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Characterization of antibacterial drugs against non-growing bacteria

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Characterization of antibacterial drugs against non-growing bacteria

Abstract

Non-growing bacterial cells, such as those found in chronic infections, are highly tolerant to antibiotics and contribute to treatment failure and relapse. A high-throughput screen of over 6,000 drugs and drug candidates from repurposing libraries identified 39 with activity against non-growing uropathogenic *Escherichia coli*. These hit compounds were subsequently tested against non-growing *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and the 24 most active candidates were selected for further evaluation.

Dose-dependent effects on regrowth delay and bacterial killing were assessed across all three species. Statistical analysis confirmed that the observed regrowth delays were significant and reproducible, with p-values below 0.0001 for the most effective treatments. The impact of treatment duration, testing conditions, and efflux pump activity on drug efficacy was also investigated. Most compounds exhibited rapid activity, with significant effects detected within the first hour of exposure. The composition of the growth medium—particularly phosphate buffering—strongly influenced drug activity, whereas bacterial strain and regrowth conditions had minimal effect. Inhibition of efflux pumps during treatment or regrowth had no clear effect.

Finally, delafloxacin—a fluoroquinolone not included in the original libraries—was tested due to its structural similarity to sitafloxacin, one of the most potent hit compounds. Despite its enhanced halogenation, delafloxacin showed inferior activity against non-growing bacteria compared to sitafloxacin.

Keywords

Nongrowing bacteria, drug repurposing, high-throughput screening, hit characterization, statistical analysis

CERCS: B230 Microbiology, bacteriology, virology, mycology

Institute name: Tartu University, Institute of Technology

Research group: Tanel Tenson's Lab

Mittekasvavate bakterite vastaste antibakteriaalsete ravimite iseloomustamine

Lühikokkuvõte

Mittekasvavad bakterirakud, mis on arvukad krooniliste infektsioonide korral, on antibiootikumide suhtes väga tolerantsed. Sageli on need ravi ebaõnnestumise ja retsidiivide põhjuseks. Ravimite taaskasutamise raamatukogudest pärit enam kui 6000 ühendi skriining tuvastas 39 ühendit, millel oli aktiivsus mittekasvava uropatogeense *Escherichia coli* vastu. Neid edukaid ühendeid testiti seejärel mittekasvava *Pseudomonas aeruginosa* ja *Staphylococcus aureus*'e vastu ning 24 kõige aktiivsemat kandidaati valiti edasiseks hindamiseks.
Kõigi kolme liigi puhul hinnati annusest sõltuvaid mõjusid bakterite töötlusejärgse kasvama hakkamise kiirusele ja mittekasvavate bakterite tapmisele. Statistiline analüüs kinnitas, et täheldatud mõju bakterite kasvama hakkamisele oli oluline ja reprodutseeritav, kusjuures kõige tõhusamate ühendite puhul jäid p-väärtused alla 0,0001. Uuriti ka ravimitöötluse kestuse, testimistingimuste ja ravimite rakust välja pumpamise (*efflux*) mõju ühendite efektiivsusele. Enamik ühendeid toimis kiiresti, juba 1 tunni jooksul. Kasvukeskkonna koostis – eriti fosfaatpuhvril põhineva söötme kasutamine – mõjutas tugevalt ravimi aktiivsust, samas kui bakteritüvel ja kasvama hakkamise tingimustel oli minimaalne mõju. Väljavoolupumpade pärssimisel ravimitöötluse ja kasvama hakkamise ajal puudus otseselt märgatav mõju.
Lõpuks testiti delafloksatsiini – fluorokinolooni, mida skriinitavate ravimite hulgas polnud – selle struktuurilise sarnasuse tõttu sitafloksatsiiniga, mis on üks tugevamaid leitud ühendeid. Vaatamata suurenemale halogeenimisele näitas delafloksatsiin mittekasvavate bakterite vastu palju nõrgemat aktiivsust kui sitafloksatsiin

Võtmesõnad:

Mittekasvavad bakterid, ravimite taaskasutamine, suure läbilaskevõimega skriining, tabamuse iseloomustamine, statistiline analüüs

CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

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TERMS, ABBREVIATIONS, AND NOTATIONS

AMR – Antimicrobial resistance

ANOVA – Analysis of Variance

CAMHB – Cation-Adjusted Mueller-Hinton Broth

CF – Cystic fibrosis

CFU – Colony forming unit

CFU assay – Colony forming unit counting assay

CFU/ml – CFU per milliliter

cftr – Cystic fibrosis transmembrane conductance regulator gene

DMSO – Dimethyl Sulfoxide

ΔSGT – The delay in regrowth between the treated and drug-free samples

EPIs – Efflux pump inhibitors

EPS – Extracellular polymeric substances

GIMP – GNU Image Manipulation Program

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HTS – High-throughput screening

IBC – Intracellular bacterial community

LB – Lysogeny Broth

LPM – Low Phosphate, Low Magnesium

LPS – Lipopolysaccharide

MES – 2-(N-morpholino)ethanesulfonic acid

MIC – Minimum inhibitory concentration

NICE – NIST's Integrated Colony Enumerator

OD600 – Optical density at 600 nm wavelength

PAβN – Phenylalanine-arginine β-naphthylamide

PAE – Post-antibiotic effect

PBS – Phosphate-Buffered Saline

RND – Resistance-nodulation division

SEM – Standard error of the mean

SGT – Start Growth Time

UPEC – Uropathogenic *E. coli*

UTI – Urinary tract infection

INTRODUCTION

Bacterial infections continue to be a leading cause of mortality worldwide; chronic and recurrent infections are becoming increasingly important due to the aging of the world population. While antibiotics have been used in bacterial infection treatment for more than 80 years, their effectiveness is largely limited to actively growing bacterial populations. Non-growing bacteria – such as those found in chronic infections, biofilms, or intracellular reservoirs – are inherently more tolerant to antibiotics and are often responsible for treatment failure and infection relapse.

This tolerance to antibacterials and the related phenomenon of persistence allow bacteria to survive exposure to bactericidal antibiotics. Distinct from antibiotic resistance, which enables bacterial growth in the presence of antibiotics, tolerance and persistence are transient phenotypic states that allow prolonged survival in temporary dormancy. These traits are especially problematic in chronic and recurrent infections, where non-growing bacteria evade immune responses and conventional therapies. Moreover, tolerance and persistence can facilitate the eventual development of resistance, compounding the challenge of effective treatment.

Despite the clinical importance of non-growing bacteria, most antibiotics in use today were discovered based on their ability to inhibit bacterial growth. As a result, compounds that are effective against non-growing cells have been largely overlooked. Addressing this gap requires not only the identification of such compounds but also a deeper understanding of the factors that influence their activity.

This thesis builds upon previous high-throughput screening efforts that identified compounds with potential activity against non-growing uropathogenic *Escherichia coli* and the work that followed in testing the hit compounds and exploring their activity against other species – *P. aeruginosa* and *S. aureus*. Here we focus on characterizing the effects of these compounds in greater detail. Specifically, we aim to assess the statistical significance of regrowth delays caused by the antibacterial treatments of the tested compounds, investigate how treatment duration and testing conditions influence drug efficacy, and evaluate the role of efflux pumps in modulating antibacterial activity. In addition, we explore the potential antibacterial activity against non-growing bacteria of a drug not part of the screening libraries – delafloxacin, which is structurally similar to a previously identified antibacterial hit compound with one of the most potent effects against non-growing bacteria.

1. LITERATURE REVIEW

1.1 Infectious disease

Infections are a historically ubiquitous health problem. They can spread between people either directly (e.g., through close contact with an infected individual) or indirectly (e.g., through contaminated water or food). Infectious diseases are caused by microscopic pathogens, such as viruses, bacteria, or fungi, that enter and propagate in the host organism, often leading to death if untreated and if the immune response is insufficient (*Infectious Disease*, n.d.).

1.1.1 Bacterial infections

One of the infectious disease types is bacterial infections, such as syphilis, tuberculosis, and pneumonia (*Infectious Disease*, n.d.). Bacterial infections were one of the leading causes of death worldwide until the 1930s (Armstrong et al., 1999; Pedersen et al., 2024; Sakai & Morimoto, 2022). Until treatment options were developed, the host's immune system was the only obstacle between dying of a bacterial infection and surviving (Runcie, 2015). The lack of treatment was partially responsible for the high mortality rate in newborns and young children, as their immune systems were less likely to deal with the infections. The first antimicrobial agent – Salvarsan, an organoarsenic with anti-syphilitic activity, was discovered in 1908 by Paul Ehrlich and Sahachiro Hata. Its toxic side effects and prolonged treatment time were very undesirable and led to optimization attempts, resulting in Neosalvarsan in 1912. Despite the reduced toxicity of Neosalvarsan, it still caused serious side effects. In 1930, it was discovered that the oxidized form of Salvarsan – marketed as Mapharsen – was the active compound. Because of its stability, it became the standard treatment choice for syphilis until the 1940s (Bosch & Rosich, 2008; Strebhardt & Ullrich, 2008). Meanwhile, the first antibiotic – penicillin – was discovered in 1928 by Alexander Fleming (Fleming, 1929). However, for a decade, its antibacterial activity was not utilized. That is, until 1939, when Ernst Chain and Howard Florey successfully purified penicillin from *Penicillium* spp. mold and treated mice infected with a viral strain of *Streptococcus*. In 1941, for the first time, a bacterial infection in a human was treated with penicillin, albeit not very successfully due to insufficient stock, sparking the use of antibiotics as a suitable treatment option (Chain et al., 1940; Gaynes, 2017).

Nowadays, various bacterial infections are still some of the most prevalent causes of death, especially in, but not limited to, low-income countries (World Health Organization, 2024). Overall bacterial infections are the 2nd leading cause of death, causing estimated 1 in every 8 deaths globally in 2019 (Institute for Health Metrics Evaluation, 2022). Of these infections 5 pathogens - *Staphylococcus aureus, Escherichia coli, Streptococcus pneumoniae,*

Klebsiella pneumoniae, and Pseudomonas aeruginosa – account for more than half of the estimated deaths (Ikuta et al., 2022). Despite treatment options existing for over 80 years, the issue of bacterial infections remains. This is further driven by antimicrobial resistance (AMR) which decreases the efficacy of existing antibacterial treatments (World Health Organization, 2023). It is estimated that 4.95 million deaths from bacterial infection in 2019 were associated with AMR, of which 1.27 million would be directly attributed to it (Murray et al., 2022).

Due to overuse, inappropriate prescriptions, lack of new antibiotics and other reasons, AMR has become a significant problem causing both welfare and economic burdens (Ventola, 2015). Projections indicate a potentially catastrophic rise in mortality if AMR trends continue. It was reported in 2016 that 700'000 deaths occur annually due to AMR and if no countermeasures are implemented, it may lead up to 10 million deaths a year by 2050 (O'Neill, 2016). Comparing this to the estimated 1.27 million deaths already in 2019, the issue becomes even more apparent.

1.1.2 Chronic bacterial infections

Chronic infections are a major concern, causing long lasting illness or relapse despite treatment. They affect patients' daily lives for extended periods, ranging from months to years (Young et al., 2002). Research has shown that significant contributors to chronic infections are biofilms (Singh et al., 2000) and bacterial persistence (Fisher et al., 2017), which allow some non-growing bacteria to survive antibiotic treatment. Not all chronic infections are difficult to treat due to a lack of treatment effectiveness. For instance, recurrent infections—a type of chronic infection—are often susceptible to antibiotic treatment; however, the survival of small subpopulations allows them to reinfect the host after a seemingly successful treatment (Fisher et al., 2017).

Urinary tract infections (UTIs) are painful bacterial infections that commonly occur in women and at a lesser rate in men (Terlizzi et al., 2017; *Urinary Tract Infections*, n.d.). Failure to treat UTIs can lead to renal failure or sepsis (Bokil et al., 2011). They are also one of the more common types on infections that can become recurrent. Up to 90% of UTIs are caused by uropathogenic *E. coli* (UPEC) (Sgarabotto et al., 2025). UPEC is capable of colonizing both extracellular and intracellular niches of the urinary tract. Intracellular bacterial communities (IBCs) are clusters of bacteria that form in the cytoplasm of superficial bladder epithelium cells. Shortly after infection, part of the UPEC population attaches to the superficial cells (Fig. 1B) and then invades them (Fig. 1C). Then they replicate in the infected cells (Fig. 1D) and may form the large biofilm-like IBCs (Fig. 1E). These IBCs can stay dormant for long periods of time. As the IBCs mature, some of the bacteria may begin to filament, but ultimately the bacteria

flux out of the cell (Fig. 1G) and restart the cycle. Alternatively, the superficial cells may be exfoliated – shed away as a host defense mechanism (Fig. 1F). The difficulty of UPEC treatment stems both from antibacterial persistence of intracellular bacteria and the formation of IBCs. These two characteristics allow for escape of both antibiotic treatment and immune response respectively, making these UTIs particularly hard to resolve completely and allowing the same species to repopulate (Schwartz et al., 2011).

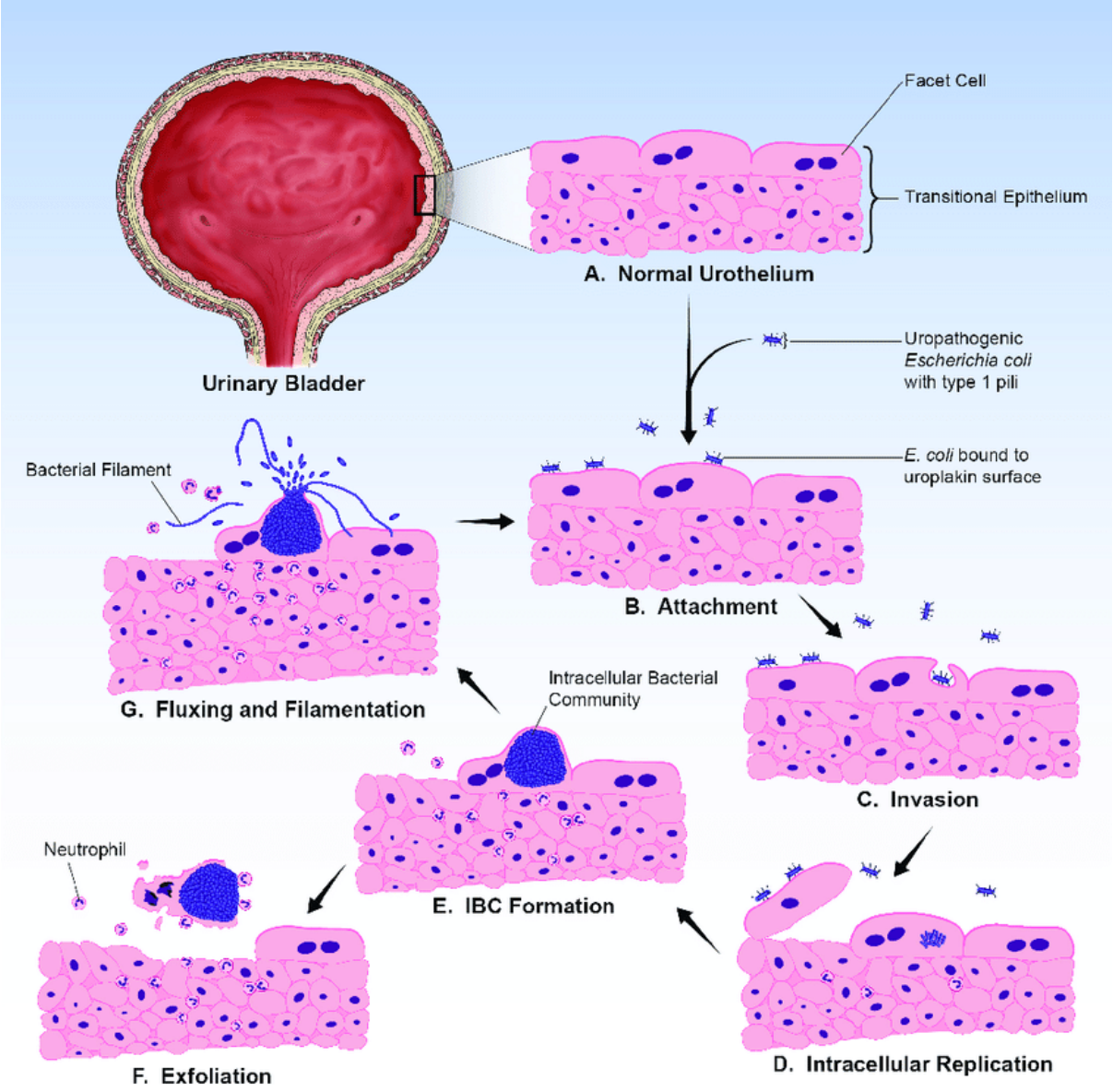

Figure 1. Adapted from Rosen et al., 2007. UPEC IBC formation pathway in murine cystitis model. The bladder urothelium (A) is lined by large facet (superficial) cells. Bacteria introduced into the bladder adhere to the bladder surface (B). Upon attachment, bacteria can invade (C) and replicate (D) within the facet cell cytoplasm. UPEC forms large biofilm-like IBCs within these cells (E). Ultimately, the bacteria flux out of their intracellular niche (G), some adopting a filamentous morphology; they then adhere to other host cells and re-enter the infectious cycle. During this process,

infected urothelial cells are sloughed into the urine (F) and neutrophils are recruited to the site of infection.

Another case where treatment against chronic infections is highly necessary is in cystic fibrosis (CF) patients. By itself, CF is an incurable genetic disease, caused by absence or malfunction of transmembrane conductance regulator (*cftr*) gene product encoding for an ion channel. The malfunction of the channel leads to production of sticky, viscous respiratory mucus. It is often accompanied by chronic lung infections as the thickened mucus reduces bacterial clearance (Alqasmi, 2024). Bacteria like *Staphylococcus aureus* and *Pseudomonas aeruginosa* are common lung infection causatives in CF patients. Both cases are difficult to treat due to their ability to form biofilms. Consequently, a pronounced antibody response is developed which then leads to chronic inflammation causing lung tissue damage over time (Boudet et al., 2021; Høiby et al., 2010).

Biofilms are complex structures that allow microorganisms to survive both treatment and immune response for prolonged periods of time. They are formed by microbial communities adhering to either biotic or abiotic surfaces and encasing themselves in extracellular polymeric substances (EPS), consisting of polysaccharides, extracellular DNA and proteins (Gondil & Subhadra, 2023). Biofilm-forming bacteria are extremely difficult to treat by standard antibiotic treatments as drugs have reduced penetrating capabilities against the biofilms (Kostakioti et al., 2013) and they contain persister subpopulations (Yan et al., 2024). These biofilms not only affect infection treatment but also colonize medical devices further driving the spread of chronic infections (Caldara et al., 2022).

1.2 How to treat infections?

Antibiotics are the primary tool for fighting bacterial infections. They are synthetic or naturally occurring compounds that specifically affect the biochemical pathways of bacteria to either inhibit the growth and division of cells or, in some cases, kill them (Brown & Wright, 2016). Additionally, antibiotics must be harmless to the patient's health to be safe to use, meaning that their targets must be unique to the bacteria.

The ability of antibiotics to inhibit bacterial growth is called bacteriostatic activity, while the ability to kill bacteria is called bactericidal activity. However, bacteriostatic agents can also kill some bacteria, just not as much as bactericidal ones. Combined with the host's natural immune system, bacteriostatic activity is often sufficient in acute infections where non-growing bacterial subpopulations are less prevalent (Pankey & Sabath, 2004). However, bacteriostatic

activity is insufficient in cases where a larger proportion of the bacterial population is non-growing (Bumann et al., 2019), such as recurrent UTIs caused by UPEC. In such cases, bactericidal activity is required, as bactericidal drugs disrupt their targets' function in a way that generates toxic effects, rather than merely inhibiting them (Lewis, 2007).

1.3 Why can antibiotic treatments be ineffective?

Treatment efficacy of bacterial infections depends on various mechanisms of the pathogen that affect susceptibility to antibacterial drug, bacterial growth stage (growing/non-growing), as well as characteristics of the drug. To assess susceptibility, in vitro assays are commonly used to characterize the drug's effects on isolated pathogens and the results are compared to standardized breakpoints specific to each species. The lowest antibiotic concentration at which bacterial growth is inhibited is referred to as the minimum inhibitory concentration (MIC). If the MIC is below the breakpoint, the bacteria are considered susceptible to the tested drug; if it exceeds the breakpoint, they are classified as resistant (Kadeřábková et al., 2024). Non-growing bacterial subpopulations are not affected by bacteriostatic activity and can evade treatment (Bumann et al., 2019). This allows tolerant and persistent bacteria (see Chapters 1.3.2 and 1.3.3) to survive bacteriostatic treatments despite having equal MIC to susceptible bacteria under standard testing conditions (Fig. 2A). However, bactericidal drugs only prolong the survival of tolerant and persistent cells (Fig. 2B and 2C) as they are killed by the bactericidal activity anyway (Balaban et al., 2019).

Drug characteristics, such as penetration into bacteria and their host cells, also influence treatment efficacy. If the drug cannot enter the cell, it cannot bind to its target (Delcour, 2009). Some resistant bacteria and persisters in biofilms have mechanisms decreasing drug diffusion and penetration into the cell (Almatroudi, 2025; Belay et al., 2024; Malki et al., 2023). Hence, drugs with improved drug penetration may be needed to breach these barriers.

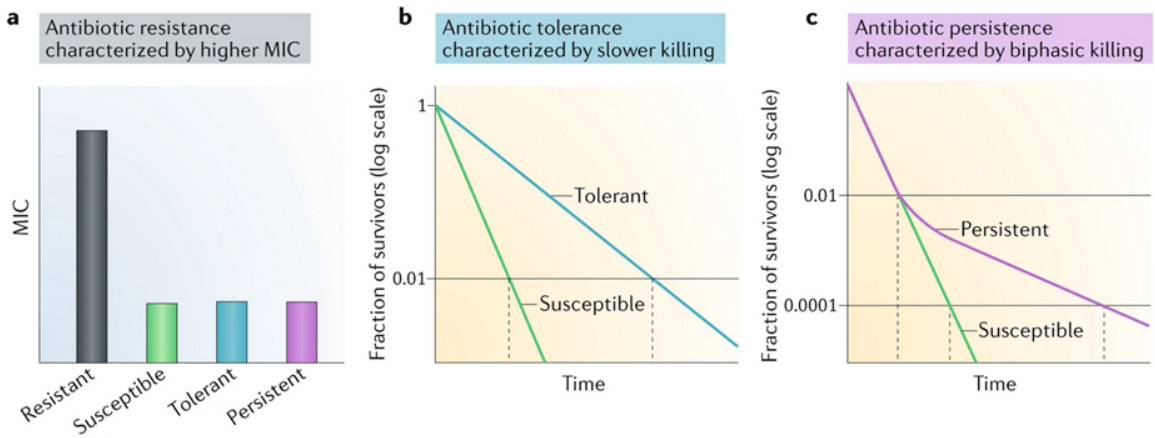

Figure 2. **Adapted from Balaban et al., 2019. Resistance, tolerance, and persistence are distinct responses to antibiotic treatment that lead to increased survival compared to susceptible cells.** (A) To inhibit the growth of resistant bacteria, a significantly higher minimum inhibitory concentration (MIC) of the antibiotic is required compared to that for susceptible strains. Importantly, persistence and tolerance do not result in elevated MIC values relative to susceptible bacteria. (B) In contrast, tolerance increases the minimum duration needed to kill the bacteria, even though the MIC remains unchanged.(C) Persistence also does not alter the MIC and shows a similar initial rate of bacterial killing as seen with susceptible strains. However, achieving near-complete eradication of the population takes much longer due to the survival of persister cells. It's worth noting that purely exponential killing of susceptible bacteria are rarely observed in practice, as most bacterial populations exhibit some degree of persistence. The data presented are illustrative and not based on actual experimental measurements (Balaban et al., 2019).

1.3.1 Antimicrobial resistance

Antimicrobial resistance is a genetically encoded ability of bacteria to evade both bacteriostatic and bactericidal activity through various mechanisms and grow in the presence of the antibiotic. It can be intrinsic, e.g., antibiotics targeting cell wall synthesis cannot affect species like *Mycoplasma* spp. which lack the cell walls (Lanao et al., 2025), or acquired (Habboush & Guzman, 2025). Some of the mechanisms conferring resistance are target alteration, reduced permeability, drug degradation and removal of the drug, which affect the drugs ability to bind the target, enter the bacteria or maintain high enough concentrations for a sufficient treatment period (Balaban et al., 2019). Bacteria can acquire resistance by horizontal transfer of resistance genes or mutations in the target genes (Habboush & Guzman, 2025). Resistance to treatment leads to significant increase in health complications and often death for sepsis patients as the infections are not curable in a timely manner (Ikuta et al., 2022).

1.3.1.1. Efflux pumps as a mechanism conferring resistance

One of the defense mechanisms in resistant bacteria is the removal of antibiotics from their intracellular environment. Efflux pumps are membrane pumps that transport toxic compounds, including antibiotics, out of the cell in an energy dependent manner. They consist of 3 protein complexes - transporter, periplasmic adaptor and outer membrane factor - spreading through the inner membrane to the outer membrane. Efflux transporters are categorized into six families of which resistance-nodulation division (RND) family contains members conferring most of the clinically relevant resistance levels in Gram-negative bacteria. Additionally, a fourth transmembrane protein, bound to the efflux transporter helps modulate substrate preference, promoting extraction of antibiotics (Blair et al., 2015; Y. Mahmood et al., 2016; Zhang et al., 2023).

Efflux pump activity can be inhibited by efflux pump inhibitors (EPIs) such as phenylalanine-arginine β-naphthylamide (PAβN). EPIs help maintain antibiotic availability in the cell by competing with the antibiotics for efflux pump binding sites, therefore allowing the antibiotics to remain inside the bacteria long enough to cause significant harm to the cell (Liu et al., 2023).

1.3.2 Antibiotic tolerance

Whereas AMR is a genetic trait, antibiotic tolerance is a phenotypic trait that enables bacteria to evade the bactericidal activity of the drug while not growing or growing at a highly reduced rate. Tolerance enables bacteria to survive longer during antibiotic treatment and to resume growth once the antibiotic stress is relieved. The main mechanisms linked to tolerance are dormancy and reduced metabolism (Balaban et al., 2019). Additionally tolerant cells may utilize various stress response mechanisms, e.g., SOS DNA damage response in presence of fluoroquinolones or upregulation of cell wall synthesis in some cases against cell wall damaging antibiotics (Dawan & Ahn, 2022; Podlesek & Žgur Bertok, 2020). By promoting bacterial survival, this trait also increases chances of developing AMR (Balaban et al., 2019).

1.3.3 Antibiotic persistence

Antibiotic persistence is a phenotypic trait similar to tolerance. It allows a small fraction of the entire, normally susceptible, population to survive the antibiotic treatment due to phenotypical heterogeneity in the bacterial population prior to the treatment. These persisters are either slow-growing or non-growing cells, which allows them to survive antimicrobial therapy. This leads to recurrent or chronic infections as at first antibiotic treatment seems to work but the bacterial population isn't completely eradicated, and a relapse occurs after the antibiotic is depleted from the environment (Balaban et al., 2013). Albeit unifying mechanisms of its action are still up for debate, a link between persistence and cellular aging has been established (Proenca et al., 2025).

1.4 Antibiotic discovery

1.4.1 History of antibiotic screening

Antibiotic discovery has a history of more than 90 years. It is preceeded by the work of Paul Ehrlich (see Chapter 1.1.1) which resulted in the discovery of Salvarsan and the "magic bullet" concept - the idea that medicines could be designed to specifically target disease-causing structures, allowing treatment with minimal to no side effects on the patient (Bosch & Rosich, 2008).

The discovery of penicillin by Fleming and streptomycin by Waksman marked the beginning of a transformative era in antimicrobial drug discovery. Waksman's approach – systematic screening of soil bacteria, particularly actinomycetes, for bioactive secondary metabolites – established a foundational platform for identifying novel antibiotics. This initiated the "golden era" of antibiotic discovery in mid 1940s, during which natural microbial scaffolds were extensively mined for their therapeutic potential. To evaluate the efficacy of these compounds, standardized measures such as MIC testing in nutrient-rich media became essential, setting a benchmark for subsequent drug development pipelines (Brown & Wright, 2016).

By late 1960s, the Waksman platform started to yield diminishing returns, as most bioactive metabolites had already been explored and new potential candidates were just rediscovered known compounds. The metabolites, while biologically active, often posed pharmacological or toxicological challenges as they were evolved to be active in the environment of their producers. At the same time, resistance to early natural antibiotics was becoming increasingly prevalent, prompting a change in drug discovery. This shift marked the rise of medicinal chemistry, which focused on synthesizing derivatives of natural scaffolds to improve activity spectra and dosage efficacy and to overcome antibiotic resistance (Brown & Wright, 2016).

The emergence of widespread antibiotic resistance in the 1990s, coupled with the lack of new antibiotic scaffolds since the 1960s, ushered in renewed discovery efforts. This period coincided with major technological breakthroughs, including recombinant DNA manipulation, high-throughput synthesis, automated screening assays, and protein-structure determination, which transformed drug development across multiple therapeutic areas. Advances in computing power and genomic technologies further drove the adoption of modern discovery models. For that, essential bacterial genes were selected as new antibiotic targets, specific inhibitors of the purified gene products were identified by high-throughput screening of chemical libraries and further developed through chemical modification. While these approaches proved successful in other therapeutic fields, they failed to yield any novel antibiotics as the new inhibitors were extruded by efflux pumps and inactive against live bacteria (Brown & Wright, 2016; Lewis, 2020).

1.4.2 The lack of drugs against non-growing bacteria.

Currently, very few antibiotics are known to effectively kill non-growing bacteria (Bumann et al., 2019). Since all antibiotics in use today have been discovered due to their ability to inhibit bacterial growth (Lewis, 2020), this indicates a significant screening bias in drug

discovery. This bias has led to a focus on compounds that target actively dividing bacteria, potentially overlooking treatments effective against non-growing cells. Consequently, the ability of antibiotics to inhibit bacterial growth does not necessarily reflect their effectiveness against non-growing subpopulations. Identifying compounds that are active against non-growing bacteria requires entirely different testing methods to address this critical gap in antibiotic efficacy.

1.4.3 Drug repurposing libraries

A possible approach to identify novel compounds with antibiotic activity is screening of already known drugs. For example, screening multiple chemical libraries led to the discovery of halicin, a non-antibiotic drug, as a novel antibiotic (Stokes et al., 2020). An extensive screen of over 1000 therapeutic drugs of all classes against 40 gut microbiome representative bacterial species also found non-antibiotic drugs showed antibacterial activity. They reported that 27% of the tested drugs inhibited the growth of at least one representative species *in vitro* (Maier et al., 2018*)*. Similarly, drug repurposing libraries can be screened for activity against non-growing bacteria. These libraries consist of drug candidates in clinical trials and drugs already approved for various therapeutic uses, not limited to just antibiotics. This provides access to drugs already characterized for use in humans or animals (Kulkarni et al., 2023). However, many of these drugs have failed due to extensive side effects (Natsheh et al., 2024), such as damage to the kidneys and liver.

1.4.4 Methods to reliably screen antibiotic activity against non-growing bacteria

To reliably screen a drug's activity against non-growing bacteria, the method must be able to distinguish between dead and living cells and detect cells that are transiently metabolically inactive. An important difference between regular antibiotic testing is that for non-growing bacteria stationary phase cultures must be used (Hazan et al., 2012).

Colony forming unit (CFU) counting (CFU assay) is a standard and often convenient method for determining number of viable cells per volume of culture. It is performed by serially diluting a culture and plating a fixed volume of the dilutions on solid growth media. After sufficient regrowth period, the colonies can be counted at the lowest dilutions where individual colonies are distinguishable. CFU per milliliter (CFU/ml) can be calculated using colony count, volume of plated sample and the dilution factor. By comparing drug-treated and drug-free controls, this assay gives an accurate representation of the tested drugs ability to reduce viable cells of non-growing bacteria. However, this method is impractical for high-throughput

screening (HTS) assays needed for screening of drug repurposing libraries due to the substantial labor and time required (Hazan et al., 2012).

A more suitable method for HTS is a Start Growth Time (SGT) assay. It uses bacterial growth determination to detect the relative number of live bacterial cells. The treated and drug-free samples are diluted into fresh media, at the same time diluting out the antibiotic residue, and optical density at 600 nm wavelength (OD600) is measured over time in a plate reader. The SGT is the time required for OD600 to reach a set threshold. The delay in regrowth between the treated and drug-free samples (ΔSGT) can be used to identify bactericidal and regrowth-delaying compounds (Hazan et al., 2012). As we discovered when validating the assay for screening, it is also possible to compare OD600 values at the timepoint when the drug-free samples have regrown and identify compounds that prevented regrowth at that stage, further simplifying the assay for use in HTS.

The SGT assay is much simpler and less labor intensive than other methods, such as flow cytometry and microscopy which would be impossible to use in HTS. However, the delay of regrowth in the SGT assay can be caused not only due to killing of non-growing bacteria, but also due to post-antibiotic effect (PAE). It refers to a period of time after the antibiotic is completely removed from both intra- and extracellular environment of bacteria during which the bacteria do not restart growth (Srimani et al., 2017). As there is no way to determine whether the antibiotic is really reducing the viable cell count or if we are detecting PAE using this assay, a further cross-validation of hit compounds is necessary using a CFU assay.

2. THE AIMS OF THE THESIS

After previously identifying 39 hit compounds active against non-growing uropathogenic *E. coli* in a high-throughput screening, we further tested the dose-dependent analysis of regrowth delay and bacterial killing of these antibacterial drugs against uropathogenic *E. coli*. Afterwards we tested these drugs against *P. aeruginosa* and *S. aureus*, selecting the 24 most active compounds for further dose-dependent analysis of regrowth delay and bacterial killing. The aims of the thesis are to:

- Assess the statistical significance of the regrowth delay caused by antibacterial drug treatments against all three species of non-growing bacteria.
- Characterize the effects on the regrowth delay caused by antibacterial drug treatment against the non-growing bacteria based on:
    - Drug treatment duration
    - Testing conditions
    - Activity of efflux pumps
- Test how to proceed with research of potential drug candidates

3. EXPERIMENTAL PART

3.1. MATERIALS AND METHODS

3.1.1. Bacterial strains

The bacterial strains used in this study were *Escherichia coli* CFT073 (UPEC), *Pseudomonas aeruginosa* DSM1117 (also known as Boston 41501, ATCC 27853), and *Staphylococcus aureus* DSM2569, all obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), as well as, *E. coli* BW25113, sourced from the Coli Genetic Stock Center (CGSC).

3.1.2. Media and growth conditions

Overnight cultures of UPEC, *P. aeruginosa* and *S. aureus* were grown in test tubes containing 3 ml of Lysogeny broth (LB) broth (~16 h), then diluted 1:100 into either 1:4 diluted Cation-Adjusted Mueller-Hinton Broth(CAMHB) medium buffered with 40mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4) or, in case of UPEC, into Low Phosphate, Low Magnesium (LPM) medium supplemented with 49 μM $MgCl_2$ and buffered with 30mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 5.5). The cultures were incubated for 24 h in non-baffled Erlenmeyer flasks at 37 °C with shaking at 200 rpm.

Overnight cultures of UPEC, *P. aeruginosa,* and *S. aureus* were grown in 3 mL of LB broth for approximately 16 hours. These cultures were then diluted 1:100 into either 1:4 diluted CAMHB medium buffered with 40 mM HEPES (pH 7.4), or, for UPEC, into Low Phosphate, Low Magnesium (LPM) medium (Coombes et al., 2004) supplemented with 49 μM $MgCl_2$ and buffered with 30 mM MES (pH 5.5). Cultures were incubated at 37 °C in non-baffled Erlenmeyer flasks with shaking at 200 rpm for 24 hours.

3.1.3. Dose-response analysis using the dilution-regrowth assay

For UPEC, dose–response analysis was conducted at the CBCS screening facility. Bacteria were cultivated, and the dilution-regrowth assay was performed according to the HTS protocol (See methods in the article in the Appendix). Compounds were dispensed into 384-well plates using the Echo® (Labcyte) system, and regrowth was assessed by measuring OD600 after 6 h of incubation at 37 °C in humidified boxes. Three biological replicates were performed.

For *P. aeruginosa* and *S. aureus*, dose–response analysis was conducted in 96-well plates with a 200 μl assay volume, following the procedure used for screening assay validation experiments. Aliquots of the 39 compounds active against non-growing UPEC were obtained

from SelleckChem and MedChem Express. Stock solutions were prepared and stored according to the manufacturers' instructions, then diluted in 10% Dimethyl Sulfoxide (DMSO) before use. Cultures were incubated for 24 h in 1:4 diluted CAMHB medium under the same conditions used for UPEC cultivation. In preliminary experiments, each compound was tested at a concentration of 20 µM in the dilution-regrowth assay with 4 technical replicates. Regrowth was monitored using a microplate reader at 37 °C with continuous shaking, and OD600 was recorded every 20min. The time at which OD600 exceeded the threshold of 0.12 was recorded as the Start-Growth-Time. Based on these results, 24 of the most active compounds were selected for further dose–response analysis, which was performed in three biological replicates using the same procedure.

3.1.4. Dose-dependent killing analysis

For survival analysis, bacteria were cultivated as described for the dose–response dilution-regrowth assay. Assays with UPEC were conducted at the CBCS screening facility using 96-well plates with a 200 µl sample volume, and compounds were dispensed using the Echo® (Labcyte) system.

P. aeruginosa and *S. aureus* samples were treated according to the protocol used for the dilution-regrowth experiments. Treated samples were serially diluted 10-fold in PBS, and 10 µl drops were spot-plated onto rectangular LB agar plates using either a Beckman NXP robot (for UPEC) or an Integra VIAFLO96 electronic pipette (for P. aeruginosa and S. aureus). The LB agar plates were incubated overnight at 37 °C and scanned. Colonies were counted at the spots of the lowest dilution where individual colonies could be accurately distinguished. Each colony was manually marked on a separate image layer using the GNU Image Manipulation Program (GIMP) and counted using NIST's Integrated Colony Enumerator (NICE 1.4) software. The experiments were performed in three biological replicates.

3.1.5. Data analysis

Statistical analysis of dilution-regrowth data was conducted using GraphPad Prism 10.2.3. Statistical significance of the drug effects was assessed using a one-way ANOVA followed by Dunnett's test, comparing each treatment to the drug-free control.

3.1.6. Time-dependent effects on drug treatment

UPEC, *P. aeruginosa* and *S. aureus* were cultivated in 1:4 diluted CAMHB (40mM HEPES, pH 7.4) for 24 h as described before. Aliquots were dispensed into a 96-well plate, and

compounds were added at a 10 μM concentration. Samples were collected at designated time points, diluted 2500-fold as described for the dilution-regrowth assay, and incubated in standing plates within a humid box at 37 °C. The OD600 of the samples was measured to assess regrowth using a microplate reader (BioTek Synergy H1) after 6 h for UPEC and 8 h for P. aeruginosa and S. aureus. The experiment was performed in triplicates.

3.1.7. Testing condition-dependent effects on drug treatment

E. coli CFT073 and BW25113 were grown in 3 ml of LB at 37 °C, shaking at 200 rpm for 16 h. The overnight cultures were then diluted either 1:100 into 40 ml of 1:4 diluted CAMHB (40mM HEPES, pH 7.4) or 1:10,000 into 40 ml of 1% LB in PBS and grown in 125 mL Erlenmeyer flasks for 24 h at 37 °C, shaking at 200 rpm. Following this, 1ml aliquots of each culture were transferred to 1.5 ml test tubes, and semapimod was added at final concentrations

of 20 μM and 50μM. DMSO was added to drug-free controls. To quantify live bacteria before treatment, a sample of each culture was serially 10-fold diluted into PBS, and 10 μL drops were spot-plated onto LB agar. The closed test tubes were incubated for 24 h at 37 °C without shaking. After treatment, the samples were diluted 2500-fold in two steps: a 50-fold dilution into PBS, followed by a 50-fold dilution into CAMHB in a 96-well plate with a total volume of 200 μl per well. The 96-well plate was then incubated in a BioTek Synergy H1 plate reader at 37 °C with continuous shaking at medium speed for 22 h with a lid. OD600 was recorded every 20 min. Additionally, the treated samples were diluted 1:200 into LB in another 96-well plate and incubated in a BioTek SynergyMx plate reader at 37 °C without shaking for 22 h, with OD600 recorded every hour. To determine the number of live bacteria after the treatment, the bacteria were pelleted, washed twice with PBS, serially diluted, and spot-plated. Plates were incubated and colonies counted as described previously. The experiment was performed in triplicates.

3.1.8. Efflux pump-dependent effects on drug treatment

E. coli CFT073 and *P. aeruginosa* were cultivated in 1:4 diluted CAMHB (40mM HEPES, pH 7.4) for 24 h as described before. Aliquots were dispensed into a 96-well plate, and compounds were added at a 2.5 μM concentration or at 2.5 μM concentration supplemented with 10 μg/ml PAβN pump inhibitor, and a drug-free control. Samples were incubated in standing plates within a humid box at 37 °C for 24 h, then diluted 2500-fold as described for the dilution-regrowth assay, and besides a sample that contained CAMHB supplemented with 10 μg/ml PAβN. The OD600 of the samples was measured every 20 minutes over 22 hours to

assess regrowth using a microplate reader (BioTek Synergy H1). The experiment was performed in triplicates.

3.2. RESULTS

3.2.1. Assessing statistical significance of regrowth delay

Previously we identified 39 compounds active against non-growing UPEC by screening 2 drug repurposing libraries (Specs Repurposing Library and Prestwick Library) using an HTS assay in 2 different growth media (See 3.1.2) and measuring the OD600 after 6 hours of regrowth. We then analyzed dose-dependent dilution-regrowth (See 3.1.3) effects of these 39 compounds against UPEC and performed additional dose-dependent killing analysis (See 3.1.4) to confirm that regrowth delays caused by these compounds are not caused by Post Antibiotic Effect (Heatmaps in Figures 3 and 4). Afterwards we tested the regrowth delay efficacy of these 39 compounds against non-growing *P. aeruginosa* and *S. aureus* using a dilution-regrowth assay similar to the HTS assay at 20 μM drug concentrations in and selected 24 compounds with the largest regrowth delay against each of the strains for further dose-dependent regrowth delay and killing analysis (Heatmaps in Figure 5). As we performed the dilution-regrowth assays against *P. aeruginosa* and *S. aureus* with a smaller number of compounds, we utilized the Start-Growth-Time parameter instead of end-point measurement (See Chapter 1.4.4) to characterize the delay of regrowth. After seeing the results of the regrowth assays, we wanted to confirm that our findings are statistically significant. To do so, I performed data analysis of the results.

The OD600 measurements after 6 hours of regrowth were compared between UPEC treated with the compounds and drug-free controls using One-Way ANOVA followed by Dunnett's test and p-values were recorded (Asterisks in Figure 3 and 4). For *P. aeruginosa* and *S. aureus* (Asterisks in Figure 5) the data analysis was performed in a similar manner, except using the 8 hour time point of regrowth, as these two species were observed to have longer division times than UPEC. This also highlights why using SGT is useful – the ΔSGT is better comparable between species compared to endpoint OD600 measurements.

From the resulting statistical analysis, we can observe that for all major delays of regrowth against all three tested species, the p-values of the tested drugs compared to the drug-free control are below 0.0001. For some drugs at concentrations where the delay is not as pronounced or is extremely minor, we still often observe p-values below 0.05.

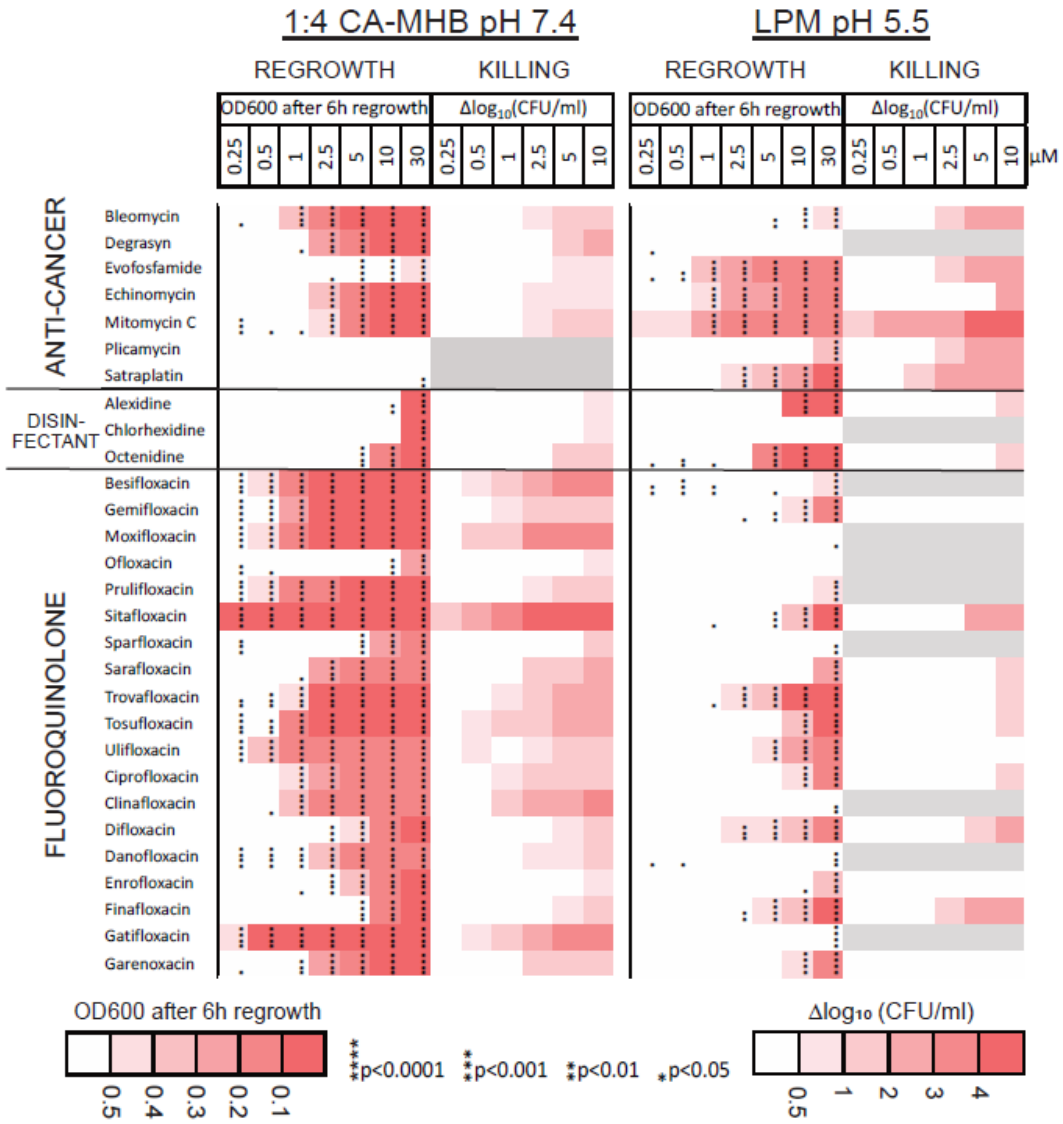

Figure 3. **Regrowth delay and killing of non-growing UPEC.**
Heatmap showing the regrowth delay and killing of non-growing UPEC CFT073 by validated hit compounds. Gray areas indicate compounds not tested for killing due to weak activity in regrowth delay experiments. Bacteria were grown in 1:4 CAMHB (pH 7.4) or LPM (pH 5.5) for 24 h and then treated with the compounds at the indicated concentrations for 24 h. Regrowth data were obtained by measuring OD600 6h after a 2,500-fold dilution of the samples into CAMHB. Killing data were determined by spot-plating serial dilutions of the treated samples on LB agar plates followed by colony counting. The differences in CFU/mL between the drug-treated samples and the drug-free control are shown. Data represents the mean of three biological replicates. The significance of the regrowth delay was assessed using one-way ANOVA followed by Dunnett's test, comparing each treatment to the drug-free control. Asterisks denote p-values from the Dunnett's test.

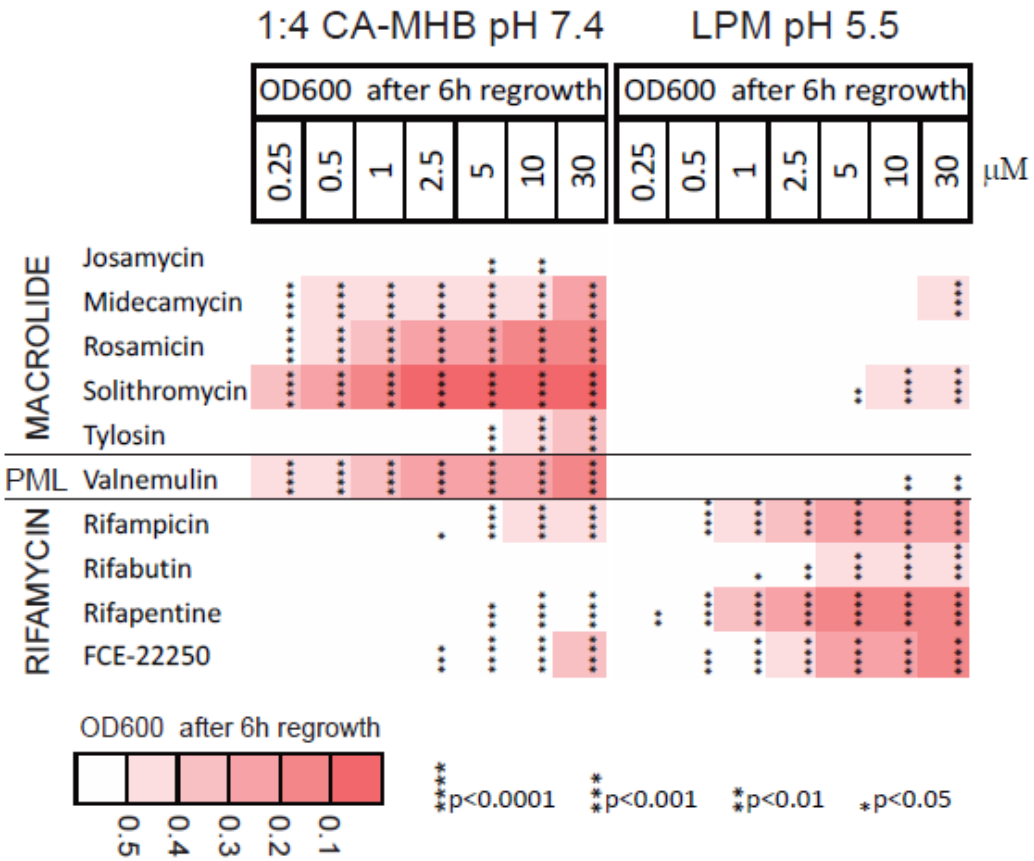

Figure 4. **The post-antibiotic effect on non-growing UPEC.**
Heatmap showing the regrowth delay of non-growing UPEC CFT073 by validated hit compounds that did not kill the stationary phase bacteria. Cultures were grown in 1:4 CAMHB (pH 7.4) or LPM (pH 5.5) for 24 h and then treated with the compounds at the indicated concentrations for another 24 h. OD600 was measured 6 h after a 2,500-fold dilution in CAMHB. PML refers to pleuromutilin. Data represents the average of three biological replicates. Regrowth delay significance was assessed using one-way ANOVA and Dunnett's test, with comparisons made against the drug-free control. Asterisks indicate p-values from Dunnett's test.

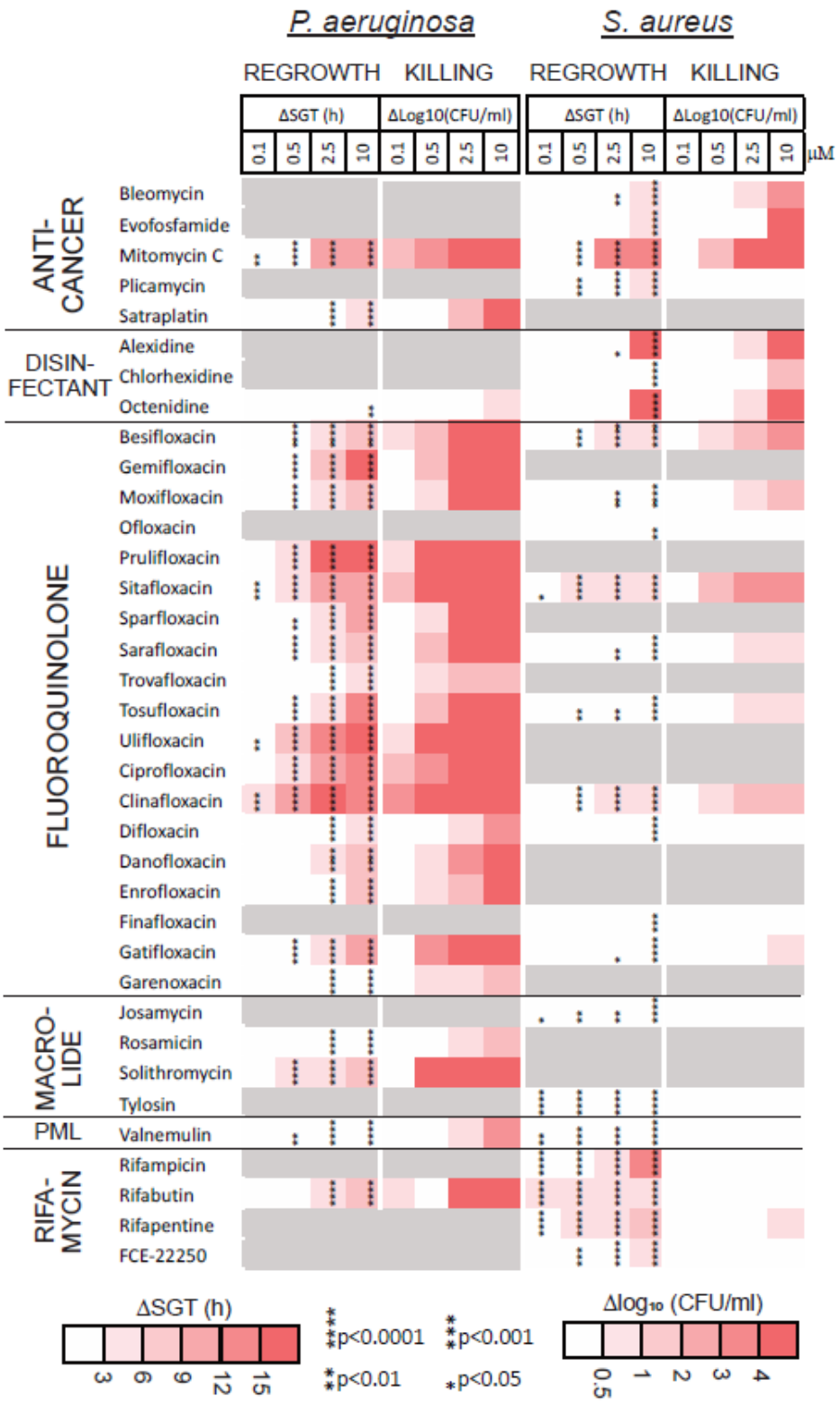

Figure 5. **Regrowth delay and killing of non-growing *P. aeruginosa* and *S. aureus*.**
Regrowth delay and killing of non-growing *P. aeruginosa* DSM1117 and *S. aureus* DSM2569 by compounds effective against non-growing UPEC. Gray areas in the heat map indicate compounds not tested due to inactivity in preliminary experiments. Cultures were grown in 1:4 CAMHB (pH 7.4) for 24 h followed by treatment with the compounds at the indicated concentrations for an additional 24 h. To assess regrowth, OD600 was measured on a microplate reader after a 2,500-fold dilution into CAMHB. The time required for cultures to reach an OD600 threshold of 0.12 is reported as the Start-Growth-Time (SGT). Bacterial killing was determined by spot-plating serial dilutions of the treated

samples on LB agar plates followed by colony counting. The differences in CFU/mL between drug treated samples and the drug-free control are presented. Data represent the average of three biological replicates. The significance of regrowth delay was evaluated using OD600 measurements taken 8 h after dilution into drug-free media. Statistical significance was assessed using one-way ANOVA followed by Dunnett's test, comparing each treatment to the drug-free control. Asterisks denote p-values from Dunnett's test.

3.2.2. Time-dependent effects on drug treatment

For both screening and dose-dependent characterization of the hit compounds, we used 24-hour incubation time to robustly capture the drug effect. However, the actual drug activity may occur earlier. To understand the speed at which the treatment affects the regrowth delay of non-growing bacteria, we modified the dilution-regrowth assay (See Chapter 3.1.6) and tested some of the drugs with strong regrowth delay against UPEC, *P. aeruginosa* and *S. aureus* at 10 μM concentration (Fig. 6). Due to constraints in laboratory equipment availability and the significant amount of time points to be tested, we returned to measuring endpoint OD600 after 6- and 8- hour regrowth for UPEC and *P. aeruginosa, S. aureus* respectively. Hence, the drugs were selected if their OD600 remained under 0.12 after the 6-/8-hour regrowth in drug-free medium, based on previous tests. We then collected samples over different timepoints of 24 hour drug incubation, diluted them 2500-fold and measured OD600 after regrowth.

As we can observe from Fig. 6, most of the tested drugs were acting rapidly, causing regrowth delay in all three organisms within the first hour of treatment. Some compounds acted more slowly, such as bleomycin and garenoxacin against *E. coli*, difloxacin, trovafloxacin and satraplatin against *P. aeruginosa.* Rifampicin acted quickly against *S. aureus.* Meanwhile, the effects of all other compounds against *S. aureus* increased over treatment time but were already apparent within the first hour.

These results show that the most effective compounds penetrate the non-growing bacteria quickly, most exhibiting antibacterial effect within one hour of treatment.

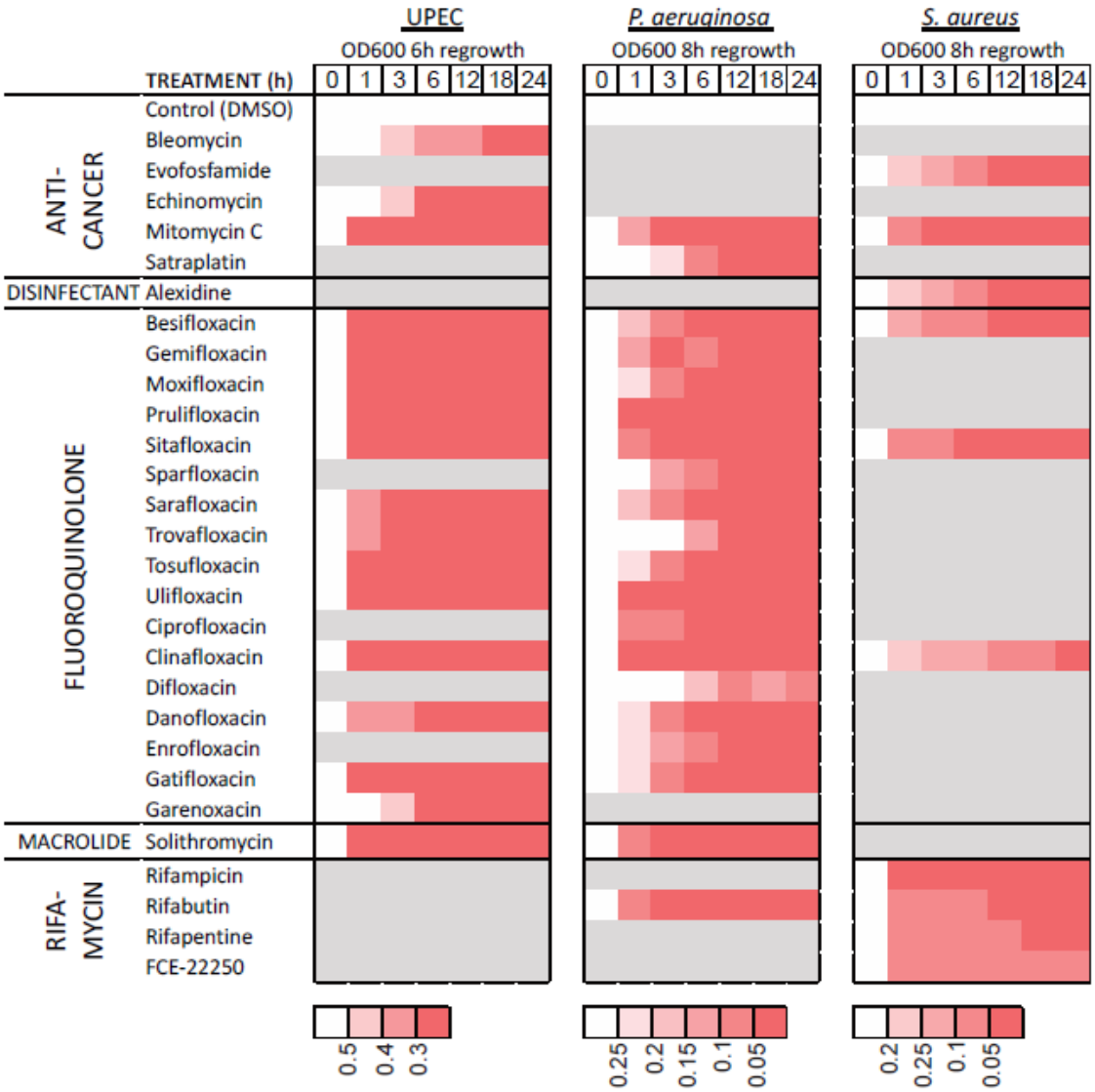

Figure 6. **Time course of antibacterial effects on non-growing bacteria.**
Bacteria were cultivated in 1:4 CAMHB (pH 7.4) for 24 h and the compounds were added at a concentration of 10 µM. Samples were taken at the indicated time points, diluted 2,500-fold into CAMHB, and OD600 was measured after 6h of incubation for UPEC CFT073 and 8h for P. aeruginosa and S. aureus. The color scale for OD600 values corresponding to each organism is shown below its respective heat map. Gray areas in the heat map indicate compounds not tested due to inactivity observed in previous experiments. Data represents the average of three biological replicates.

3.2.3. Testing condition-dependent effects on semapimod treatment

A similar study on non-growing bacteria (Zheng et al., 2024) identified semapimod (an investigational new drug) as having activity against non-growing *E. coli* BW25113. Despite semapimod being in the drug libraries we screened, it wasn't among our hit compounds. To investigate this situation, we combined the testing assay they used and our own (see Chapter 3.1.7.). We identified major differences between our assay and the assay used by Zheng et al. Namely, bacterial strain (CFT073 vs BW25113), drug concentration (20 vs 50 µM), growth media (1:4 CAMHB buffered with HEPES pH7.4 vs PBS+1% LB) and regrowth media along

with shaking conditions (CAMHB with shaking vs LB in standing plate). We tested both the delay of regrowth and the killing of the non-growing bacteria to compare all these different testing conditions (Fig. 7).

We found no differences between the strains and regrowth conditions, and a minor difference between drug concentrations. However, we observed that the bactericidal effect of semapimod against non-growing *E. coli* is heavily influenced by growth media, specifically the effect caused by the phosphate buffer-based medium.

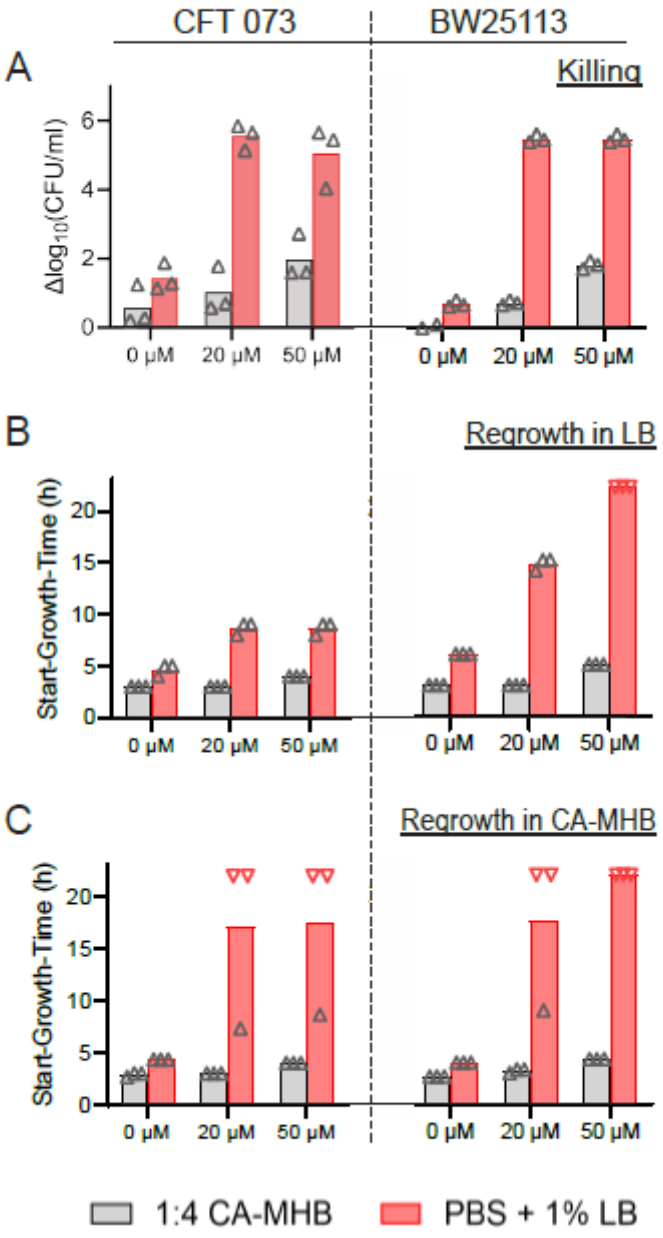

Figure 7. ***The b*****actericidal effect of semapimod on non-growing *E. coli* depends on a phosphate buffer-based culture medium.**

E. coli strains CFT073 and BW25113 were cultivated in either 1:4 CAMHB pH 7.4 (gray columns) or PBS supplemented with 1% LB (pink columns) for 24 h, followed by treatment with 20 μM or 50 μM semapimod for an additional 24 h.
(A) To estimate survival, samples were serially diluted and spot-plated on LB agar. The differences in CFU/ml before and after treatment are presented. The average log bacterial densities before treatment (log_{10}CFU/ml ± SEM) were 9.97 ± 0.63 for CFT073 grown in 1:4 CAMHB, 9.49 ± 0.01 for BW25113 grown in 1:4 CAMHB, 7.74 ± 0.05 for CFT073 grown in PBS + 1% LB, and 7.49 ± 0.07 for BW25113 grown in PBS + 1% LB. Red symbols represent individual experiments where bacterial counts fell below the detection limit (no colonies from the undiluted sample). The maximum detectable decrease in CFU/ml for these samples is indicated and used for calculating average Δ log10CFU/ml.
(B, C) To assess regrowth ability, samples were diluted 2,500-fold into LB broth (B) and CAMHB (C). OD600 was measured periodically for 22 h post-dilution. The time cultures reached an OD600 threshold of 0.12 is reported as the Start-Growth-Time (SGT). Red symbols represent individual experiments where cultures did not reach the threshold during 22 h. For these samples, an SGT of 22 h is indicated and used to calculate the average SGT. Data are presented as means for n=3.

3.2.4. Efflux pump-dependent effects on drug treatment

Efflux pumps are membrane protein complexes that may confer resistance to bacteria by removing drugs from the cell cytoplasm (See chapter 1.3.1.1). To test if their activity is also involved in drug efficacy against non-growing bacteria, we performed a dilution-regrowth assay with gatifloxacin and solithromycin – 2 antibiotics that showed an increase in regrowth delay at 2.5 μM concentration against non-growing UPEC and *P. aeruginosa* (See chapter 3.1.8). We tested four conditions by using an efflux pump inhibitor PAβN:

- PAβN was supplemented during drug treatment, but diluted out before regrowth.
- PAβN wasn't supplemented during drug treatment and wasn't present during regrowth.
- PAβN wasn't supplemented during drug treatment, but was added to regrowth media.
- Drug-free control where efflux pump inhibitor PAβN wasn't supplemented either during drug incubation time or during regrowth.

The drug-free control acts as a baseline to determine whether regrowth delay happens. As we see from Fig. 8, there is no difference between the regrowth delay of the non-growing bacteria using the pump inhibitor during drug treatment versus regular drug treatment for either drug or strain. However, a minor difference is seen when the pump inhibitor is added to the regrowth media following a regular drug treatment. These findings suggest that while the efflux pumps have some effect on the regrowth of the bacteria if present in the media after the treatment, however, there is no evidence that this mechanism works during the treatment the non-growing bacteria.

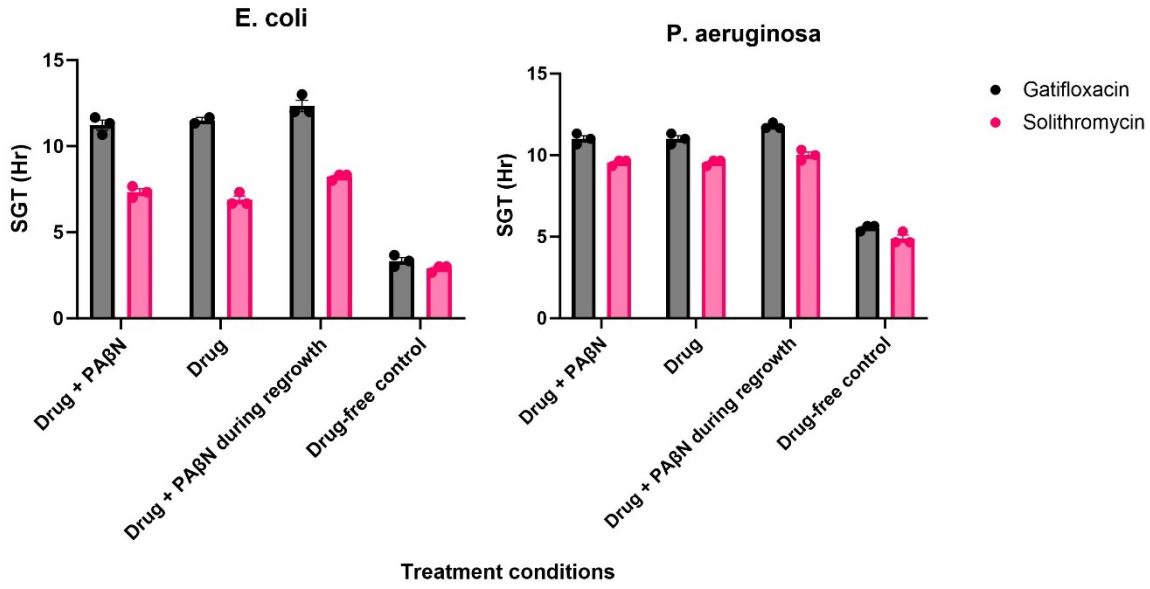

Figure 8. **Delay of regrowth caused by antibacterial drug and supplementation of pump inhibitor PAβN during different stages of testing.**
UPEC and *P. aeruginosa* were cultivated in 1:4 CAMHB (pH 7.4) for 24 h and the compounds were added at a concentration of 2.5 μM or a concentration of 2.5 μM supplemented with 10 μg/ml PAβN, alongside a drug-free control. Samples were taken after 24 hour incubation, diluted 2,500-fold into CAMHB or CAMHB supplemented with 10 μg/ml PAβN and incubated in a plate reader. OD600 was measured every 20 minutes and the time required for cultures to reach an OD600 threshold of 0.12 is reported as the Start-Growth-Time (SGT ± SEM). Symbols represent individual experiment replicate results. Gray bars indicate treatment with gatifloxacin and pink bars – solithromycin. Data represents the average of three biological replicates, except for gatifloxacin-only treated *E. coli*, where the data represents the average of two biological replicates.

3.2.5. Delafloxacin treatment effect against non-growing bacteria

In an attempt to further research potential drug candidates and how to identify them, we considered the difficulty of drug penetration into non-growing bacteria as one of the major obstacles in drug efficacy against non-growing bacteria. The largest class of the hit compounds discovered by the initial screen were fluoroquinolones (19 out of 39) (Fig. 3.) which have been shown to have increased penetration due to halogenation of the compound (Rusu et al., 2021). Therefore, we decided to test a fluoroquinolone antibiotic delafloxacin which is approved in US and EU and which wasn't present in either of the tested drug repurposing libraries. Delafloxacin has an additional fluorine atom compared to sitafloxacin - a fluoroquinolone with the strongest antibacterial effects against non-growing UPEC and *S. aureus* and second strongest effects against *P. aeruginosa* amongst the 19 hit fluoroquinolones (Fig. 3 and 5). We hypothesized that perhaps delafloxacin may yield better potency of treatment against non-growing bacteria compared to sitafloxacin due to the extra fluorine atom.

To assess this hypothesis, we performed dose-dependent analysis using dilution-regrowth and killing assays against UPEC, *P. aeruginosa* and *S. aureus* with both delafloxacin

and sitafloxacin (Fig. 9). The repetition of sitafloxacin testing was done to ensure equal testing conditions.

We found that delafloxacin treatment against non-growing UPEC had some bactericidal effect at the two highest tested concentrations, but much less than sitafloxacin. Against non-growing *P. aeruginosa* delafloxacin had no antibacterial effect at all. Meanwhile, against the non-growing *S. aureus*, the bactericidal effect was minor, effectively reducing the viable bacterial count by approximately 1 $\log_{10}$(CFU/ml) at 10 µM concentration, and the delay of regrowth, while also minor, was still comparable to that of sitafloxacin. These results clearly show that delafloxacin is inferior to sitafloxacin in treatment against non-growing bacteria. Note that we cannot say this is because of the extra fluorine atom, as the compounds have other structural differences.

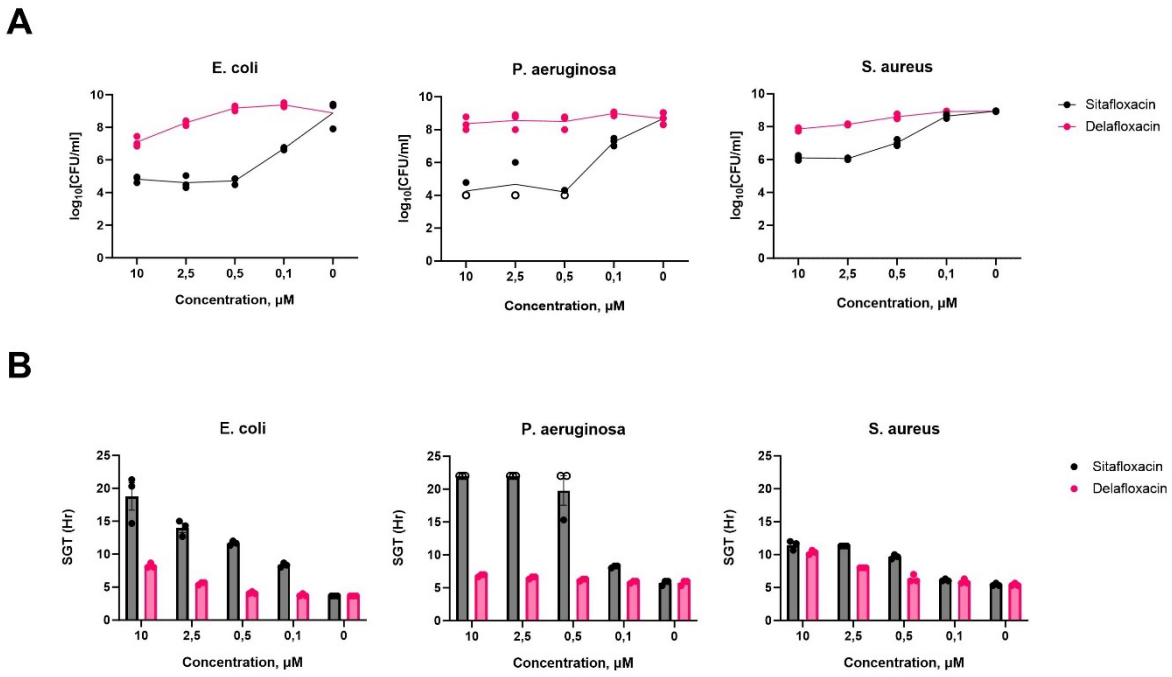

Figure 9. **Regrowth delay and killing of non-growing bacteria treated with delafloxacin and sitafloxacin.**
Regrowth delay and killing of non-growing *E. coli* CTF073, *P. aeruginosa* and *S. aureus* by delafloxacin and sitafloxacin. Cultures were grown in 1:4 CAMHB (pH 7.4) for 24 h followed by treatment with the compounds at the indicated concentrations for an additional 24 h. (A) Bacterial killing was determined by spot-plating serial dilutions of the treated samples on LB agar plates followed by colony counting. Data represent the average of three biological replicates. Symbols indicate individual replicates, with non-filled symbols representing replicates where bacterial counts fell under the limit of detection and as such are set to be the limit of detection ($\log_{10}$(CFU/ml) = 4) for purposes of calculating the mean. (B) To assess regrowth, OD600 was measured on a microplate reader after a 2,500-fold dilution into CAMHB. The time required for cultures to reach an OD600 threshold of 0.12 is reported as the Start-Growth-Time (SGT). Data represent the average of three biological replicates. Symbols indicate individual replicates, with non-filled symbols representing replicates where OD600 measurement didn't reach the set threshold during the 22 hours of regrowth, and as such are set to be the limit of incubation time (SGT = 22 h) for the purpose of calculating the mean.

3.3. DISCUSSION.

This study aimed to deepen our understanding of antimicrobial efficacy against non-growing bacteria, a critical area often overlooked in traditional antibiotic development. Our approach involved repurposing existing drugs, testing and analyzing their efficacy based on dose-dependent regrowth delays and bacterial killing, analyzing their time-dependent effects, investigating the influence of testing conditions on semapimod, exploring the role of efflux pumps, and evaluating the impact of specific structural modifications on drug potency.

Questioning the necessity for statistical analysis when the regrowth delays of the more effective drugs are as major as in our case is quite valid. Indeed, for the strongest drug effects, the visual evidence might seem sufficient. However, it is important to understand the difference between the magnitude of an effect (effect size) and its reliability (statistical significance). While effect size reflects the practical importance or magnitude of differences found, statistical significance examines how likely the observed results are due to random chance. For all major regrowth delays across the three non-growing bacteria, p-values were consistently below 0.0001, demonstrating the robustness and reproducibility of the assay. Even for drugs and concentrations that had smaller regrowth delays , there were cases where p-values were below 0.05. This shows that the drug effects at these concentrations are genuine and consistent, although the scale of the effect for some drugs at lower concentrations was relatively too small for us to consider the treatment to be effective.

For dose-dependent regrowth delay analysis, we used single-timepoint OD600 measurements instead of Start-Growth-Time (SGT). While SGT provides an easily understandable visual representation of the regrowth, statistical analysis complexity increases as it is a value representing time intervals of 20 minutes during which we detect if the OD600 has reached a threshold (OD600 = 0.12), whereas the OD600 are simple values measured at the same time. The choice of One-Way ANOVA followed by Dunnett's test was deliberate and well-suited for our experimental design, as we were bound to run into the multiple testing problem with how many samples we wanted to compare to the control group. The method allowed for robust comparison of numerous treatment groups against a single drug-free control while accounting for the multiple testing problem. For dose-dependent bacterial killing analysis, we deliberately decided not to perform statistical analysis, as comparing the CFU counts over different dilutions introduces the need to account for additional errors, such as pipetting and counting errors, and stochastic bacterial counts in samples, overcomplicating the analysis (Christen & Parker, 2020; Martini et al., 2024).

The time-course experiments provided insights into the speed of drug action against non-growing bacteria. Most tested compounds exhibited rapid effects, inducing regrowth delay in all three organisms within the first hour of treatment. While some compounds, such as bleomycin and garenoxacin against *E. coli*, and difloxacin, trovafloxacin, and satraplatin against *P. aeruginosa*, acted more slowly, the swift action observed for the majority of effective compounds is highly promising from a therapeutic perspective.

The aim of antibiotic development is ultimately to provide effective therapies. For this, the pharmacokinetic (PK) and pharmacodynamic (PD) characteristics of a drug must be integrated. Antibiotics, based on their bactericidal activity, generally fall into one of two major patterns: time-dependent killing or concentration-dependent killing (Jacobs, 2001). If an antibacterial effect is achieved rapidly, as observed for most of the tested compounds, it can allow for more flexible dosing regimens in clinical practice. This could mean administering smaller doses or less frequently, as there would be less needed to maintain high plasma concentrations of the drug in the patient for extended periods. Consequently, this rapid action can lead to reduced overall toxicity and fewer side effects, significantly improving patient outcomes.

While our initial screen did not identify semapimod as a hit, a similar study by Zheng et al. (2024) reported its activity against non-growing *E. coli*. We identified differences between bacterial strain, drug concentrations, growth media, and regrowth media/shaking conditions by comparing our assay conditions with theirs. Our findings indicated no substantial differences attributable to bacterial strain or regrowth conditions, and only minor differences due to drug concentration. However, the most striking observation was the heavy influence of the growth medium, particularly the phosphate buffer-based medium, on semapimod's bactericidal effect against non-growing *E. coli*.

This suggests that the presence of a phosphate buffer-based medium likely enhances semapimod's activity. This enhancement is possibly due to the huge excess of phosphate in the buffer, which can chelate divalent cations like Mg^{2+} from the bacterial outer membrane. Electrostatic interactions between divalent cations and phosphate substituents of the lipid A backbone provide overall stability to the supramolecular structure of lipopolysaccharide (LPS) in Gram-negative bacteria (Clifton et al., 2015). By titering out these Mg^{2+} ions, the phosphate buffer damages the LPS, rendering the outer membrane more vulnerable to membrane-targeting compounds like semapimod. This observation is consistent with findings from Zhang et al. (2024), who reported that semapimod’s activity is abolished in the presence of excess Mg^{2+}. These results underscore the paramount importance of carefully standardized and reported

assay conditions, as even subtle differences in treatment environment can impact the observed efficacy of antibacterial drugs.

Efflux pumps are well-established mechanisms of antibiotic resistance in actively growing bacteria, functioning to expel drugs from the cell cytoplasm. To determine their involvement in drug efficacy against non-growing bacteria, we conducted experiments using the efflux pump inhibitor PAβN with gatifloxacin and solithromycin. Our results indicate that the activity of efflux pumps during the treatment phase of non-growing bacteria appears limited. We observed no significant difference in regrowth delay when PAβN was supplemented during drug treatment but diluted out before regrowth, compared to regular drug treatment. This suggests that efflux pumps may not be highly active or may not play a dominant role in drug removal under non-growing conditions.

A limitation of this particular experiment is that MIC measurements for growing cells in the presence and absence of PAβN were not presented for these specific drugs, which would have provided direct evidence of whether gatifloxacin and solithromycin are indeed substrates of the efflux pump targeted by PAβN in actively growing bacteria. This information would further strengthen the interpretation of their role in non-growing cells. Interestingly, a minor increase in regrowth delay was observed when PAβN was added to the regrowth media after the drug treatment. This subtle effect suggests that efflux pumps might exert either some toxic activity or some residual drugs are not removed due to efflux pump inhibition during the initial stages of bacterial regrowth, potentially impacting the overall delay. However, the primary conclusion remains that their contribution during the treatment of non-growing bacteria is minimal. Future studies involving genetic knockouts of efflux pump-encoding genes could provide more definitive insights into their role in non-growing bacterial populations.

Fluoroquinolones constituted the largest class of hit compounds (19 out of 39) in our initial screen. A characteristic often attributed to fluoroquinolones is their enhanced membrane penetration facilitated by halogenation (Rusu et al., 2021). To further investigate the relationship between heavily halogenated fluoroquinolones and antibacterial potency against non-growing bacteria, we tested delafloxacin, a fluoroquinolone with an additional fluorine atom compared to sitafloxacin (Fig. 10), which demonstrated strong effects in our initial screen. We hypothesized that having an additional halogen might confer delafloxacin better activity against the non-growing bacteria.

However, our dose-dependent analysis revealed that delafloxacin was generally inferior to sitafloxacin in treating non-growing bacteria. While delafloxacin showed some bactericidal effect against *UPEC* at the highest concentrations, it was considerably less potent than sitafloxacin. Against non-growing *P. aeruginosa*, delafloxacin exhibited no antibacterial effect. For non-growing *S. aureus*, its bactericidal effect was minor (approximately 1 $\log_{10}$ CFU/mL reduction at 10 μM), and the regrowth delay, though also minor, was comparable to sitafloxacin. These findings demonstrate that delafloxacin is less effective than sitafloxacin against the tested non-growing bacterial strains.

The structural evolution from first-generation quinolones like nalidixic acid (without fluorine) to later-generation fluoroquinolones such as ciprofloxacin, sitafloxacin, and delafloxacin (with increasing numbers of halogen atoms) (Fig. 10), typically correlates with improved membrane penetration and enhanced potency (Ball, 2003; Rusu et al., 2023). However, as delafloxacin is structurally different from sitafloxacin, it is impossible to assess what is causing the difference in treatment activity without further testing.

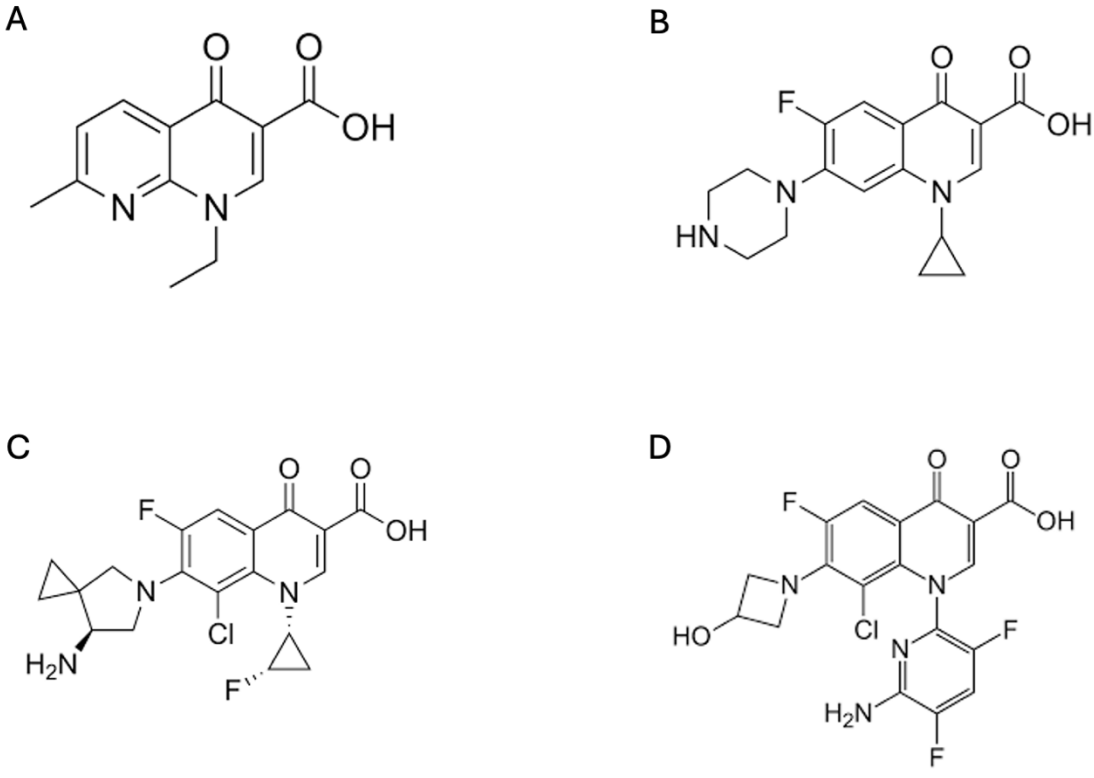

Figure 10. Chemical structures of quinolones and fluoroquinolones. (A) Nalidixic acid – first generation quinolone, without any halogen atoms. (B) Ciprofloxacin – second generation fluoroquinolone, 1 fluorine atom. (C) Sitafloxacin – fourth generation fluoroquinolone, 2 fluorine and 1 chlorine atoms. (D) Delafloxacin – fourth generation fluoroquinolone, 3 fluorine and 1 chlorine atom.

4. SUMMARY

Previously, in an attempt to find antibacterial drugs exhibiting activity against non-growing bacteria, prevalent in chronic and recurrent infections, we screened over 6400 drugs and drug candidates from Prestwick and SPECS drug repurposing libraries. We identified 39 hit compounds active against non-growing uropathogenic *E. coli* in neutral and/or acidic media, mimicking extracellular and acidic intravacuolar conditions. We then further tested dose-dependent regrowth delay and bacterial killing effects of these compounds against UPEC, as well as tested 24 of the most active compounds against *P. aeruginosa* and *S. aureus*

By assessing the statistical significance of the regrowth delay caused by the antibacterial drug treatments against all three tested non-growing bacteria, we show that the assay for dose-dependent analysis of dilution-regrowth is sufficiently robust to claim that the results of strong drug activity were not stochastic. We observed p-values below 0.0001 for all major delays of regrowth, displaying that the drug effects at these concentrations where the delays occur are both robust and reproducible. As we evaluate the scale of the effect size by the entire dataset, we sometimes observe p-values below 0.05 for minor delays of regrowth, confirming that there is consistent drug activity at these concentrations, yet simply the delays of some other drugs or some other concentrations are much higher.

The default drug incubation duration was set to 24 hours for both screening and the subsequent dose-dependent testing of hit compounds. We then characterized the effects that drug treatment duration had on regrowth delay caused by some of the compounds exhibiting the strongest activity at 10 μM concentrations in previous experiments. We found that most of the tested drugs were acting within the first hour of treatment, causing major regrowth delay in UPEC, *P. aeruginosa* and *S. aureus*. We also observed some compounds acting more slowly, exhibiting their effects over 3 to 6 hours of treatment, e.g., bleomycin and garenoxacin against *E. coli* and difloxacin and trovafloxacin against *P. aeruginosa*. Meanwhile, all tested drugs against *S. aureus* had visible decrease of regrowth within the first hour, and for all the tested drugs except rifampicin, the effectiveness was gradually increasing over the treatment duration. Rifampicin however exhibited close to maximum efficacy already at the 1-hour timepoint.

In a similar study on non-growing bacteria by Zheng et al., 2024), they discovered that semapimod has antibacterial activity against non-growing *E. coli*. While semapimod was also present in the drug libraries we screened, it was not amongst our hit compounds. We identified the four major differences between testing conditions in our screening assays. Namely, bacterial strain, growth media, semapimod concentration and regrowth conditions. We compared all these testing conditions by performing regrowth delay and bacterial killing assays. As both tested concentrations were considerably high, the drug concentration had a minor effect on regrowth delay and bacterial killing, but strain and regrowth conditions had none. Meanwhile, the growth media which is also the environment where drug treatment was done, accounted for all of the major differences in semapimod activity. The phosphate buffer-based medium being responsible for the increased regrowth delay and bacterial killing.

Efflux pumps, protein complexes responsible for removing toxic compounds from the cell cytoplasm, may also confer resistance to bacteria if substrate specificity allows binding of the antibiotic. To test whether efflux pump activity is also involved in drug efficacy against non-growing bacteria, we performed tested regrowth delay of two hit compounds – gatifloxacin and solithromycin – against non-growing UPEC and *P. aeruginosa* in presence of efflux pump inhibitor PAβN. We tested whether the inhibition of the efflux pumps have an effect during the drug treatment or during the regrowth following normal drug treatment. Compared to the normal drug treatment, the inhibition of efflux pumps during treatment had no effect in either species. The addition of the pump inhibitor during regrowth following a regular drug treatment yielded a minor increase in regrowth delay, suggesting some increase of toxicity during growth phase of these bacteria.

In an attempt to further research and identify potential candidates for activity against non-growing bacteria, we considered drug permeability into the non-growing bacteria as one of the mechanisms limiting drug efficacy against non-growing bacteria. As fluoroquinolones, known to have increased penetration due to halogenation of compound, were the most represented class of compounds among the hits of the initial screen, we decided to test another fluoroquinolone which wasn't present in either of the drug libraries – delafloxacin. Delafloxacin has an extra fluorine atom compared to sitafloxacin – the fluoroquinolone with highest tested antibacterial activity against non-growing UPEC and *S. aureus* and second highest against *P. aeruginosa*. We tested delafloxacin in dose-dependent regrowth delay and bacterial killing assays against UPEC, *P. aeruginosa* and *S. aureus*, hoping to see higher potency of treatment compared to sitafloxacin. And, while treatment with delafloxacin produced some delays of regrowth and reduction of viable bacteria at the highest concentrations against non-growing

UPEC and *S. aureus,* and essentially no delay of regrowth against *P. aeruginosa*, the effects were much weaker than those of sitafloxacin.

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7. APPENDIX

npj | antimicrobials & resistance

Article

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Antibacterial compounds against non-growing and intracellular bacteria

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Slow- and non-growing bacterial populations, along with intracellular pathogens, often evade standard antibacterial treatments and are linked to persistent and recurrent infections. This necessitates the development of therapies specifically targeting nonproliferating bacteria. To identify compounds active against non-growing uropathogenic *Escherichia coli* (UPEC) we performed a drug-repurposing screen of 6454 approved drugs and drug candidates. Using dilution-regrowth assays, we identified 39 compounds that either kill non-growing UPEC or delay its regrowth post-treatment. The hits include fluoroquinolones, macrolides, rifamycins, biguanide disinfectants, a pleuromutilin, and anti-cancer agents. Twenty-nine of the hits have not previously been recognized as active against non-growing bacteria. The hits were further tested against non-growing *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Ten compounds – solithromycin, rifabutin, mitomycin C, and seven fluoroquinolones—have strong bactericidal activity against non-growing *P. aeruginosa*, killing >4 $\log_{10}$ of bacteria at 2.5 μM. Solithromycin, valnemulin, evofosfamide, and satraplatin are unique in their ability to selectively target non-growing bacteria, exhibiting poor efficacy against growing bacteria. Finally, 31 hit compounds inhibit the growth of intracellular *Shigella flexneri* in a human enterocyte infection model, indicating their ability to permeate the cytoplasm of host cells. The identified compounds hold potential for treating persistent infections, warranting further comparative studies with current standard-of-care antibiotics.

All currently used antibiotics were discovered based on their ability to inhibit bacterial growth[1]. However, only a few can effectively kill non-growing bacteria, which are prevalent in chronic and recurrent infections[2]. The ability of bacteria to survive treatment with bactericidal antibiotics without proliferation is referred to as antibiotic tolerance or antibiotic persistence. These terms describe bacterial survival in the presence of a killing drug, enabling evasion of conventional antibacterial therapies. Importantly, tolerance and persistence are temporary, transient phenotypic traits that manifest in the absence of growth. This contrasts with antibiotic resistance, a genetically acquired trait that allows bacteria to grow in the presence of the drug[3]. Notably, evasion of killing through tolerance or persistence can increase the likelihood of resistance development[4,5]. Non-growing bacteria in stationary-phase cultures are inherently tolerant to most antibiotics. In growing bacterial cultures, a subpopulation of non-growing cells, known as persisters, can transiently survive exposure to bactericidal antibiotics and resume growth once the drug pressure is removed[6–8]. However, the connection between these in vitro persisters and persistent infections in the clinical settings remains uncertain and is challenging to investigate[9,10]. Furthermore, chronic infections are often caused by intracellular bacteria as well as by biofilms, which both evade the immune defense and antibiotics[11–14]. To effectively treat these infections and curb the spread of resistance, there is an urgent need for drugs that selectively target non-growing and intracellular bacteria. These drugs must be devoid of severe side effects and either possess antibacterial activity on their own or enhance conventional antibiotics.

In this study, we performed a high-throughput screening to identify compounds that selectively target non-growing Gram-negative bacteria using a dilution-regrowth assay[15]. Specifically, drug-treated stationary phase

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culture samples were diluted into fresh medium, and the time to outgrowth was measured as a proxy for the number of bacteria that survived the treatment. For this purpose, we utilized drug repurposing, a method of assessing previously approved drugs or candidates for new medical applications. Using a library of approved and candidate drugs with known safety and pharmacokinetic properties excluded reactive and toxic compounds that might otherwise come up as false hits in the screen.

In our primary screen we used uropathogenic *Escherichia coli* (UPEC), a clinically important pathogen and a model organism for other Gram-negative pathogens. UPEC is a prevalent cause of recurrent urinary tract infections (UTIs)[16], which are among the most common outpatient bacterial infections[17] and exhibit high treatment failure rates[18,19]. UPEC can reside both extracellularly and intracellularly, forming persistent intracellular bacterial reservoirs in urinary epithelial cells and macrophages[20,21]. Reactivation of quiescent bacteria in the urinary tract may be one cause of recurrent UTIs, along with potential reinfection originating from the host's intestinal tract[22–24]. To identify drug candidates targeting intracellularly persisting, intravacuolar bacteria, the screen was performed in an acidic, low-phosphate, and low-magnesium medium that is designed to mimic conditions in intravacuolar reservoirs, where quiescent UPEC resides within host cells[22,25].

After characterizing the effects of the hit compounds on non-growing UPEC, we tested their activities against stationary phase cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* to explore their potential broader use. These two pathogens were selected due to their prevalence in chronic biofilm infections[26,27]. Finally, to assess the capacity of the candidate compounds to permeate through the host cell cytoplasmic membrane barrier, we characterized their inhibitory activity against *Shigella flexneri*. Unlike UPEC, which resides in acidified vacuoles, *S. flexneri* replicates in the cytosol of intestinal epithelial cells, where the pH is neutral rather than acidic[28]. In the absence of a licensed *Shigella* vaccine, antibiotics remain essential for controlling mortality in vulnerable populations, including children under five and the elderly in low- and middle-income countries. Unfortunately, over the past two decades, multidrug-resistant and extensively drug-resistant *S. flexneri* and *Shigella sonnei* strains have emerged globally, including recently in sexually transmitted infections in the global North[29]. As a consequence, in 2024 the WHO included *Shigella* on its list of high-priority antibiotic-resistant pathogens[30].

Using stationary phase culture as a model for non-growing condition, we identified 29 candidate compounds that were not previously recognized for their ability to affect non-growing bacteria. These compounds either killed stationary-phase UPEC or delayed its outgrowth without causing bacterial death. Notably, many of these compounds were even more bactericidal against non-growing *P. aeruginosa*, while some were active also against *S. aureus*.

Results

A dilution-regrowth screen identifies compounds that delay the regrowth of non-growing UPEC

We conducted a screen of 6454 registered drugs and drug candidates, aiming to identify compounds effective against non-growing UPEC strain CFT073[31]. This collection comprised 1200 compounds from the Prestwick Library and 5254 from the Specs Repurposing Library. The Prestwick Library primarily consists of off-patent, regulator-approved drugs from diverse therapeutic classes, including 144 antibiotics and 12 antiseptics, while the Specs Library contains commercially available drugs, drug candidates in clinical development phases 1 through 3, and compounds in preclinical development. All compounds were tested at a final concentration of 20 μM.

Bacteria were cultured in either 1:4 diluted cation-adjusted Mueller-Hinton broth (CA-MHB) at pH 7.4 or in an acidic, low-phosphate, low-magnesium medium (LPM) at pH 5.5. CA-MHB was diluted to reduce bacterial density in the stationary phase culture, which, based on our observations, we anticipated to minimize bacterial clumping and thereby reduce the pipetting error. Acidic LPM was chosen to simulate conditions in UPEC-inhabited vacuoles[22,25]. Given that variation in antibiotic tolerance decreases when cultures remain in the stationary phase for extended periods or are inoculated from such cultures[32,33], we used a 24-h cultivation and treatment time in both the screening and validation experiments to minimize stochasticity and variability.

The screening assay involved treating a stationary-phase culture with each compound for 24 h, followed by monitoring the delay in bacterial outgrowth after a 2500-fold dilution into drug-free growth medium (Fig. 1A)[15]. This dilution reduced the compound concentration to 8 nM, which was expected to be below their minimum inhibitory concentration (MIC), thus allowing the regrowth of surviving bacteria. This assumption was confirmed, as the lowest measured MIC for all hit compounds was 12.5 nM (clinafloxacin, see Dataset 1). However, we cannot rule out the possibility that the remaining 8 nM drug residue might partially inhibit growth in certain cases.

To validate the assay, we used gatifloxacin (GAT) and finafloxacin (FIN) as positive controls, while mock-treated samples (1% DMSO without antibiotics) served as negative controls (Fig. 1B, Fig. S1). GAT has previously been shown to kill UPEC persisters[34], while FIN has bactericidal activity at acidic pH and kills effectively intracellular UPEC in a cell culture model[35,36]. Preliminary experiments confirm that both GAT and FIN have bactericidal effects against stationary-phase UPEC cultures at a concentration of 20 μM. The Z'-factor, indicating assay robustness and discrimination between positive controls and mock treatments, was above 0.5 between 5 and 8 h after dilution (Fig. S1), demonstrating excellent assay reliability within this timespan.

In the course of the screening, we measured the optical density of regrowing samples 6 h post-dilution, defining wells with an $OD_{600} < 0.1$ as hits. GAT, FIN, and DMSO controls were included on every screening plate to ensure consistency (Fig. 1C, D). After eliminating duplicates—compounds present in both libraries or those appearing in different chemical forms (e.g., the hydrochloride salt and free base of the same fluoroquinolone)—we identified 54 hits in 1:4 CA-MHB (pH 7.4) and 38 hits in LPM (pH 5.5), with 23 overlapping hits active in both media.

Hit validation and dose–response analysis

Hits from the primary screen were validated through a dilution-regrowth assay at 20, 10, and 5 μM concentrations in both 1:4 CA-MHB pH 7.4 and LPM pH 5.5 (Fig. S2). Compounds that delayed regrowth, resulting in an OD_{600} at least 50% lower than the drug-free control at one or more of these concentrations after 6 h of incubation in regrowth medium, were selected for further testing at concentrations ranging from 30 to 0.25 μM. We identified 39 compounds of six different classes that significantly delayed regrowth at one or more of these concentrations (Fig. 1E). Of these compounds, 37 were active in 1:4 CA-MHB, 17 in acidic LPM and 15 in both media (Figs. 1F, 2, and 3).

Of the 39 compounds, 29 are well-known antibiotics. While ofloxacin, ciprofloxacin[37,38], tosufloxacin, sparfloxacin, moxifloxacin, gatifloxacin, enrofloxacin, sarafloxacin[34,39] as well as rifampicin[40,41] have been previously identified as active against non-growing bacteria, to the best of our knowledge, this has not been demonstrated for the remaining 20 antibiotics that were hits from the screen. The largest group consists of 19 fluoroquinolones, including 18 hits from the screen, as well as finafloxacin which was used as a positive control. Other antibiotic classes include protein synthesis inhibitors: five macrolides and one pleuromutilin (valnemulin), as well as four RNA synthesis-inhibiting rifamycins. The list also includes three antiseptics and disinfectants: the biguanide octenidine, and the bisbiguanides alexidine and chlorhexidine.

Hit compounds that are neither antibiotics nor cationic surfactants, are anti-cancer agents with varying mechanisms of action and at different stages of development. Mitomycin C (MMC)[42], bleomycin (BLE)[43], satraplatin (SAT)[44], and evofosfamide (EVO)[45] are known genotoxic compounds targeting DNA integrity and synthesis. Echinomycin and plicamycin (also known as mithramycin) are DNA-intercalating antitumor drugs that block transcription[46]. A deubiquitinase inhibitor Degrasyn (DGS, WP1130) is a thiol-reactive compound that modifies cysteine residues[47,48].

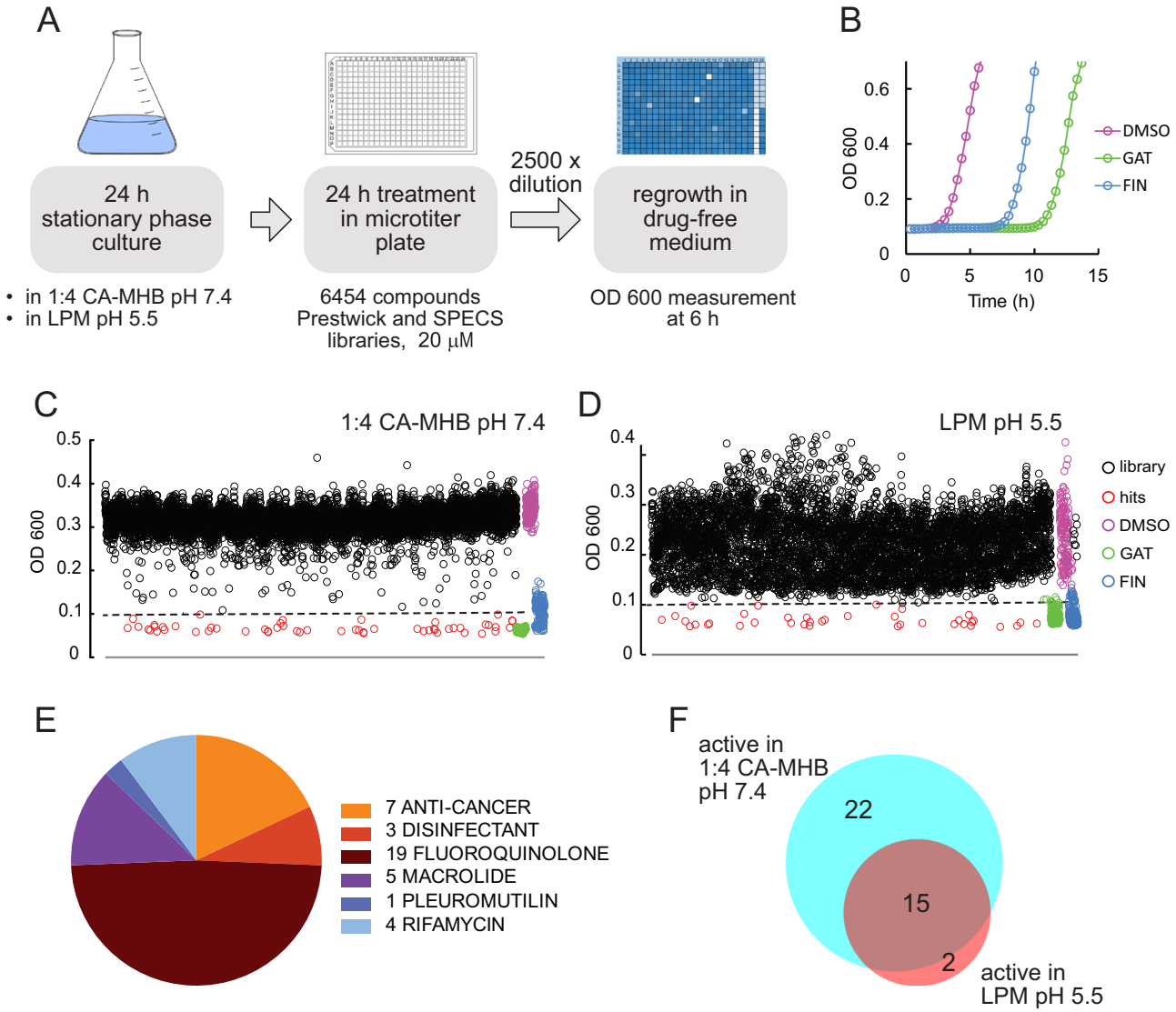

Fig. 1 | A dilution-regrowth-based screen identifies compounds that delay the regrowth of stationary-phase UPEC. A Schematic of the screening assay. A 24-h culture of UPEC strain CFT073 was dispensed into a microtiter plate and treated with 20 μM compounds from the collection for 24 h. The samples were then diluted 2500-fold into drug-free CA-MHB (pH 7.4), and OD_{600} was measured after 6 h. **B** Regrowth delay following bactericidal treatment. For assay validation, UPEC CFT073 was cultivated for 24 h in 1:4 diluted CA-MHB (pH 7.4) and treated for 24 h with 20 μM gatifloxacin (GAT) or finafloxacin (FIN), which kill non-growing bacteria and served as positive controls for screening. After a 2500-fold dilution into drug-free CA-MHB, the regrowth of the drug-treated samples was delayed compared to that of the drug-free control (DMSO). **C**, **D** Screening of the combined Prestwick and SPECS collections identified drugs that delayed bacterial regrowth in 1:4 diluted CA-MHB (pH 7.4) (**C**) and LPM (pH 5.5) (**D**). A cutoff of $OD_{600} < 0.1$ (dashed line) was used to identify hits (red). Most of the tested compounds (black) showed no effect compared to the drug-free control (purple). GAT (green) and FIN (blue) were included as positive controls in each plate. **E** Pie chart illustrating the distribution of validated hits across different drug groups. **F** Venn diagram depicting the overlap of validated hits identified in 1:4 CA-MHB (pH 7.4) and LPM (pH 5.5).

In summary, our screen identified numerous compounds that were previously unknown to be active against non-growing UPEC.

Both killing and the post-antibiotic effect contribute to delaying UPEC regrowth

While setting up the screen, we anticipated that the killing of non-growing bacteria would be the primary cause of delayed regrowth. However, an alternative explanation could be the post-antibiotic effect (PAE), in which the regrowth of individual bacteria is temporarily delayed after the antibiotic is removed from the extracellular environment[49]. It is also possible that both bacterial killing and PAE occur simultaneously.

To differentiate between these scenarios, we used CFU counting by agar plating to assess the bactericidal effect on non-growing bacteria. A reduction in CFU indicates bacterial killing and suggests that the delay in regrowth is due to the loss of viable cells. Conversely, if no significant reduction in CFU is observed, the regrowth delay is due to PAE without killing. This analysis was performed for all compounds that demonstrated activity in the dilution-regrowth assay at a concentration of 10 μM (Fig. S2). Fluoroquinolones, anti-cancer agents, and disinfectants do kill a fraction of stationary phase UPEC (Fig. 2), whereas macrolides, rifamycins, and valnemulin are not bactericidal against *E. coli* but rather cause PAE (Fig. 3, Dataset 2). The occurrence of PAE indicates that these drugs penetrate the non-growing bacteria and remain there for hours, inhibiting targets and postponing regrowth. Fluoroquinolones are known to cause PAE after treatment of growing bacterial cultures[50]. We compared the bactericidal activity of each compound based on the log killing at a 2.5 μM concentration and regrowth-inhibiting activity by determining the EC_{50} values, which were calculated using the OD_{600} readings from treated and diluted stationary phase samples after a 6 h incubation period. Here, EC_{50} represents the concentration of a compound that causes a regrowth delay, halfway

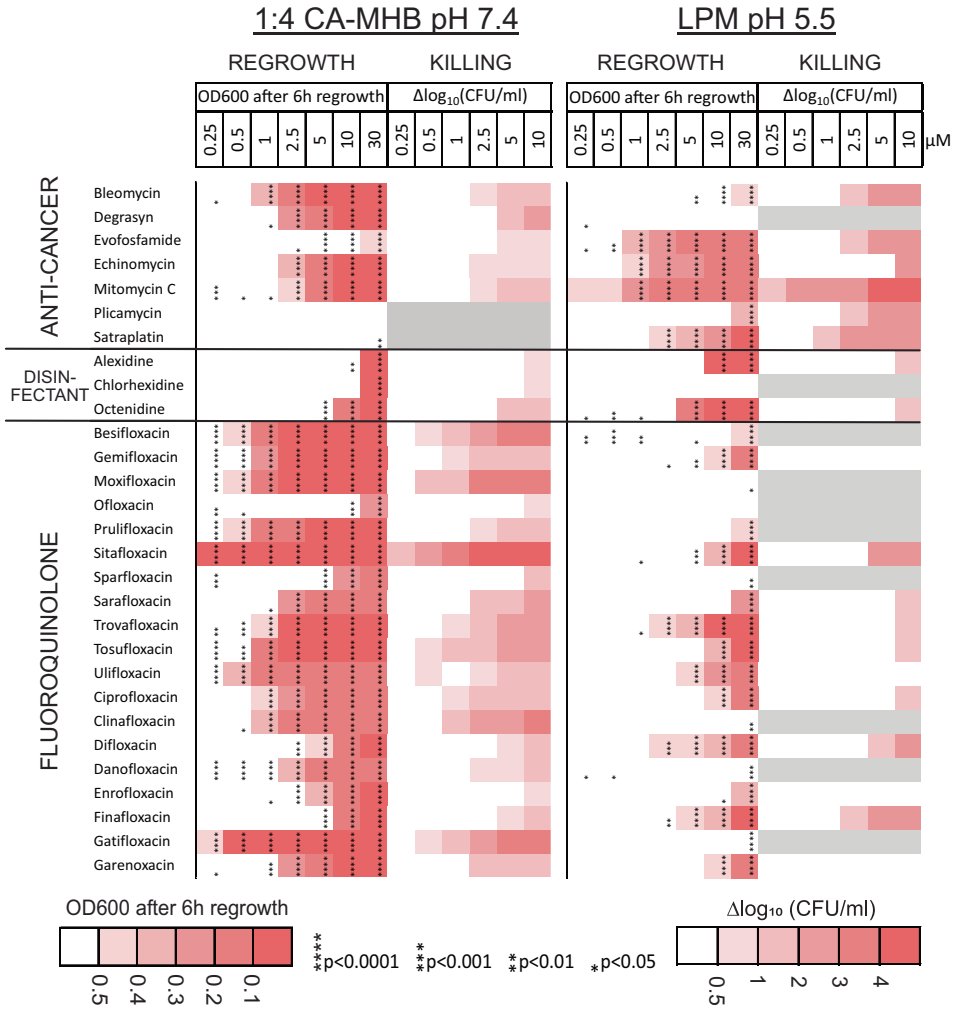

Fig. 2 | Regrowth delay and killing of non-growing UPEC. Heatmap showing the regrowth delay and killing of non-growing UPEC CFT073 by validated hit compounds. Gray areas indicate compounds not tested for killing due to weak activity in regrowth-delay experiments. Bacteria were grown in 1:4 CA-MHB (pH 7.4) or LPM (pH 5.5) for 24 h and then treated with the compounds at the indicated concentrations for 24 h. Regrowth data were obtained by measuring OD_{600} 6 h after a 2500-fold dilution of the samples into CA-MHB. Killing data were determined by spot-plating serial dilutions of the treated samples on LB agar plates followed by colony counting. The differences in CFU/mL between the drug-treated samples and the drug-free control are shown. Data represent the mean of three biological replicates. The significance of the regrowth delay was assessed using one-way ANOVA followed by Dunnett's test, comparing each treatment to the drug-free control. Asterisks denote *p*-values from the Dunnett's test.

between the baseline (no regrowth inhibition, with OD_{600} of the drug-free control) and the maximum effect (complete inhibition of regrowth, with OD_{600} of the sterile medium) (Dataset 1). We found that fluoroquinolones with similar EC_{50} exhibit different bactericidal activity, although these two factors are correlated ($r = -0.67$; 95% CI = −0.86 to −0.29; $p = 0.0025$) (Fig. S3A). This indicates that both reduction of the inoculum by killing and the PAE of the surviving bacteria contributed to the observed regrowth delay by fluoroquinolones (Fig. 2). Finally, we found that sitafloxacin is the most potent and bactericidal compound against non-growing UPEC at pH 7.4, considerably exceeding ciprofloxacin, which is recommended for the treatment of uncomplicated pyelonephritis and complicated UTIs[51,52].

Impact of pH and medium composition on activity against non-growing UPEC

The majority of the hit compounds were less effective in LPM (pH 5.5) compared to 1:4 CA-MHB (pH 7.4). Several fluoroquinolones, macrolides, valnemulin, and chlorhexidine were active at pH 7.4 and completely inactive in the acidic medium (Figs. 2 and 3). In particular, the bactericidal activity of all the fluoroquinolones, except finafloxacin, drops significantly at acidic pH (Fig. 2, Fig. S3A,B). Conversely, the rifamycins, alexidine, and octenidine, as well as the anti-cancer agents evofosfamide, mitomycin C, satraplatin, and plicamycin, are potentiated by the low pH of the medium (Figs. 2 and 3). In total, eleven compounds are more active in LPM (pH 5.5) than in 1:4 CA-MHB (pH 7.4) (Fig. S4).

Recently, the Collins lab identified semapimod, an anti-inflammatory drug with anti-membrane activity, as effective against stationary-phase *E. coli* and *Acinetobacter baumannii* using a similar screening procedure[53]. Although semapimod was included in our library, it was not among our hits. Zheng and colleagues treated stationary-phase *E. coli* cultures grown in 1% LB diluted in phosphate-buffered saline (PBS)[53] while our experimental setup differed in several aspects, including the *E. coli* strains used, screening concentrations, and the dilution factor of the stationary-phase culture. To investigate how the screening procedure influences the results, we tested semapimod using both protocols and strains. As shown in Fig. S5, the growth and treatment medium appear to play a key role in semapimod activity. Semapimod exhibits strong bactericidal activity (Fig. S5A) and delays regrowth (Fig. S5B, C) in 1% LB diluted in PBS but not in 1:4 CA-MHB. Other factors had comparatively minor effects.

Thus, the use of different media by Zheng and colleagues, compared to our 1:4 CA-MHB, likely explains the differing screening results and aligns with their finding that semapimod's activity is influenced by divalent cation concentration, as the addition of 21 mM Mg^{2+} abolished its activity[53].

In summary, we observed that the activity of antimicrobials against non-growing UPEC is strongly influenced by the composition of the testing medium, particularly its pH.

Hit compounds have different activity profiles against non-growing *P. aeruginosa* and *S. aureus*

To determine the spectrum of activity of the compounds that were effective against non-growing UPEC, we further assessed their efficacy against the well-characterized Gram-negative and Gram-positive reference strains *P. aeruginosa* DSM1117 and *S. aureus* DSM2569, respectively. The cultures were grown and treated for 24 h in 1:4 CA-MHB (pH 7.4). Twenty four compounds that were identified as the most active in preliminary dilution-

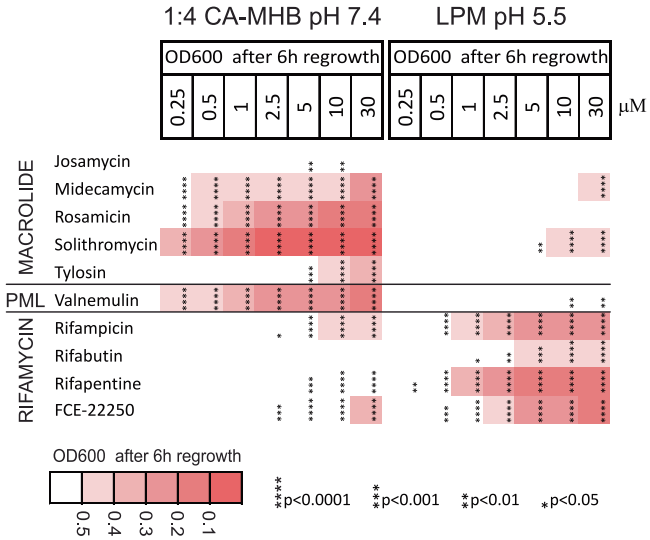

Fig. 3 | The post-antibiotic effect on non-growing UPEC. Heatmap showing the regrowth delay of non-growing UPEC CFT073 by validated hit compounds that did not kill the stationary phase bacteria. Cultures were grown in 1:4 CA-MHB (pH 7.4) or LPM (pH 5.5) for 24 h and then treated with the compounds at the indicated concentrations for another 24 h. OD_{600} was measured 6 h after a 2500-fold dilution in CA-MHB. PML refers to pleuromutilin. Data represent the average of three biological replicates. Regrowth delay significance was assessed using one-way ANOVA and Dunnett's test, with comparisons made against the drug-free control. Asterisks indicate *p*-values from Dunnett's test.

regrowth tests at a concentration of 10 µM were further characterized for regrowth delay and bactericidal activity at concentrations ranging from 10 to 0.1 µM (Fig. S2).

For better characterization of the compounds causing the strongest regrowth delay, we performed the regrowth step of the dilution-regrowth experiments on a plate reader, measuring the OD_{600} at 20-min intervals for over 20 h instead of using an endpoint measurement. This approach allowed us to capture the time when cultures reached a threshold optical density (start-growth-time, SGT[15]).

P. aeruginosa is notorious for causing resilient infections that do not respond well to antibiotics[26]. Therefore, we were surprised to find that many drugs, especially fluoroquinolones, were active against non-growing *P. aeruginosa* DSM1117 (Fig. 4). Strikingly, the bactericidal activity of the tested drugs against this *P. aeruginosa* strain is more potent compared to their activity against UPEC CFT073 (Figs. 2 and 4, Fig. S3A,C). Of the 24 compounds that are most active against *P. aeruginosa* based on preliminary regrowth-dilution results, all are also effective at killing nongrowing bacteria of this species, including those that induced only a post-antibiotic effect (PAE) without reducing CFU in UPEC.

Notably, solithromycin (a macrolide) and rifabutin (a rifamycin) were highly bactericidal against non-growing *P. aeruginosa*, reducing bacterial counts by more than four logs at concentrations of 10 µM and 2.5 µM, respectively. In contrast, these drugs did not kill UPEC but only induced PAE (Fig. 3). Solithromycin kills more than four logs of stationary phase *P. aeruginosa* at a concentration as low as 0.5 µM, similarly to the most bactericidal fluoroquinolones: clinafloxacin, ulifloxacin, sitafloxacin and prulifloxacin. Altogether, 10 compounds (including the above-listed, as well as mitomycin C, besifloxacin, gemifloxacin, and ciprofloxacin) reduce bacterial counts by more than four logs at a concentration of 2.5 µM. In several individual samples, the bacterial count is reduced to undetectable levels: no colonies were observed on agar from the smallest plated dilution, and/or the diluted bacteria did not regrow in liquid medium during the observation period (Dataset 2).

The activity profile of the hit compounds against non-growing *S. aureus* differed significantly from those against both UPEC and *P. aeruginosa* (Fig. 4, Fig. S3D). In the regrowth-dilution assay, most fluoroquinolones and macrolides are inactive, exhibit weak activity, or show activity only at the highest concentration tested, 10 µM. While macrolides do not kill non-growing *S. aureus*, some of the fluoroquinolones reduce bacterial counts by one to two logs, with sitafloxacin showing the most activity. Rifamycins do not kill non-growing *S. aureus*, but they demonstrate an increased activity in delaying regrowth compared to their effects on the Gram-negative organisms. Together with the disinfectants alexidine and octenidine, the anti-cancer agents mitomycin C and evofosfamide are the most bactericidal compounds in our set, reducing non-growing *S. aureus* below the limit of detection at a concentration of 10 µM (Fig. 4).

Diverse hit compounds are active against non-growing bacteria at sub-MIC concentrations

The bactericidal effect of antibiotics correlates positively with bacterial growth rate[37,54–56], implying that slow-growing bacteria are less sensitive to these drugs. Non-growing bacteria are typically tolerant to β-lactams, other cell-wall inhibitors, and aminoglycosides, which did not emerge as hits. In contrast, rapid bacterial growth has been found to reduce macrolide accumulation and efficacy[57]. To characterize the activity of the hit compounds against growing bacteria, we measured their MIC values and found that some had MIC values much higher than the concentrations needed to kill non-growing bacteria or delay their regrowth (Dataset 1).

The MIC of midecamycin against UPEC in a standard assay (in CA-MHB) is over 160 µM, yet it effectively delays UPEC regrowth at 0.5 µM (Fig. 3). Similarly, solithromycin has a MIC of 80 µM against *P. aeruginosa* but kills almost four logs of stationary phase cells at 0.5 µM (Fig. 4). Comparing the activity of hit compounds against growing bacteria (MIC) and non-growing bacteria (EC_{50} for regrowth delay) shows considerable differences between organisms, groups of compounds, and testing conditions (Fig. 5). Anti-cancer agents are inactive based on MIC (EVO, Fig. 5A, B, D; SAT, Fig. 5C) or have an MIC far higher than EC_{50} (MMC in *P. aeruginosa*, Fig. 5C). In Gram-negative *E. coli* and *P. aeruginosa*, the MIC of macrolides and valnemulin considerably exceeds EC_{50} in 1:4 CA-MHB medium (Fig. 5A, C). There was no statistically significant correlation between MIC and EC_{50} for regrowth delay across the entire set of the hit compounds (see Fig. 5 legend for statistical details). For fluoroquinolones, the largest group of compounds, EC_{50} exceeded MIC manyfold for UPEC in 1:4 CA-MHB pH 7.4, indicating that growing *E. coli* is more sensitive to these compounds than non-growing cells (Fig. 5A). For *P. aeruginosa*, however, MIC values are higher than EC_{50} for all fluoroquinolones, suggesting that non-growing bacteria in this strain are more sensitive to these compounds than growing bacteria, which is unexpected (Fig. 5C).

Comparing UPEC MIC values at pH 7.4 and 5.5, we found that the anti-cancer agents, which were potentiated against non-growing UPEC in acidic LPM, also have lower MIC in this medium. All rifamycins and three fluoroquinolones (finafloxacin, trovafloxacin, and difloxacin) display lower MIC at pH 5.5 compared to pH 7.4 (Dataset 1). For trovafloxacin and difloxacin, this does not translate into superior activity against non-growing UPEC in acidic medium; however, both drugs are active against non-growing UPEC in both media (Fig. 2).

The standard medium for MIC determination is CA-MHB, which we also initially used to determine the MIC of the hit compounds; however, we cultured and treated the stationary-phase bacteria in a diluted medium with a fourfold reduction in divalent cation concentration. The composition of the testing medium significantly impacts both the MIC values and the bactericidal rates[58]. To determine if the disparity between high MIC and activity against non-growing cultures was due to different testing media, we measured the MIC of selected compounds in 1:4 CA-MHB pH 7.4. We found that extremely high MICs generally do not decrease in the diluted medium. Exceptions included two rifamycins: rifabutin (in UPEC and *P. aeruginosa*) and FCE-22250 (in *P. aeruginosa*), whose MICs drop in the diluted medium. Additionally, the MIC of most fluoroquinolones in *P. aeruginosa* remain the same in diluted CA-MHB, or even increase, as in the

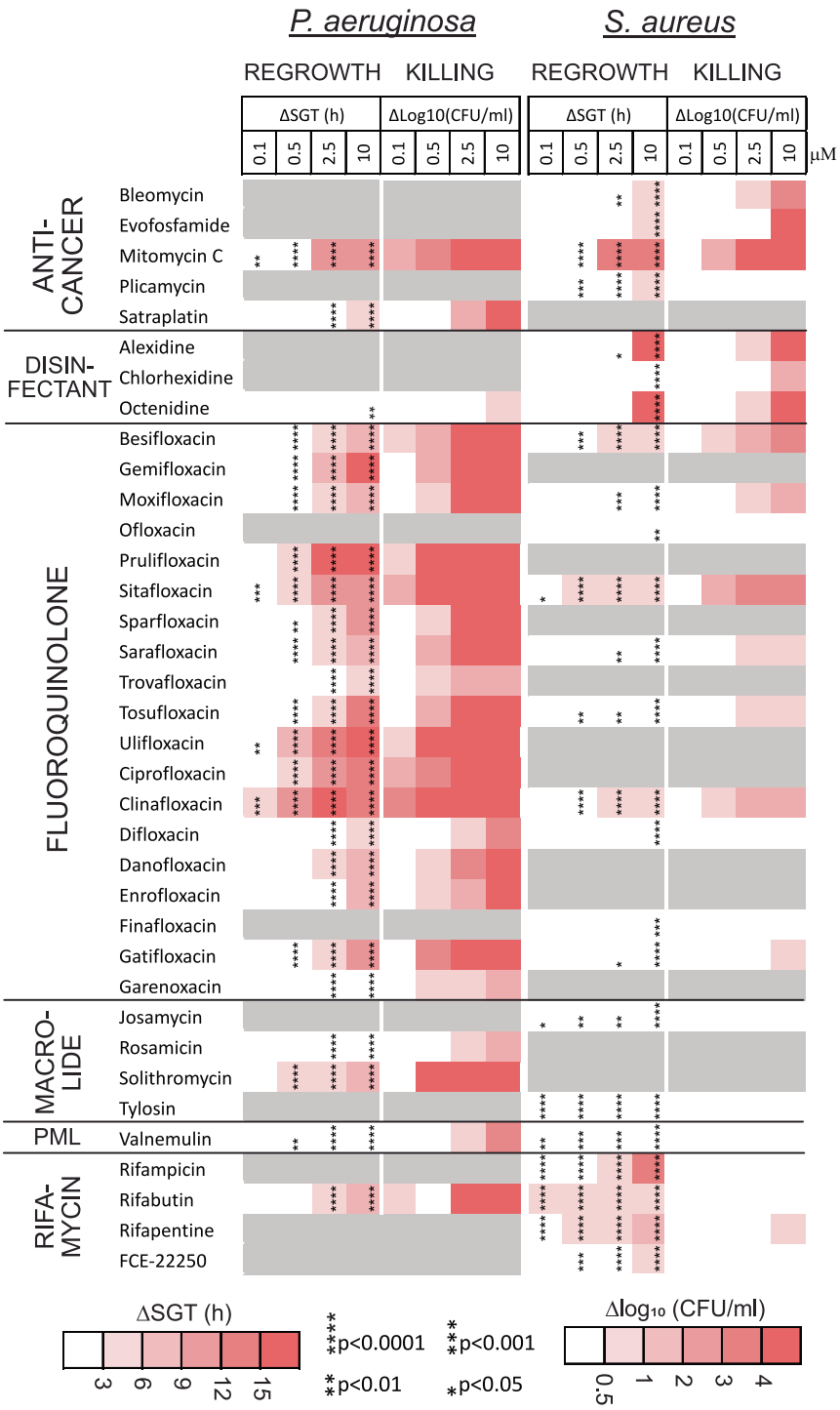

Fig. 4 | Regrowth delay and killing of non-growing *P. aeruginosa* and *S. aureus*. Regrowth delay and killing of non-growing *P. aeruginosa* DSM1117 and *S. aureus* DSM2569 by compounds effective against non-growing UPEC. Gray areas in the heat map indicate compounds not tested due to inactivity in preliminary experiments. Cultures were grown in 1:4 CA-MHB (pH 7.4) for 24 h followed by treatment with the compounds at the indicated concentrations for an additional 24 h. To assess regrowth, OD_{600} was measured on a microplate reader after a 2500-fold dilution into CA-MHB. The time required for cultures to reach an OD_{600} threshold of 0.12 is reported as the start-growth-time (SGT). Bacterial killing was determined by spot-plating serial dilutions of the treated samples on LB agar plates followed by colony counting. The differences in CFU/mL between drug-treated samples and the drug-free control are presented. Data represent the average of three biological replicates. The significance of regrowth delay was evaluated using OD_{600} measurements taken 8 h after dilution into drug-free media. Statistical significance was assessed using one-way ANOVA followed by Dunnett's test, comparing each treatment to the drug-free control. Asterisks denote *p*-values from Dunnett's test.

case of trovafloxacin and besifloxacin (four and twofold, respectively; Dataset 1).

We investigated whether the high activity of certain compounds against non-growing bacteria, combined with their lack of activity against growing cells, could be attributed to efflux pump activity. Since efflux pumps are energized and active in growing bacteria, we measured the MIC of these compounds in the presence of the broad-spectrum efflux pump inhibitor phenylalanine-arginine β-naphthylamide (PaβN). Our results showed that PaβN did not reduce the MIC of compounds with the highest MIC values, indicating that efflux pump activity does not account for their elevated MICs (Dataset 1).

Hit compounds display different kinetics of antimicrobial activity against non-growing bacteria

For screening and concentration-dependent characterization of the hit compounds, we used a 24-h incubation to capture their effects, although the actual onset of their activity may occur much sooner. To assess the speed of action, we treated stationary-phase cultures of three organisms with compounds that had previously shown a strong regrowth delay at 10 μM. We selected compounds that, in earlier tests, strongly delayed regrowth, such that diluted cultures remained below an OD_{600} of 0.12 (indicating no growth) after 6 h for UPEC or 8 h for *P. aeruginosa* and *S. aureus* in drug-free medium.

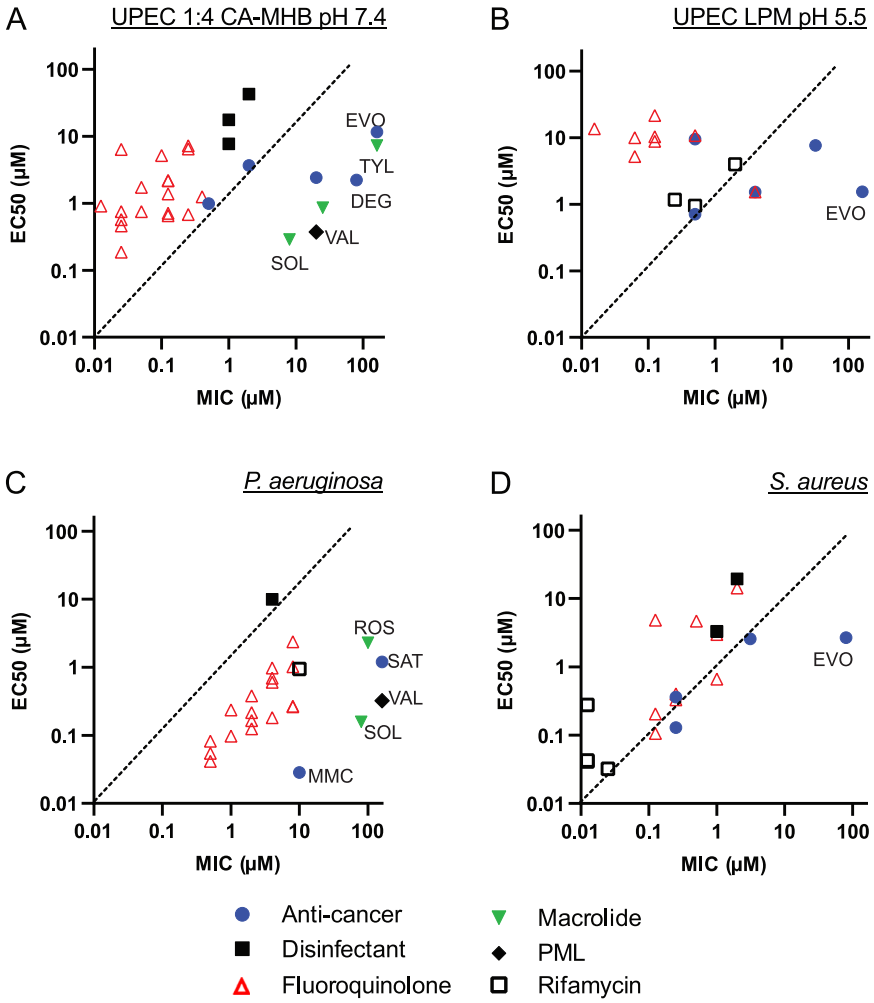

Fig. 5 | Activity of hit compounds against growing and nongrowing bacteria. The activity against growing bacteria was assessed by determining the minimal inhibitory concentration (MIC) using the broth dilution method. The activity against non-growing bacteria was evaluated by calculating the EC_{50} based on regrowth delay, measured by OD_{600} readings at 6 h (**A**, **B**) or 8 h (**C**, **D**) after dilution of treated bacteria into drug-free growth media. EVO evofosfamide, DEG degrasyn, MMC mitomycin C, SOL solithromycin, TYL tylosin, ROS rosamicin, VAL valnemulin. **A**, **C**, **D** 1:4 CA-MHB (pH 7.4) was used for cultivation and treatment of non-*growing* UPEC CFT073, *P. aeruginosa* DSM1117, and *S. aureus* DSM2569. 1:4 CA-MHB (pH 7.4) and CA-MHB (pH 7.4) were used for MIC measurement (Dataset 1). **B** LPM (pH 5.5) was used in experiments with non-growing *E. coli* CFT073 and MIC determination (Dataset 1).

We collected samples at different times during the 24-h incubation, diluted them 2500-fold, and measured the OD_{600} after 6 or 8 h of further incubation, respectively. As seen in Fig. 6, most of the compounds cause the growth delay after just 1 h incubation in all three organisms. A few compounds act more slowly, such as garenoxacin in *E. coli*; difloxacin, satraplatin, trovafloxacin, and sparfloxacin in *P. aeruginosa*. Rifampicin acts quickly against non-growing *S. aureus*, while the effects of the other compounds increase gradually during incubation but are already apparent within the first hour of treatment.

In summary, these results indicate that most compounds penetrate non-growing bacteria rapidly, exhibiting an antibacterial effect after one hour of incubation.

Evaluating hit compounds for growth inhibition of intracellular *S. flexneri*

Nonproliferating bacterial pathogens frequently inhabit intracellular environments, but establishing relevant infection models for high-throughput testing is challenging. Therefore, we firstly tested ability of the hit compounds to cross the eukaryotic cytoplasmic membrane barrier and inhibit growth of *S. flexneri* within the cytoplasm of the TC7 human colon adenocarcinoma cell line[59]. To this end, we developed a new, fluorescence-based method that enables real-time monitoring of growth of intracellular *Shigella*. We used *S. flexneri* 5a M90T strain producing the AfaE adhesin that enhances bacterial adhesion to epithelial cells and allows synchronization of invasion. The strain was further modified to constitutively express super-folder GFP (sfGFP), which enables monitoring bacterial growth by fluorescence measurement. The epithelial cells were grown to confluence and infected with bacteria at an MOI (multiplicity of infection) of 50. Gentamicin was added to inhibit extracellular bacteria with no effect on intracellular bacteria. Following bacterial adhesion and invasion, the cells were incubated with varying concentrations of the hit compounds and intracellular bacterial growth was tracked by measuring green fluorescence. At concentrations of antibacterials that permit bacterial growth, fluorescence intensity steadily increased over the 14-h incubation period, as exemplified by solithromycin in Fig. 7A. To quantify the effect of the compounds, we calculated the IC_{50}, which represents the concentration at which intracellular bacterial growth was inhibited by 50%. This calculation was based on the endpoint fluorescence intensity values provided in Dataset 3, ranging from baseline (no inhibition, fluorescence intensity similar to the drug-free control) to maximum effect (complete inhibition, with no increase in fluorescence). An example of this analysis is illustrated by the solithromycin inhibition curve in Fig. 7B.

To evaluate the potentially toxic impact of the hit compounds on mammalian cell viability, we incubated TC7 cells with each drug in the absence of bacteria. At the MIC concentrations, satraplatin, chlorhexidine, alexidine, and valnemulin were toxic to epithelial cells (Fig. S6). Consequently, these compounds were excluded from further analysis. We observed that most compounds inhibited intracellular *Shigella*, with the exception of bleomycin, which was inactive (Dataset 1). A comparison of intracellular IC_{50} values with MICs revealed a strong correlation between intracellular and extracellular antibacterial activities ($r = 0.79$; 95% CI = 0.59–0.89; $p < 0.0001$) (Fig. 7C). Fluoroquinolones with low MIC values were also among the most effective against intracellular *S. flexneri*

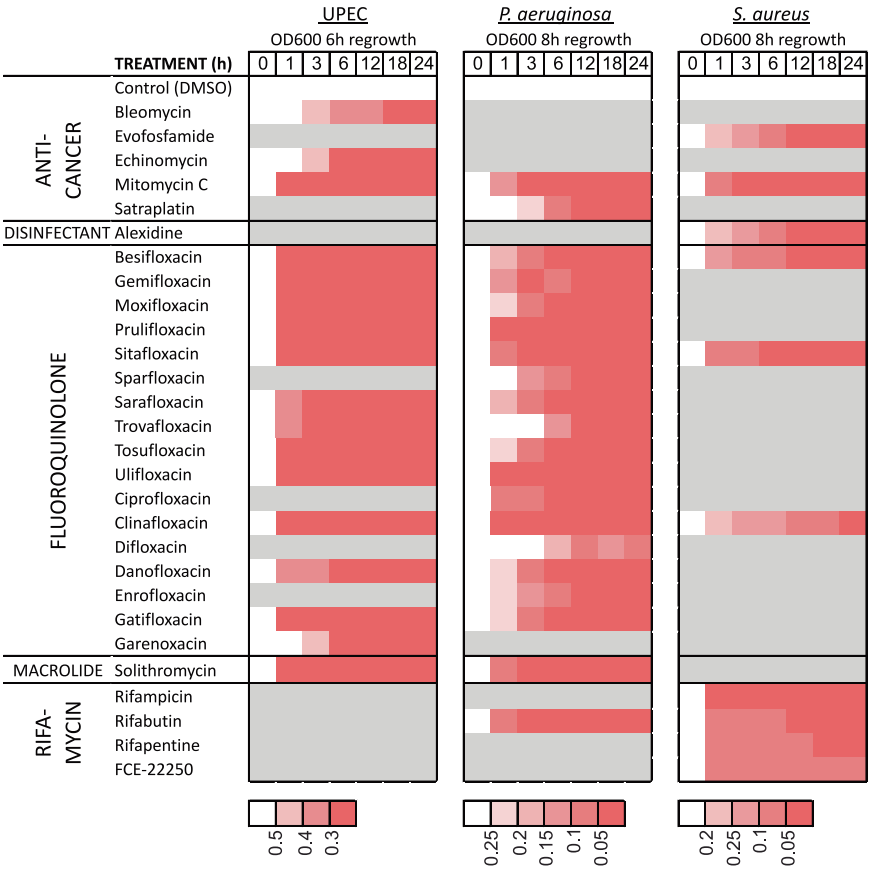

Fig. 6 | Time course of antibacterial effects on non-growing bacteria. Bacteria were cultivated in 1:4 CA-MHB (pH 7.4) for 24 h and the compounds were added at a concentration of 10 µM. Samples were taken at the indicated time points, diluted 2500-fold into CA-MHB, and OD_{600} was measured after 6 h of incubation for UPEC CFT073 and 8 h for *P. aeruginosa* and *S. aureus*. The color scale for OD_{600} values corresponding to each organism is shown below its respective heat map. Gray areas in the heat map indicate compounds not tested due to inactivity observed in previous experiments. Data represent the average of three biological replicates.

(Fig. 7C). The MIC:IC_{50} ratios are generally close to one, with the exception of rifamycins, which showed notably higher ratios (Fig. 7D). All rifamycins have MIC values at least 2-fold higher than their IC_{50} values, with rifabutin exhibiting a an MIC:IC_{50} > 10. Apart from rifamycins, solithromycin, josamycin, sparfloxacin, and mitomycin C also have an MIC:IC_{50} ratio >2, indicating enhanced activity against intracellular bacteria. In contrast, ulifloxacin, prulifloxacin, and evofosfamide display a slightly weaker intracellular activity, with MIC:IC_{50} ratios below 0.5 (Fig. 7D).

In summary, we found that, with a few exceptions, the compounds active against non-growing bacterial cultures also inhibit bacterial growth within the cytoplasm of enterocytes, demonstrating their ability to cross the eukaryotic cytoplasmic membrane barrier.

DNA damage is a possible anti-bacterial mechanism of the anti-cancer compounds

Most of the anti-cancer agents (MMC, BLE, SAT and EVO) that exhibit bactericidal activity against non-growing bacteria are genotoxic, which makes their development into antimicrobials unlikely. The other two drugs of this group, echinomycin (ECH) and plicamycin are DNA-intercalating transcription blockers[46]. We aimed to evaluate the genotoxicity of ECH and PLI by detecting the possible induction of the SOS response. Degrasyn was excluded from further testing due to its chemical instability.

The SOS response is induced by DNA damage that leads to double-strand breaks and RecA activation. The SOS regulon consists of several operons that are controlled by the LexA repressor, which is degraded in response to RecA activation[60]. We aimed to quantify SOS induction using the pANO1::*cda*' reporter plasmid, which expresses the fluorescent GFPmut3* protein from the SOS-inducible *cda* promoter[61]. MMC and GAT, a fluoroquinolone, were used as control compounds. MMC induces DNA interstrand and intrastrand cross-links between adjacent guanine bases[62]. In bacteria, the induction of SOS regulon proteins is required to repair this damage[63]. GAT induces DNA damage and SOS as a secondary response to topoisomerase inhibition in bacteria, while it is not genotoxic to eukaryotic cells, which lack type II topoisomerases.

Green fluorescence was induced in response to GAT and MMC in growing cultures of UPEC CFT073 bearing pANO1::*cda*'. However, as the cultures approached the stationary phase, the drug-free control began to produce GFP as well. After 10 h, the fluorescence normalized to OD_{600} in the control culture surpassed that of the treated samples (Fig. S7A). Since we aimed to test SOS induction in non-growing cultures, it was essential to use a reporter with no leaky expression in the stationary phase. Therefore, we constructed the plasmid pAED2, based on the low copy-number plasmid pSC101, which contains the *cda*' promoter in front of the mScarlet-I-encoding reporter gene. During a 6-h test, this reporter effectively lacked leaky expression and was induced by fluoroquinolones several thousand-fold, thus meeting our requirements (Fig. S7B).

Using pAED2 as a reporter, we tested the SOS response to anti-cancer agents in the medium where these agents exhibited higher antibacterial activity (MMC, BLE, and ECH in 1:4 CA-MHB pH 7.4, PLI, SAT, and EVO in LPM pH 5.5). MMC and SAT, as well as BLE, EVO, and ECH induced the SOS response to different extents when added to the growing UPEC cultures (Fig. 8). Induction of SOS by PLI was not detected (Fig. 8B). In accordance with their high MICs (Dataset 1), 20 µM PLI, EVO, and SAT cause only a slight growth inhibition compared to the effects of MMC, BLE and ECH (Fig. S7C,D). When added to stationary phase cultures, none of the compounds induced SOS (Fig. 8). This might indicate that the stationary phase bacteria are unable to induce a detectable SOS response because of the protein synthesis inhibition and are killed during the treatment. However, another possibility is that the bacteria are poisoned during the stationary phase treatment but are subject to DNA damage and initiate the SOS response only during regrowth after dilution into drug-free medium. This has been demonstrated for fluoroquinolone persisters that required functional SOS regulon for survival but induced a strong SOS response only during recovery, after the ofloxacin removal[64–66]. We tested this possibility

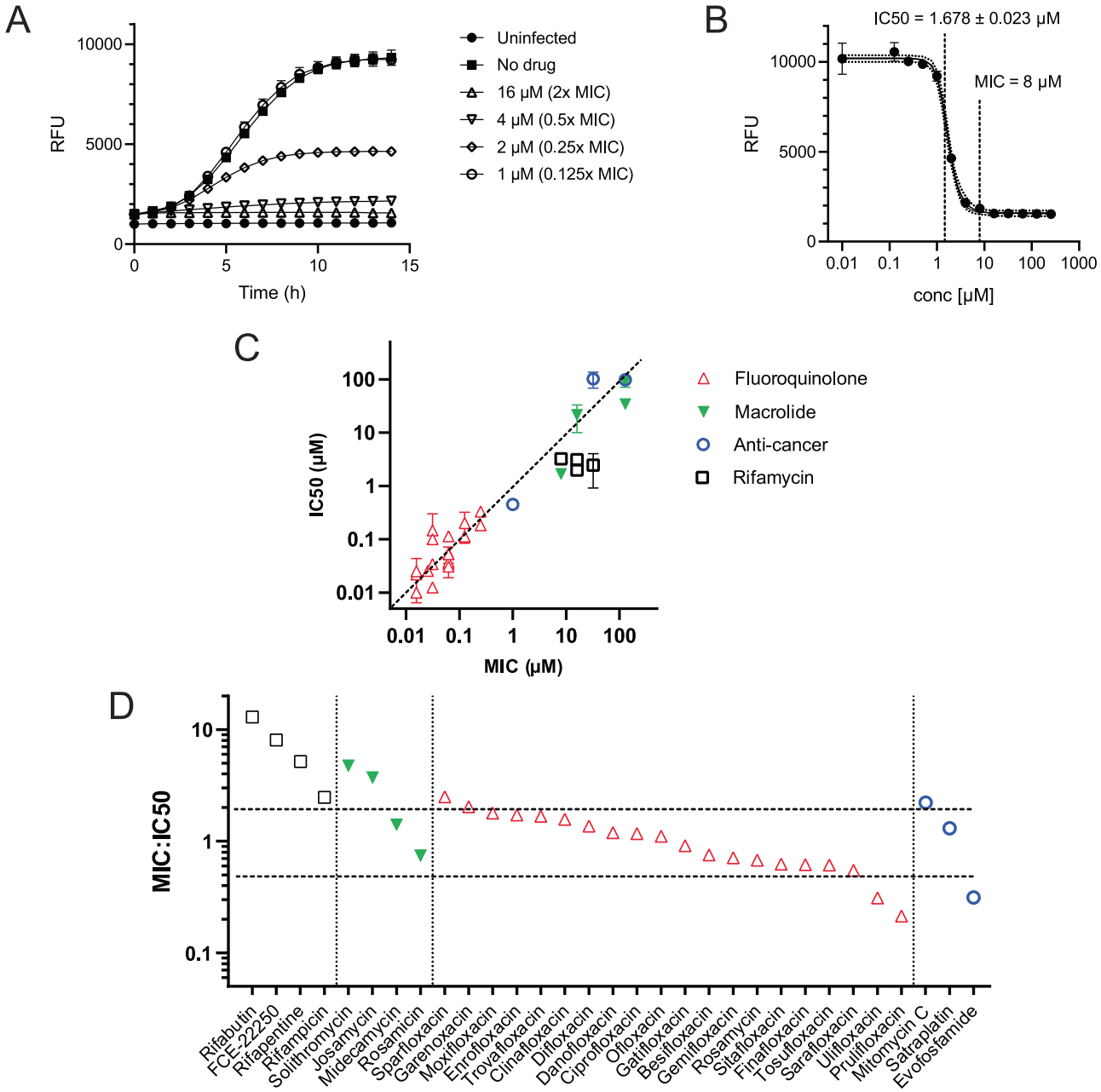

Fig. 7 | Inhibition of intracellular growth of *Shigella* in TC7 cells. TC7 cells were infected with *S. flexneri* constitutively expressing sfGFP and treated with various concentrations of the hit compounds. Gentamicin was used to eliminate extracellular bacteria and intracellular bacterial growth was monitored by measuring GFP fluorescence. RFU relative fluorescence units. The symbols for antibiotic classes are shown next to panel (**C**). **A** Intracellular growth curves of *S. flexneri* in the presence of solithromycin at different concentrations as a representative example of a set of growth inhibition curves. Data are presented as means ± SD for $n = 3$. **B** Solithromycin inhibition curve. Intracellular growth inhibition was assessed by GFP fluorescence after 14 h treatment. Data are presented as mean ± SD for $n = 3$. A five-parameter logistic equation was used for asymmetric sigmoidal curve fitting and IC_{50} determination in GraphPad Prism. For comparison, the MIC obtained by treating extracellular bacteria with the drug is also indicated. **C** Comparison of MIC and intracellular IC_{50} values. Error bars represent 95% confidence intervals (CI). **D** MIC:IC_{50} ratios with horizontal dashed lines indicating twofold differences.

and observed no SOS induction despite the characteristic compound-specific regrowth delay (Fig. 8, Fig. S7E, F). At the same time, a control experiment demonstrated SOS induction during recovery of the ofloxacin-treated non-growing UPEC (Fig. 8A), confirming the published finding[66].

In summary, we found that in growing bacterial cultures, DNA-intercalating agents either do not induce the SOS response (plicamycin) or induce it to a lesser extent (echinomycin) compared to other anticancer agents. None of the tested compounds induced an SOS response after the removal of the drug and the transfer of bacteria into drug-free growth medium, as observed with fluoroquinolones[66].

Discussion

The development of antibiotics specifically targeting non-growing bacteria and chronic bacterial infections has been largely neglected. As a first step to address this gap, we conducted a drug-repurposing screening to identify antimicrobial agents that are primarily effective against quiescent Gram-negative pathogens. Importantly, we focused on compounds that have already undergone or are currently undergoing clinical trials, ensuring that their safety and pharmacokinetic properties are at least partially characterized. We also sought compounds active against Gram-negative pathogens residing in acidified intracellular compartments. This screen

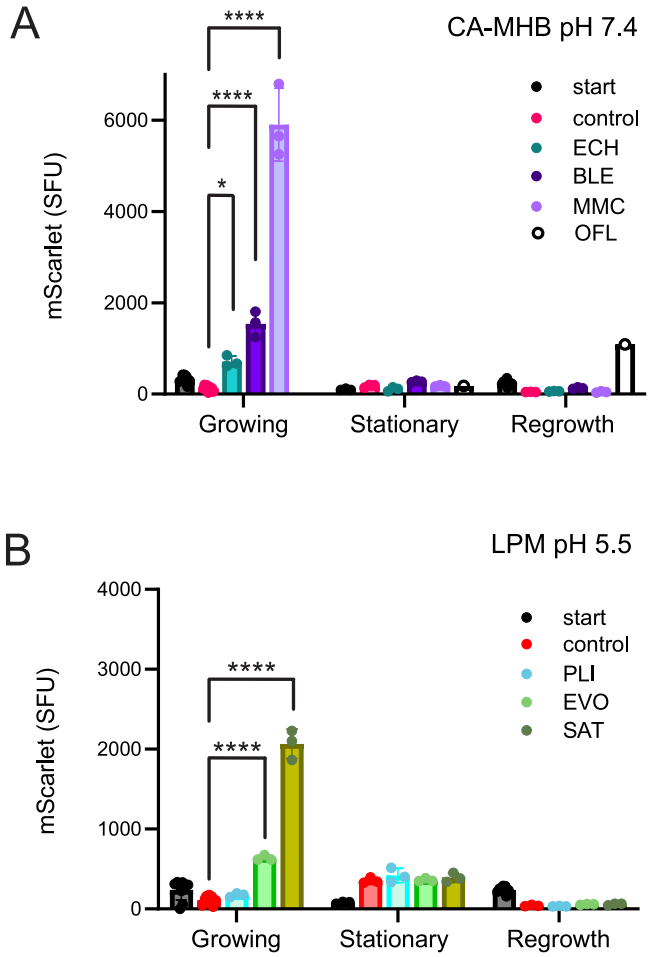

Fig. 8 | Induction of the SOS response by anti-cancer agents. UPEC CFT073 harboring pAED2 was cultivated in 1:4 CA-MHB (pH 7.4) to assess SOS response induction by echinomycin (ECH), bleomycin (BLE), mitomycin C (MMC), and ofloxacin (OFL) (**A**), and in LPM (pH 5.5) to evaluate plicamycin (PLI), evofosfamide (EVO), and satraplatin (SAT) (**B**). The expression of the mScarlet-I reporter, controlled by the SOS-inducible cda' promoter, was measured in a microplate. Growing cultures were treated with 20 μM of each compound for 6 h, while stationary-phase cultures were treated with 10 μM for 24 h. mScarlet fluorescence and OD_{600} were recorded at the start (start) and end of the incubation for both drug-free controls (control) and treated samples. Following treatment, stationary-phase bacteria were collected by centrifugation, washed, diluted 1:7 in CA-MHB, and incubated in a microplate. mScarlet fluorescence was recorded at the start of the incubation (start) and when OD600 reached approximately 0.5 for both the drug-free controls (control) and treated samples. Specific fluorescence units (SFU) were calculated by normalizing fluorescence (arbitrary units, AU) to cell density (OD600). Data are presented as means ± SEM for $n = 3$. Statistical significance was assessed using one-way ANOVA followed by Dunnett's test, comparing each treatment to the drug-free control. Asterisks indicate p-values: ****$p < 0.0001$ and *$p < 0.05$. A single control experiment was performed using OFL.

led to the identification of several antimicrobials with previously unrecognized potent activity against non-growing bacterial pathogens. While previous screening projects aimed at finding drugs against dormant bacteria have yielded somewhat different results—likely due to technical variations in screening and follow-up assays or differing criteria for prioritizing hit compounds[34,38,39,53]—our approach offers new candidates that warrant testing against larger panels of clinical isolates.

A previous repurposing screening aimed at identifying bacterial growth inhibitors found that 24% of drugs targeting human cells, spanning all therapeutic classes, inhibited the growth of certain gut bacterial strains[67]. In contrast, we found that compounds effective against non-growing bacteria in this study were limited to a few antibiotic classes, with only select drugs within these classes demonstrating activity. For instance, among the quinolones included in the library, less than half showed bactericidal activity against non-growing bacteria. Among the 11 macrolides in the SPECS collection, five were active, while six—including widely used erythromycin, clarithromycin, and azithromycin—were not. Among the 14 DNA-alkylating agents, only three—mitomycin C, satraplatin and evofosfamide—exhibited activity against non-growing UPEC, and out of the 19 annotated DNA synthesis inhibitors, only bleomycin demonstrated such activity. The number of disinfectants and antiseptics (14 annotations) in the collection is difficult to determine due to inconsistent classification based on their complementary mechanisms of action (e.g., alexidine dihydrochloride is annotated as a phosphatidylglycerophosphatase inhibitor). Among these, only three biguanides and bisbiguanides with membrane-disrupting activity were active[68], while other membrane-targeting compounds, such as the last-resort antibiotic colistin, were not identified as hits. As compounds within the same class target the same biological process, the formation of these two subgroups cannot be attributed to differences in target activity or corruption. We hypothesize that the observed variation is likely attributable to differences in the permeability or uptake of these drugs into non-growing versus growing bacteria, which are influenced by their chemical properties. The hit compounds display diverse chemical and physical characteristics (Table S1, Fig. S8), despite half of them being fluoroquinolones, which are chemically similar to one another. The median hydrophobicity (partition coefficient, XLogP) of the hit compounds is slightly lower than that of the full Prestwick collection (Fig. S8A). In contrast, their median topological polar surface area (TPSA) and molecular complexity are significantly higher than those of the Prestwick collection (Fig. S8B,D), reflecting the inclusion of large molecules such as macrolides, rifamycins and bleomycin. Notably, the polar surface area of 15 hit compounds (over one-third), including the highly active solithromycin and MMC, exceeds 140 $Å^2$—a threshold often associated with reduced membrane permeability[69]. In summary, the initial analysis of the chemical properties of the candidate compounds does not explain their activity against non-growing bacteria.

We identified several compounds with high MIC values, indicating poor efficacy against growing bacteria, which were nonetheless highly effective against the nongrowing cells of the same strain. For instance, 0.5 μM solithromycin, a macrolide antibiotic of the ketolide sub-class, killed about four logs of stationary phase *P. aeruginosa*, despite its MIC being 80 μM. The discrepancy, once again, is likely attributable to differences in drug uptake between growing and non-growing bacterial cells. In contrast, telithromycin, another ketolide included in the library, showed no activity. The enhanced uptake of solithromycin into non-growing bacteria may be related to the presence of a fluoride atom, which is absent in telithromycin. Based on its MIC alone, solithromycin would not be considered a viable drug candidate against *P. aeruginosa*. However, it could be effective when used in combination with another drug that targets proliferating bacteria. In such a combination, solithromycin could be administered at a dose lower than the therapeutic concentration required to exceed the MIC, focusing instead on its activity against non-growing bacterial cells. The identification of solithromycin as a candidate for such synergistic therapy highlights the critical need for specialized testing procedures to uncover antibiotics with activity against persistent infections.

The different bactericidal activity of solithromycin against *P. aeruginosa* and *E. coli* may be attributed to variations in their ribosomal structures and, potentially, the slower dissociation of the drug from the *P. aeruginosa* ribosome. While bacteriostatic and bactericidal macrolides bind to the ribosome with comparable affinity, bactericidal drugs exhibit significantly slower dissociation. This prolonged inhibition of translation can damage bacterial cells, ultimately leading to death[70]. The slow dissociation of ketolides is attributed to their extended alkyl-aryl side chain, which establishes additional interactions with the ribosome. This side chain acts as a potential pharmacophore, offering opportunities to enhance the bactericidal properties of the drug.

In conclusion, purposefully modifying drugs to improve their penetration into non-growing bacteria and enhance their bactericidal effect could

be a promising approach for developing agents against persistent infections. This strategy could enable the development of drugs specifically targeting non-growing bacteria (antiNG-antibiotics) and facilitate their synergistic use with antibiotics targeting actively growing pathogens (antiG-antibiotics, such as beta-lactams) in combination therapies. Notably, antiNG-antibiotics would not aim to achieve the MIC but instead would be administered at a dose sufficient to reach the effective antiNG concentration.

A significant challenge in developing drugs for dormant pathogens is managing toxicity[38,53,71]. Half of the hits of our screen were fluoroquinolones, which have drawn increasing attention due to their severe adverse effects, and are no longer recommended as first-line antibiotics[72]. Sitafloxacin and clinafloxacin, which were among the most effective against non-growing bacteria, carry risks due to their extra halogen atoms, which enhance the penetration of lipid membranes but also increase undesirable accumulation in adipose tissue. Moreover, although sitafloxacin is observed to be more potent than ciprofloxacin, its efficacy is significantly reduced in acidic environments, where intravacuolar UPEC persisters are thought to reside. Oral sitafloxacin is currently approved in Japan and Thailand, while clinafloxacin was withdrawn from development due to phototoxicity, a common issue among fluoroquinolones with a halogen at the C-8 position[73]. Topical application can mitigate the systemic toxicity of fluoroquinolones, as demonstrated by gatifloxacin, which is safely used for eye infections despite its oral form being banned[74]. To assess an antibiotic's suitability for the topical treatment of chronic infections, such as chronic wound infections, targeted drug development and specialized testing are necessary.

We successfully employed our new GFP-based method to monitor the intracellular growth of *Shigella* upon challenge with drugs. All hit compounds, except those that were toxic to TC-7 cells or that could not be tested because of their high extracellular MIC values, were evaluated for their ability to inhibit intracellular *Shigella*. All but one compound (bleomycin) demonstrated activity, indicating they are able to permeate the cytoplasm of epithelial cells. However, it remains unclear whether rifapentine, solithromycin, and other drugs with higher MIC-to-intracellular IC_{50} ratios accumulate in the cytoplasm of TC-7 cells, or if intracellularly located *S. flexneri* is more sensitive to these drugs compared to its sensitivity in TSB broth.

Notably, our screening did not identify any non-antibacterial human drugs with generally new mechanisms of antibacterial activity. The findings included certain anti-cancer compounds with known nonspecific DNA-modifying activity, as well as the DNA-intercalating agents echinomycin and plicamycin, which have the potential to interfere with both transcription and replication. The antibacterial properties of these compounds likely rely on these same mechanisms. MMC is already known for its anti-persister activity[75–78], and, when used in combination therapy, is effective in vivo against multidrug-resistant (MDR) *P. aeruginosa*[79]. The antimicrobial activity of DGS was identified through a repurposing screen[80] and it is effective against multidrug-resistant *S. aureus* by modifying several essential cysteines and altering multiple pathways[48]. Echinomycin is active against *S. aureus*, including methicillin-resistant strains (MRSA)[81].

Testing the extent and timing of bacterial DNA damage induced by these anti-cancer compounds using an SOS reporter provided useful clues but left open questions requiring further investigation. Based on their weak or absent propensity to induce the SOS response in growing UPEC, echinomycin and plicamycin appear to be less genotoxic than other DNA-targeting agents, warranting further exploration for antibacterial use.

Surprisingly, treatment of non-growing bacteria with anti-cancer compounds, including MMC, did not trigger an SOS response during resuscitation, in contrast to the robust SOS induction observed in fluoroquinolone-treated persisters[64–66]. Despite the lack of detectable SOS induction, the bacteria remained capable of (delayed) outgrowth. Resumption of DNA replication and regrowth in these cells is impossible without repairing the inter-strand cross-links caused by MMC, and the *uvrABC* genes of the SOS regulon are essential for *E. coli* survival following MMC treatment[75]. Reduced DNA repair capacity accompanied by increased mutagenesis have been observed as a survival strategy both in certain tumor cells[82] and in bacteria, where error-prone mutagenesis is induced in response to antibiotics[83,84]. These parallels underscore the importance of further investigating the mechanisms underlying damage survival in both tumor cells and recovering bacterial persisters.

In conclusion, the dilution-regrowth screen proved to be an effective tool for identifying compounds with potential activity against persistent Gram-negative infections, highlighting several candidates for further study. We identified 39 compounds active against non-growing bacteria, including 29 previously unrecognized for this activity. These compounds either killed stationary-phase bacteria or delayed their outgrowth without inducing cell death. Notably, some compounds that delayed regrowth in one pathogen exhibited lethality in another, underscoring the potential for species-specific effects. These findings encourage continued research to identify targeted agents for clinically significant chronic infections.

This study has certain limitations that should be considered when interpreting the findings. We found that the assumption that the time to outgrowth measurement correlates with the number of bacteria surviving treatment does not hold due to the post-antibiotic effect (PAE), which can delay outgrowth independently of bacterial killing. This limitation can be addressed using standard colony-forming unit (CFU) counting as an orthogonal assay, although employing colony counting as the primary screening method is labor-intensive.

Only reference strains were used in this study; therefore, the findings need to be validated using panels of clinical isolates of UPEC, *P. aeruginosa*, *S. aureus* and *S. flexneri*. The complexity of the in vivo environment and the uncertainty regarding how well acidic, low-phosphate, and low-magnesium media mimic these natural niches highlight the preliminary nature of the in vitro data. These findings require further validation in vivo.

This study did not aim to investigate the mechanistic basis for the activity of particular agents against non-growing bacteria. Future experiments involving transcriptomic or proteomic analyses will be necessary to identify the molecular targets affected by the compounds tested here.

Methods

Bacterial strains and plasmids

E. coli CFT073, *P. aeruginosa* DSM1117 (also known as Boston 41501, ATCC 27853), and *S. aureus* DSM2569 were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). *E. coli* BW25113 was from Coli Genetic Stock Center (CGSC). pANO1::*cda*'[61] was a gift from Dr. Lars Hestbjerg Hansen. pAED2, a low-copy, ampicillin-resistant (AmpR) plasmid based on the pSC101 backbone, was constructed using circular polymerase extension cloning (CPEC)[85] in two steps. First, the *trpT* terminator region from pSC101-CAM-bioreporter[86] was inserted between *gfp*-mut2 and *mScarlet*-I genes in pSC101-GFPmut2-mScarlet-I[87], resulting in plasmid pAED1. Next, the *cda* promoter from pANO1::*cda*' was inserted upstream of the mScarlet-I gene, resulting in pAED2. Due to difficulties amplifying the *ampR* gene, the vector was amplified in two fragments. The primers used for cloning are listed in Table S2. *S. flexneri* 5a M90T contained a chromosomal insertion of constitutively expressed super-folder *gfp* gene at the attn::Tn7 site, downstream the *glmS* gene, and was constructed as detailed previously[88,89]. *S. flexneri* M90T expressed the adhesin AFA-I (also known as AfaE[90,91]) from a plasmid conferring spectinomycin resistance.

Media and growth conditions

Overnight cultures of UPEC, *P. aeruginosa* and *S. aureus* were grown in test tubes containing 3 ml of LB broth (~16 h), then diluted 1:100 into either 1:4 diluted CA-MHB medium buffered with 40 mM HEPES (pH 7.4) or, in case of UPEC, into LPM medium[25] supplemented with 49 μM $MgCl_2$ and buffered with 30 mM MES (pH 5.5). The cultures were incubated for 24 h in non-baffled Erlenmeyer flasks at 37 °C with shaking at 200 rpm.

Dilution-regrowth screen

The high-throughput screening (HTS) procedure was based on the protocol by Hazan and colleagues[15] and conducted at the Chemical Biology

Consortium Sweden (CBCS) screening facility. Compounds from the Prestwick Chemical Library (1200 compounds) and the Specs Repurposing Library (5254 compounds) were dispensed using the Echo® (Labcyte) system directly into 384-well plates; 200 nl of 10 mM solution in DMSO was added per well. Each plate included eight wells with 200 nl of 10 mM gatifloxacin, finafloxacin, and kanamycin solutions, along with 8-wells containing 200 nl of DMSO as controls. After 24 h incubation,100 µl of the culture was dispensed into each well of the 384-well plates for screening at a final concentration of 20 µM. The plates were sealed with parafilm and incubated at 37 °C for 24 h in closed humidified boxes, with wet paper towels added to prevent evaporation. After treatment, samples were first diluted 50-fold, using Beckman NX^P robot to transfer 2 µl of the sample to 98 µl of phosphate-buffered saline (PBS), followed by a second 50-fold dilution into CA-MHB using the same volumes, resulting in a total 2500-fold dilution. The plates were incubated at 37 °C for 6 h in humidified boxes, then OD_{600} was measured using a microplate reader. Compounds were considered active if the $OD_{600} < 0.1$. Initially, six plates were normalized using the drug-free control, but since this normalization had no effect on the results, it was later omitted.

For hit validation, bacteria were cultivated, and the dilution-regrowth assay was conducted following the same protocol used in the primary screen. Hit compounds were dispensed into 384-well plates using the Echo® (Labcyte) system, with 200, 100, or 50 nl of a 10 mM solution in DMSO added per well, yielding final screening concentrations of 20, 10, and 5 µM, respectively. OD_{600} values of regrowing samples were normalized to the mean OD_{600} of the drug-free control. The testing was conducted in two replicates. Compounds were considered active if their OD_{600} was at least 50% lower than that of the drug-free control at one or more concentrations in both replicates.

For screening assay validation in pilot experiments, bacteria were cultivated as later in the screening assays. Aliquots of 180 µl from the stationary-phase culture were transferred to 96-well plates, and 20 µl of a 200 µM antibiotic solution (gatifloxacin or finafloxacin) in 10% DMSO or 10% DMSO alone for drug-free controls were added. After 24 h treatment, 4 µl of the sample was diluted in 196 µl of PBS, followed by a second dilution of 4 µl into 196 µl of CA-MHB. For assay validation in 384-well plates, 90 µl of the stationary-phase culture was mixed with 10 µl of the 200 µM antibiotic solution or 10% DMSO. After the treatment, 2 µl of the sample was diluted in 98 µl of PBS, followed by a second dilution of 2 µl into 98 µl of CA-MHB. To monitor regrowth, the plates were incubated in a microplate reader (BioTek Synergy Mx) at 37 °C with continuous shaking, and OD_{600} was recorded every 20 min. For regrowth validation in standing plates, the plates were incubated at 37 °C in humidified boxes, and OD_{600} was measured after 5, 6, 7, and 8 h. The assays were carried out in triplicates.

Dose–response analysis using the dilution-regrowth assay

For UPEC, dose–response analysis was conducted at the CBCS screening facility. Bacteria were cultivated, and the dilution-regrowth assay was performed according to the HTS protocol. Compounds were dispensed into 384-well plates using the Echo® (Labcyte) system, and regrowth was assessed by measuring OD_{600} after 6 h of incubation at 37 °C in humidified boxes. Three biological replicates were performed.

For *P. aeruginosa* and *S. aureus*, dose–response analysis was conducted in 96-well plates with a 200 µl assay volume, following the procedure used for screening assay validation experiments. Aliquots of the 39 compounds active against non-growing UPEC were obtained from SelleckChem and MedChem Express. Stock solutions were prepared and stored according to the manufacturers' instructions, then diluted in 10% DMSO before use. Cultures were incubated for 24 h in 1:4 diluted CA-MHB medium under the same conditions used for UPEC cultivation. In preliminary experiments, each compound was tested at a concentration of 20 µM in the dilution-regrowth assay with 4 technical replicates. Regrowth was monitored using a microplate reader at 37 °C with continuous shaking, and OD_{600} was recorded every 20 min. The time at which OD_{600} exceeded the threshold of 0.12 was recorded as the Start_Growth-Time. Based on these results, 24 of the most active compounds were selected for further dose–response analysis, which was performed in three biological replicates using the same procedure.

Dose-dependent killing analysis

For survival analysis, bacteria were cultivated as described for the dose–response dilution-regrowth assay. Assays with UPEC were conducted at the CBCS screening facility using 96-well plates with a 200 µl sample volume, and compounds were dispensed using the Echo® (Labcyte) system. *P. aeruginosa* and *S. aureus* samples were treated according to the protocol used for the dilution-regrowth experiments. Treated samples were serially diluted 10-fold in PBS, and 10 µl drops were spot-plated onto rectangular LB agar plates using either a Beckman NXP robot (for UPEC) or an Integra VIAFLO96 electronic pipette (for *P. aeruginosa* and *S. aureus*). The LB agar plates were incubated overnight at 37 °C and scanned. Colonies were counted at the spots of the lowest dilution where individual colonies could be accurately distinguished. Each colony was manually marked on a separate image layer using the GNU Image Manipulation Program, and counted using NIST's Integrated Colony Enumerator (NICE 1.4) software[92]. The experiments were performed in three biological replicates.

Susceptibility testing

Minimal inhibitory concentrations (MICs) were determined using a standard microdilution assay protocol[92]. CA-MHB medium buffered with 40 mM HEPES (pH 7.4) was used to determine the MICs for UPEC, *P. aeruginosa*, and *S. aureus*. For compounds with high MIC values, the measurements were repeated in 1:4 diluted CA-MHB (40 mM HEPES, pH 7.4) and in CA-MHB (40 mM HEPES, pH 7.4) supplemented with 10 µg/ml phenylalanine-arginine β-naphthylamide (PaβN). MICs for UPEC were also determined in LPM buffered with 30 mM MES (pH 5.5). For *S. flexneri*, MICs were determined in tryptic soy broth (TSB). The assay was performed in triplicates.

SOS response induction analysis

Overnight cultures of CFT073 harboring a reporter plasmid were grown in 3 ml of LB supplemented with 100 µg/ml carbenicillin (Cb), then diluted 1:100 into the medium used for testing. For validation of the pANO1::cda' reporter, an overnight culture was diluted into CA-MHB (supplemented with 40 mM HEPES, pH 7.4, and 100 µg/ml Cb) and grown to an $OD_{600} \approx 0.5$ at 37 °C with shaking at 200 rpm in an Erlenmeyer flask. Aliquots of 200 µl were dispensed into a 96-well plate and treated with 0.1 and 0.5 µM gatifloxacin, mitomycin C, or left drug-free. Empty wells were filled with water. The plate was incubated in a microplate reader (BioTek Synergy H1) at 37 °C with continuous shaking for 20 h. OD_{600} and GFPmut3* fluorescence ($\lambda_{ex} = 485 \pm 9$ nm, $\lambda_{em} = 508 \pm 9$ nm) were recorded every 10 min.

For pAED2 validation, the overnight culture was diluted into 3 ml of LB supplemented with 100 µg/ml Cb and incubated at 37 °C with shaking at 200 rpm for 1 h. Aliquots of 200 µl were dispensed into a 96-well plate and incubated for 6 h with 0.1 and 0.3 µM gatifloxacin, ofloxacin, or left drug-free. OD_{600}, GFPmut2 fluorescence ($\lambda_{ex} = 485 \pm 9$ nm, $\lambda_{em} = 508 \pm 9$ nm), and mScarlet fluorescence ($\lambda_{ex} = 569 \pm 9$ nm, $\lambda_{em} = 594 \pm 9$ nm) were measured using a BioTek Synergy H1 microplate reader. The experiment was performed in triplicate.

For analysis of the SOS induction by anti-cancer compounds, the overnight cultures were diluted either into 1:4 diluted CA-MHB (40 mM HEPES, pH 7.4) or LPM (30 mM MES, pH 5.5) that were supplemented with 100 µg/ml Cb. For analysis of the effect on growing bacteria, the cultures were incubated at 37 °C with shaking at 200 rpm in Erlenmeyer flasks for 90 min, to an $OD_{600} \approx 0.5$. Then, 180 µl aliquots were transferred to 96-well plates, and 20 µl of the 200 µM drug solution was added to achieve a final concentration of 20 µM. The plates were incubated in a microplate reader (BioTek Synergy H1) at 37 °C with continuous shaking for 6 h. OD_{600} and mScarlet fluorescence were recorded throughout the incubation.

For analysis of the effect of anti-cancer compounds on stationary phase- and regrowing bacteria, the cultures were incubated for 24 h at 37 °C

with shaking at 200 rpm in Erlenmeyer flasks. For the drug treatment, 475 µl of the culture was mixed with 25 µl of a 200 µM drug solution to achieve a final concentration of 10 µM, which had been demonstrated to be effective against stationary-phase bacteria. The samples were incubated for 24 h at 37 °C in standing 1.5 ml tubes. Before and after the drug treatment, 25 µl aliquots were taken and diluted by mixing with 175 µl PBS. OD_{600} and mScarlet fluorescence were recorded using a microplate reader (BioTek Synergy H1).

After the treatment, stationary-phase bacteria were harvested by centrifugation at 5000 × *g* for 5 min at 20 °C, washed twice with PBS, and resuspended in 475 µl PBS. Then, 25 µl aliquots were transferred to 96-well plates, and 175 µl CA-MHB was added. The plates were incubated in a microplate reader at 37 °C with continuous shaking for 14 h. OD600 and mScarlet fluorescence were recorded throughout the incubation. Specific fluorescence was calculated by dividing fluorescence intensity (in relative fluorescence units, RFU) by optical density. The experiments were performed in three biological replicates.

Culture of the TC7 intestinal epithelial cells

TC7 cells (a human cell line derived from Caco-2 colon adenocarcinoma cells[59]) were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco, catalog number 21885-025) supplemented with 10% heat-inactivated serum, 1× amino acid solution prepared from Gibco Minimum Essential Medium Non-Essential Amino Acids (100×) (catalog number 11140-035), and 100 U/ml penicillin–streptomycin (Gibco, catalog number 15140-122). The cells were split using trypsin, seeded in 96-well plates for infection, and grown to confluence, corresponding to approximately 40,000 cells per well.

In vitro infection of cell culture and susceptibility testing of intracellular *S. flexneri*

A single colony of *S. flexneri* was inoculated into TSB supplemented with 50 µg/ml spectinomycin and incubated overnight at 37°C with agitation. The overnight culture was diluted 1:200 in fresh TSB, and the bacteria were grown at 37 °C until the OD_{600} reached 0.3–0.4, corresponding to approximately 2×10^7 CFU/ml. Bacteria were pelleted by centrifugation, washed twice with sterile PBS, and resuspended in infection solution (serum-free DMEM supplemented with 20 mM HEPES, pH 7.4). Just prior to infection, the medium was withdrawn, and the cells were washed twice with sterile PBS. The bacterial inoculum (MOI 50, 2×10^7 CFU/ml) was added to all wells except the no-infection control. The plates were incubated at room temperature for 15 min to allow bacterial adhesion, followed by a 1 h incubation at 37 °C with 5% CO_2 to facilitate bacterial invasion.

Twofold serial dilutions of the drugs were prepared in infection solution containing 50 µg/ml gentamicin in a 96-well plate. After the invasion period, the bacterial inoculum was removed, and the serial dilutions of the drugs were added to the wells, except for the no-drug control. The plates were incubated in a Spark multi-mode plate reader (Tecan) at 37 °C with 5% CO_2 for 12 h. Green fluorescence from intracellular bacteria was recorded hourly for 15 h at $\lambda_{ex} = 475$ nm and $\lambda_{em} = 510$ nm, measuring 12 distinct regions of each well. The experiments were performed in triplicates on separate days.

Determination of cellular viability upon drug exposure

TC7 cells were grown to confluence in transparent 96-well cell culture plates (TPP) in the same cell culture medium used for the in vitro infection assay. Just prior to the experiment, the medium was discarded, and the cells were washed twice with sterile PBS to remove any traces of medium or dead cells. A total of 100 µl of infection solution containing MIC concentrations of individual drugs was added to duplicate wells. The plate was incubated at 37 °C in a CO_2 incubator for 12 h, under the same conditions as the infection assay.

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega), according to the manufacturer's instructions. Briefly, 20 µl of CellTiter 96 AQueous One Solution Reagent was added to each well, and the plate was incubated for 2 h at 37 °C in a CO_2 incubator. The OD_{490} of the samples was measured using a Spark multimode plate reader (Tecan). The raw values were normalized to the control wells incubated with infection buffer only. The experiment was performed in duplicate.

For compounds that strongly absorb light at 490 nm (FCE-22250, rifampicin, mitomycin C, rifabutin, rifapentine), a lactate dehydrogenase (LDH) release assay was performed instead of the MTS assay to assess cytotoxicity. The LDH assay was conducted using the CytoTox-ONE Homogeneous Membrane Integrity Assay kit (Promega), according to the manufacturer's instructions. Briefly, cells in positive control wells were lysed with 10 µl of 10× lysis solution. Then, 50 µl of supernatant from each well was transferred to a black 96-well microplate (Greiner), and 50 µl of CytoTox 96 Reagent was added to each sample. The plate was incubated in the dark for 10 min at room temperature, followed by the addition of 50 µl of Stop Solution. Fluorescence was measured at $\lambda_{ex} = 560$ nm and $\lambda_{em} = 590$ nm using a Spark multimode plate reader (Tecan). The raw values of the samples were normalized to the average of the total lysis controls, which was set to 100% cell death.

Time-dependent effects of drug treatment

Bacteria were cultivated in 1:4 diluted CA-MHB (40 mM HEPES, pH 7.4) for 24 h. Aliquots were dispensed into a 96-well plate, and compounds were added at a 10 µM concentration. Samples were collected at designated time points, diluted 2500-fold as described for the dilution-regrowth assay, and incubated in standing plates within a humid box at 37 °C. The OD_{600} of the samples was measured to assess regrowth using a microplate reader (BioTek Synergy H1) after 6 h for UPEC and 8 h for *P. aeruginosa* and *S. aureus*. The experiment was performed in triplicates.

Semapimod assay

E. coli CFT073 and BW25113 were grown in 3 ml of LB at 37 °C, shaking at 200 rpm for 16 h. The overnight cultures were then diluted either 1:100 into 40 ml of 1:4 diluted CA-MHB (40 mM HEPES, pH 7.4) or 1:10,000 into 40 ml of 1% LB in PBS and grown in 125 mL Erlenmeyer flasks for 24 h at 37 °C, shaking at 200 rpm. Following this, 1 ml aliquots of each culture were transferred to 1.5 ml test tubes, and semapimod was added at final concentrations of 20 µM and 50 µM. DMSO was added to drug-free controls. To quantify live bacteria before treatment, a sample of each culture was serially 10-fold diluted into PBS, and 10 µL drops were spot-plated onto LB agar. The closed test tubes were incubated for 24 h at 37 °C without shaking.

After treatment, the samples were diluted 2500-fold in two steps: a 50-fold dilution into PBS, followed by a 50-fold dilution into CA-MHB in a 96-well plate with a total volume of 200 µl per well. The 96-well plate was then incubated in a BioTek Synergy H1 plate reader at 37 °C with continuous shaking at medium speed for 22 h with a lid. OD600 was recorded every 20 min. Additionally, the treated samples were diluted 1:200 into LB in another 96-well plate and incubated in a BioTek Synergy Mx plate reader at 37 °C without shaking for 22 h, with OD_{600} recorded every hour.

To determine the number of live bacteria after the treatment, the bacteria were pelleted, washed twice with PBS, serially diluted, and spot-plated. Plates were incubated and colonies counted as described previously. The experiment was performed in triplicates.

Data analysis

The statistical analysis of all data, as well as EC_{50} and IC_{50} determination, was conducted using GraphPad Prism 10.2.3. Statistical significance of the drug effects was assessed using a one-way ANOVA followed by Dunnett's test, comparing each treatment to the drug-free control. Statistical significance of the differences in chemical characteristics between the hit compounds and the Prestwick library was assessed using a Mann–Whitney test. Statistical significance was set at $P \leq 0.05$. A five-parameter logistic equation was used for asymmetric sigmoidal curve fitting and for EC_{50} and IC_{50} determination. The number of biological replicates and the meaning of the error bars are provided in the figure legends. The Venn diagram in Fig. 1F was prepared using BioVenn web application[62]. Characteristics of the

chemical and physical properties of Prestwick library compounds were obtained from Supplementary Table S1 from reference[67]. Characteristics of the hit compounds not included in the Prestwick library were retrieved from the PubChem database[93].

Data availability

The dataset supporting the conclusions of this article are included within the article and supplementary information. The plasmid pAED2 generated for this project is available through Addgene (accession number 234599).

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Author contributions

N.K., T.T., V.H. and A.P. conceived the project. N.K. and S.B.F. performed the screening and analyzed the data. N.B., S.B.F., and N.K. performed the follow-up characterization of antimicrobials. A.S. and A.P. conceived and performed the *Shigella* and mammalian cell toxicity testing. N. Ch. A. performed the SOS response testing. J.A. and M.H. constructed the SOS reporter plasmid. N.K. wrote the main manuscript, N.K., N.B. and N. Ch.A. prepared the figures. All authors reviewed the paper.

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Competing interests

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Additional information

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