. aureus* VISA isolates. Fitness was estimated by measuring the optical density at 600 nm (OD_{600}) of the population during 48 hours of growth in an antibiotic-free medium. ULD1/ULD2 resistant clones displayed a statistically significant decrease in growth compared to the wild type strain and formed tiny, slow-growing colonies on agar plates. These data indicate that long-term exposure to ULD1 and ULD2 yields mutants with limited resistance and high associated fitness costs in an antibiotic-free environment.

Based on our prior experience regarding the link between gepotidacin and ciprofloxacin resistance, it was rational to hypothesize that novobiocin-resistant clinical isolates might interfere with the antibacterial effects of ULD1 and ULD2. To investigate potential cross-resistance, the MIC of ULD1 and ULD2 have been tested against 9 independently evolved novobiocin-resistant isolates. These strains have been found to display no cross-resistance to ULD2, and only a modest, up to 6-fold decrease in ULD1 susceptibility has been detected compared with the corresponding wild type strain.

Based on the potent antibacterial activities of ULD1 and ULD2, we have finally tested their in vivo efficacy in mice models of *S. aureus* infections. First, a murine model of human

staphylococcal SSTI has been utilized. This preclinical model is extensively used to characterize the pharmacokinetic and pharmacodynamic properties of antistaphylococcal agents, as well as to predict their human clinical efficacy. (Kugelberg et al., 2005; Ling et al., 2015; Vingsbo Lundberg & Frimodt-Møller, 2013) Topical ULD1 and ULD2 treatments (in the form of ointments) were tested against *S. aureus* USA300 MRSA (BAA1556) and VISA and VRSA clinical isolates. These 3 strains together are resistant to at least 9 distinct classes of antibiotics, including mupirocin, a last-resort antibiotic against SSTIs caused by multidrug-resistant *S. aureus*. Topical application of ULD1 and ULD2 has exerted a potent antibacterial activity, comparable to that of mupirocin. Subsequent pharmacokinetic (PK) analyses indicate efficient skin penetration for both drugs, reaching a concentration of up to 300-times the MIC of ULD1 and ULD2 in wild-type *S. aureus* VISA.

We have also tested ULD1 in a neutropenic model of murine thigh infection. Intravenous (IV) administration of the drug resulted in potent antibacterial activity against *S. aureus* VISA infection. Notably, the antibacterial activity of ULD1 was comparable to that of linezolid, a widely used clinical agent against systemic MRSA infections. (Watkins et al., 2012) Taken together, these in vivo efficacy data indicate that the ULD molecules could serve as a basis for successful future drug development efforts against both topical and systemic *S. aureus* infections.

In summary, our work critically examined the concept of multi-targeting antibiotics in relation to yielding less resistance-prone antibiotics. We demonstrated that despite a balanced *in vivo* targeting of multiple proteins, resistance can rapidly emerge to antibiotics when the drug molecule's inhibitory effect depends merely on interactions with a few indispensable amino acids. Moreover, based on adaptive laboratory evolution and clinical data, we propose that target gene mutations conferring resistance to fluoroquinolones can facilitate resistance evolution to novel topoisomerase-targeting antimicrobials, including gepotidacin. This conclusion does not necessarily hold for the ULD molecules that have no related antibiotics in clinical rotation. Based on that multi-targeting might be a favorable approach if the antibiotic inhibits one or two completely novel targets, but scenarios like this should be examined in the future.

These findings might be relevant for other antibiotics currently in development. One example might be zoliflodacin (ETX0914), a novel bacterial topoisomerase inhibitor in human clinical trials. It shows promising activity against multidrug-resistant infections, including those

caused by *N. gonorrhoeae*. (Basarab et al., 2015; Taylor, Marrazzo, et al., 2018) However, in this species, mutations in GyrB have been reported to confer zoliflodacin resistance. One of these zoliflodacin-resistant mutants (D429N) has already been detected in clinical populations and naturally occurring zoliflodacin-resistant variants were also found to be highly resistant to fluoroquinolones. (Damião Gouveia et al., 2018; Foerster et al., 2015) Another relevant example outside the DNA gyrase – topoisomerase inhibitor family is SCH79797 and its derivative, Irresistin-16. SCH79797 is a repurposed drug that, according to recent data, shows antimicrobial activity with a unique mode of action, by simultaneously targeting folate metabolism and membrane integrity. In line with these findings, frequency of resistance against the drug in these first experiments was low and SCH79797 resistance did not develop while passaging the bacteria on sublethal concentration. (Martin et al., 2020) However, based on our experiences, these data worth revisiting in different bacterial species or with different methodologies.

References:

Jim O'Neill: Tackling Drug-Resistant Infections Globally: Final Report and Recommendations (2016). Retrieved 6 January 2021, from https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf

Aminov, R. I. (2009). The role of antibiotics and antibiotic resistance in nature. *Environmental Microbiology*, *11*(12), 2970–2988. https://doi.org/10.1111/j.1462-2920.2009.01972.x

Antibiotic Hybrids: The Next Generation of Agents and Adjuvants against Gram-Negative Pathogens? | Clinical Microbiology Reviews. (n.d.). Retrieved 7 January 2021, from https://cmr.asm.org/content/31/2/e00077-17

Antibiotics Currently in Global Clinical Development. (n.d.). Retrieved 6 January 2021, from http://pew.org/1YkUFkT

Basarab, G. S., Kern, G. H., McNulty, J., Mueller, J. P., Lawrence, K., Vishwanathan, K., Alm, R. A., Barvian, K., Doig, P., Galullo, V., Gardner, H., Gowravaram, M., Huband, M., Kimzey, A., Morningstar, M., Kutschke, A., Lahiri, S. D., Perros, M., Singh, R., … Newman, J. V. (2015). Responding to the challenge of untreatable gonorrhea: ETX0914, a first-in-class agent with a distinct mechanism-of-action against bacterial Type II topoisomerases. *Scientific Reports*, *5*(1), 11827. https://doi.org/10.1038/srep11827

Bell, G., & MacLean, C. (2018). The Search for 'Evolution-Proof' Antibiotics. *Trends in Microbiology*, *26*(6), 471–483. https://doi.org/10.1016/j.tim.2017.11.005

Biedenbach, D. J., Bouchillon, S. K., Hackel, M., Miller, L. A., Scangarella-Oman, N. E., Jakielaszek, C., & Sahm, D. F. (2016). In Vitro Activity of Gepotidacin, a Novel Triazaacenaphthylene Bacterial Topoisomerase Inhibitor, against a Broad Spectrum of Bacterial Pathogens. *Antimicrobial Agents and Chemotherapy*, *60*(3), 1918–1923. https://doi.org/10.1128/AAC.02820-15

Bisacchi, G. S., & Manchester, J. I. (2015). A New-Class Antibacterial—Almost. Lessons in Drug Discovery and Development: A Critical Analysis of More than 50 Years of Effort toward ATPase Inhibitors of DNA Gyrase and Topoisomerase IV. *ACS Infectious Diseases*, *1*(1), 4–41. https://doi.org/10.1021/id500013t

Champoux, J. J. (2001). DNA Topoisomerases: Structure, Function, and Mechanism. *Annual Review of Biochemistry*, *70*(1), 369–413. https://doi.org/10.1146/annurev.biochem.70.1.369

Collin, F., Karkare, S., & Maxwell, A. (2011). Exploiting bacterial DNA gyrase as a drug target: Current state and perspectives. *Applied Microbiology and Biotechnology*, *92*(3), 479–497. https://doi.org/10.1007/s00253-011-3557-z

Damião Gouveia, A. C., Unemo, M., & Jensen, J. S. (2018). In vitro activity of zoliflodacin (ETX0914) against macrolide-resistant, fluoroquinolone-resistant and antimicrobial-susceptible Mycoplasma genitalium strains. *Journal of Antimicrobial Chemotherapy*, *73*(5), 1291–1294. https://doi.org/10.1093/jac/dky022

D'Costa, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W. L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G. B., Poinar, H. N., & Wright, G. D. (2011). Antibiotic resistance is ancient. *Nature*, *477*(7365), 457–461. https://doi.org/10.1038/nature10388

Farrell, D. J., Sader, H. S., Rhomberg, P. R., Scangarella-Oman, N. E., & Flamm, R. K. (2017). In Vitro Activity of Gepotidacin (GSK2140944) against Neisseria gonorrhoeae. *Antimicrobial Agents and Chemotherapy*, *61*(3). https://doi.org/10.1128/AAC.02047-16

Flamm, R. K., Farrell, D. J., Rhomberg, P. R., Scangarella-Oman, N. E., & Sader, H. S. (2017). Gepotidacin (GSK2140944) In Vitro Activity against Gram-Positive and Gram-Negative Bacteria. *Antimicrobial Agents and Chemotherapy*, *61*(7). https://doi.org/10.1128/AAC.00468-17

Foerster, S., Golparian, D., Jacobsson, S., Hathaway, L. J., Low, N., Shafer, W. M., Althaus, C. L., & Unemo, M. (2015). Genetic Resistance Determinants, In Vitro Time-Kill Curve Analysis and Pharmacodynamic Functions for the Novel Topoisomerase II Inhibitor ETX0914 (AZD0914) in Neisseria gonorrhoeae. *Frontiers in Microbiology*, *6*. https://doi.org/10.3389/fmicb.2015.01377

Fujimoto-Nakamura, M., Ito, H., Oyamada, Y., Nishino, T., & Yamagishi, J. (2005). Accumulation of Mutations in both gyrB and parE Genes Is Associated with High-Level Resistance to Novobiocin in Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy*, *49*(9), 3810–3815. https://doi.org/10.1128/AAC.49.9.3810-3815.2005

Gross, C. H., Parsons, J. D., Grossman, T. H., Charifson, P. S., Bellon, S., Jernee, J., Dwyer, M., Chambers, S. P., Markland, W., Botfield, M., & Raybuck, S. A. (2003). Active-Site Residues of Escherichia coli DNA Gyrase Required in Coupling ATP Hydrolysis to DNA Supercoiling and Amino Acid Substitutions Leading to Novobiocin Resistance. *Antimicrobial Agents and Chemotherapy*, *47*(3), 1037–1046. https://doi.org/10.1128/AAC.47.3.1037-1046.2003

Hughes, D., & Andersson, D. I. (2015). Evolutionary consequences of drug resistance: Shared principles across diverse targets and organisms. *Nature Reviews Genetics*, *16*(8), 459–471. https://doi.org/10.1038/nrg3922

Ince, D., Zhang, X., Silver, L. C., & Hooper, D. C. (2002). Dual Targeting of DNA Gyrase and Topoisomerase IV: Target Interactions of Garenoxacin (BMS-284756, T-3811ME), a New Desfluoroquinolone. *Antimicrobial Agents and Chemotherapy*, *46*(11), 3370–3380. https://doi.org/10.1128/AAC.46.11.3370-3380.2002

Jackson, N., Czaplewski, L., & Piddock, L. J. V. (2018). Discovery and development of new antibacterial drugs: Learning from experience? *Journal of Antimicrobial Chemotherapy*, *73*(6), 1452–1459. https://doi.org/10.1093/jac/dky019

Klahn, P., & Brönstrup, M. (2017). Bifunctional antimicrobial conjugates and hybrid antimicrobials. *Natural Product Reports*, *34*(7), 832–885. https://doi.org/10.1039/C7NP00006E

Kugelberg, E., Norström, T., Petersen, T. K., Duvold, T., Andersson, D. I., & Hughes, D. (2005). Establishment of a Superficial Skin Infection Model in Mice by Using Staphylococcus aureus and Streptococcus pyogenes. *Antimicrobial Agents and Chemotherapy*, *49*(8), 3435–3441. https://doi.org/10.1128/AAC.49.8.3435-3441.2005

Ling, L. L., Schneider, T., Peoples, A. J., Spoering, A. L., Engels, I., Conlon, B. P., Mueller, A., Schäberle, T. F., Hughes, D. E., Epstein, S., Jones, M., Lazarides, L., Steadman, V. A., Cohen, D. R., Felix, C. R., Fetterman, K. A., Millett, W. P., Nitti, A. G., Zullo, A. M., … Lewis, K. (2015). A new antibiotic kills pathogens without detectable resistance. *Nature*, *517*(7535), 455–459. https://doi.org/10.1038/nature14098

Martin, J. K., Sheehan, J. P., Bratton, B. P., Moore, G. M., Mateus, A., Li, S. H.-J., Kim, H., Rabinowitz, J. D., Typas, A., Savitski, M. M., Wilson, M. Z., & Gitai, Z. (2020). A Dual-Mechanism Antibiotic Kills Gram-Negative Bacteria and Avoids Drug Resistance. *Cell*, *181*(7), 1518-1532.e14. https://doi.org/10.1016/j.cell.2020.05.005

Nyerges, Á., Csörgő, B., Draskovits, G., Kintses, B., Szili, P., Ferenc, G., Révész, T., Ari, E., Nagy, I., Bálint, B., Vásárhelyi, B. M., Bihari, P., Számel, M., Balogh, D., Papp, H., Kalapis, D., Papp, B., & Pál, C. (2018). Directed evolution of multiple genomic loci allows the prediction of antibiotic resistance. *Proceedings of the National Academy of Sciences*, *115*(25), E5726–E5735. https://doi.org/10.1073/pnas.1801646115

Nyerges, A., Tomašič, T., Durcik, M., Revesz, T., Szili, P., Draskovits, G., Bogar, F., Skok, Ž., Zidar, N., Ilaš, J., Zega, A., Kikelj, D., Daruka, L., Kintses, B., Vasarhelyi, B., Foldesi, I., Kata, D., Welin, M., Kimbung, R., … Pal, C. (2020). Rational design of balanced dual-targeting antibiotics with limited resistance. *PLOS Biology*, *18*(10), e3000819. https://doi.org/10.1371/journal.pbio.3000819

Oldfield, E., & Feng, X. (2014). Resistance-resistant antibiotics. *Trends in Pharmacological Sciences*, *35*(12), 664–674. https://doi.org/10.1016/j.tips.2014.10.007

Payne, D., Gwynn, M., Holmes, D., & Pompliano, D. (2007). Drugs for Bad Bugs: Confronting the Challenges of Antibacterial Discovery. *Nature Reviews. Drug Discovery*, *6*, 29–40. https://doi.org/10.1038/nrd2201

Ramakrishnan, K., Salinas, R. C., & Higuita, N. I. A. (2015). Skin and Soft Tissue Infections. *American Family Physician*, *92*(6), 474–483.

Scangarella-Oman, N. E., Ingraham, K. A., Tiffany, C. A., Tomsho, L., Horn, S. F. V., Mayhew, D. N., Perry, C. R., Ashton, T. C., Dumont, E. F., Huang, J., Brown, J. R., & Miller, L. A. (2020). In Vitro Activity and Microbiological Efficacy of Gepotidacin from a Phase 2, Randomized, Multicenter, Dose-Ranging Study in Patients with Acute Bacterial Skin and Skin Structure Infections. *Antimicrobial Agents and Chemotherapy*, *64*(3). https://doi.org/10.1128/AAC.01302-19

Silver, L. L. (2007). Multi-targeting by monotherapeutic antibacterials. *Nature Reviews Drug Discovery*, *6*(1), 41–55. https://doi.org/10.1038/nrd2202

Spellberg, B., Powers, J. H., Brass, E. P., Miller, L. G., & Edwards, J. E., Jr. (2004). Trends in Antimicrobial Drug Development: Implications for the Future. *Clinical Infectious Diseases*, *38*(9), 1279–1286. https://doi.org/10.1086/420937

Strahilevitz, J., & Hooper, D. C. (2005). Dual Targeting of Topoisomerase IV and Gyrase To Reduce Mutant Selection: Direct Testing of the Paradigm by Using WCK-1734, a New Fluoroquinolone, and Ciprofloxacin. *Antimicrobial Agents and Chemotherapy*, *49*(5), 1949–1956. https://doi.org/10.1128/AAC.49.5.1949-1956.2005

Szili, P., Draskovits, G., Révész, T., Bogár, F., Balogh, D., Martinek, T., Daruka, L., Spohn, R., Vásárhelyi, B. M., Czikkely, M., Kintses, B., Grézal, G., Ferenc, G., Pál, C., & Nyerges, Á. (2019). Rapid Evolution of Reduced Susceptibility against a Balanced Dual-Targeting Antibiotic through Stepping-Stone Mutations. *Antimicrobial Agents and Chemotherapy*, *63*(9). https://doi.org/10.1128/AAC.00207-19

Tari, L. W., Li, X., Trzoss, M., Bensen, D. C., Chen, Z., Lam, T., Zhang, J., Lee, S. J., Hough, G., Phillipson, D., Akers-Rodriguez, S., Cunningham, M. L., Kwan, B. P., Nelson, K. J., Castellano, A., Locke, J. B., Brown-Driver, V., Murphy, T. M., Ong, V. S., … Finn, J. (2013). Tricyclic GyrB/ParE (TriBE) inhibitors: A new class of broad-spectrum dual-targeting antibacterial agents. *PloS One*, *8*(12), e84409. https://doi.org/10.1371/journal.pone.0084409

Taylor, S. N., Marrazzo, J., Batteiger, B. E., Edward W. Hook, I. I. I., Seña, A. C., Long, J., Wierzbicki, M. R., Kwak, H., Johnson, S. M., Lawrence, K., & Mueller, J. (2018). Single-Dose Zoliflodacin (ETX0914) for Treatment of Urogenital Gonorrhea. *New England Journal of Medicine*. https://doi.org/10.1056/NEJMoa1706988

Taylor, S. N., Morris, D. H., Avery, A. K., Workowski, K. A., Batteiger, B. E., Tiffany, C. A., Perry, C. R., Raychaudhuri, A., Scangarella-Oman, N. E., Hossain, M., & Dumont, E. F. (2018). Gepotidacin for the Treatment of Uncomplicated Urogenital Gonorrhea: A Phase 2, Randomized, Dose-Ranging, Single-Oral Dose Evaluation. *Clinical Infectious Diseases*, *67*(4), 504–512. https://doi.org/10.1093/cid/ciy145

Tomašič, T., Katsamakas, S., Hodnik, Ž., Ilaš, J., Brvar, M., Solmajer, T., Montalvão, S., Tammela, P., Banjanac, M., Ergović, G., Anderluh, M., Mašič, L. P., & Kikelj, D. (2015). Discovery of 4,5,6,7-Tetrahydrobenzo[1,2-d]thiazoles as Novel DNA Gyrase Inhibitors Targeting the ATP-Binding Site. *Journal of Medicinal Chemistry*, *58*(14), 5501–5521. https://doi.org/10.1021/acs.jmedchem.5b00489

Vickers, A. A., O'Neill, A. J., & Chopra, I. (2007). Emergence and maintenance of resistance to fluoroquinolones and coumarins in Staphylococcus aureus: Predictions from in vitro studies. *Journal of Antimicrobial Chemotherapy*, *60*(2), 269–273. https://doi.org/10.1093/jac/dkm191

Vingsbo Lundberg, C., & Frimodt-Møller, N. (2013). Efficacy of topical and systemic antibiotic treatment of meticillin-resistant Staphylococcus aureus in a murine superficial skin wound infection model. *International Journal of Antimicrobial Agents*, *42*(3), 272–275. https://doi.org/10.1016/j.ijantimicag.2013.05.008

Watkins, R. R., Lemonovich, T. L., & File, T. M. (2012). An evidence-based review of linezolid for the treatment of methicillin-resistant Staphylococcus aureus (MRSA): Place in therapy. *Core Evidence*, *7*, 131–143. https://doi.org/10.2147/CE.S33430