UiT The Arctic University of Norway

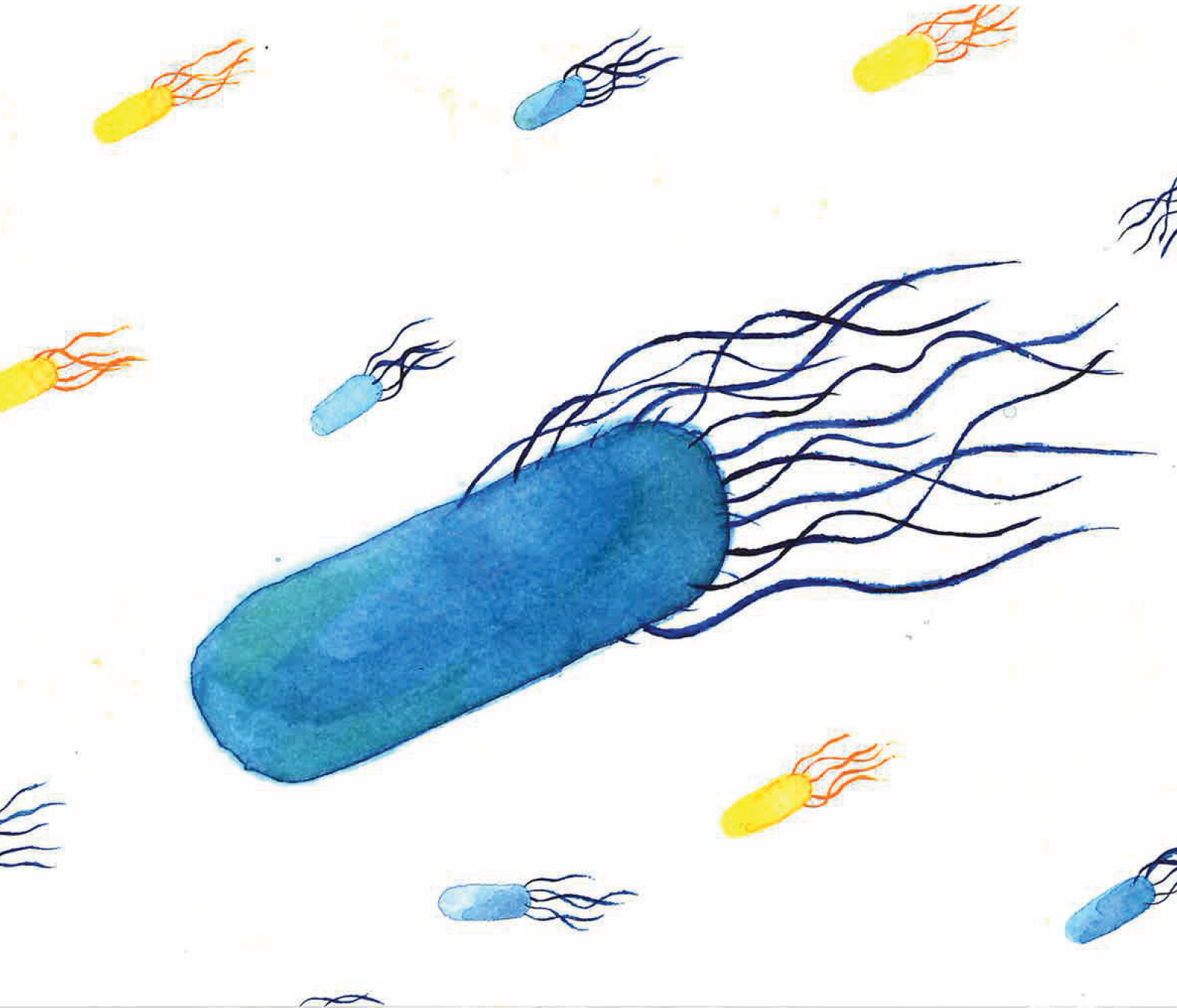
Faculty of Health Sciences

Department of Pharmacy

Cancer drugs as drivers of antibiotic resistance

Jónína Sæunn Guðmundsdóttir

A dissertation for the degree of Philosophiae Doctor - June 2023



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Cancer drugs as drivers of antibiotic resistance

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Tromsø - June 2023

Microbial Pharmacology and Population Biology Research Group
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Front page content: “Bakteríurnar hennar Nínu” – a watercolour painting by Helga Guðrún Guðmundsdóttir.

Illustrations in this thesis were created by Jónína Sæunn Guðmundsdóttir with the use of Adobe Illustrator.

“Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.”

- Marie Curie

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Guðmundsdóttir, J. S.**, Fredheim, E. G. A., Koumans, C. I. M., Hegstad, J., Tang, P., Andersson, D. I., Samuelsen, Ø., Johnsen, P. J. (2021). The chemotherapeutic drug methotrexate selects for antibiotic resistance. *EBioMedicine,* 74, 103742. https://doi.org/10.1016/j.ebiom.2021.103742

II **Guðmundsdóttir, J. S.**, Hansen, J. U., Samuelsen, Ø., Johnsen, P. J. (2023). Maintenance of a multidrug resistance plasmid in NMRI mice exposed to methotrexate: a pilot study. *Manuscript.*

III **Guðmundsdóttir, J. S.**, Gama, J. A., Fredheim, E. G. A., Harms, K., Samuelsen, Ø., Johnsen, P. J. (2023). Antineoplastic drug selection for antimicrobial resistance. *Manuscript.*

Articles not included in the thesis:

Knopp, M., **Gudmundsdottir, J. S.**, Nilsson, T., König, F., Warsi, O., Rajer, F., Ädelroth, P., Andersson, D. I. (2019). *De novo* emergence of peptides that confer antibiotic resistance. *mBio* 10:e00837-19. https://doi.org/10.1128/mBio.00837-19

Knopp, M., Babina, A. M., **Gudmundsdóttir, J. S.**, Douglass, M. V., Trent, M. S., & Andersson, D. I. (2021). A novel type of colistin resistance genes selected from random sequence space. *PLOS Genetics, 17*(1), e1009227. https://doi.org/doi:10.1371/journal.pgen.1009227

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Abbreviations

ALL	Acute lymphoblastic leukaemia
AMR	Antimicrobial resistance
ARG	Antimicrobial resistance genes
ATC	Anatomical Therapeutic Chemical
AUC	Area under the curve
bp	Base pairs
CCNSC	The Cancer Chemotherapy National Service Center
DDD	Defined daily doses
DHF	Dihydrofolic acid
DHFR	Dihydrofolate reductase
DHP	Dihydropteroate
DHP-PPi	Dihydropteridine pyrophosphate
DHPS	Dihydropteroate synthetase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
GI	Gastrointestinal
GTP	Guanosine triphosphate
HGT	Horizontal gene transfer
IV	Intravenously
MDR	Multi-drug resistance
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*

MTX	Methotrexate
NCI	The National Cancer Institute
OD	Optical density
OM	Outer membrane
PABA	*para*-amino benzoic acid
PBP	Penicillin binding protein
RFC-1	Reduced-folate transporter 1
RNA	Ribonucleic acid
SERM	Selective estrogen receptor modulator
THF	Tetrahydrofolic acid
TMP	Trimethoprim
TS	Thymidylate synthase
UNN	Universitetssykehuset Nord-Norge
UTI	Urinary tract infection
WHO	Word Health Organization
wt	Wild type
WWI/II	World War I/II

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"*Ber er hver að baki, nema sér bróður eigi*" - Brennu-Njáls saga.

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Tromsø, June 2023

Jónína S.

Abstract

Antibiotics are the cornerstone for modern medicine, and their introduction into clinical use has made common medical procedures such as surgeries and cancer chemotherapy possible. The consequences of antimicrobial resistance (AMR), if it continues to rise on a global scale at its current speed, are expected to be staggering. It is well-known that antibiotics drive the evolution and spread of AMR, but the extent to which non-antibiotic drugs can do the same remains largely unknown.

In this thesis, I have investigated whether drugs used in cancer therapy may drive AMR evolution in the common gut bacteria *Escherichia coli.* I screened a panel of 73 oncology compounds against 11 common AMR mechanisms, looking for combinations where expressing AMR gives bacteria fitness advantages in the presence of antineoplastic agents. Of the 23 strongest combinations identified in the screen, an in-depth study looking into the effects on bacterial evolution and the underlying molecular mechanisms has been conducted for one agent. I show that the widely used cytotoxic drug methotrexate (MTX), used both in the treatment of cancer as well as for many autoimmune diseases, can not only cause high-level trimethoprim (TMP) resistance at a wide range of concentrations. Furthermore, I have demonstrated that selection for TMP resistance takes place at MTX concentrations well below the concentrations known to inhibit growth. This is especially problematic when TMP resistance is plasmid-mediated, as MTX exposure will then select for practically any AMR determinant co-expressed on the same plasmid.

With this work, we provide valuable insights into the effects that drugs used in cancer chemotherapy have on AMR evolution. A better understanding of the drivers of resistance, especially those directly affecting vulnerable patient groups, is essential if we hope to curb the spread and evolution of AMR.

Útdráttur á Íslensku

Sýklalyf eru einn af hornsteinum læknavísindanna. Þau umbyltu meðhöndlun smitsjúkdóma og hafa gert flóknari skurðaðgerðir, meðhöndlun nýbura og krabbameinsmeðferðir að möguleika sem ekki þekktist áður. Það er því mikið áhyggjuefni þegar sýklalyf hætta að virka sem skyldi og sýklalyfjaónæmi er eitt af þeim stærri lýðheilsuvandamálum sem heimsbyggðin stendur frammi fyrir í dag. Það hefur verið þekkt lengi að notkun sýklalyfja valdi og auki sýklalyfjaónæmi, það sem hefur hinsvegar verið minna rannsakað er hvaða áhrif önnur lyf en sýklalyf hafi á þá þróun.

Í doktorsverkefninu mínu hef ég rannsakað hvort að krabbameinslyf geti haft áhrif á þróun sýklalyfjaónæmis í bakteríum. Ég hef beitt þekktum aðferðum innan örveru- og þróunarfræði til að rannsaka hvaða áhrif meðhöndlun með krabbameinslyfjum hafi á þarmabakteríuna *Escherhicia coli.* Áhrif 73 krabbameinslyfja á 11 algenga ónæmisferla voru könnuð, í leit að samsetningum þar sem að sýklalyfjaónæmi gerir ónæmu bakteríunum kleift að vaxa betur en en þeim næmu. Af þeim 23 samsetningum sem ég fann, þar sem sýklalyfjaónæmi jók hæfni ónæmra bakteríu, hef ég rannsakað ítarlega hvaða erfða- og sameindalíffræðilegu ferlar liggja að baki fyrir eitt parið. Í þeirri rannsókn sýni ég að methotrexate (MTX), lyf sem er mikið notað í meðferðum gegn bæði krabbameini og gigtarsjúkdómum, geti valdið klínísku sýklalyfjaónæmi gegn sýklalyfinu trimethoprim (TMP). Í þeirri rannsókn sýni ég að valið sé fyrir auknu ónæmi í bakteríum við MTX lyfjastyrki langt undir þeim sem þarf til að hafa áhrif á bakteríuvöxt. Þessar niðurstöður eru sérstaklega áhyggjuvaldandi í ljósi þess að TMP ónæmi er oft að finna á plasmíðum, sem leiðir af sér að lágir styrkir af MTX geti í verstu tilfellum valið fyrir fjölónæmum bakteríum þegar mörg ólík ónæmisgen er að finna á einu og sama plasmíði.

Með mínu framlagi vonast ég til þess að gefa aukna innsýn inn í þau áhrif sem krabbameinslyf hafa á þróun sýklalyfjaónæmis í bakteríum. Aukinn skilningur á drifkröftum lyfjaónæmis, sérstaklega þeim sem hafa bein áhrif á þegar viðkvæma sjúklingahópa, er nauðsynlegur ef við eigum að eiga von um að hægja á þróun og útbreiðslu sýklalyfjaónæmis á farsælan máta.

Preface

In this thesis, I present three investigations conducted by me and my co-authors during my doctoral studies. They all aim to address the same question: do drugs used in cancer chemotherapy act as drivers of antibiotic resistance? I first heard of this idea during my interview for the position, and I knew it was a once-in-a-lifetime opportunity worth chasing to (what sometimes has felt like) the end of the earth. It has been a privilege to have been entrusted with bringing a mere idea to an up-and-running research project. A process that has allowed me to grow into an independent and confident scientist. I chose to conduct my research using *Escherichia coli* because of its status as the flagship bacterium of molecular biology. It is well-described and understood and easy to manipulate and play around with. A substantial part of my time in Tromsø has been spent working with the cancer drug methotrexate, where I have had the opportunity to utilise many traditional molecular and evolutionary biology methods to study how the drug affects bacteria. However, I have also been allowed to establish my own ways in the lab, trying out various new methods and designing my own, all while working as a part of a solid team with my supervisors to bring our vision for this project to reality through trial and error. This process has not only been challenging but also tremendous fun. I strongly believe that the key to battling antibiotic resistance is to slow down the evolution and spread of resistance and that to do so, we need a better understanding of the underlying drivers. It is my hope that the studies presented in this work make a substantial contribution to our understanding of how drugs used in cancer chemotherapy can contribute towards resistance formation in gut bacteria and that with this better understanding, we may be better equipped to deal with the challenge ahead.

1. Background

"The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."

- Sir Alexander Fleming, Nobel Lecture, December 11, 1945.

1.1. Antibiotics

Those were the words of Alexander Fleming, spoken when he was awarded the Nobel Prize in Physiology or Medicine for the discovery of penicillin. For he foresaw already then, when the rest of the world saw hope during times when a small scratch could easily become a lethal wound, the threat antimicrobial resistance (AMR) evolution had the potential to become. It was several years earlier, in 1928, that Fleming discovered the antimicrobial effects of the fungus *Penicillium notatum* during an accidental contamination event in his laboratory[1]. As Fleming struggled with the purification of penicillin, years would pass from its discovery until a collaborative effort between a team of Oxford scientists led by Howard Florey and Ernst Chain (who both shared the 1945 Nobel Prize with Fleming) and the US military led to its purification and mass production during World War II (WWII)[2,3]. Noteworthy is that at that time, in collaboration with Edward Abraham, Chain had already discovered and described penicillinase, a bacterial enzyme able to break down penicillin[4].

1.1.1. The origin of antibiotics

Despite penicillin being the first natural antibiotic on the market, earlier milestones in the treatment of infectious diseases should not go unmentioned. The use of natural remedies to treat infectious diseases is ancient and vastly outdates the modern antibiotic era. In fact, there is evidence that the ancient Egyptians used honey, mouldy bread, garlic, essential oils and other natural mixtures for the treatment of infectious diseases[5,6], and remedies described in medical texts from the Anglo-Saxon period have been shown to be effective against the same diseases

they were described as a treatment for[7]. The start of the modern antibiotic era, however, is commonly credited to the Nobel Laureate Paul Ehrlich. In the early 1900s, Erlich began a systematic search for a drug against syphilis [5]. Ehrlich envisioned finding a "magic bullet" in the form of a chemical agent that could selectively kill a disease-causing microbe without harming the body itself. He screened hundreds of different compounds. His 606th compound, later named Salvarsan, became the first synthetic antibiotic to be put on the market in 1910[8,9]. The screening approach Ehrlich applied became widely adopted, and a similar screen resulted in the discovery of sulphonamides a couple of decades later[10]. Penicillin became publicly available shortly thereafter, replaced salvarsan and neosalvarsan as treatment for syphilis, and properly marked the dawn of the antibiotic era.

The word *antibiotic* was first used by Selman Waksman in 1942. In those early days, it exclusively referred to chemical substances produced naturally by microorganisms, shown to inhibit bacterial growth[11]. However, its definition has broadened and is used more extensively for all chemical substances with antibacterial properties, no matter their origin. Waksman, who studied actinomycetes, realized that many of the soil-derived bacteria he studied could produce secondary metabolites conferring antimicrobial properties and started a systematic search for antibiotic-producing soil bacteria. He and his students screened thousands of different microbes using a systematic agar overlay process known today as the "Waksman platform". Their efforts resulted in the isolation and identification of more than 15 new antibiotics, and to this day a vast majority of known antibiotics are secondary metabolites of a natural origin[12,13].

1.1.1.1. Antibiotics in nature

It is estimated that microorganisms have been producing antibiotics for as long as 2 billion years, suggesting that these compounds predate multicellular life as we know it[14,15]. Several explanations for why bacteria and yeast produce antibiotics have been proposed. The two main hypotheses are their role in microbial warfare (i.e., that secondary metabolites with antibiotic properties serve as ecological weapons when fighting for space and nutrition) and as signalling molecules[16-19]. Antibiotic concentrations found in nature are often too low to have an antibacterial effect[20] but have been shown to effect global transcription patterns[21,22], regulate the expression of genes associated with virulence[23-25], induce the SOS response in bacteria[26,27] and play a role in quorum sensing and host-parasite interactions[25,28,29]. Beyond revolutionizing infectious disease treatment, antibiotics play a key role in enabling several medical procedures. These include but are not limited to cancer chemotherapy, organ transplants, replacement

surgeries and neonatal care[30]. Consequently, antibiotics have become some of the most widely used medicines in the world.

1.1.1.2. Global consumption and usage of antibiotics

There is currently little information on antibiotic use in low-income countries, and the need for a better understanding of antibiotic consumption has led to global efforts to address the issue. Recent work has shown that antibiotic usage has increased worldwide, with an estimated increase from 9.8 defined daily doses (DDD) per 1000 population per day in the year 2000 to an estimated consumption of 14.3 DDD/1000/day globally in 2018[31]. This translates to every human being on earth taking between 1.5 to 10 individual antibiotic doses a year on average[32,33]. Concurrently, antibiotic use in agriculture has exploded: they are not only used to treat and prevent diseases in animals and crops but are, in many countries, still used as growth promoters[34]. It has been estimated that more than 70% of antibiotics sold worldwide are used in livestock animals, with global sales steadily increasing[35].

1.1.2. Antibiotic classes and mechanisms of action

Antibiotics comprise a large and diverse group of molecules traditionally classified as bactericidal (i.e., drugs that kill) or bacteriostatic (i.e., drugs that inhibit growth). However, this classification is not always clear and can be affected by outside factors such as nutritional and oxygen availability, resulting in drugs that can be bactericidal at times but bacteriostatic under different conditions[36,37]. Other common ways of distinguishing antibiotics are to group them based on their chemical structures, origin, and activity spectrum. Antibiotics able to kill or inhibit the growth of both Gram-positive and Gram-negative bacteria are termed broad-spectrum, whereas those with effects limited to a single Gram group are classified as narrow-spectrum. Bacteria are divided into one of two Gram groups based on their cell wall structure (illustrated in Figure 1)[38].

Most antibiotics work through disruption of cell wall integrity or by interrupting essential molecular processes within the bacterial cells. In order to get to their targets within the cell, antibiotics need to cross the bacterial cell envelope, which represents a first line of defence for bacterial cells against antibiotics. Of the two Gram groups, the cell wall of Gram-negatives has been proven to be the more difficult to cross[39]. The antibiotics on the market today can roughly be divided into five main groups based on their targets within the bacterial cell: drugs targeting the cell wall, drugs targeting nucleic acid synthesis (both DNA and RNA), drugs targeting protein synthesis and drugs targeting metabolic processes within the cell (Figure

1). These groups can be further subdivided into 15 major antibiotic classes based on their mechanism of action[40,41].

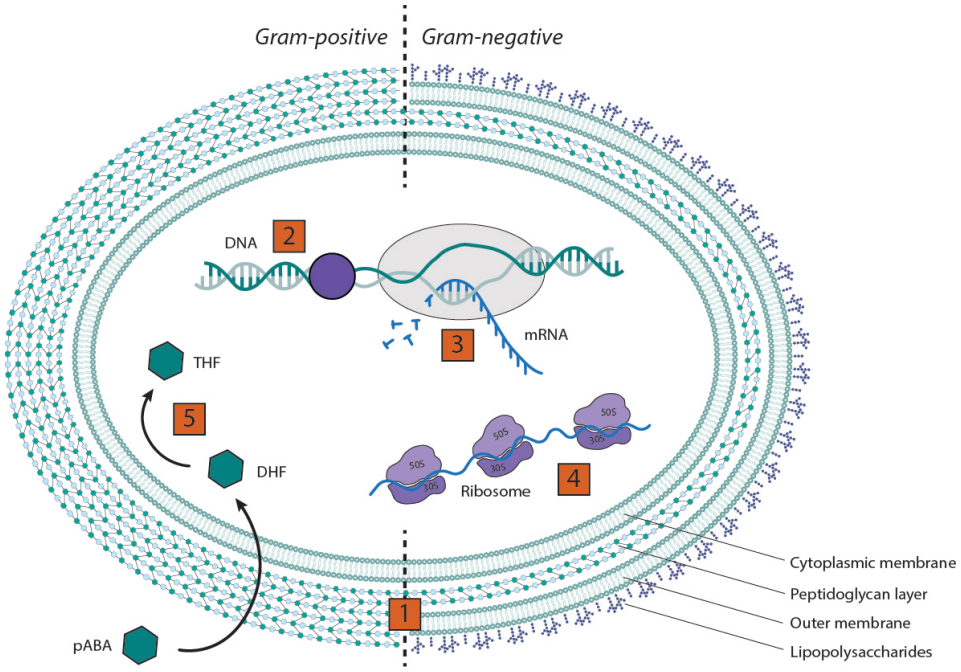

Figure 1: Targets of antibiotics within the bacterial cell. The major antibiotic classes can be divided into five different groups based on their targets: 1) cell wall synthesis and/or integrity 2) DNA synthesis, 3) RNA synthesis, 4) protein synthesis and 5) metabolic processes, here exemplified by folic acid synthesis. The left half of the figure shows the structure of a Gram-positive cell wall, composed of a cytoplasmic membrane and a thick peptidoglycan layer. The right half of the figure shows the Gram-negative cell wall, composed of a cytoplasmic membrane, thin peptidoglycan layer in the periplasmic space and an outer membrane adorned with lipopolysaccharides.

1.1.2.1. Drugs targeting the cell wall

Of the antibiotic classes targeting the cell wall, the β-lactam antibiotics are undoubtably the most important. They represent the most widely used antibiotic class worldwide, are used both against Gram-positive and Gram-negative bacteria, and are easily distinguishable by their chemical structure[42]. All β-lactam antibiotics and most β-lactamase inhibitors share a common core comprising a four-membered β-lactam ring[43]. They bind to the penicillin binding proteins (PBPs), inhibiting cross-linking of the peptidoglycan layer, disturbing cell wall synthesis and causing cell death. β-lactams can be divided into four main sub-classes (penicillins, cephalosporins, carbapenems and monobactams) varying in both structures and activity spectrums[44].

The glycopeptides are another important antibiotic class inhibiting the cell wall synthesis in Gram-positive bacteria. Unlike other antibiotic classes, the glycopeptides bind directly to the substrate of cell-wall biosynthesis (in contrast to active-site inhibition) and cause cell death through its inhibition[45]. Their inability to cross the outer membrane (OM) of Gram-negative bacteria limits use to Gram-positive bacteria. But vancomycin as well as newer

glycopeptides on the market remain highly relevant and have become “last-resort antibiotics” (i.e., should only be used when all other possibilities have been exhausted) for treatment of serious Gram-positive infections[46].

Other antibiotic classes targeting the cell wall are lipopeptides (e.g., daptomycin), polymyxins (e.g., colistin), and some antimicrobial peptides. Both classes work through Ca^{2+} dependent mechanisms targeting cell wall integrity. Colistin binds to the lipopolysaccharide of Gram-negative bacteria, while daptomycin inserts into the cell membrane of Gram-positive bacteria and cannot penetrate the Gram-negative OM. The few antimicrobial peptides currently approved for human medicine are considered last-resort antibiotics and have been associated with severe toxic side effects[47,48].

1.1.2.2. Drugs targeting nucleic acid synthesis

There are two main antibiotic classes targeting nucleic acid synthesis, one for each nucleic acid class: the quinolones targeting DNA synthesis and the rifamycins targeting RNA synthesis. The quinolones in clinical use today are fluorinated derivates of nalidixic acid referred to as fluoroquinolones. Of those, ciprofloxacin remains one of the clinically most important antibiotics worldwide, decades after it first came on the market. Not only is ciprofloxacin able to penetrate the cell wall of both Gram-positives and Gram-negatives, but it became the first quinolone effective for treating diseases other than urinary tract infections (UTIs). Quinolones work by inhibiting the bacterial type II topoisomerases (DNA gyrase and topoisomerase IV), preventing the DNA helix from unwinding, resulting in DNA strand breaks and cell death[49]. Rifamycins, on the other hand, work by inhibiting a downstream step in nucleic acid synthesis, namely DNA-dependent RNA synthesis. They make up one of the most broad-spectrum antibiotic classes on the market, binding to the DNA-dependent RNA polymerase in bacteria while leaving the mammalian RNA polymerases be. This binding sterically blocks the elongation of the mRNA chain and consequently prevents downstream protein synthesis within the bacterial cell[50,51].

1.1.2.3. Drugs targeting protein synthesis

After DNA has been transcribed into mRNA, the mRNA binds to the ribosomal 30S subunit which decodes its genetic information. Subsequently, the larger 50S subunit can dock onto the smaller subunit and form a functional 70S ribosome. Protein synthesis is initiated, and the nucleic acid sequence is translated into amino acids forming a peptide chain until the ribosome comes to a stop codon. A release factor binds to the stop codon, signalling protein synthesis to

be terminated and the ribosomal subunit to release the nascent polypeptide. The 70S ribosome is then dissembled and its components recycled for the next round of initiation[52]. Protein synthesis is a multiplex process where every step represents a potential target for antibiotics; consequently, it has become the molecular process most antibiotic classes work through inhibiting[41]. Aminoglycosides and tetracyclines bind to the 30S ribosomal subunit, which results in either mistranslated/truncated proteins or complete inhibition of protein synthesis, respectively[41,53]. Some major antibiotic classes inhibiting translation and resulting in truncated peptide chains by binding to the 50S subunit are streptogramins, lincosamides, macrolides and chloramphenicol[54,55]. The oxazolidinones bind to the same position in the ribosome but work through a slightly different mechanism, inhibiting the formation of the initiation complex and thus preventing the formation of a functional 70S subunit[56].

1.1.2.4. Drugs targeting metabolic processes

A cellular process that has been central to the work conducted in this thesis is folic acid synthesis. It makes for a great target for antibiotics but is also a known target in cancer chemotherapy. The different drugs targeting this pathway in bacteria and cancer cells are the focus of **Paper I** and **II,** and their mechanisms of action are therefore described in more detail both in this chapter and chapter 1.2.1.1.

While higher eukaryotes, including humans, are unable to make folic acid and take it up from the diet, many bacteria can generate folic acid from *para*-amino benzoic acid (PABA). Dihydropteroate synthetase (DHPS) catalyzes a reaction converting dihydropteridine pyrophosphate (DHP-PPi) and PABA into dihydrofolic acid (DHF). DHF is then further reduced to tetrahydrofolic acid (THF) by dihydrofolate reductase (DHFR). As THF is an essential precursor for nucleic acid synthesis (both DNA and RNA), enzymes associated with the folic acid synthesis pathway make for an excellent antibiotic target[57]. A summary of the folic acid metabolism in bacteria and cancer cells can be seen in Figure 2. The two antibiotic classes targeting the pathway work by inhibiting the two major steps in it: sulphonamides by inhibiting the DHPS[58] and pyrimidines (e.g., trimethoprim (TMP)) by inhibiting the DHFR[59]. TMP and sulphonamides are often given in combination because of the synergy between the two drugs[57,60].

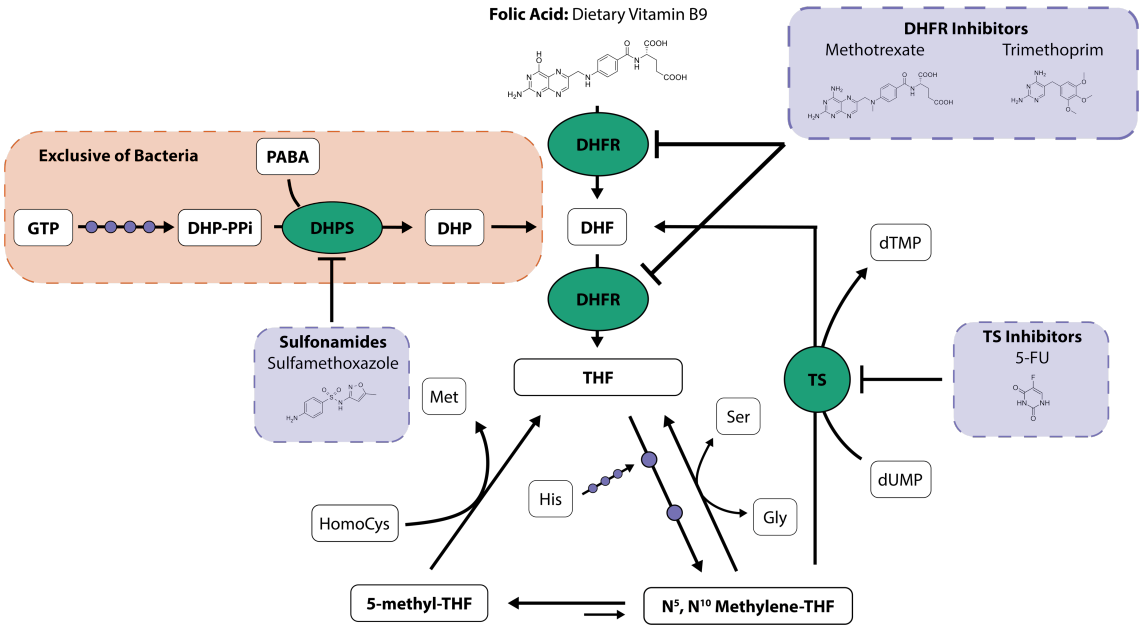

Figure 2: Summarised pathway of folic acid metabolism in bacteria and cancer cells. Main antifolate drugs discussed in this thesis along with their chemical structures are listed in purple boxes. The enzymes they inhibit are highlighted in green circles. Small purple circles over the arrows indicate an enzymatic reaction. While humans need to obtain folic acid from their diet, bacteria can synthesise their own from PABA. This process has been separated from the rest by an orange box. Abbreviations: DHP = dihydropteroate, DHP-PPi = dihydropteroate pyrophosphate, DHPS = dihydropteroate synthase, DHF = dihydrofolate, DHFR = dihydrofolate reductase, Gly = glycine, GTP = guanosine triphosphate, His = histidine, HomoCys = homocysteine, Met = methionine, PABA = p-aminobenzoic acid, Ser = serine, THF = tetrahydrofolate, and TS = thymidylate synthase. Adapted, with permission, from[61].

1.1.3. Current developments in antibiotic discovery

Having discussed the different molecular mechanisms applied by different antibiotics, one cannot help but circle back to Ehrlich's concept of a "magic bullet" and wonder what the "perfect antibiotic" would look like. What properties would that compound have? Does such an antibiotic exist, and if not, how far off is the scientific field from achieving such a goal?

A perfect antibiotic would have to meet several requirements. It should be bactericidal and able to kill a broad range of different bacterial species, both Gram-negative as well as Gram-positive. It should simultaneously be target-specific, harming only the pathogenic bacteria causing disease while leaving both the harmless bacteria residing in the body and the mammalian host cells be. Last but not least, the bacteria should not be able to find a way to work around the drug, and resistance evolution should not become a problem[62]. Such an antibiotic does not and has never existed. The disheartening truth is that when it comes to antibiotic development in the 21st century, the struggles of developing the perfect antibiotic have given way to struggles with developing new antibiotics in general. The problem lies not in finding new compounds that can inhibit bacterial growth, but in finding new compounds fulfilling enough of the requirements of the perfect antibiotic to make them worth developing[63].

More than half of the different antibiotic classes in clinical use today were discovered during the so-called golden era of antibiotic discovery, starting in the 1940s and lasting through the 1960s. While the Waksman platform resulted in a boom of new antibiotics reaching the market, the reality scientists were forced to face only a couple of decades later is that the number of unique antibiotic scaffolds derived from easily culturable soil bacteria is finite[63,64]. In the 1970s, when the antibiotic discovery rate dwindled, the focus shifted from finding new antibiotics to improving the antibiotics already available. The period from 1987 onwards is frequently referred to as "the discovery void". Very few antibiotics made it to the market during this time and those that did belonged to previously known antibiotic classes[62]. The timeline of antibiotic discovery can be seen in Figure 3.

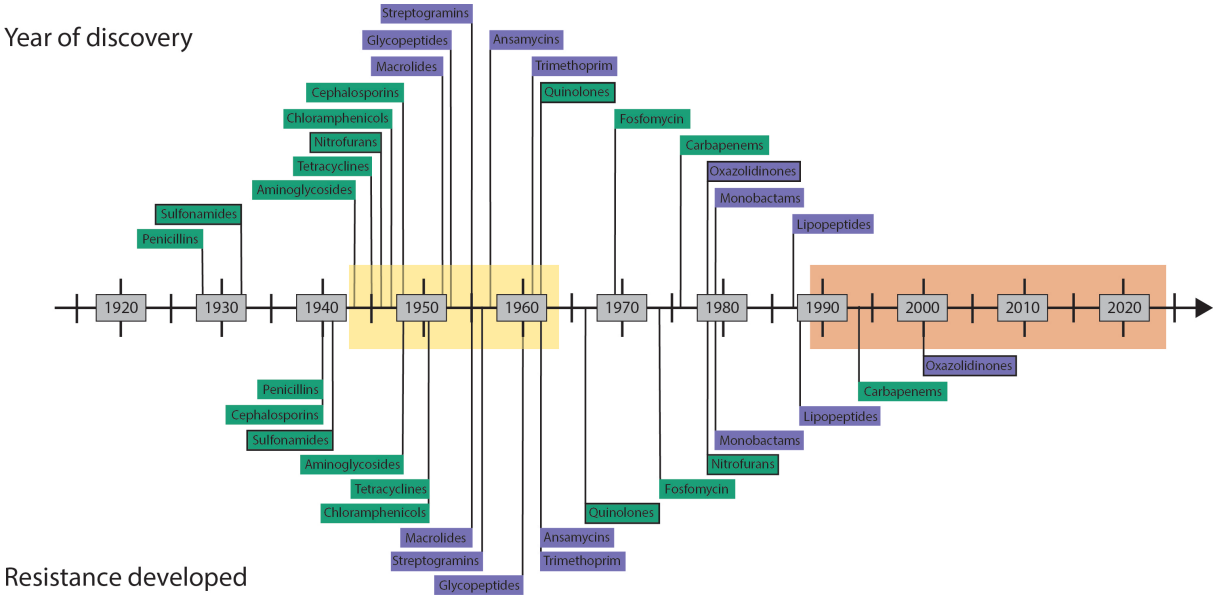

Figure 3: The timeline of antibiotic discovery, highlighting the rapid development of antibiotic resistance development, the golden era, and the discovery void. Top: year of discovery. Bottom: year when clinical resistance towards the antibiotic class was identified. Green boxes represent broad-spectrum antibiotic classes and purple boxes narrow-spectrum. Natural antibiotic classes do not have a box boarder while synthetic antibiotics are displayed using a thick box boarder. The golden era of antibiotic discovery between 1940 and through 1960 highlighted in yellow while the discovery void lasting from 1987 to current date is highlighted in orange. Adapted, with permission, from[63] and[65].

Why is it that, while the need for new and novel antibiotics is urgent and technical advantages have revolutionised the field of drug discovery, the big players in the pharmaceutical industry show little or no interested in antibiotic development? This has been attributed to several factors. Drug development, in general, is an exceedingly expensive and time-consuming process. Developing a new antibiotic is estimated to take between 10 and 20 years from the time of Hit discovery until the compound reaches the market, costing more than US$1.5 billion[66]. For a pharmaceutical company to invest that kind of time and money in a

product, it needs to be profitable. AMR development will be discussed in further detail in chapter 1.3, but due to the emerging global health threat, new antibiotics that make it through clinical trials and onto the market quickly become last-resort antibiotics. Drugs held in reserve instead of sold result in a broken business model where big pharmaceutical companies (big-pharma) focus their efforts on developing more profitable drugs while smaller biotech companies still working on antibiotic discovery go bankrupt in the process[66]. The biopharmaceutical company Achaogen is a daunting example of this, but the company was forced to file for bankruptcy in 2019, less than a year after the FDA approved their new antibiotic, plazomicin[67]. This was followed by another successful antibiotic developer, Melinta, filing for bankruptcy later in the year[68]. As more than 70% of all institutions actively working on the development of new antibiotics are considered small enterprises, the fact that they are repeatedly proven unsuccessful in surviving the development stage of their primary Hit raises a big concern for the future of the antibacterial pipeline.

The preclinical antibacterial pipeline currently consists of 217 antibacterial projects across 121 institutions. The high number of institutions whose sole focus is a single drug candidate is unsurprising, given that most players involved are small enterprises. However, there is a rapid decrease in numbers when looking at the number of compounds that have made it further along the clinical antibacterial pipeline. The current clinical pipeline contains 77 antibacterial agents, of which 45 are considered traditional antibiotics. Of those, 27 are reported to be active against pathogens on the list of priority pathogens (a list compiled by the World Health Organization (WHO) listing pathogens where the need for new and effective antibacterial agents is the most urgent), and only six can be considered novel in any way. This number is far from what is required to meet the urgent need for novel antibacterial agents. It is not expected to rise without a new finance model for the antibiotic industry[69,70], something that will not happen unless policymakers in the western world become more involved in the topic of financial incentives for antibiotic development and make it possible for scientists and pharmaceutical companies to stay (and become) involved in antibiotic development[70-72].

1.1.4. Effects of antibiotics on the human gut microbiota

Many drugs, including antibiotics, have negative side effects. The most common side effects of antibiotics stem from the gastrointestinal (GI) tract, resulting in nausea, diarrhea, vomiting and stomach pain. This is commonly credited to the disruption of the normal human gut microbiota, as the effects of antibiotics are not limited to disease-causing microbes[73]. The human gut microbiota refers to all microorganisms living in a person's GI tract. Microbial

colonization starts during birth, and the average healthy adult is estimated to have roughly 10^{13} microorganisms living in their intestinal tract[74,75].

The relationship between humans and their microbiota is largely symbiotic. Bacteria depend on the human host for nutrition, while the commensal bacteria living in our gut play a role in human health. They are known to prevent exogenous pathogens from colonising the gut, play an important role in the development of the gut immune system, and have a role in gut metabolism[76]. The role of the microbiota in human health and disease is an exceedingly complicated topic and beyond the scope of this thesis. However, there are things worth considering when discussing the effects of antibiotics on the microbiota. While technological advances made in the last decade have made research on a population level possible, resulting in a great interested in research focusing on changes to the microbiota, a huge challenge to the field remains distinguishing between association and causation[77]. A well-known example of this being the impact of the gut microbiota on obesity and other diseases. While it has become a well-established fact that changes in the microbiota composition can be correlated with obesity, studies attempting to establish causality wildly contradict each other[78-80].

1.1.4.1. The composition of the gut microbiota

The microbiota composition varies from one individual to the next, with countless factors playing a role in the unique bacterial composition, e.g., diet, lifestyle, drug use, stress, disease, age and race[77]. There are two divisions of bacteria which undoubtably dominate in the healthy human gut: the Bacteroidetes and the Firmicutes, which together represent more than 90% of the total population of the gut microbiota[81-83]. When the natural balance between the different bacterial species residing in the gut is altered, with one or more bacterial species/families increasing or decreasing in abundance, it is commonly referred to as dysbiosis. Dysbiosis has been associated with the onset of various diseases[84-86].

Antibiotics are known to directly affect both the number of bacterial species in the gut and total cell numbers. While the bacterial abundance in the gut will normally stabilise to pre-treatment numbers within a few weeks after treatment ends, we now know it can be with permanent changes to the taxonomy, resistome (i.e., all resistance genes found in the gut community) and the metabolic output of the bacteria residing in the gut[87]. The general consensus for a long time has been that broad-spectrum antibiotics have a more negative impact on the gut microbiota than narrow-spectrum antibiotics, based on the postulation that the more commensal bacteria that are removed, the larger the space that opens up in their habitat for opportunistic strains to invade. A well-established example of this is *Clostridioides difficile*

infections. While *C. difficile* is a common member of the normal gut microbiota, treatment with broad-spectrum antibiotics such as clindamycin is known to be one of the biggest risk factors for contracting *C. difficile*-associated diarrhea. This is a result of *C. difficile* grabbing the opportunity to multiply in numbers and invade a larger part of the gut, going from a harmless part of the commensals to a disease-causing pathogen[88,89]. However, there is no one-size-fits-all for those kinds of dynamics, and recent studies have described the differential effects of antibiotics on bacterial abundance and microbiota composition. While broad-spectrum antibiotics may lower the general bacterial abundance during treatment, the whole community then has an equal chance of regrowing once treatment stops. Hence antimicrobial treatment has a relatively large, short-time effect on bacterial abundance but minor long-term effects on diversity. On the other hand, narrow-spectrum antibiotics remove a few but specific species from the population, allowing other members of the microbiota to take over during the treatment period. This can result in a more long-term effect on the population dynamics as the eliminated species do not have the same chance to regrow[90,91].

1.1.5. The role of antibiotics in the history of cancer chemotherapy

Ehrlich, who is often referred to as the father of chemotherapy and best known for his contributions to the field of immunology, was the first person to use the word chemotherapy, defining it as any use of a chemical to treat any disease. This showcases how Ehrlich's vision of a "magic bullet" as a chemical agent that could selectively kill a disease-causing entity while leaving its host unharmed did not stop at microbes. Finding a chemical agent able to selectively kill cancer cells while leaving the body's normal cells unharmed must have seemed like a Sisyphean task for clinicians and scientists of the time[9,92]. Today, while its official definition remains the same, the word chemotherapy is generally used for cancer treatment.

One can speculate on how much Ehrlich and Fleming may have inspired each other's work at the time, but in 1909 Fleming was one of the few doctors Ehrlich had entrusted with salvarsan and that actively used the drug in the treatment of syphilis[93]. However, one thing that leaves no room for speculation is that the development of penicillin was not the only chemotherapy breakthrough brought on by warfare. As a physician during WWI, Fleming realised that the chemicals used to treat sepsis were highly toxic and affected the growth of white blood cells much more than bacterial cells[3]. In the same period, a research project led by Louis S. Goodman and Alfred Gilman investigated the effects of nitrogen mustard on lymphoma[94]. When hundreds of people were exposed to mustard gas during an air raid on Bari in WWII, they combined

victim autopsies from Bari with their own research on mice and conducted the first clinical trial of nitrogen mustard as a therapeutic agent in 1943[95].

1.1.5.1. Sidney Farber – the father of modern chemotherapy

Another important research project in cancer chemotherapy originated from nutritional research during WWII, investigating the effects of vegetable intake on bone marrow. The project, led by Sidney Farber, identified folic acid analogues as a potential cancer treatment, and in 1947 the drug candidate, aminopterin, became the first drug to cause temporary remissions in acute lymphoblastic leukaemia (ALL) in children[96,97]. When a closely related antifolate methotrexate (MTX) was shown to have a better therapeutic index than aminopterin[98] the clinical use of the latter drug was abandoned in favour of MTX, which became the first drug to be used to cure a solid tumour[99].

Farber knew of the work of Waksman and his screening platform. In the summer of 1954, a discussion between the two led to Waksman sending some of his more toxic antibiotics to Farber, who wanted to see if any of them could be repurposed as antitumour agents even if too toxic to justify the use for the treatment of infectious diseases. Their interaction led to actinomycin D, an antibiotic originally discovered by Waksman in 1940, becoming the first antibiotic to be shown to have antitumour activities and used in the treatment of cancer[100,101]. Farber concluded in his publication on the first clinical trials that he expected Waksman's research to result in the discovery of multiple new antibiotics with potential as antitumour agents, a prospect Waksman himself shared[101,102]. The Cancer Chemotherapy National Service Center (CCNSC) was formed at the National Cancer Institute (NCI) in the US, and a bacterial screening system was used to actively look for more cytostatic drugs[103,104]. Simultaneously, scientists at the Institute of Microbial Chemistry in Japan extended their search for antibiotics from microbial metabolites and started to actively look for antitumor antibiotics within their collections[105], resulting in the discovery of sakromycin in 1952[106] and bleomycin a decade later[107]. Thus, parallel to the golden age of antibiotic discovery, the era of cancer chemotherapy had begun. Even though antibiotic and cancer chemotherapies have become two separate fields of research and are today rarely associated with one another, their shared history gives background and rationale for the many commonalities between the two drug classes.

1.2. Cancer chemotherapy

While the oldest descriptions of cancer date back to ancient Egypt, it is only during the last few centuries that cancer has gone from being a rare disease without a name to becoming one of the world's largest health problems. There are a couple of things to consider when discussing the increased prevalence of cancer. One is the technical advances in the field of medicine. A recent study of the prevalence of bone cancer in skeletons from the 6th – 16th century suggests that cancer was considerably more widespread during medieval times than previously believed. However, it was first during the 17th century that doctors had the tools to diagnose cancer, if not yet understand it[108]. In addition, the lifetime risk of developing cancer increases annually, with more than 10 million deaths credited to cancer every year due to an ageing population. In countries where communicable diseases have been managed, life expectancy has increased, but it has done so in parallel to the prevalence of cancer and other non-communicable diseases. However, age is not the only risk factor responsible for the geographical patterns observed in global health and diseases: Westernized lifestyle has increased the prevalence of early-onset cancer in given parts of the world[109,110].

As technology has advanced, so has our understanding of the molecular mechanisms of cancer and our abilities to treat it. Unfortunately, the initial breakthroughs in cancer chemotherapy in the 1950s did not become the same beacon of hope the first antibiotics presented. The brutal side effects of chemotherapy, combined with the realisation that remission was short-lived at best, resulted in most clinicians of the time being critical towards those who persevered in their search for chemotherapeutic cures for cancer. The few doctors actively administrating those drugs to patients were considered radical (even cruel) by some and on the verge of fanatical by others. The common consensus was that while those chemotherapeutic agents might be able to actively kill cancer cells, cancer chemotherapy still did more harm than good to the patients being treated[97]. Despite that, unprecedented amounts of money were being invested in CCNSC in a combined effort between scientists and politicians to find a drug that could cure cancer, and the field of cancer chemotherapy steadily moved forward.

In contrast to the antibiotic discovery field, which has remained relatively stagnant since the late 1980s, the late 1980s and early 1990s marked a turning point in cancer chemotherapy. Scientists gained a better understanding of the molecular pathways distinguishing cancer cells from their healthy cell counterparts. It transpired that in addition to targeting the cancer cells themselves, one could target different proteins and signalling pathways imperative to cancer growth. The pharmaceutical industry suddenly saw the potential, and the market got flooded

with new drugs targeting different growth factors, modulators of apoptosis and other pivotal molecular targets of cancer[111]. This resulted in an immuno-oncology drug pipeline consisting of more than 2000 agents in either preclinical or clinical development, as immuno- and targeted therapies take up a continuously larger space in the field of oncology[112].

Targeted therapy completely changed the chemotherapeutic drug landscape, and today antineoplastic agents used to treat cancer can be divided into four major groups. (i) Traditional chemotherapy using cytostatic or cytotoxic drugs inhibiting cancer growth, (ii) hormonal therapy interfering with hormone production to slow down or stop cancer growth, (iii) targeted therapy using drugs to reverse the molecular changes making it possible for cancer cells to grow and divide and (iv) immunotherapy helping the body's own immune system to fight the cancer. However, most cancer patients require combination therapy combining one or more drug types, often with surgery and radiation[97,111,113].

1.2.1. Antineoplastic drug classes and their mechanisms of action

Whereas antibiotics can be classified based on their mechanisms of action and targets within a single-celled organism, the complexity level increases when discussing drugs used in cancer chemotherapy. There are hundreds of different types of cancer, some that stay local to specific organs and body parts and others that become metastatic and spread to different parts of the body[113]. The Anatomical Therapeutic Chemical (ATC) classification system is an internationally recognised classification system used to categorise drugs into different classes according to which part of the body they act on, as well as their therapeutic, pharmacological, and chemical properties. According to the ATC system, drugs used in the treatment of cancer, no matter the type of cancer, belong to the L group: Antineoplastic and immunomodulating agents[114]. Those are further divided into four subgroups based on their mechanism of action: L01 antineoplastic agents, L02 endocrine therapy, L03 immunostimulants and L04 immunosuppressants. The L01 drug class contains drugs used in cytostatic chemotherapy and targeted therapy. The L02 drug class contains drugs used for hormonal cancer therapy, and classes L03 and L04 are drugs used for immunotherapy. Notably, one of the seven L01 subgroups, L01D, is named "Cytotoxic antibiotics and related substances" and contains cytostatic and cytotoxic drugs of microbial origin, emphasising the historical connection between cytostatic drugs and antibiotics[115].

The work presented in **Papers I** and **II** focuses on the antifolate MTX, belonging to subgroup L01B, and its effects on the evolution of AMR in bacteria. A more in-depth background of MTX and its mechanism of action will be given in chapter 1.2.1.1. For the

experimental work done in **Paper III** a commercially available screening library, containing 72 drugs belonging to the therapeutic group "oncology" from the Prestwick Chemical Library® was used. The 72 drugs include 51 drugs belonging to ATC group L (divided unevenly between all four subgroups), 10 drugs belonging to other ATC groups and 12 drugs that do not have an ATC code. Of the antineoplastic and immunomodulating agents tested in **Paper III**, 18 drugs were identified as potential drivers of AMR. The drugs identified belong to various subgroups within the L01 and L02 groups of the ATC system and work through various mechanisms, most as antimetabolites, topoisomerase inhibitors or hormone antagonists, subgroups that will be described in more depth in chapters 1.2.1.1-1.2.1.3.

1.2.1.1. Antimetabolites

Antimetabolite drugs make up one of the oldest antineoplastic drug classes, encompassing drugs mimicking the natural building blocks of DNA: purines, pyrimidines and folic acid. The cancer cells, unable to distinguish the antimetabolites from their natural counterparts, will attempt to incorporate them into their DNA instead, inhibiting DNA synthesis. They can be divided further into subclasses based on which building block they mimic: purine antagonists, pyrimidine antagonists and antifolates[116].

MTX is arguably the most prominent antimetabolite on the market, and some would go as far as calling it one of the pharmaceutical industry's greatest success stories[117]. It remains in active use more than 70 years after its initial discovery, having gone from being the first drug used to cure a solid tumour to become the first-line drug in the treatment of rheumatoid arthritis, and is frequently used for the treatment of various cancers as well as autoimmune diseases. MTX has thus secured a spot on the list of essential medications by the WHO[111,118,119]. MTX works through different pathways in cancer and autoimmune diseases, inhibiting cell growth in cancer, but displaying anti-inflammatory effects at lower concentrations utilized in treating autoimmune diseases[118]. As autoimmune diseases remain outside the scope of the work presented in this thesis, this chapter will focus on its mechanism of action in cancer.

MTX is an antimetabolite of the antifolate type, a class of drugs working through the inhibition of folic acid metabolism and, consequently, DNA and protein synthesis (see Figure 2). MTX resembles the antibiotic TMP. Both drugs are structural analogues of folic acid, share a common 2,4-diamino pyrimidine moiety and work through DHFR inhibition. MTX can be taken up by cancer cells through at least two different mechanisms. The main mechanism is active transport by the reduced-folate transporter 1 (RFC-1), but during high-dose treatment MTX is known to enter the cell through passive diffusion as well. The latter mechanism is the

only one at play in MTX-resistant cells with malfunctioning RFC-1[120]. As bacteria make their own folic acid from PABA, they do not have a folic acid transporter. TMP easily enters bacteria cells by passive diffusion, but the high negative charge of MTX makes for a limited permeability compared to eukaryotic cells. However, at high concentrations MTX is known to enter bacteria cells through passive diffusion[121,122]. Inside the cancer cells MTX is polyglutamated, and both versions of the drug, MTX and MTX$(Glu)_n$, bind with high affinity to the DHFR and effectively stop the conversion of oxidized folates [FH_2] to their active reduced form [FH_4], resulting in the inhibition of both DNA and RNA synthesis[120,123]. Inside the bacterial cell, MTX binds to the bacterial DHFR in the same way as it does the human one, although with somewhat less affinity, giving the drug antimicrobial properties as well as antineoplastic ones[124-126].

1.2.1.2. Topoisomerase inhibitors

The DNA topoisomerases are enzymes crucial to all cellular life and are found in eukaryotes, prokaryotes, and archaea, where they play an indispensable role in DNA replication by breaking and re-joining the double-stranded DNA. They are generally divided into sub-groups based on their activity and structures, where type I topoisomerases cause single-strand breaks (type IA on the 5'-end, type IB/C on the 3'-end) while type II topoisomerases cause double-strand breaks[127]. Their ubiquitous and indispensable nature makes them an ideal drug target, and various topoisomerase inhibitors have been shown to confer both antineoplastic and antimicrobial properties[128].

The human DNA topoisomerase IB plays a large role in cancer; its overexpression in cancer cells results in faster cell replication, making it an ideal target for antineoplastic drugs. An example of a clinically important topoisomerase I inhibitor is the plant alkaloid camptothecin and its derivates[129]. Additionally, numerous antineoplastic agents work by inhibiting the human topoisomerase II. Those can be divided into catalytic inhibitors and topoisomerase II poisons based on their mechanism of action, with the latter group further divided into intercalating and non-intercalating poisons[128]. A substantial number of the drugs identified as potential drivers of AMR in **Paper III** were found to be topoisomerase II poisons, mainly drugs belonging to the anthracycline family (intercalating poisons). The mechanism of action of anthracycline in cancer cells remains a quite controversial topic, as various mechanisms have been credited with the observed effects and the antineoplastic effects of those drugs were known long before they were identified as topoisomerase II inhibitors. What is undisputable is that they intercalate into DNA where they, among other things, form complexes

with topoisomerase II, ultimately disrupting DNA replication and synthesis[130]. Markedly, a non-intercalation poison, etoposide, was also identified as a potential driver of resistance in **Paper III**, but etoposide works by inhibiting DNA religation[131].

1.2.1.3. Hormone antagonists

While most of the antineoplastic drugs relevant to this thesis are cytostatic or cytotoxic drugs used in traditional chemotherapy, a fair share of the drugs used in **Paper III** are used in hormonal therapy. One of the greater landmarks in cancer research was the realisation that prostate, breast and ovarian cancers can be hormone-dependent[132,133]. This in turn led to the realisation that the growth of these tumours can be inhibited by hormones and their analogues, hormone antagonists or other drugs inhibiting hormone synthesis. Hormone agonists are widely used to treat sex-steroid-dependent cancers, where they inhibit target tissues by downregulating hormone receptors necessary for cancer growth[134,135]. Hormone antagonists, on the other hand, work by inhibiting hormones from binding to the hormone receptors, leading to the arrest of cancer growth in hormone-dependent cancers. Hormone antagonists can be further divided into sub-classes based on the type of receptor they bind to, such as anti-estrogens, anti-androgens, and aromatase inhibitors[134]. Through the years there have been numerous reports about the antimicrobial effects of selective estrogen receptor modulators (SERMs)[136]. The exact mechanism underlying those effects is not fully understood but is believed to be through the disruption of the membrane potential of bacteria[137,138].

1.2.2. Effects of cancer chemotherapy on the human microbiota

While the effects of antibiotics on the human microbiota have been known for a long time, less attention has been given to the effects of non-antibiotic drugs. This has started to change, with drug use generally being recognised as a major factor in shaping the gut microbiota[77]. Recent studies have associated drugs used in the treatment of diabetes[139], GI diseases[140,141], mental disorders[142,143], cancer[144,145] and anti-inflammatory drugs[146] with changes in the human gut microbiota composition[147,148]. It has been known for a long time that drugs used in cancer chemotherapy confer antimicrobial properties[125,149,150], but if and how those drugs affect the human microbiota has remained largely unknown. A seminal study by Maier *et al*., published in 2018, became the first study to address the impact non-antibiotic drugs have on human gut bacteria on a large scale. The effects of 835 human-targeted drugs were tested on a panel of 38 different bacterial species, which aimed to represent the human gut microbiota as closely as possible *in vitro*. Of the 40 most active drugs, 12 (30%) are used in cancer chemotherapy. When

looking at drugs belonging to the ATC group L (antineoplastic and immunomodulating agents), half of the tested drugs (28/57) had antimicrobial activities *in vitro*[126].

When it comes to studies looking at the effects of cancer chemotherapy on the gut microbiota in cancer patients during treatment, the results point in the same direction. The microbiota composition as well as the total bacterial abundance in the gut is dramatically altered during cancer treatment, with dysbiosis and GI mucositis amongst common side effects of cancer chemotherapy[151-153]. How much of that can be attributed to antineoplastic drugs is hard to say, as most patients undergoing cancer treatment receive other types of medication before and during treatment, often including prophylactic antibiotic treatment. However, it is clear that the combination of cancer chemotherapy, antibiotics and hospitalisation has a large effect on the gut microbiota, but how much each factor contributes to the total effect is impossible to say[151,154-157]. Microbial dysbiosis in the gut of children undergoing cancer treatment has been associated with further health complications later in life, though establishing direct causality remains a common limitation of those studies[158]. Furthermore, while concerns have been raised that drugs used in cancer chemotherapy may drive antibiotic resistance evolution[126,159] the effects of cancer chemotherapy on the microbiota resistome remains a topic largely unexplored.

1.3. Drug resistance

Drug resistance refers to the ability of microorganisms or cancer cells to survive exposure to chemical agents that have previously been shown to kill them or inhibit cellular growth and has been described for both traditional drugs as well as vaccines[160]. Drug resistance in cancer and infectious diseases have many commonalities. As touched upon in previous chapters, various antineoplastic drugs work through the same molecular mechanisms as known antibiotics and have the same molecular targets. Concomitantly, drug resistance in cancer cells and bacteria can arise and be addressed in similar ways. Combination therapy is an example of a strategy used to battle drug resistance evolution in both bacteria and tumour cells[111]. What makes AMR particularly treacherous is the fact that bacteria are evolving clinical resistance that is spreading on a global scale faster than new antibiotics are making it to the market. So, while our abilities to cure cancer improve on yearly bases, our abilities to fight AMR bacteria are dwindling at an alarming rate, representing a major threat to global public health[161].

Global life expectancy has more than doubled in the last 150 years, from 29 to 73 years. The increase is attributed to a combination of factors: a better understanding of epidemiology

and the importance of sanitation in healthcare, a decrease in child mortality, and the introduction of antibiotics into clinical use. While antibiotics alone were not responsible for the shift, global life expectancy is estimated to drop down to about 50 years in the modern world without antibiotics[162,163]. Infectious diseases remain one of the leading causes of death worldwide, with 7.7 million deaths being linked to bacterial infections alone in 2019. Of those, 1.27 million deaths are directly attributed to infections caused by AMR bacteria[164,165]. This number is expected to rise in the years to come as global antibiotic use continues to increase while clinical resistance has been identified towards every single antibiotic on the market, while the antibiotic pipeline runs dry. It is thus clear that we need to gain a better understanding of what drives the evolution, selection and spread of AMR in order to slow it down.

1.3.1. Mechanisms of antibiotic resistance

AMR can be either intrinsic or acquired. Intrinsic resistance refers to species-specific traits conferring natural resistance towards an antibiotic class in most if not all, members of a given species. A good example of this is Gram-negative bacteria's resistance to antibiotics that cannot cross the Gram-negative cell wall. Conversely, acquired resistance tends to be bacteria's defence towards antibiotic exposure in its environment. It occurs when a previously susceptible bacteria gains increased resistance towards an antibiotic through mutations in one of the genes already present in the bacterial genome, gene amplifications, or the acquisition of new genes conferring AMR[166-168]. Cross-resistances between two or more antibiotics can arise if resistance towards one antibiotic simultaneously confers resistance towards another drug due to shared mechanisms of AMR. How those resistance mutations and genes are passed on from one bacterium to the next can be either from mother cell to its offspring via vertical inheritance, or via horizontal gene transfer (HGT) where genetic material is shared between unrelated cells. The three main mechanisms of HGT are transformation (i.e., where bacteria take up free DNA from their environment), conjugation (i.e., conjugative plasmids or transposons are moved from one cell to another through a procedure requiring cell-to-cell contact) and transduction (i.e., bacteriophages move resistance genes from one bacteria to another). Conjugative plasmids are circular DNA molecules, independent of the chromosome, that can express one or more AMR genes. They are transferred horizontally between bacteria, not restricted to a specific species, and represent one of the most important drivers of AMR[169]. Once a bacterium has acquired a new resistance gene through HGT, it can be further passed on through HGT as well as through vertical inheritance to any future offspring[167].

The basic mechanisms of AMR can roughly be divided into three groups: (i) prevention of access to target, (ii) target modification and (iii) alterations of the antibiotic (Figure 4). It can be through a single pathway or be the cumulated result of more than one. For example, aminoglycoside resistance has been described through active drug efflux, target alterations, as well as through acetylation of the drug itself. A single bacteria can have multiple mutations and genes encoding more than one of these mechanisms[170,171].

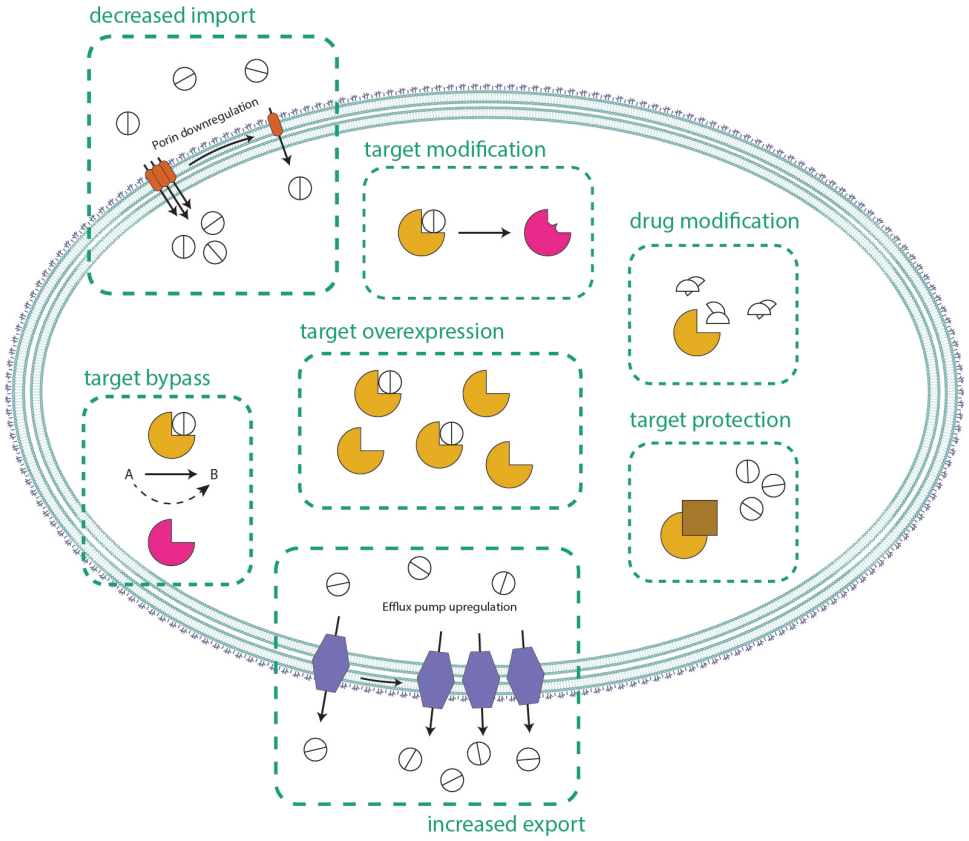

Figure 4: The basic mechanisms of AMR. AMR mechanisms can be roughly divided into three groups: i) prevention of access to the target, either through decreased import by changes to the porins or increased export through active efflux, ii) target modification, an umbrella term also covering target overexpression, target bypass and target protection and iii) drug modification.

1.3.1.1. Prevention of access to target

Most antibiotics have intracellular targets they need to cross the cell wall to gain access to (see Figure 1). If antibiotics are hindered from entering the cell or actively exported back out of the cell before reaching their intracellular target, the result will be increased AMR[41]. Resistance due to reduced permeability is generally associated with Gram-negative bacteria, although changes in the cytoplasmic membrane, affecting fluidity, have been shown to lead to increased resistance towards daptomycin in Gram-positives[172].

The Gram-negative OM is rich in water-filled channels called porins, used for nutrient transfer from the environment into the cell. Porins are usually not very selective, and many

small, hydrophilic antibiotics use them to cross the OM. Porin downregulation, loss or mutations affecting structure, substrate specificity or expression can increase AMR[171,173]. Should an antibiotic cross the cytoplasmic membrane, all bacteria have a well-preserved defence system, efflux pumps, that actively remove toxic substances from the cytoplasm. The various efflux pumps can be divided into six families and are either single-component transporters (found in Gram-positives or crossing the cytoplasmic membrane in Gram-negatives) or three-component tripartite systems spanning the whole Gram-negative cell wall. They can be compound-specific (for example, the Tet pumps) or able to transport a wide range of compounds out of the cell (known as multi-drug resistance (MDR) efflux pumps) and while all bacteria have some encoded on their chromosome, many are found on mobile genetic elements[41,174,175]. The most widely studied efflux system in *Escherichia coli* is the AcrAB-TolC efflux pump. It is substrate unspecific and has been shown to effectively transport various drugs, bile salts, dyes, detergents, biocides, and other compounds out of the cell. It has been hypothesised that bile salts may be the natural substrate of AcrAB-TolC and its original role may have been in GI colonisation[176]. Increased resistance due to efflux is often a result of changes in gene regulation and over-expression of already present efflux pumps. Conversely, knocking out genes encoding for AcrAB-TolC will increase susceptibility to various compounds (such as antibiotics and cancer drugs)[121,173,177,178].

1.3.1.2. Target modification

Resistance caused by target modification is an umbrella term used for AMR mechanisms involving target modifications, target protection, target overexpression and target bypass. As antibiotic binding is often quite specific, it can take as little as a single nucleotide change in the target to alter its protein structure enough to inhibit antibiotic binding without loss of functionality. This is a well-described resistance mechanism for multiple antibiotics and their targets. Some examples are resistance towards β-lactam antibiotics associated with genetic changes in genes coding for PBPs[179,180], and fluoroquinolone resistance due to mutations in the DNA gyrase or DNA topoisomerase IV genes[181]. Furthermore, alterations of the ribosomal 30S subunit, known to occur through mutations but most commonly seen via enzymatic modification by methyltransferases such as ArmA, will result in aminoglycoside resistance[182], while mutations in the 50S subunit are a well-described resistance mechanism towards macrolides[183].

Another way bacteria can prevent antibiotic binding is through a protection protein blocking access to the target. Target protection on its own can be considered a rather weak

resistance mechanism, but it is often seen in combination with target modifications, resulting in high-level clinical resistance. A common example of this is the Qnr proteins coupled with mutations in the DNA gyrase[184]. Increasing the copy number of the drug target, either through overexpression or gene amplification, can result in AMR. Even if the antibiotic successfully binds to its target, the extra copies will be able to execute its normal functions in the cell[185,186]. In a similar manner, expressing an alternative target able to fulfil the same role within the cell as the target the antibiotic inhibits will zero out its inhibitory effects. Some well-known examples of this kind of target bypass are genes coding for alternative DHFR and PBPs[187,188].

1.3.1.3. Antibiotic alteration

Bacteria can acquire AMR by producing enzymes capable of degrading or inactivating antibiotics before reaching their target, which generally requires less energy from the bacteria than mechanisms involving changes to the molecular components of the cell[41]. Examples of clinically relevant mechanisms involving drug degradation are the β-lactamases, enzymes able to hydrolyse the β-lactam ring of β-lactam antibiotics[189], and the tetracycline-inactivating enzymes (e.g., the Tet(X) enzymes) that catalyse the oxidation of tetracycline and confer resistance towards tigecycline[190]. Antibiotics can also be rendered useless by drug-modifying enzymes transferring chemical groups on the molecule, resulting in the loss of its antimicrobial properties. Different types of drug-modifying enzymes have been identified for different types of antibiotics. Aminoglycoside resistance can for example be mediated by antibiotic modification by acetyltransferases, phosphotransferases as well as by nucleotidyltransferases[182,191] and enzymes of the same classes have been recognised that can modify macrolides, rifamycins, streptogramins, lincosamides, quinolones and phenicols as well[184,188,192,193].

1.3.2. Evolution of antibiotic resistance

Experimental evidence detecting clinically relevant AMR genes (ARGs) in permafrost samples dating back more than 30,000 years suggests that AMR may have been evolving for as long as bacteria have existed[14]. However, it is clear that human use and the ensuing and ongoing environmental pollution with antibiotics have dramatically affected the evolution and spread of AMR. However, to properly explain and describe the drivers of AMR, one must first address basic concepts of evolutionary microbiology: the plasticity of bacterial genomes, selection, bacterial fitness and compensatory evolution.

When comparing bacteria to eukaryotes, the most obvious differences lie in their size and complexity. While eukaryotic genomes vary a lot in size, ranging from about 10^7-10^{11} base pairs (bp), bacterial genomes are more compact and show less size variation, with most falling into the 2-5 Mbp category[194]. Given the genomic plasticity of bacteria (i.e., their ability to rapidly alter their genomes to survive environmental changes), their small genome size is quite unexpected but can be explained by an inherited deletional bias. Noncoding DNA and genes not actively selected for are simply lost from the genome as more advantageous genes are selected for, resulting in genomes that keep changing and acquiring new genes without the genome size changing much in the long run[195]. The idea of the principle of natural selection dates back to Charles Darwin and his work "On the Origin of Species", where he defines it as "the principle by which each slight variation [of a trait], if useful, is preserved" [196]. At its most basic, this means that any trait making an organism more fit than other members of its species will result in that individual having more offspring than the rest. This will in turn lead to those conferring the superior trait taking over the population in time, as the trait gets selected for and those that have it end up outnumbering those that do not. Today, we commonly refer to this as "survival of the fittest".

Fitness is a measurable unit of how likely an individual is to survive and reproduce in a given population and environment and is greatly dependent on both. A specific mutation can give different fitness effects in the same bacteria at different growth conditions[197], and the same plasmid can give different fitness effects in different strains of the same bacterial species grown under the same growth conditions[198]. Similarly, an AMR mechanism shown to confer high fitness cost in a drug-free environment can come with fitness benefits when the same bacterial strain is grown in the presence of antibiotics[199-201], and the phenotypic effects observed for a given resistance mutation can depend on the strain background and epistatic interactions with other mutations on the chromosome[202,203]. When referring to fitness costs and benefits of a given trait in bacteria, it is often the relative fitness being discussed and not the absolute fitness. That is to say that the fitness of one genotype (often the wild type (wt) or the parental strain grown in rich-medium without selection) is set to 1, and the fitness of all other genotypes and conditions is normalized to the wt.

1.3.2.1. Evolutionary trajectories to AMR

The dynamics of AMR evolution is a complex interplay between positive selection and the cost of resistance. Those dynamics are made even more complicated by the influence of epistasis and compensatory evolution. Epistasis describes how the same mutation or gene can

give different phenotypes depending on the genomic context. That is to say that a complex interplay commonly takes place between genes on the chromosome, and the AMR phenotype can be highly dependent on the genomic background[203]. Meanwhile, compensatory evolution can be viewed as nature's response to genetic changes that confer significant fitness cost, but are for some reason selected for in the population. Then, instead of reversion and loss of function, secondary mutations will occur that increase fitness without losing the beneficial effect of the first mutation[203,204]. This has been observed for both the acquisition of chromosomal mutations and plasmids[204-206]. The most common way of measuring a mutant's resistance level is to measure its minimum inhibitory concentration (MIC), which is defined as the lowest drug concentration where bacterial growth is visibly inhibited[200]. Selection at low antibiotic concentrations has the potential to select for a much larger number of mutations, many of which confer smaller effects than observed during selection at higher concentrations. This results in very different evolutionary dynamics and enrichment patterns at low and high antibiotic concentrations. While fitness is the major deciding factor for which mutants become enriched under weak selection, resistance levels are the deciding factor under strong selection[203,207]. This mutational space for AMR in a bacterial population is visualised in Figure 5.

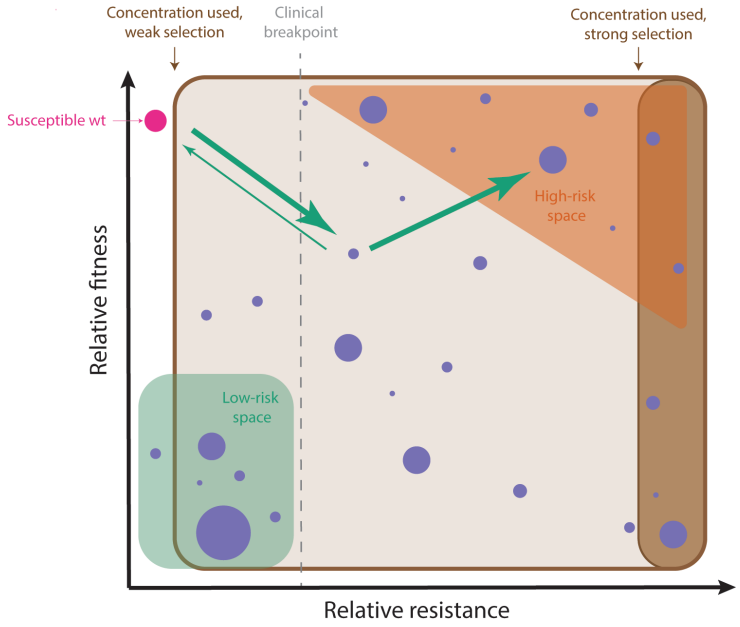

Figure 5: Mutational space for AMR. Each purple dot represents a resistant mutant popping up in a population. Its placement on the y-axis is based on its fitness and its placement on the x-axis on its resistance level. The pink circle represents the susceptible ancestor wild type (wt) Each purple circle represents one specific resistant mutant, and dot size represents the rate it grows at. The grey, dashed line represents the concentration of the clinical breakpoint. Weak selection at low concentrations can select for various mutations, differing a lot in both relative fitness and resistance. Meanwhile, strong selection at high concentrations can select for fewer mutants, but will select for mutations giving larger effects on relative resistance. Green arrows visualise compensatory evolution, where a first step mutation confers increased resistance but comes with a fitness cost that is then rescued by a secondary mutation. Adapted, with permission, from[203].

An increase in MIC does not necessarily come with clinical implications, even if the measured resistance has increased, which is why clinical breakpoints are set for clinically important antibiotics and pathogens every year. The clinical breakpoint of a drug is the MIC where treatment failure has become the most likely outcome even at high-dose treatment[208]. For a long time, the general assumption was that selection for resistance would only take place at antibiotic concentrations between the MICs of a resistant mutant and its wt, a concentration span referred to as the mutant selective window[209,210]. The hypothesis suggested that if growth was not inhibited, resistance would not be selected for, and the traditional selective window was set at concentrations between the MICs of a susceptible strain and its resistant mutant. It has now been well established that this is not the case and that concentrations below the MIC (sub-MIC) can not only select for high-level resistance mutations, but that they select for mutations that can be very different from the ones selected for above the MIC. The sub-MIC selective window can be much wider than the traditional one, ranging from the minimum selective concentration (MSC) to the MIC of the susceptible strain (Figure 6). Furthermore, it has been shown that sub-MIC concentrations of antibiotics and heavy metals can select for and contribute towards maintaining MDR-plasmids[200,211,212].

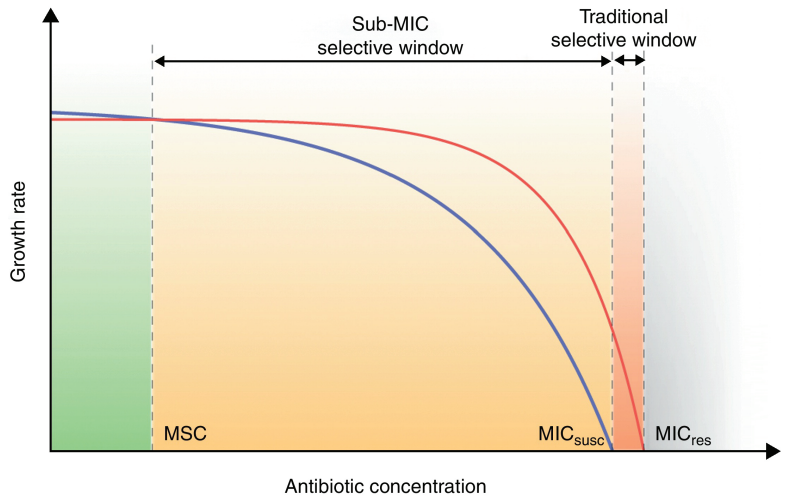

Figure 6: The mutant selective window. Traditionally the concentration span where selection for AMR was thought to take place was set between the MICs of the susceptible wt strain and the MIC of the resistant mutant (traditional selective window). It is now known that selection can take place at concentrations below the MIC of the wt, referred to as the sub-MIC selective window that ranges from the MSC to the MIC of the wt. The sub-MIC selective window can be much wider than the traditional window and go down to concentrations hundred times lower than the MIC of the susceptible strain. Figure re-printed, with permission, from[213].

1.3.2.2. Drivers of AMR

While the development of resistance was predicted, the unprecedented speed of the evolution and spread of AMR bacteria has taken was not. The genomic plasticity of bacteria in combination with their low generation time, made for a solid foundation for AMR evolution, which was then dramatically accelerated by human use of antibiotics. A daunting example of the direct changes caused by human use is the emergence and spread of MDR-plasmids. Conjugative plasmids have existed in nature for a very long time, and studies of historical isolates from the pre-antibiotic era have given valuable insights into how the introduction of antibiotics affected those. Plasmids in the Murray collection (strains collected between 1917 and 1954) are all resistance-free, whereas many of the same plasmids isolated from the 1950s and onwards had gained resistance genes[214,215]. The misuse and overuse of antibiotics in human health may be the most recognised driver of AMR, but it has become increasingly clear that other factors play a role. Other well-documented drivers are the misuse and overuse of antibiotics in agriculture, environmental contamination, health-care transmission, suboptimal antibiotic dosing, suboptimal diagnostics, suboptimal vaccinations, globalisation, and increased travelling[216,217].

Why is misuse and overuse of antibiotics in human health care still an issue when we know of the consequences it brings? One major problem in addressing this is the improper use of antibiotics to treat viral infections. A large portion of antibiotics being prescribed globally are for the treatment of upper respiratory tract infections, usually caused by viruses, not bacteria. The increase in global antibiotic sales during the COVID-19 pandemic is a good example of this problem[218]. Lack of diagnostics is another big contributor to human misuse and overuse of antibiotics. Doctors often prescribe the most practical antibiotic instead of the most appropriate one. A clinical example where this is starting to have dramatic consequences is the treatment of gonorrhoea. As patients undergoing treatment for sexually transmitted diseases rarely show up for a follow-up appointment, doctors need to be sure that the first-line antibiotics they prescribe work. This has led to the current first-line treatment of gonorrhoea being with last-resort cephalosporins, with pan-resistant (i.e., clinically resistant towards all known antibiotics) gonorrhoea on the rise worldwide[219,220]. Also, the human microbiota seems to act as a reservoir for ARGs, from which they can be transmitted from one healthy individual to the next at a population level and not just within smaller communities such as families and hospitals[221,222]. A clear correlation has been described between the abundance of ARGs in the

microbiome of healthy individuals and the per capita consumption rates of their country of residence[221], underpinning the importance of this factor.

While much attention goes to the consequences of antibiotic use in human health care, the fact remains that between 70 and 80% of the global antibiotic production ends up in animal husbandry for food production[223], amounting to about 100,000 tonnes of active antibiotics being used yearly in the production of cattle, sheep, chicken, and pigs alone. This number is expected to increase by at least 8% by 2030[35,224]. Assuming that approximately half of those antibiotics will be excreted in urine in their active form and released into the environment, an ecological network is formed where antibiotics and ARGs continuously circle from one environment to another[20,225]. Ecology may thus play an even bigger role in driving the evolution of AMR than antibiotic consumption and human exchanges[226]. A clinical example is the livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA), which is a growing cause of human infections in Europe. In the space of just 10 years (2008 to 2018), the proportion of MRSA-positive pig farms in Denmark increased from <5% to 90%, with a significant increase in human infections at the same time. Upon confirming the identity of the same MRSA-linages circulating between pig herds and hospitalised patients, the Danish Health Authority identified pig farmers and their families as a risk-groups likely to act as reservoirs of MRSA[227,228]. This kind of human-environment-human transmission is an exceedingly problematic scenario in low-income countries where limited access to clean water and proper sewage systems, in combination with poor infection control and awareness, results in high prevalence of human-to-human transmission via the oral-faecal route[161,217,229]. A recent study followed the real-time dynamics of AMR acquisition through direct person-to-person transmission from a common source in the gut microbiota of medical students travelling from Europe to Laos. Of the 20 students, four became sick with diarrhea during their stay in Laos, one took antibiotics, and every single one became colonised with extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria during the stay[230]. As cross-continent travelling becomes more common, it opens a direct route of transmission from countries where the prevalence of MDR-bacteria is already a serious problem to higher-income countries where AMR does not currently pose the same clinical threat.

1.3.2.3. Non-antibiotic drugs as drivers of AMR

The realisation that drivers of AMR evolution are not limited to antibiotics and antibiotic exposure in various forms is a particularly alarming one. The list of non-antibiotic chemicals known to affect AMR evolution is continuously growing, including but not limited to heavy

metals, disinfectants, microplastics, herbicides and non-antibiotic pharmaceuticals. Many of these are agents heavily used in our daily lives, in agriculture, and are found in the environment as pollution in large quantities[212,225,231-234].

While the antimicrobial properties of various chemicals and non-antibiotic drugs have been recognised for a long time, it is only in the last few years that the potential role of non-antibiotic drugs as drivers of AMR has started to gain attention beyond vague speculations[217]. An opinion piece published at the end of 2017 raised the question of whether cancer chemotherapy could drive AMR evolution by induction of the SOS response[159]. This was followed by an extensive study examining the impact of non-antibiotic drugs on the microbiota, recognising their potential role as drivers of AMR and looking at how different antibiotic resistance mechanisms can protect *E. coli* against human-targeted drugs[126]. Since then, more experimental data has been published supporting this hypothesis. These studies have shown that many types of commonly prescribed antidepressants, nonsteroidal anti-inflammatory drugs, the lipid-lowering drug gemfibrozil and the beta-blocker propranolol can induce the bacterial SOS-system, increase mutation rates (leading to a detrimental increase in intracellular reactive oxygen species as well as increased efflux) or enhance transformation and conjugation[235-238]. Moreover, the antipsychotic drug Quetiapine has been shown to cause MDR through mutations increasing efflux[239]. These examples are likely just the tip of the iceberg[126].

While the question of whether drugs used in cancer chemotherapy act as drivers of AMR has, until recently, flown under the radar, experimental evidence pointing in that direction has existed for a while. Cancer drugs have been used in studies investigating the SOS-response in bacteria for decades, but the focus of those studies has been on the connection between the induction of the SOS-response and AMR, not digging deeper into the potential implications of cancer drugs as drivers of resistance[240-242]. However, that has started to change, and multiple studies have been published in the last five years focusing on how exposure to cancer drugs can lead to induction of the bacterial SOS-system, concurrently increasing mutation rates and driving AMR evolution. This has been shown in vitro for multiple chemotherapeutic drugs of different classes, including platinum-based drugs, anthracyclines and more[243-245]. The underlying mechanism has been investigated in detail for etoposide, showing that exposure to etoposide can induce the production of reactive oxygen species, cause oxidative stress, and confer ciprofloxacin resistance in *Pseudomonas aeruginosa.* Furthermore, etoposide-evolved bacterial biofilms have been shown to promote tumour progression by protecting tumour cells from etoposide killing[246]. These studies make it clear that drugs used in cancer chemotherapy may influence and drive the evolution of AMR. To what extent, and if this occurs in the human

gut microbiota during cancer treatment remains a subject that the scientific community has yet to address properly.

1.3.3. Current strategies for fighting AMR

With more and more antibiotics becoming unusable for the treatment of bacterial infections due to increasing AMR at the same time that very few new antibiotics make it to the market, the scientific and medical communities have come together in search of alternative solutions to this problem[247]. Some of the main strategies are the continuous efforts looking for new antibiotics[248,249], the repurposing of old antibiotics that had previously been discarded from clinical use[250], the use of antibiotic adjuvants and combination therapy to prolong the life of the antibiotics we do have[251-254] and the use of alternative therapies. Some of the alternatives currently being investigated are vaccines[255], immunotherapeutic strategies aimed to help the immune system to fight off the infection[256,257], bacteriophages[258,259], antibodies targeting the bacterial surface or virulence factors[260,261], probiotics and the use of the commensal bacteria[262,263] and the repurposing of non-antibiotic drugs and agents for the treatment of bacterial infections[264-267]. An alternative to antibiotics of special relevance for the work presented in this thesis is the idea that repurposing drugs used in cancer chemotherapy as antibiotics may be used to battle AMR. This idea started to surface in recent years as people have started to become more aware of the antimicrobial effects of antineoplastic agents[137,138,268,269]. However, this strategy may have limited chances of succeeding if those same agents are able to drive AMR evolution.

1.3.4. The consequences of AMR for cancer treatment

The patient groups at increased risk for contracting life-threatening diseases from bacterial infections are concurrently extremely vulnerable to AMR bacteria. One of those major risk groups is patients undergoing cancer treatment. Not only does cancer chemotherapy often severely impair the immune system, is also often given in combination with other treatments such as major surgeries, prolonged hospitalisation, medications impairing the human gut microbiota and the presence of catheters or other foreign devices in the body[270]. Consequently, cancer patients are more than three times more likely to die from a fatal infection than the general public, with infectious diseases being one of the leading causes of death in cancer patients[271,272].

As one in five cancer patients relies on antibiotics while undergoing treatment, the potential consequences of AMR for cancer treatment can be fatal. The loss of effective

antibiotics could result in certain cancer types currently considered treatable losing that status. This would be the case for acute myeloid leukaemia and all cancer forms treated with high-dose treatment with autologous stem cell support, not to mention the consequences for major surgeries, including those to remove solid tumours[273]. To put some numbers to those kinds of scenarios, a reduction in the efficacy of antibiotic prophylaxis is estimated to result in between 40,000 and 280,000 additional surgical site infections and infections after chemotherapy each year in the USA alone (40,000 as a result of 10% reduction in efficacy, 280,000 as a result of 70% reduction). Between 2,100 and 15,000 of those infections are likely to be fatal[273,274]. In addition, there is the added burden on our healthcare systems in the form of prolonged hospital stays, increased treatment costs and economic consequences owing to lost productivity to consider[272]. It is clear that while the evolution of AMR remains a global health threat with potentially dire consequences for all of humanity, the consequences for patients undergoing cancer treatment are considerably larger than for the cancer-free population. It is, therefore, of the utmost importance that the question raised by Papanicolas *et al.* in 2018, "Is cancer chemotherapy driving AMR?" be properly addressed so that we may better understand the drivers of AMR and prolong the lifetime of the care we can give those patients today.

2. Aim of the current thesis

The main aim of this thesis was to address the hypothesis that drugs used in cancer chemotherapy play an underrecognized role as drivers of AMR. While the objective of the thesis was to test this hypothesis as broadly as possible, we initially designed a proof of concept study based on what we predicted to be the most likely combination of cancer drug and AMR determinant that could demonstrate positive cancer drug-mediated selection of AMR. This allowed us to establish a methodology and scale up our approaches. Our study design was based on the following questions:

1) Is there a relationship between the cytotoxic drug MTX and TMP resistance in *E. coli*?
2) How many cancer drug and AMR combinations can we identify?
3) Can the results obtained *in vitro* be replicated in an *in vivo* mouse model at clinically relevant drug concentrations?

The long-term goal of this work is to make a contribution towards optimising antibiotic treatment in cancer patients and, through that, contribute towards curbing the spread and evolution of AMR.

3. Methodological considerations

The materials and methods used in this thesis have been described in detail in **Papers I - III**. The following chapter aims not to repeat the information available there, but to give a more in-depth description of experimental details and considerations vital to the work presented.

3.1. *E. coli* as a model organism

The work in this thesis has principally been conducted using different strains of the model bacteria *E. coli*. The only exception is in **Paper I**, where parts of the obtained results were replicated using a different bacterial species, *Klebsiella pneumoniae*. For more than a century, scientists all around the world have used *E. coli* as their model organism of choice for experiments in different fields of research, making it the most widely studied organism on the planet and the one we understand the best[275]. In many ways, the simplicity of *E. coli* became the basis for its success over other model organisms. It is a facultative anaerobic, rod-shaped Gram-negative bacterium belonging to the *Enterobacteriaceae* family. It is known to be both a common part of the human gut microbiota and a highly versatile pathogen. *E. coli* is the leading causes for bloodstream and urinary tract infections worldwide, making it a clinically relevant species to conduct the work in[276-278]. The versatility of the species may have been a contributing factor to the diversity of *E. coli* strains. Its wild population is estimated to include 10^{20} different strains and scientists working with some of the most common lab strains, such as *E. coli* K-12 MG1655, know that even closely related lab strains can display vast differences in both phenotypes and genotypes[279]. The *E. coli* genome, which varies significantly in size, consists of approximately 5,000 genes and is about 5 Mbp in length. While its core genome is estimated to consist of about 3,000 genes, varying up and down depending on how the core genome is defined, the most recent datasets on the ever-growing pan-genome of *E. coli* are made up of >55,000 different genes. This great genetic diversity is the most simple explanation for the versatility and diversity of the species[280]. This is something we observed ourselves in **Paper I**, where more than 16-fold differences were measured in susceptibility profiles of different laboratory strains compared to a panel of clinical strains.

As the fluorescently tagged strain pair used in **Papers I - III** was readily available upon the project start, choosing *E. coli* K-12 MG1655 as our model organism of choice seemed

straightforward at the time. Considering the amount of genetic construction work needed in the project, we aimed to capitalize on the numerous molecular tools available for genetic engineering in *E. coli* K-12 MG1655. However, those are often strain specific and give little or no success in clinical strains of *E. coli* or other species. The biggest drawback of using *E. coli* K-12 MG1655 was the intrinsic resistance towards various chemotherapeutic drugs due to active efflux[121,236], a factor with ramifications we vastly underestimated during the project's planning stages. This is evident in the high drug concentrations used throughout **Paper I**, often resulting in MICs and parameters exceeding the experimental limit. As that is a problem fairly easily circumvented by using Δ*tolC* knockouts lacking the AcrAB-TolC efflux pump system, the advantages largely outweighed the detriments.

3.1.1. Strain constructions

After confirming the extreme effect the AcrAB-tolC efflux system had on MTX susceptibility in **Paper I**, and knowing that it would likely not be the only cancer drug actively pumped out of the bacteria cells by the system, it was decided to follow the example of others and conduct the initial screen in a Δ*tolC* strain pair[126,281]. This was done to identify resistance genes giving bacteria fitness benefits in the presence of different drugs without efflux potentially masking the effects. The first step in strain construction to set up our screen was to take the fluorescently tagged *E. coli* MG1655 strains used in **Paper I** and **II** and knock out the *tolC* gene from their chromosomes to inactive the AcrAB-tolC efflux pump. One of the most widely established methods for gene deletion in *E. coli* has long been crossing the knocked-out allele from the strain of interest in the Keio collection into the target strain by P1 transduction[282,283]. Despite repeated efforts, where dozens of clones of *E. coli* JW5503 were isolated from three individual sources (two individual copies of the KEIO collection as well as an individually bought copy of the strain), I was unable to isolate a clone containing the Δ*tolC::nptII* allele and not a fully functional *tolC*, indicating widespread contamination of some sort, specific for the JW5503 strain in the KEIO collection. Having had to give up on the P1 transduction, we developed an approach based on homologous recombination with a merodiploid assay (a more detailed description of the method can be found in **Paper III**) and were able to construct a new pair of "parent strains" to use when constructing the different resistance strain pairs to use in the screen (strain numbers MP26-19 and MP26-20). Given the multiple cloning steps and growth cycles involved in the strain constructions leading up to this stage, the strains were whole genome sequenced to confirm their isogenicity. I confirmed that the *tolC* gene was successfully removed from both strains, the *IS150* element replaced with YFP (GenBank: KM018300) in MP26-19

and BFP (GenBank: KM018299) in MP26-20, and that no relevant point mutations had arisen on the chromosome during cloning (a total of two mutations were identified differing between the strains: a synonymous variant of Leu375Leu in the *entE* gene and a missense variant of Ile419Ser in the *treF* gene of MP26-20).

Different resistant variations of the fluorescently tagged strain pair were constructed following a miniTn*7* conjugation protocol described in **Paper III**. When resistant clones were recovered at the end of each selection step, phenotypic variations between colonies were observed for some of the resistance genes included in the screen. Size difference was the most common, as well as differences in measured MICs and relative growth rates. In those cases, multiple clones of both yellow and blue were isolated and characterized before the strains included in the screen were chosen. This was done to ensure isogenic strain pairs and to exclude clones that had picked up any unknown point mutations during the selection step affecting resistance and bacterial fitness. The results from those characterizations have been summarized in Table 1.

3.2. Measuring bacterial fitness

There are numerous methods that can be applied to measure bacterial fitness, each associated with a number of benefits and drawbacks. The work presented in this thesis has relied on both growth curve data obtained from monocultures, as well as competition experiments in mixed populations.

Growth curves: Possibly the most widely-adopted method for measuring bacterial fitness, used in both **Papers I** and **III**, is the determination of maximum growth rate. Here, optical density (OD) is measured in a growing culture and plotted as a function of time. The exponential growth phase of the resulting growth curve can then be used to calculate doubling time. By comparing the doubling time of a mutant to its wt, conclusions can be drawn regarding the relative fitness cost of the mutation. Some downsides to the method are the lack of sensitivity and the fact that it only looks at differences in the exponential growth phase, disregarding differences in other phases of growth and the differences in carrying capacity. A different way of analysing the growth rate data, used in **Paper III**, is to compare the area under the curve (AUC) for different strains at different growth conditions, considering the initial population size and the carrying capacity and not just exponential growth rates.

Table 1: Bacterial strains constructed for the semi high-throughput screen. Summary from strain characterization assays conducted. MIC fold changes and relative growth rates comparing the strain to the parent strain it originated from. NA = Data not available. gyrA/parC mutant refers to a GyrA S83L D87N ParC S80I E84V chromosomal quadruple mutant.

MP-number	Fluorescence marker	tolC	AMR gene	Resistance marker	MIC [µg/mL]	MIC fold change	Relative growth rate	Cost of AMR gene [%]
26-23	sYFP2	Δ*tolC*	*aac(6')-Ib-cr*	Kanamycin	8	21	0.79	-21.5
26-37	mTagBFP2	Δ*tolC*	*aac(6')-Ib-cr*	Kanamycin	8	21	0.75	-24.7
26-69	sYFP2	*tolC*+	*aac(6')-Ib-cr*	Kanamycin	12	16	0.73	-27.2
26-70	mTagBFP2	*tolC*+	*aac(6')-Ib-cr*	Kanamycin	12	16	0.72	-27.7
26-24	sYFP2	Δ*tolC*	*armA*	Kanamycin	>256	>512	0.94	-6.03
26-38	mTagBFP2	Δ*tolC*	*armA*	Kanamycin	>256	>1024	0.95	-5.2
26-71	sYFP2	*tolC*+	*armA*	Kanamycin	>256	>341	0.94	-5.9
26-72	mTagBFP2	*tolC*+	*armA*	Kanamycin	>256	>341	0.94	-6.0
26-25	sYFP2	Δ*tolC*	*catA1*	Chloramphenicol	128-256	170-341	1.01	+1.3
26-39	mTagBFP2	Δ*tolC*	*catA1*	Chloramphenicol	128-256	128-256	0.99	-1.5
26-28	sYFP2	*tolC*+	*catA1*	Chloramphenicol	>256	NA	0.98	-2.1
26-42	mTagBFP2	*tolC*+	*catA1*	Chloramphenicol	>256	NA	0.97	-3.2
26-26	sYFP2	Δ*tolC*	*dfrA1*	Trimethoprim	>32	>128	NA	NA
26-40	mTagBFP2	Δ*tolC*	*dfrA1*	Trimethoprim	>32	>84	NA	NA
26-34	sYFP2	*tolC*+	*dfrA1*	Trimethoprim	>32	>84	NA	NA
26-48	mTagBFP2	*tolC*+	*dfrA1*	Trimethoprim	>32	>64	NA	NA
26-27	sYFP2	Δ*tolC*	*qnrS1*	Ciprofloxacin	0.25	83	NA	NA
26-41	mTagBFP2	Δ*tolC*	*qnrS1*	Ciprofloxacin	0.38	63	NA	NA
26-29	sYFP2	*tolC*+	*qnrS1*	Ciprofloxacin	0.125	10	0.96	-3.9
26-43	mTagBFP2	*tolC*+	*qnrS1*	Ciprofloxacin	0.125-0.19	8-12	0.96	-3.6
26-30	sYFP2	Δ*tolC*	*tetA*	Tetracycline	24	96	0.98	-2.3
26-44	mTagBFP2	Δ*tolC*	*tetA*	Tetracycline	32	84	0.97	-2.8
26-33	sYFP2	*tolC*+	*tetA*	Tetracycline	>256	NA	0.99	-0.1
26-47	mTagBFP2	*tolC*+	*tetA*	Tetracycline	>256	NA	0.97	-3.1
26-36	sYFP2	Δ*tolC*	ble_{MBL}	Bleomycin	300 IU[1]	500	1.00	+0.1
26-50	mTagBFP2	Δ*tolC*	ble_{MBL}	Bleomycin	150 IU[1]	250	0.98	-2.1
26-31	sYFP2	Δ*tolC*	$bla_{CTX-M-15}$	Ampicillin	>256	>128	NA	NA
26-45	mTagBFP2	Δ*tolC*	$bla_{CTX-M-15}$	Ampicillin	>256	>128	NA	NA
26-32	sYFP2	Δ*tolC*	bla_{NDM-1}	Ampicillin	>256	>128	NA	NA
26-46	mTagBFP2	Δ*tolC*	bla_{NDM-1}	Ampicillin	>256	>128	NA	NA
26-35	sYFP2	Δ*tolC*	bla_{TEM-1}	Ampicillin	>256	>128	NA	NA
26-49	mTagBFP2	Δ*tolC*	bla_{TEM-1}	Ampicillin	>256	>128	NA	NA
26-67	sYFP2	Δ*tolC*	*gyrA/parC* mutant	Ciprofloxacin	6	2000	0.99	-1.3
26-68	mTagBFP2	Δ*tolC*	*gyrA/parC* mutant	Ciprofloxacin	8	2000	1.01	0.5

[1]IU = International units. Bleomycin is traditionally given in potency-based doses of international units of bleomycin. As the only concentrations available for the drug solutions used were IU/mL, MIC estimates for bleomycin have been given in IU.

Competition experiments: How bacteria behave in a monoculture does not account for ecology and does not necessarily reflect how it will behave in a mixed culture with other bacteria. In **Paper I**, competition experiments were used to address the question of whether harbouring TMP resistance determinants would give bacteria fitness benefits in the presence of MTX. In our setup, isogenic strains differing only in fluorescence and resistance markers were mixed at equal starting ratios and passaged for 30 generations of growth. A fluorescence cell sorter was used to count 100,000 cells every 10 generations to monitor any changes in the resistant:susceptible ratios, making it possible to visualize fitness differences as small as 0.5% between the competing strains. The biggest drawbacks to the method are that it requires expensive machinery and, as the instrument (FACS Aria™ III) used in **Paper I** reads single sample tubes and not 96-well plates, it rapidly becomes time-consuming and laborious. In **Paper III**, a regular plate reader able to read fluorescence signal as well as OD was used to detect shifts in the fluorescence signal over time, making it possible to screen through much larger sample numbers than possible using a cell sorter. The biggest drawback of the method, described in more detail in chapter 3.3.2, is that it can only give an idea of where the largest fitness differences may come into play due to a large technical variation in the fluorescence reads.

Noteworthy, I observed large discrepancies when comparting the effects of AMR on fitness using different methods. While *armA,* an ARG I had estimated to confer 5-6% fitness cost had a smaller colony morphology than the wt and other resistant strains, *aac(6')-Ib-cr*, which I had estimated to confer 22-28% fitness cost (a fitness cost noticed and commented on by others[284]), showed no visual change in growth on plates. Simultaneously, I noticed that the *armA* strain had a much lower carrying capacity than the others, implying a bigger effect on total growth than observed when looking at the exponential phase specifically. For that reason, we decided to use relative AUCs for our estimations. There, *armA* was estimated to confer a 10% fitness cost, explaining the smaller colony phenotype. Meanwhile, the fitness cost of *aac(6')-Ib-cr* dropped from 27% to 2%, a number more in line with the observed phenotype. Interestingly, the enormous differences in the estimated fitness cost of the strains when using different methods for calculation are plainly visualised when looking at the relative AUC of the strains at different timepoints (Figure 7). While relative AUC sets the fitness cost at 2% after 18 hours, the same calculations done on data from the first 7 hours sets the fitness cost at 24%. Demonstrating quite elegantly how different methods can give very different results depending on which factors and growth phases they look at.

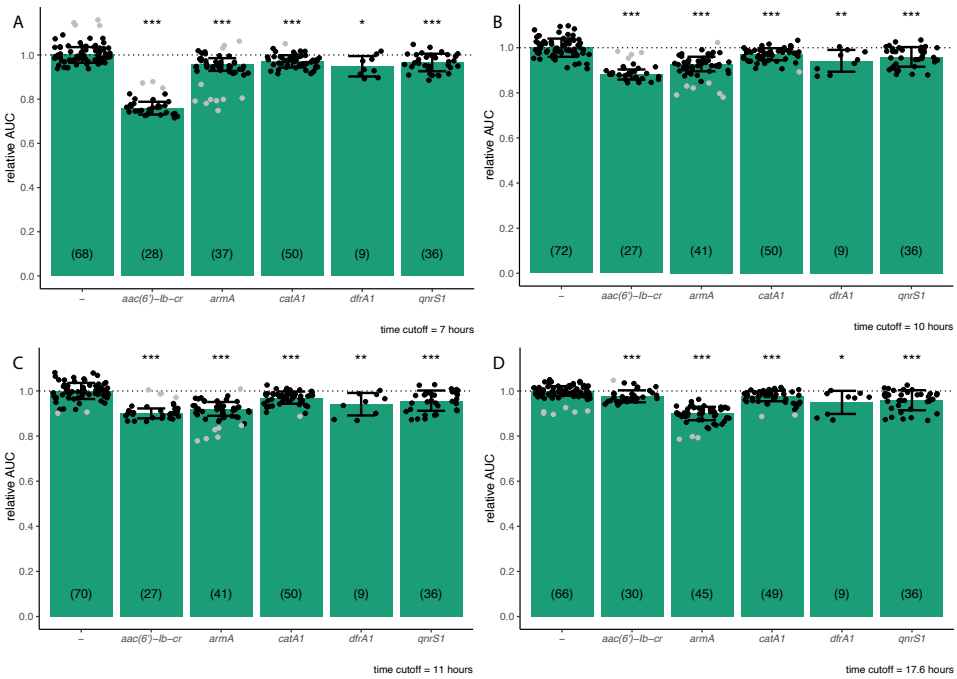

Figure 7: Relative AUC of *E. coli* K-12 MG1655 DEL(IS150)::CP25-sYFP2-T expressing the different ARGs in absence of drugs. Bar heights represent the mean AUC relative to the wt (MP18-01) and the error bars represent the standard deviation. Each point represents a biological replicate, and results from the median of its three technical replicates, black points were used for calculations whereas grey points represent outliers excluded from the calculations. The number of biological replicates included in the calculations are written on the bar of each gene.

3.3. Method design for a semi high-throughput screen

The work in **Papers I** and **II** was planned and carried out for a single cytostatic drug/AMR mechanism combination. This combination was chosen based on chemical structures and mechanisms of action as a combination likely to give cross-resistances. Conversely, in **Paper III**, we wanted to test as many pairs as possible in order to address how common cross-resistances between antibiotics and cancer drugs really are. To do so, we designed a semi high-throughput method for screening a larger number of possible cytostatic drug/AMR mechanism combinations. We used a panel of 73 oncology compounds (Prestwick Chemical Library®), prepared for customers working with anticancer drugs. All drugs came as a ready-made 10 mM stock solution dissolved in dimethyl sulfoxide (DMSO), allowing for a final concentration of 0.1 mM in our screen (resulting in a final DMSO concentration of 1%, as DMSO at higher concentrations is known to impact bacterial growth[285]). The 73 oncology compounds were then tested against a panel of 11 different resistance mechanisms in *E. coli* MG1655, looking for combinations where an AMR gene would give the bacteria growth benefits in the presence of the drug. When choosing which resistance mechanisms to include in the screen, the aim was to cover as many different mechanisms as possible, from all the main groups of AMR mechanisms described in Figure 4. We included three different β-lactamase genes, $bla_{\text{NDM-1}}$, $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-15}}$, which all work by hydrolysing β-lactam antibiotics but have different spectrums

and properties, as well as the bleomycin resistance gene *ble*$_{MBL}$, that is often found co-expressed with *bla*$_{NDM-1}$. Other drug modification enzymes included in the screen were the chloramphenicol acetyltransferase *catA1* and the aminoglycoside 6'-N-acetyltransferase *aac(6')-Ib-cr*. Another aminoglycoside resistance gene included was the *armA,* a gene known to confer resistance through target modification. A tetracycline efflux pump (*tetA*) was included in the screen as well as the dihydrofolate reductase *dfrA1* known to confer trimethoprim resistance. Lastly, two different fluoroquinolone resistance mechanisms were included, the *qnrS1* gene and a GyrA S83L D87N ParC S80I E84V chromosomal quadruple mutant. Our selection gives a good representation of the various clinically relevant AMR mechanisms.

3.3.1. Method optimization and validation using Tecan Spark® plate reader to detect population changes over time

In **Paper I,** we showed that while MTX had been assumed to have dismissible effects on bacteria[286-288] and would have no visible effect on a growing monoculture of *E. coli,* it can dramatically affect the population structure in a mixed population of resistant and susceptible strains. Therefore, we chose to conduct the screen in **Paper III** in a competition setting similar to the one we had applied when investigating the fitness benefits conferring TMP resistance in the presence of MTX. A big factor when designing the experimental setup for the screen became the number of competing cultures. The combination of *E. coli* strains expressing 11 different resistance determinants being grown in the presence of 73 oncology compounds amounted to >800 combinations, excluding replicates and any types of controls. Therefore, using the same method we did for the MTX/TMP combination in **Paper I**, growing single cultures in individual tubes, and measuring single samples daily, quickly became unfeasible. We, therefore, started looking into ways to do the competitions and following fluorescence measurements using a 96-well setup. We landed on using the Tecan Spark® multimode microplate reader capable of conducting a fluorescence top and bottom reading of a 96-well plate in a matter of minutes. Other groups have started to apply similar screening approaches. An example is the Kishony lab that recently published an antibiotic combination screen using an automated macroscope device to take images of mixed cultures of fluorescently tagged *S. aureus* spotted on microplates, quantifying the differences in strain ratios from competing cultures at different timepoints[289].

Our competitions were grown in 1 mL deep well plates, allowing for a maximum capacity of three resistance determinants and seven drugs on each plate, including positive and

negative controls. After serial passaging for 30 generations, the cultures were diluted 1:200 in 1xPBS and taken to the Tecan Spark® for a fluorescence top reading. First, the yellow signal was measured (480 nm excitation wavelength and 527 nm emission wavelength), followed directly by a measurement of the blue signal (399 nm excitation wavelength and 454 nm emission wavelength). To make sure that the machine was sensitive enough to distinguish between the yellow and the blue fluorescent strains, we measured mixtures of the two susceptible strains at different ratios with the Tecan Spark® and plotted the calibration curves (Figure 8).

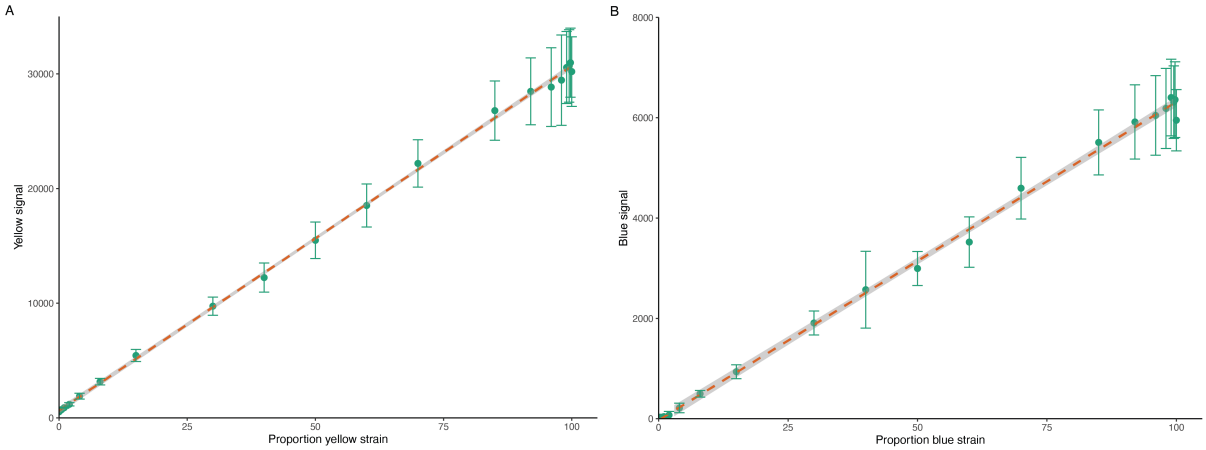

Figure 8: Calibration curves for fluorescence signal on the Tecan Spark® at different ratios of yellow:blue strain mixtures. Monocultures grown over night of MP18-01 and MP18-02 were mixed at different ratios before being diluted and taken to the Spark® for fluorescence measurements. Proportion of the strain having its fluorescence signal read can be found on the x-axis with fluorescence signal on the y-axis. Error bars represent standard deviation of the mean.

As the measured fluorescence signal had a large numeric variation between technical replicates, we worried that the "noise" in the measurements would not only mask the smaller fitness effects, but potentially result in false negative/false positive results. To avoid this, some additional measures were taken: for every resistance determinant included, two strains were constructed expressing different fluorescence markers, assuming that conducting the screen in independent strain pairs, excluding effects only observed in one dye swap, would limit the number of false positive hits.

To bypass the limitation presented by the "noisy" measurements and optimize the number of combinations identified as hits, the initial screen was conducted in Δ*tolC* strains, enlarging any fitness effects of resistance observed in the screen itself. Positive hits were identified as combinations where expressing one of the AMR determinants gave fitness advantages in the presence of the drug tested. A follow-up verification assay was designed to

verify that the 216 combinations identified as hits in the initial screen were not solely a result of knocking out the *tolC* gene. For all relevant AMR determinant-drug combinations identified, monocultures of a *tolC*+ strain expressing the same resistance gene were grown at various concentrations of the drug and growth was measured over 18 hours, identifying combinations where the resistant strain grew significantly better in the presence of the drug than the wt.

4. Current investigations: Results and discussions

As presented in the background chapter, it has become increasingly clear in recent years that non-antibiotic drugs affect the growth and evolution dynamics of bacteria in more ways than previously credited or properly investigated. The investigation included in this thesis aimed to contribute towards filling that knowledge gap, investigating the effects of drugs used in cancer chemotherapy on AMR dynamics in *E. coli*. The following summaries give an overview of the rationale behind and key results obtained from the work in each paper, positioning the current investigations within the research field as a whole.

Paper I:

The chemotherapeutic drug methotrexate selects for antibiotic resistance

As mentioned in the preface, the work presented in this thesis marked a start of a new research project within my group. We hypothesised that cancer chemotherapy plays an unexplored role as a driver of AMR. We established the existence of a knowledge gap with a literature search conducted in the first weeks of my PhD work, and the next step became to establish our proof of concept. We chose an antibiotic–anticancer drug pair with structural and mechanistic similarities, presuming that if our hypothesis were correct, that would be the easiest pair to show cross-resistances in. The pair we chose was MTX and TMP.

Here we have shown that pre-existing TMP resistance determinants, both chromosomal mutations in the *folA* gene as well as plasmid-mediated resistance stemming from *dfrA* genes on the MDR plasmid pG06-VIM-1, confer growth advantages to *E. coli* in the presence of MTX. The resistant strains have increased MTX MICs compared to their susceptible wt strains and have stable relative growth rates at MTX concentrations way above those where the growth of the wt strains was inhibited. Moreover, we looked at the effects of MTX exposure on pan-susceptible strains, showing that MTX exposure results in *de novo* emergence of mutations conferring TMP resistance. We showed this for both single-step resistance evolution at high MTX concentrations and for sub-MIC evolution over 300 generations of growth at a

concentration 10 times below the measured MIC. These experiments resulted in the isolation of *folA* mutants conferring not only MTX resistance but cross-resistance towards TMP as well, with TMP MICs that, in many cases, crossed the clinical break point of TMP[208]. Sub-MIC concentrations of MTX were even shown to promote the invasion of TMP resistant strains when rare, with resistant strains increasing in numbers under competition settings starting from 1:10000 starting ratios.

When discussing MTX and bacteria, I would argue that a lot of (what could have been considered rather obvious) effects have been disregarded due to general misconceptions about concentrations. It was assumed that since bacteria lack the RFC-1 receptor, MTX could not cross the Gram-negative cell wall and consequently could not affect bacterial growth[286]. However, there is no denying that limited amounts of MTX successfully cross the cell wall via passive diffusion at high extracellular contractions[121,287,288]. These concentrations have been overlooked as irrelevant, given the large amounts of MTX necessary to obtain physiologically relevant intracellular concentrations[122]. However, a consequence of the various uses of MTX is that it is a drug given at varying concentrations and schedules, which in some cases exceed the lethal daily dose, with concentrations at >1000 mg/m^2 [290]. MTX is known to accumulate in various human tissues, including the small intestine, following high-dose MTX treatment[291], but the actual concentrations found in the gut during treatment remain unknown. Given our improved understanding of how selection for resistance takes place, and a much larger (sub-MIC) selection window than previously acknowledged[200], I feel it is timely that we stop looking solely at concentrations affecting growth and start talking about concentrations affecting selection for resistance. We show clearly in this paper that selection for TMP resistance can take place at concentrations way below the measured MTX MIC, at concentrations below those we estimated to be present in the gut of patients undergoing MTX treatment. This opens up for the question if MTX treatment may be selecting for TMP resistance in the gut of such patients.

A comment we encountered when working on publishing the presented results was the statement that "MTX does a lot, really a lot, of things, and this is just one more", disregarding all the presented findings as irrelevant in a single sentence. I therefore think it is important to address the question of why we should care if an unrecognised side-effect of MTX treatment turns out to be that it can drive AMR evolution in the gut of patients undergoing high-dose MTX treatment. The background chapters have introduced how various agents can cause AMR and how selection pressures are needed to maintain those in a population. It is easy to conclude that even if resistance development takes place in the gut during treatment, it is not a problem as the resistant bacteria will disappear from the population with time once treatment is

discontinued. However, the real threat is not the appearance of resistant bacteria in the gut, it is the chain reaction that can take place if said bacteria relocates to different parts of the body. Most UTIs are caused by uropathogenic *E. coli* residing in the gut that found their way up to the urethra and then spread onward to the bladder, kidneys and (in the worst cases) the bloodstream[292,293]. A common complication of cancer therapy is urinary diversion and associated UTIs, of which approximately 20% are associated with sepsis[270]. As one of the leading causes of death in cancer patients is sepsis, the accumulation of TMP-resistant bacteria in the gut can therefore have potentially fatal consequences.

Another aspect worth keeping in mind is the fact that TMP resistance is often plasmid mediated [294], meaning that as long as MTX selects for a MDR-plasmid, co-selection takes place and resistance towards all other antibiotics the plasmid expresses ARGs for is selected for. Potentially making the problem a much larger than one than for just the single combination.

The presented investigation became one of the first examples showing how antineoplastic drugs can not only cause AMR through specific molecular mechanisms, but select for resistance and apply selective pressure on bacteria. The study's biggest limitation is that all effects are observed *in vitro,* while whether those are replicable *in vivo* during treatment remains unknown.

Paper II:

Maintenance of a multidrug resistance plasmid in NMRI mice exposed to methotrexate: a pilot study

Having investigated MTX and TMP resistance from various angles *in vitro*, an obvious next step became to conduct an *in vivo* study investigating if the effects we had seen could be replicated in the mouse gut. As conducting those experiments ourselves at UiT became impossible, we outsourced the animal experiments to collaborators in Denmark. Unfortunately, the pilot experiments aimed at establishing a working protocol were fruitless. Co-colonization was not obtained, and the lack of a baseline makes it impossible to draw significant conclusions from the data collected.

What we were able to establish, however, was a working treatment protocol, showing that while a daily oral dose of 10 mg/kg MTX for 4 days negatively affected the health of the animals, no animal had to be sacrificed prematurely as a result. The main aim of the study was to co-colonize the mouse gut with an isogenic strain pair, where one strain expressed a TMP

resistance determinant and the other strain was its susceptible wt strain, looking at the effects of MTX treatment on the population dynamics *in vivo*. When the susceptible strain unexpectedly was not detected at all in faecal samples collected from mice inoculated with a mixture of both strains, comparison of treatment groups became impossible. However, we did observe a trend where the plasmid-carrying strain (MP18-08) seemed to be eradicated slower than its susceptible sister strain (MP18-01). Our two main theories as to what could explain the disappearance of the susceptible strain in the initial pilot were human error and inoculation effect. To test this and rule out human error, a follow-up pilot was conducted with a new inoculum/treatment scheme. There, we aimed to investigate if including inocula groups with 1:100 starting ratios of the resistant:susceptible strains would allow us to detect the susceptible strain and confirm with experimental data that the mice had indeed been inoculated with both strains. Disquietingly, human error resulted in the strains being mixed up and the follow-up study conducted with the new treatment groups were inoculated at 100:1 starting ratios as opposed to the planned 1:100. Unsurprisingly, the wt strain that was not detected at 1:1 ratios was also not recovered from faecal samples where the starting ratios were 100:1 of resistant:susceptible bacteria. Realising no conclusions could be drawn from the mice inoculated with a mixture of the two strains, we looked at if any differences could be seen on enrichment in samples from animals inoculated with a monoculture of MP18-08. The same trend was observed, and MTX treatment seemed to slow down the clearance of the plasmid-harbouring strain compared to samples from mice being treated with water as a control.

While the data obtained from the first pilot experiments presented in this paper may not have given us the information we hoped for, some important lessons were learned along the way. We now know that our fluorescently tagged strain pair is unable to co-colonize the NRMI gut and that we will have to select a more appropriate pair moving forward with the project. This will include abandoning the use of fluorescently tagged strains and rather using a classical plating approach and a clinical strain, for example *E. coli* K56-75 ST69, which is known to be a successful human pathogen[295]. There is also no going around it that human errors discovered during data analysis and not underway significantly affect the data and our abilities to draw conclusions. The only solid conclusion is that the experiments need to be redone to determine which effects can be ascribed to how the bacteria respond to drug exposure and which effects result from unanticipated experimental artefacts.

Paper III:

Antineoplastic drug selection for antimicrobial resistance

Having shown for MTX that not only can exposure to cancer drugs result in *de novo* AMR arising, but that it can actively select for both chromosomal as well as plasmid-mediated AMR at sub-MIC concentrations, way below the estimated MIC, our next step become to address how common those effects might be.

To do this, we bought a set of oncology compounds, stemming from the Prestwick Chemical Library®, that we considered our best option for a drug library giving a wide variety of drug used in cancer chemotherapy. The 73 compounds from the library were screened against a panel of 11 different AMR determinants, covering the main groups of AMR resistance mechanisms. The screen was competition based[289] (30 generations) and aimed at identifying combinations of cancer drugs and AMR determinants resulting in positive AMR selection. During method optimization of the screen, it was clear that fluorescence-based detection of shifts in populations composition displayed relatively noisy data output. This was in part the reason for why the screen was conducted using a hypersensitive Δ*tolC* genetic background. Performing the screen in a *tolC* deficient background also allowed for the identification of selective effects that would otherwise have been masked by *tolC* mediated efflux[121,126].

Of the 792 combinations included in the screen, 208 were categorised as hits, identifying drugs from eight different sub-classes of antineoplastic drugs (ATC-L), as well as experimental agents, as possible drivers of AMR. Of the hits identified, 95% were for five of the eleven AMR determinants, all ARGs known for conferring resistance towards antibiotics working through inhibition of DNA and/or protein synthesis, processes well conserved between both prokaryotic and eukaryotic cells.

To verify the hits identified and examine for which combinations the effects were strong enough to be replicated in a *tolC*+ background, five of the ARGs for which strong hits had been identified (*aac(6')-Ib-cr, armA, catA1, dfrA1* and *qnrS1*) were moved into the parental strain. Dejectedly, I was unsuccessful in constructing a wt variant of the remaining gene, ble_{MBL}, so only 67 of the 99 strong hits identified were moved on to the next set of experiments. Those I grew in monocultures, at increasing concentrations of the drugs they had been paired with, for 18 hours while measuring changes in OD_{600} to calculate the effects of ARG expression and drug exposure on *E. coli*. For the drugs administrated intravenously (IV), concentrated drug solutions were purchased directly from the pharmacy at our University Hospital (UNN). For the drugs orally administrated the active compounds was bought in a powder from where stock solutions

were made. For a large portion of the drug stocks, a visual precipitation of the drug was observed when mixed with growth media. While cation adjusted MHII gave a somewhat better visual solubility than others tested for differences (LB and M9), this did affect the OD_{600} measurements considerably. Even so, for most drugs this is something that could be adjusted for during analyses, with the exceptions of mitoxantrone (dark blue solution) and mitotane (crystalises and falls out in the media solution) where data obtained was unreadable. Additionally, as the antimicrobial properties observed for 5-fluorouracil using a drug solution from UNN were much stronger than those observed using the solution from the Prestwick Chemical Library® I added measurements for two ARGs for which beneficial effects were not observed in the initial screen, this to rule out false negatives for those combinations. In the end, a total of 24 combinations, distributed between the 5 ARGs and 18 cancer drugs, were identified, where expressing one of the ARGs was shown to confer growth benefits to the strain at one or more drug concentrations tested. The presented data suggest that ARGs expressing enzymes conferring AMR through drug modification may confer cross-resistances towards multiple known cancer drugs. That is something we have not looked further into at this point, and any mechanistical connections made for the pairs identified remains at a speculative stage at this point, except for MTX and TMP where mechanism was addressed in **Paper I**.

In this paper, we have demonstrated that a number of widespread ARGs, known to work through drug modification, and inhibition of DNA/protein synthesis, confer growth advantages to *E. coli* in the presence of various antineoplastics agents used in the treatment of cancer. While the work of addressing the underlying molecular mechanisms explaining the observed effects remains undone, the resulted presented in this manuscript imply that drugs used in cancer chemotherapy may play a much larger role as drivers of AMR than previously credited.

5. Future perspectives

It is my hope that the studies I have presented in this thesis will not be the last once conducted as a part of the ongoing cytostatic project in my research group, as it is a project that has a great potential for follow up studies. I would like to use this opportunity to highlight some that I feel would be worth pursuing.

The first one being taking MTX and other final hits from Paper III and looking at the effects those drugs may or may not have on plasmid maintenance over a longer time period, for example 300 generations. This could be combined with a studly looking at the effects of sub-MIC evolution of the drugs identified as potential drivers of AMR in Paper III, properly addressing, not only if they can cause *de novo* AMR to evolve, but simultaneously if sub-MIC concentrations can cause AMR to arise in a susceptible population.

One of the ARGs giving the most hits in the initial screen was the bleomycin resistance gene *ble*$_{\text{MBL}}$. It would be interesting to look into the molecular mechanism of *ble*$_{\text{MBL}}$, addressing if it works through mechanisms specific for bleomycin or if more universal mechanisms are at play.

Another study just waiting to happen is to take the drugs giving the strongest effects into an *in vivo* model and looking at how they affect resistance formation in the gut. This we attempted with limited success in Paper II, but it is my hope that a better co-colonization model would give valuable information on if MTX and other drugs used in cancer treatment cause the same effects I have observed *in vitro* in the gut during treatment.

A more long-time vision for the project would then be to take it a step further and conduct a clinical study, collecting feacal samples from patients before, during and after cancer treatment to look at if and how treatment influences the gut microbiota resistome. Should one obtain access to a collection of such samples, there are number of potential side projects one could conduct with the data obtained. Deep sequencing could give insights into how the population structure of the gut microbiota is affected during treatment and one could look to see if specific species and/or clones of bacteria turn out to be more fit during cancer treatment.

6. Concluding remarks

The problem of AMR is a multifaced one and will not be solved or even explained by looking at just one side of it. To curb the ongoing development where AMR continues to limit our abilities to treat infectious diseases, a better understanding on the drivers of AMR is urgently needed. The work presented in this thesis has aimed to contribute towards filling this knowledge-gap, providing new insights into if and how drugs used in cancer chemotherapy can cause and drive AMR in gut bacteria.

I have aimed to address this as broadly as possible, looking at the effects of 72 cancer drugs on bacterial fitness, and **Paper III** demonstrates how multiple drugs used in cancer chemotherapy can select for various ARGs. While the underlying molecular mechanisms have not yet been solved for most combinations, we did conduct an in-depth study into one of the combinations identified in the screen. **Paper I** looks at how bacterial exposure to the widely used drug MTX, used amongst other things for the treatment of various cancer types, can not only result in *de novo* TMP resistance but actively select for resistance in mixed populations over time *in vitro*. If cancer drugs assert those selective effects and drive AMR evolution at clinically relevant concentrations is a question that remains to a large extent unanswered. Our work in **Paper II** was aimed at addressing this, but sadly the experiments presented in that work need to be replicated in order to draw any firm conclusions.

Increasing amounts of experimental evidence has been published in recent years, showing how drugs used in cancer chemotherapy induce bacterial SOS-response and cause AMR to arise through increased mutation rates[243-246]. To the best of our knowledge, the work presented in this thesis is so far the only study looking more explicitly on selection. Distinguishing between the two is, in the context of microbial evolution, quite important, as if cancer drugs can cause *de novo* resistance evolution, where AMR mutants are generated at low but detectable frequencies, but do not select for the same mutations in a population, the mutants will only increase in frequency if antibiotic treatment downstream of cancer treatment matches the specific resistant determinants. On the contrary, we show in our work that not only can MTX cause *de novo* AMR evolution through mutagenesis (**Paper I**), but various drugs used in the treatment of cancer can drive AMR evolution by selecting for pre-existing resistance determinants in a mixed population (**Papers I** and **III**).

Whereas it has been observed, and reported, that the effects of *tolC* mediated efflux clearly affect *E. coli* responses to antineoplastic drugs[121,126], the data presented here provide a more complete picture on to which extent and puts the observed effects into the context of AMR selection. The ubiquitous nature of efflux pumps belonging to the resistance-nodulation-cell division superfamily (including the AcrAB-TolC efflux pump), which are extremely well conserved across a wide range of bacterial species[173,174], has raised the question if those efflux pumps play a larger role in bacterial survival than just antibiotic clearance. This is supported by the fact that AcrAB-TolC has indeed multiple non-antibiotic substrates, including but not limited to bile salts, fatty acids, aromatic hydrocarbons (the basic structure to many antineoplastics compounds), detergents and dyes[173,176,178]. It is believed that the active efflux of bile salts and other host-derived substrates may likely have been the natural role of AcrAB-TolC and related efflux systems, allowing for bacterial survival in the natural environment of enteric bacteria, the GI-trackt[176,177,296]. It leaves the question open that, while it is known that MDR efflux pumps play an important role in bacterial gut colonization and pathogenicity[178], it does not fully explain the extent of cross-resistance towards antineoplastic drugs. Leaving one to wonder if this extensive export of cancer drugs may perhaps be actively selecting for bacteria in the gut during treatment, giving this active efflux of cancer drugs a more descriptive role.

In conclusion, the work presented in this thesis raises the question if cancer chemotherapy may cause bacteria in the GI-trackt to develop AMR during treatment and/or affect the microbiota resistome in other ways. If you have cancer the goal is to beat the cancer and survive, consequences be damned, and while the research fields of anticancer and antimicrobial chemotherapies may have been historically intertwined, they are today widely separate branches of medical research. I strongly believe this is something we may need to take a step back from and that the potential effects of cancer chemotherapy on AMR evolution in the gut is a topic that warrants further investigation, not only from a microbial evolutionary perspective but also from the clinical side. This due to the imminent consequences AMR may have on cancer patients in particular, if we understand better how treatment alters the gut microbiota, the consequences of that alterations can more easily be predicted and addressed.

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The chemotherapeutic drug methotrexate selects for antibiotic resistance

Jónína S. Guðmundsdóttir[a,*], Elizabeth G.A. Fredheim[a], Catharina I.M. Koumans[b], Joachim Hegstad[a,c], Po-Cheng Tang[d], Dan I. Andersson[d], Ørjan Samuelsen[a,e], Pål J. Johnsen[a,*]

[a] Department of Pharmacy, Faculty of Health Sciences, UiT The Arctic University of Norway, Tromsø, Norway
[b] Institute of Biology, Leiden University, Leiden, Netherlands
[c] Research and Development Division, Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway
[d] Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden
[e] Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway

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ABSTRACT

Background: Understanding drivers of antibiotic resistance evolution is fundamental for designing optimal treatment strategies and interventions to reduce the spread of antibiotic resistance. Various cytotoxic drugs used in cancer chemotherapy have antibacterial properties, but how bacterial populations are affected by these selective pressures is unknown. Here we test the hypothesis that the widely used cytotoxic drug methotrexate affects the evolution and selection of antibiotic resistance.

Methods: First, we determined methotrexate susceptibility (IC_{90}) and selective abilities in a collection of *Escherichia coli* and *Klebsiella pneumoniae* strains with and without pre-existing trimethoprim resistance determinants. We constructed fluorescently labelled pairs of *E. coli* MG1655 differing only in trimethoprim resistance determinants and determined the minimum selective concentrations of methotrexate using flow-cytometry. We further used an experimental evolution approach to investigate the effects of methotrexate on *de novo* trimethoprim resistance evolution.

Findings: We show that methotrexate can select for acquired trimethoprim resistance determinants located on the chromosome or a plasmid. Additionally, methotrexate co-selects for genetically linked resistance determinants when present together with trimethoprim resistance on a multi-drug resistance plasmid. These selective effects occur at concentrations 40- to >320-fold below the methotrexate minimal inhibitory concentration.

Interpretation: Our results strongly suggest a selective role of methotrexate for virtually any antibiotic resistance determinant when present together with trimethoprim resistance on a multi-drug resistance plasmid. The presented results may have significant implications for patient groups strongly depending on effective antibiotic treatment.

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1. Introduction

Global overuse and misuse of antimicrobial drugs in combination with dwindling discovery rates of new antimicrobials have led to the current antibiotic resistance crisis [1]. It is also increasingly clear that non-antibiotic natural and anthropogenic substances affect antibiotic resistance evolution in bacterial populations and exacerbates the problem. These include biocides, metals and non-antibiotic drugs that may either directly select for antibiotic resistance, play important roles as co-selective agents, influence horizontal gene transfer (HGT) or mutation rates, and potentiate the effect of low antibiotic concentrations [2–5]. To effectively launch global initiatives to reduce antibiotic resistance there is an urgent need to identify novel drivers of resistance evolution. Antibiotic resistance is a major risk factor for patients with impaired immunity, such as cancer patients, and often a patient's survival depends on antibiotic treatment to reduce the risk for hospital-acquired infections during chemotherapy

* Corresponding author at: UiT The Arctic University of Norway, Faculty of Health Sciences, Department of Pharmacy, Sykehusveien 44, 9019 Tromsø, Norway.
E-mail addresses: jonina.gudmundsdottir@uit.no (J.S. Guðmundsdóttir), paal.johnsen@uit.no (P.J. Johnsen).

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Research in context

Evidence before this study

We searched PubMed and Web of Science for the following search terms: Methotrexate + trimethoprim + resistan*; Cytostatic* + resistan* + antibiotic*; Cytostatic* + cross + resistan* + antibiotic; Chemotherapy + driving + resistan* + antibiotic*; drivers/driving + antimicro* + resistan*; fecal + cancer + trimethoprim in May 2020. We searched DataCite, Google Dataset Search and BASE in August 2021. The existing literature shows that many cytotoxic drugs, including methotrexate, a widely used drug for treatment of cancer and inflammatory diseases inhibit bacterial growth. Many of those drugs are also known to share molecular targets with commonly used antibiotics (e.g. methotrexate and trimethoprim). Further, it is proposed that cytotoxic drugs may drive antibiotic resistance evolution due to microbiome alterations, overlapping intrinsic resistome, and SOS induced mutagenesis.

Added value of this study

To our knowledge, we show for the first time that methotrexate directly selects for acquired trimethoprim resistance determinants on the chromosome as well as on a clinical multi-drug resistance plasmid. These selective and co-selective effects occur at methotrexate concentration ranges expected to represent intestinal concentrations during clinical use. Thus, we provide new evidence on how a cytotoxic drug can affect the evolution, selection, and spread of acquired antibiotic resistance determinants.

Implications of all the available evidence

The current antibiotic resistance crisis can have serious consequences for cancer treatment since these patients display higher risk of bacterial infections and consequently depend on antibiotic treatment. The indications that drugs used in cancer chemotherapy may drive resistance evolution through the same and/or similar resistance mechanisms as antibiotics is potentially of great concern for both cancer patients and the general society. This report represents a first step that will enable us to target drug combinations where resistance evolution is less likely to be an undesired side effect of cancer treatment.

[6,7]. Several cytotoxic drugs used in cancer chemotherapy are known to both elevate bacterial mutation rates and have direct antimicrobial properties [8,9]. It has been proposed that cancer chemotherapy may drive *de novo* antibiotic resistance evolution through SOS induced mutagenesis [10], and some reports have provided support for this hypothesis [11,12]. Recently, the effects of non-antibacterial drugs on bacteria typically found in the human gut were thoroughly explored and cytotoxic drugs were reported to cause the most severe alterations of the microbiota [2]. Taken together, these studies suggest that cytotoxic drugs affect survival of human gut commensals, they may increase the evolvability of bacterial populations, and lead to reduced bacterial susceptibility towards drugs used to treat cancer. How bacterial populations respond to selective and co-selective pressures exerted by individual cytotoxic drugs and the implications for antibiotic resistance selection and spread is unknown. Thus, there is an urgent need to understand these potential collateral effects of cancer chemotherapy to ensure effective antibiotic treatment for a large group of immunocompromised patients. Moreover, cytotoxic drugs may constitute a previously unrecognized target for intervention to limit the selection and spread of antibiotic resistance.

Methotrexate (MTX) is widely used in treatments including but not limited to; cancer of the breast, skin, head, neck, and lung as well as many inflammatory diseases, such as rheumatoid arthritis [13]. We specifically targeted resistance towards trimethoprim (TMP), since both drugs are structurally similar (Figure S1) and act through inhibiting the dihydrofolate reductase enzyme in bacteria and eukaryotic cells, central in DNA synthesis [14]. TMP in combination with sulfamethoxazole is among the most frequently used antibiotics in the treatment of urinary tract infections and is recommended as first line treatment internationally [15]. Our main target organism in this study is *Escherichia coli*, the most common agent of nosocomial infections world-wide [16]. *E. coli* is known to display intrinsic resistance towards MTX through AcrAB-TolC mediated efflux [17], however TMP is not a substrate for this efflux system.

Previous studies have focused on the abilities of MTX and other non-antibiotics to inhibit bacterial growth [2]. These approaches have provided valuable insights on the effects of non-antibiotics as modulators of the intestinal flora, but lacked the necessary resolution to detect more subtle selective effects on acquired antibiotic resistance determinants.

Here, we hypothesize that despite the demonstrated *E. coli* intrinsic MTX resistance [17], MTX can affect antibiotic resistance evolution in *E. coli*, due to the shared molecular target with TMP. We show that MTX selects for acquired bacterial TMP resistance (TMP^R) and co-selects for other antibiotic resistance determinants when co-residing on a mobile genetic element. Exposure to a wide concentration range of MTX selects for mutations identical to those emerging during TMP selection in clinical isolates of *E. coli*. Moreover, we show that the minimum selective concentrations (MSCs) of MTX and positive selection for chromosomal and plasmid-mediated TMP^R determinants occurs at concentrations 40- and >320-fold below the MTX minimum inhibitory concentrations (MICs), respectively.

2. Methods

2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table S1. All incubations of liquid cultures were performed with orbital shaking (225 rpm) at 37 °C, unless otherwise specified. Overnight cultures were grown in Miller Difco Luria-Bertani (LB) broth/agar (Becton, Dickinson and Co.). We used cation adjusted Mueller-Hinton II Broth (MHIIB, Becton, Dickinson and Co.) for assays with drugs supplemented to the media. When appropriate, media were supplemented with: 100 mg/L ampicillin (Sigma-Aldrich), 12.5 mg/L chloramphenicol (Sigma-Aldrich), 7.5 mg/L tetracycline (Sigma-Aldrich), 100 mg/mL Teva/Ebetrex (MTX) (Pharmachemie B.V./Ebewe Pharma Ges.m.b.H Nfg.KG). Methotrexate (MTX) was used in the form of a hydroxide solution ready for i.v. therapy. For strains harbouring the pBAD30 expression vector, cultures were supplemented with 0.2% (w/v) arabinose (Sigma-Aldrich) for induction. Generalized transduction using the P1*vir* [18] were used to move chromosomal markers between strains. For selection against cells expressing *sacB*, sucrose selection plates were used. For long-term storage, strains and populations were mixed with glycerol at a final concentration of 20% (v/v) and frozen at −80 °C.

2.2. Strain constructions

A promoter-levansucrase-chloramphenicol resistance-promoter cassette (P_{CP25}-*sacB*-*cat*-P_{J23101}) was first constructed by amplifying the *sacB*-*cat*-P_{J23101} cassette (GenBank: KM018298) by using primers with homologies to each end of the *insKJ* and partially *mokA* genes in the IS*150* region on the *E. coli* MG1655 chromosome (Table S2). The

construct was introduced onto the chromosome by λ Red recombineering [19,20] in a strain carrying the pSIM5 plasmid [21] with tetracycline as the antibiotic selection marker (pSIM5-*tet*, DA45134). Chloramphenicol resistance was used to select for the inserted construct.

Fluorescent protein encoding: *bfp* (*cat*-PJ23101-*mtagBFP2*, blue; [4]; GenBank: KM018299), *yfp* (*cat*-PJ23101-*SYFP2*, yellow; [4]; GenBank: KM018300) were PCR amplified from previous strains [4]. PCR amplifications were carried out using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). Reaction primers were designed with one of the 40 bp homology to the disrupted IS*150* locus whilst the other retained the P_{CP25} promoter (Table S2). Reaction products were purified using the GeneJet Purification Kit (Thermo Scientific) and introduced onto the chromosome by λ Red recombineering by counter-selection on sucrose agar medium. This resulted in [P_{CP25}-*sYFP2*] and [P_{CP25}-*mtagBFP2*] constructs.

Dup-In methodology of the IS*150* locus was carried out on all previous constructs [22] using the *sacB-cat*-P_{J23101} cassette (GenBank: KM018298) and chloramphenicol resistance as the antibiotic selection. P1*vir* lysates for both fluorescent markers were prepared and transduced into a common background (DA4201) by generalized transduction and segregation of Dup-Ins. Briefly, transduced colonies were picked from plates first with chloramphenicol resistance to transfer the Dup-In with the IS*150* locus, then single colonies were patched on sucrose plates for loss of the *sacB-cat*-P_{J23101} cassette but retaining of the IS*150* locus. For screening of the final strains constructed and generation of templates for Sanger sequencing, DreamTaq PCR Master Mix (Thermo Scientific) was used.

The fluorescently tagged strains were further engineered to obtain TMP^R derivatives. Two point mutations associated with *folA* (one in the *folA* gene, W30R, and the second 58 bp upstream of *folA* within the promotor region, C>T) were introduced onto the chromosome of the strains using a double MAGE cycle with the pORTMAGE-2 plasmid (RRID:Addgene_72,677) as described by previously [23]. The pG06-VIM-1 [24], was transformed into the fluorescently tagged strains as well as *Klebsiella pneumoniae* using room temperature electroporation [25].

To verify the role of *dfrA* genes in both TMP^R as well as methotrexate resistance (MTX^R), both *dfrA1* and *dfrA12* were PCR amplified using Phusion High-Fidelity DNA Polymerase (New England BioLabs Inc.)(Table S2), purified using QIAquick PCR Purification Kit (QIAGEN), phosphorylated using T4 Polynucleotide Kinase (Thermo Scientific) and cloned using T4 DNA Ligase (Thermo Scientific) into the pBAD30 [26] vector at the *Sma*I site. Thus, gene expression was under a tightly inducible control by the P_{BAD} promotor when in the presence of arabinose [26]. The purified ligation reactions were transformed into electrocompetent DH5-α cells with electroporation and clones carrying the vector-born *dfrA* genes isolated.

2.3. Susceptibility testing

Due to the bacteriostatic activities of MTX and a lack of a gold standard for MTX microbiological assays we define the MIC of MTX in this study as the 90% inhibitory concentration (IC_{90}). This allows for a high resolution and has previously been used as a proxy for the MIC [27,28]. The IC_{90} values for TMP and MTX were determined as described previously with minor changes [29]. Briefly, 96-well plates were incubated at 300 rpm when containing MTX and 700 rpm when containing TMP (3 mm stroke) for 18 h at 37 °C before the OD_{600} was measured using an Epoch 2 Microplate Spectrophotometer (BioTek Instruments, Inc.)/VersaMax™ ELISA Microplate (Molecular Devices®). Internal controls were included on all plates. Percent inhibition was calculated as previously described [30]. At least three biological replicates were used and the MIC was set as the most read (modal) value on a two-fold scale of replicates that met quality control standards. For characterization of TMP^R mutants isolated from the MTX sub-MIC evolution, TMP MIC was determined by gradient diffusion strips following the manufacturer's guidelines (Liofilchem). Measurements were done using two to four biological replicates, where the MIC was set as the most read (modal) value.

2.4. Growth rate measurements

Growth rates were determined using a Bioscreen C MBR reader (Oy Growth Curves Ab, Ltd). A minimum of five independent overnight cultures of each strain were diluted to ~5×10^6 CFU/mL in MHIIB containing MTX at concentrations ranging from 0 to 8 mg/mL. Two 300 μL aliquots of each dilution were transferred into sterile Honeycomb plates (Oy Growth Curves Ab, Ltd). The samples were grown at 37 °C with continuous shaking for 18 h and OD_{600} values were measured every 4 min. The growth curves from the Bioscreen C measurements were analysed and growth rate calculations done using the statistical software R [31]. In short, the R package Bioscreen Analysis Tool BAT 2.1 [32] was used to calculate the doubling time of each well by fitting a straight line to the logarithmic phase (OD_{600} values between 0.02 and 0.1). Relative growth rates were then calculated by dividing the mean doubling time of the reference strain grown without any drug present by the mean doubling time of the strain and condition being tested.

2.5. Competition experiments

Competition experiments were performed using the fluorescently tagged strain pairs, both for *folA* and pG06-VIM-1 mediated TMP^R. A susceptible strain tagged with either *yfp* or *bfp* was mixed at 1:1 ratio with the constructed TMP^R strains harbouring the disparate fluorescence marker to initiate a head-to-head competition, at different MTX concentrations. Six independent cultures (~5×10^9 CFU/mL) of each strain were used to start 12 competitions, i.e. six biological replicates for each color arrangement in a dye-swap set-up. Every 24 h for three to four days the competing strains were passaged by a 1:1000 dilution into fresh medium and the mutant to wild type (wt) ratio measured by counting 10^5 cells using a fluorescence-activated cell sorter (BD FACS Aria III). For safety reasons, all cultures were washed in fresh drug-free MHIIB in order to remove MTX from the cultures before FACS analysis. Cells were pelleted at 5000 rcf at 4 °C for 5 min, MTX containing supernatant removed and cells resuspended in fresh MHIIB.

Selection coefficients were calculated according to the regression model $s=[lnR(t)/R(0)]/t$, as previously described [33], where R is the mutant to wt ratio and t is the time measured in generations of growth. The minimum selective concentration (MSC) is defined as the concentration where the selection coefficient equals zero (where the regression line crosses the x-axis) [34].

In a similar way, six individual cultures of a susceptible *yfp* strain was competed against the *bfp* resistant strains in 1:1, 1:10, $1:10^2$, $1:10^3$ and $1:10^4$ starting ratios of TMP^R:TMP^S strains at concentrations slightly above the estimated MSCs (400 μg/mL for *folA* mutant, 75 μg/mL for p06-VIM-1).

To assess the stability of the pG06-VIM-1 in the presence of MTX, three independent lineages of K56−75 harbouring the plasmid (MP05−31) were serially passaged for 50 generations (1:100-dilution) in 1 mL MHII batch cultures with 400 μg/mL MTX. The lineages were then plated on non-selective agar. One hundred colonies from each lineage was replica plated and reduced susceptibilities towards ampicillin, TMP, streptomycin and spectinomycin determined by patching on MHII agar supplemented with 100 μg/mL ampicillin, 25 μg/mL TMP, 40 μg/mL streptomycin or 40 μg/mL spectinomycin as well as MHII agar.

2.6. Selective plating on high concentrations of MTX

Single MTX resistant mutants of K56−2 (MP06−01) were selected at lethal MTX concentrations. Dense overnight cultures grown in

drug-free LB was concentrated 10 ×, and 100 μL spread on LB agar plates supplemented with 4, 8 and 16 mg/mL MTX. Mutants were picked after 48 to 96 h and purified on non-selective plates. Additionally, an overnight culture in LB containing MTX at the estimated MIC concentration was concentrated 10 × and 100 μL spread on LB agar plates with and without MTX 32 mg/mL. After 48 h incubation, mutants were purified on non-selective plates. The MTX and TMP MICs for all mutants isolated were determined as previously described by IC_{90} testing [29] and the *folA* gene, its promotor area and the *marR* gene sequenced with Sanger sequencing and analyzed using the CLC Main Workbench (QIAGEN).

2.7. Laboratory evolution at sub-MICs of MTX

To examine the effect of MTX on TMP^R evolution, strain K56−2 (MP06−01) was serially passaged in liquid cultures with MTX supplemented at concentration slightly above the estimated MSC. Initially, 10 independent overnight cultures were started from independent colonies on separate agar plates from which ~10^3 cells were used to start ten independent lineages in 1 mL MHIIB containing 400 μg/mL MTX (lineages 1−10). Every 12 h for 25 days, the lineages were serially passaged by 1000-fold dilution in 1 mL batch cultures, allowing for ~500 generations of growth. Every ~50 generations the populations were frozen down at −80 °C. In parallel, three independent control lineages were simultaneously sampled for TMP resistance under the same experimental conditions except for MTX exposure (lineages 11−13). After ~500 generations of growth end-point populations were plated on MHII agar plates containing 32 mg/mL MTX. From lineages 1−10, 20 colonies were isolated from each and tested for TMP^R with no increase in TMP resistance detected compared to the parental strain. The frozen populations were gently thawed on ice and dilution series plated on both MHII agar with TMP 4 μg/mL and without drug, and frequencies of TMP resistant mutants calculated. From each plate where mutants grew, up to five colonies were randomly isolated, their susceptibility towards TMP measured, and the *folA* gene and its promotor area sequenced with Sanger sequencing and analysed using the CLC Main Workbench (QIAGEN). No MTX^R or TMP^R colonies were isolated from the lineages grown without drug (lineages 11−13).

2.8. Whole genome sequencing

To investigate the possibility of additional genetic changes during MTX selection, other than the TMP^R determinants shown to be associated with reduced susceptibility towards MTX, five isolates from the lethal selection were chosen (MP18−13, MP18−17, MP18−20, MP18−26 and MP18−28) based on their different susceptibility profiles and subjected to whole genome sequencing (WGS). Bacteria were grown overnight and genomic DNA prepared using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich) following the manufacturer's instructions with slight adaptions. In brief, 1.5 mL of dense culture (OD_{600}: 0.8−1.0) was pelleted by centrifugation at 13000 rpm and supernatant removed. The pellet was resuspended in 200 μL lysozyme solution (100 mg/mL) and incubated for 30 min at 37 °C, before 20 μL of RNase A solution was added and incubated for 2 min at room temperature. Following, 20 μL of Proteinase K (20 mg/mL) and 200 μL of Lysis solution C were added to the mixture and incubated at 55 °C for 10 min after being thoroughly vortexed. To each pre-assembled GenElute Miniprep Binding Column, 500 μL of the Column Preparation Solution were added, 200 μL of ethanol (95−100%) was then added to the lysate and thoroughly mixed before the lysate was carefully loaded onto the binding column, centrifuged at 13000 rpm for 1 min and then washed 2 × with 500 μL of Wash Solution. Genomic DNA was eluted in 100 μL of 10 mM Tris-base and purity and concentration determined using NanoDrop One (Thermo Scientific) and Qubit (Thermo Scientific) respectively. Next-generation sequencing libraries were prepared from the bacterial genomic DNA samples and sequenced on an Illumina NovaSeq with a 2 × 150 bp configuration (GENEWIZ). Average whole genome coverage per sample was approximately 700. Analysis of the fastq files obtained from Illumnia sequencing was performed using an in-house bioinformatic pipeline (Table S3) to compare the mutant sequences to the previously published wt strain (available at NCBI, BioSample SAMN08095529). Where single-nucleotide polymorphisms (SNPs) were identified with a coverage below 100, the evidence was considered insufficient and the SNPs were removed from the analysis. Raw sequence reads were deposited under BioProject PRJNA677979.

2.9. Statistics

Means and standard deviations were estimated using the software R (version 4.1.0) and RStudio (version 1.4.1717).

2.10. Role of the funders

The funders had no role in study design, data collection, data analysis, interpretation, or writing of the report.

3. Results

3.1. Methotrexate selects for pre-existing TMP^R determinants

We initially determined the MICs of MTX in clinical [35] and laboratory strains of *E. coli* (Table S1). Initial experiments revealed variable, but high MTX MICs, ranging from 4 to 32 mg/mL in the different genetic backgrounds, with the exception of <0.25 mg/mL for *E. coli* W3110 Δ7NR*tolC* (Tables S4-S5) [36]. This being consistent with previous reports demonstrating that *E. coli* displays intrinsic resistance towards MTX due to AcrAB-TolC mediated efflux [2,17]. We also observed that the MTX MIC was dependent on the presence of TMP^R determinants. All isolates with a functional TMP^R determinant and increased TMP MIC showed consistently higher MTX MICs (>32 mg/mL) than TMP susceptible (TMP^S) isolates (4−32 mg/mL), indicating possible co-selective abilities of the two drugs. This included strains of both *E. coli* as well as *K. pneumoniae* ATCC13883 harbouring the clinical multi-drug resistance (MDR) plasmid pG06-VIM-1. Strains of both species harbouring the plasmid displayed reduced susceptibility towards MTX as well as TMP (Table S4-S5).

Antibiotic resistance selection and co-selection have traditionally been assumed to occur between the MICs of susceptible and resistant isolates within a bacterial population (known as the selective window) [34]. However, several reports unequivocally show that antibiotic resistance selection and co-selection can occur at concentrations several hundredfold below the MIC of a susceptible isolate (known as sub-MIC) [4,34,37]. To test how sub-MICs of MTX affect bacterial fitness, we measured exponential growth rates for two pairs of clinical isogenic TMP^R and TMP^S *E. coli* across a wide MTX concentration span. One pair with TMP^R located on the chromosome (one intragenic point mutation *T>A* (W30R) in the *folA* gene, and one in its promotor region (P_{folA}, C>T 58 base pairs (bp) upstream of the gene)(MP06−01) [29] and one pair with TMP^R (*dfrA*) located on the MDR plasmid pG06-VIM-1 (MP05−31)(24). TMP^S strains displayed sharply declining growth rates between 1 and 2 mg/mL of MTX, whereas the TMP^R strains remained unaffected (Figure S2, Table S6). These results suggest a selective benefit during MTX exposure for TMP^R strains at concentrations below the observed MTX MIC of the TMP^S clinical isolates. The same effect was observed in the nosocomial pathogen *K. pneumoniae* ATCC13883 where a dose response curve comparing the strain with and without pG06-VIM-1 shows a clear difference in susceptibility already at concentrations below 2 mg/mL (Figure S3, Table S7-S8).

The MSC describes pharmacodynamically the lowest concentration where selection for resistance occurs [34]. To determine the MSC for

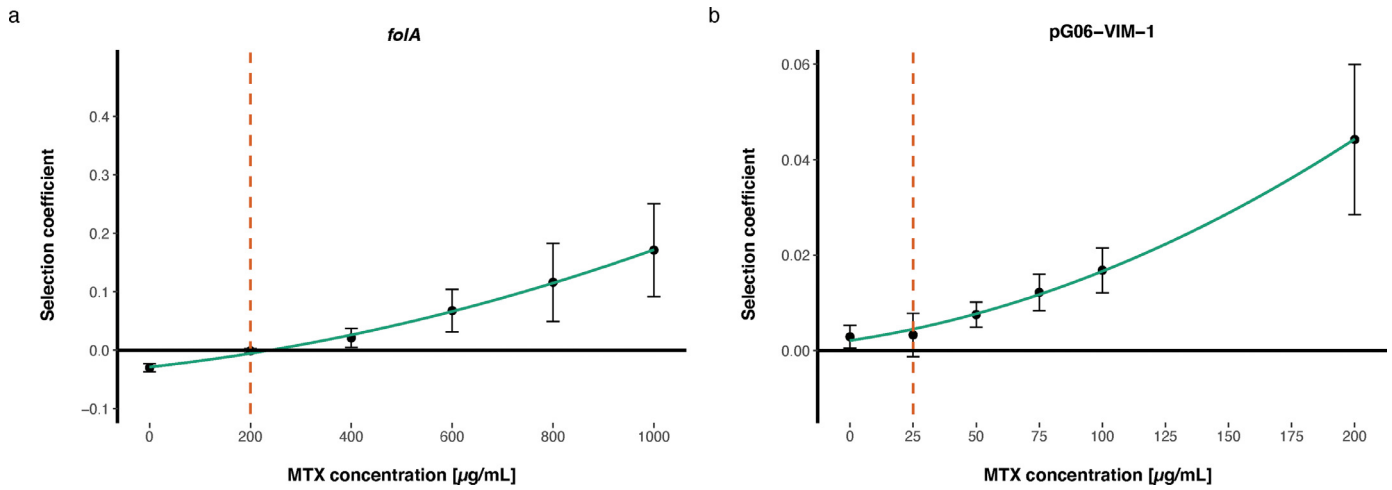


Fig. 1. Selection coefficients as functions of MTX concentrations from competition experiments between TMP^R and TMP^S isogenic strains. The MSC is defined as the concentration where the selection coefficient equals zero. The MSC of *E. coli* MG1655 harboring (a) two chromosomal *folA* mutations (MP18−04 and MP18−07) is set to 200 μg/mL, and (b) the MDR pG06-VIM-1 plasmid encoding *dfrA12* (MP18−05 and MP18−08) is conservatively set at 25 μg/mL. Dashed lines represent the set MSC, bullets the average selection coefficients based on 12 individual replicates and error bars the standard deviations.

MTX, we constructed a fluorescently tagged pair of *E. coli* MG1655 strains to enable accurate separation between the two in mixed populations. In these backgrounds, we introduced TMP^R, either through mutations (*folA*) using genome engineering or the pG06-VIM-1 plasmid. The isogenic TMP^R and TMP^S strain pairs were competed head-to-head by serial passage for 30 generations and the ratio of TMP^R:TMP^S was determined over time using flow cytometry. From this data the MSC was estimated (Tables S9-S10 [34]. Chromosomal *folA* mutations reduced fitness in *E. coli* MG1655 with 3.01% (+/- 0.71, SD) (Table S9) and displayed an MSC of 200 μg/mL (1/40 of the MIC of MTX) (Fig. 1). The MDR plasmid pG06-VIM-1 was selectively neutral (potentially slightly beneficial) displaying a 0.29% (+/- 0.24, SD) increase in fitness (Table S10). The latter estimates of relative fitness were close to the detection limit of the assay [34], and we conservatively estimated the MSC to be <25 μg/mL (less than 1/320 of the MIC of MTX) (Fig. 1). Taken together, our data strongly suggest that selection for TMP^R occurs at MTX concentrations far below the estimated MTX MIC.

3.2. Sub-MICs of methotrexate promotes invasion of TMP^R determinants even when rare in E. coli populations

Exploring MTX-selective dynamics further, we asked if TMP^R determinants could invade the population at lower initial densities to exclude potential bias from the 1:1 ratio in the competition experiments. We started competition experiments from frequencies as low as 10^{-4} of the TMP^R strains, at concentrations slightly above the estimated MSC of MTX (400 μg/mL for *folA* mediated resistance and 75 μg/mL for pG06-VIM-1 mediated resistance) and followed the change in ratios over 30 generations of growth (Fig. 2, Table S11). Both chromosomal and plasmid mediated TMP^R determinants were able to invade, even when initially rare in their respective populations, strongly suggesting that the MTX selective effects are independent on initial frequencies of resistant and susceptible strains during competition experiments.

3.3. Methotrexate co-selects for resistance determinants on a multidrug resistance plasmid

The *dfr*-genes represent a common TMP^R mechanism in *E. coli* and these genes are frequently located on mobile genetic elements such as integrons and plasmids. Given that MTX selects for *dfr*-mediated TMP^R, co-selection of other genetically linked resistance genes is likely. To show this, we used the MDR pG06-VIM-1 plasmid harboring *dfrA1* and *dfrA12* along with multiple resistance determinants including four aminoglycoside resistance genes and the bla_{VIM-1} carbapenemase gene conferring resistance to broad-spectrum β-lactams

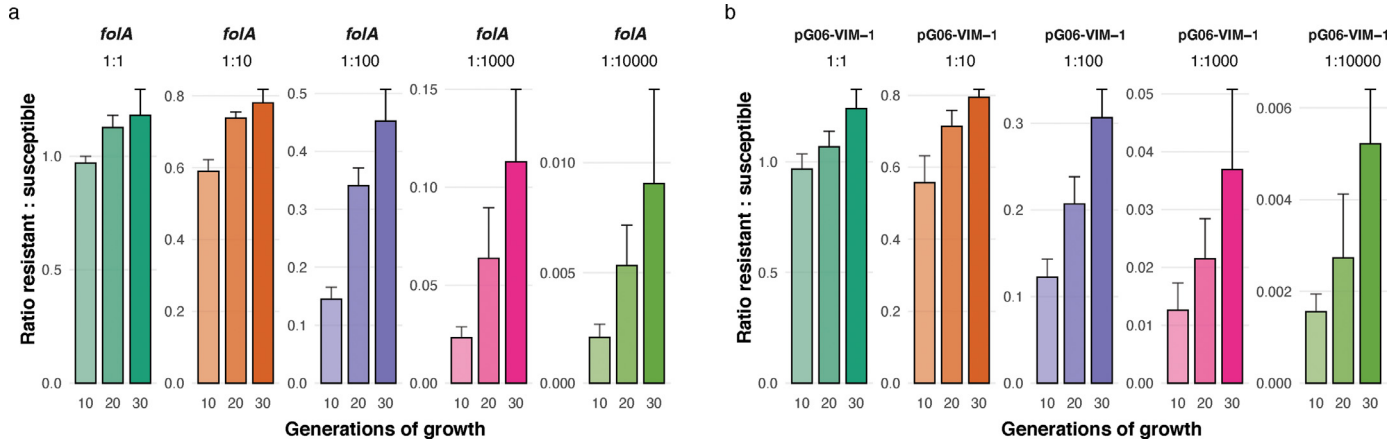


Fig. 2. Competition experiments during sub-MIC MTX exposure at different initial frequencies of TMP^R strains. The change in TMP^R:TMP^S ratios over 30 generations of growth at MTX concentrations slightly above the MSCs where *E. coli* MG1655 harbors (a) two *folA* mutations (MP18−07), and (b) the MDR pG06-VIM-1 plasmid (MP18−08) were competed against a differently tagged, isogenic susceptible strain (DA56507) at 1:1, $1:10^{-2}$, $1:10^{-3}$ and $1:10^{-4}$ starting ratios. Error bars represent the standard deviation of the average ratio, based on 6 individual replicates.

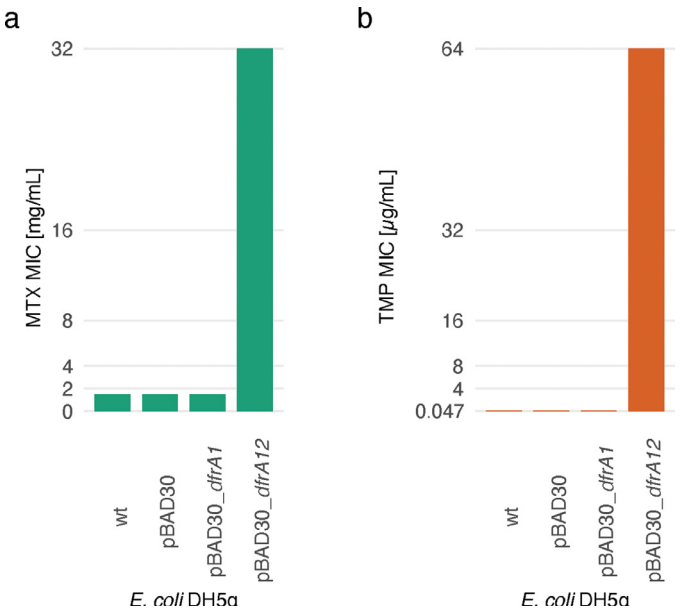


Fig. 3. MTX and TMP MIC of *E. coli* DH5α expressing *dfrA1* or *dfrA12*. The MTX (a) and TMP (b) MIC for the wild type (wt) *E. coli* DH5α (MP18−09) compared to the strain harboring the empty pBAD30 (MP18−10) expression vector as well as strains with pBAD30 with different *dfrA* genes expressed under the inducible expression control by the pBAD promotor (MP18−11 and MP18−12). The detection limit of the assay is 64 μg/mL for TMP and 32 mg/mL for MTX. For both drugs, the MIC of *E. coli* DH5α expressing *dfrA*12 (MP18−12) exceeded the detection limit whereas the strain expressing *dfrA1* (MP18−11) has the same MICs as the wt strain. Showing that MTX and TMP resistance conferred by the pG06-VIM-1 plasmid is caused by the *dfrA12* gene.

including carbapenems [24]. To assess the stability of pG06-VIM-1 in our strains competing in the presence of MTX, *E. coli* K56−75 harboring the plasmid (MP05−31) was serially passaged in batch cultures with 400 μg/mL MTX supplemented for 50 generations. The lineages were then plated on non-selective agar and 100 colonies from each lineage tested for reduced susceptibility towards ampicillin, TMP, streptomycin and spectinomycin. The results revealed complete phenotypic stability across all three lineages, confirming MTX mediated co-selection of plasmid-mediated MDR.

To verify that the TMP^R determinants on the MDR pG06-VIM-1 plasmid is the primary mediators of MTX resistance and selection, both *dfrA1* and *dfrA12* were isolated from the plasmid (Table S2) and cloned onto an expression vector and the effects of the individual genes measured. Of the two genes, only *dfrA12* was shown to give the same resistance pattern for TMP as well as MTX as the pG06-VIM-1 plasmid (Fig. 3), and the lack of detectable phenotype for *dfrA1* (MP18−11) is likely due to a start codon frameshift mutation [24].

3.4. *Methotrexate selects for* de novo *TMP^R*

We further examined whether exposure to MTX could lead to *de novo* TMP^R evolution. We selected spontaneous mutants from overnight cultures with and without exposure to MTX, plated on selective agar at high MTX concentrations and tested for TMP cross-resistance (Figure S4, Table S12). *E. coli* K56−2 isolated at 16 and 32 mg/mL MTX (MP18−17 to MP18−28) displayed increased MICs of TMP close to or above the clinical breakpoint [38], clearly demonstrating selection for TMP^R by MTX. DNA sequencing of the resistant isolates revealed two different mutations in the *folA* promoter, previously reported to result in TMP^R [39], as well as a single mutation in the *marR* gene (Tables S12-S17).

Finally, we asked if exposure to sub-MICs of MTX close to the estimated MSCs would select for *de novo* TMP^R mutations in a susceptible *E. coli* population. Starting from 1000 cells to minimize the probability of pre-existing mutants, we grew ten independent lineages of the *E. coli* K56−2 strain at 400 μg/mL MTX for 500 generations. The frequency of TMP^R was determined every 50 generations. TMP^R ascended in frequency in 2/10 lineages at different rates and timepoints during the first 250 generations before they were outcompeted by a different set of mutants with reduced susceptibility to MTX and no cross-resistance to TMP (Fig. 4, Table S18). These experiments show that MTX exposure can select for *de novo* TMP^R, both at high and sub-MIC concentrations. Arguably, the emergence of *folA* mutations in only 2 lineages is likely due to a larger mutational target within AcrAB-TolC, resulting in reduced susceptibility towards MTX (and not TMP).

3.5. *Pharmacokinetic approximations*

To assess pharmacokinetic relevance, we attempted to estimate the MTX concentration range likely to be found in the intestine of patients undergoing MTX treatment. Limited information is available on gut MTX concentrations following intravenous administration during cancer treatment, as pointed out by others [2]. Pharmacokinetic data reveal that up to 90% of administered MTX is renally excreted [40] and we assume that the remaining ~10% of the dose constitutes the upper limit of the concentration range present in the human intestine. The lower limit is set to 2% of the dose based on the mean ^{3}H labelled MTX concentrations measured in stool samples from nine patients receiving MTX intravenously [41]. From this, we set a 24 hour transition time in a total volume of 0.6 L [2] and calculated the dose (d) required to achieve MSC in the human intestine from:

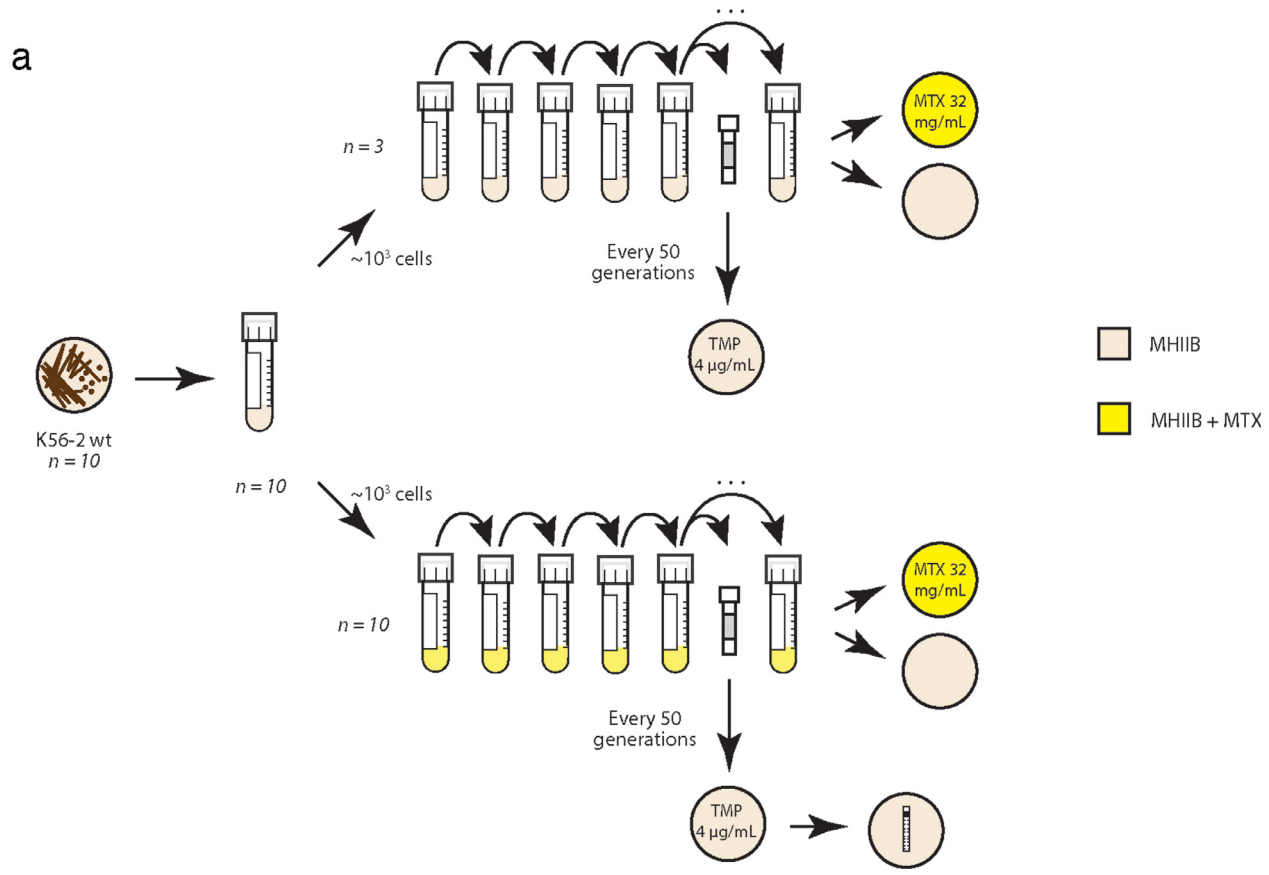
$$\frac{d}{0.6L}\ x\ (0.1\ or\ 0,02) = MSC$$

Estimated doses needed to reach intestinal MSCs assuming 2% and 10% fecal MTX concentrations were from 0.15 g to 0.75 g for plasmid-mediated TMP^R and from 1.2 g to 6 g for chromosomal *folA* mutations. Thus, assuming close to 2m^2 body surface in grown-up patients [42] estimates of MSC for plasmid-mediated TMP^R translates to dosing regimens from 75 to 375 mg/m^2 and from 0.6 to 3 g/m^2 for the chromosomal *folA* mutations. These approximations indicate that our MSC estimates are relevant for patients receiving high dose MTX treatment (1−12 g/m^2) [43]. A recent study, also using a literature-based approach but with slight differences, estimates gut MTX concentrations following oral administration during treatment of rheumatoid arthritis [44]. Their data suggested MTX concentrations as high as 100 μg/mL are found in the lower intestine, suggesting that our estimated MSC for plasmid-mediated antibiotic resistance determinants (25 μg/mL) is well within this concentration range.

4. Discussion

Here we show that exposure to the cytotoxic drug MTX affects selection and evolution of TMP^R determinants at clinically relevant concentrations. Notably, MTX can mediate selection of any antibiotic resistance determinant in *E. coli* when TMP^R is co-localized on a mobile genetic element across a wide concentration gradient. Transferring the MDR plasmid pG06-VIM-1 into a *K. pneumoniae* strain resulted in reduced susceptibility towards MTX, suggesting that our findings are relevant beyond *E. coli*. Arguably, this potentially important side-effect of MTX treatment has been previously unrecognized, as studies on the effects of non-antibiotic drugs, including MTX, have either focused on bacterial growth inhibition or used drug concentrations around the MIC [2,45−47], with a few exceptions [4,48].

Using the approaches outlined here, including high resolution mixed culture competition experiments, allow determination of the true MTX selective window ranging from the MSC to the MIC [34].



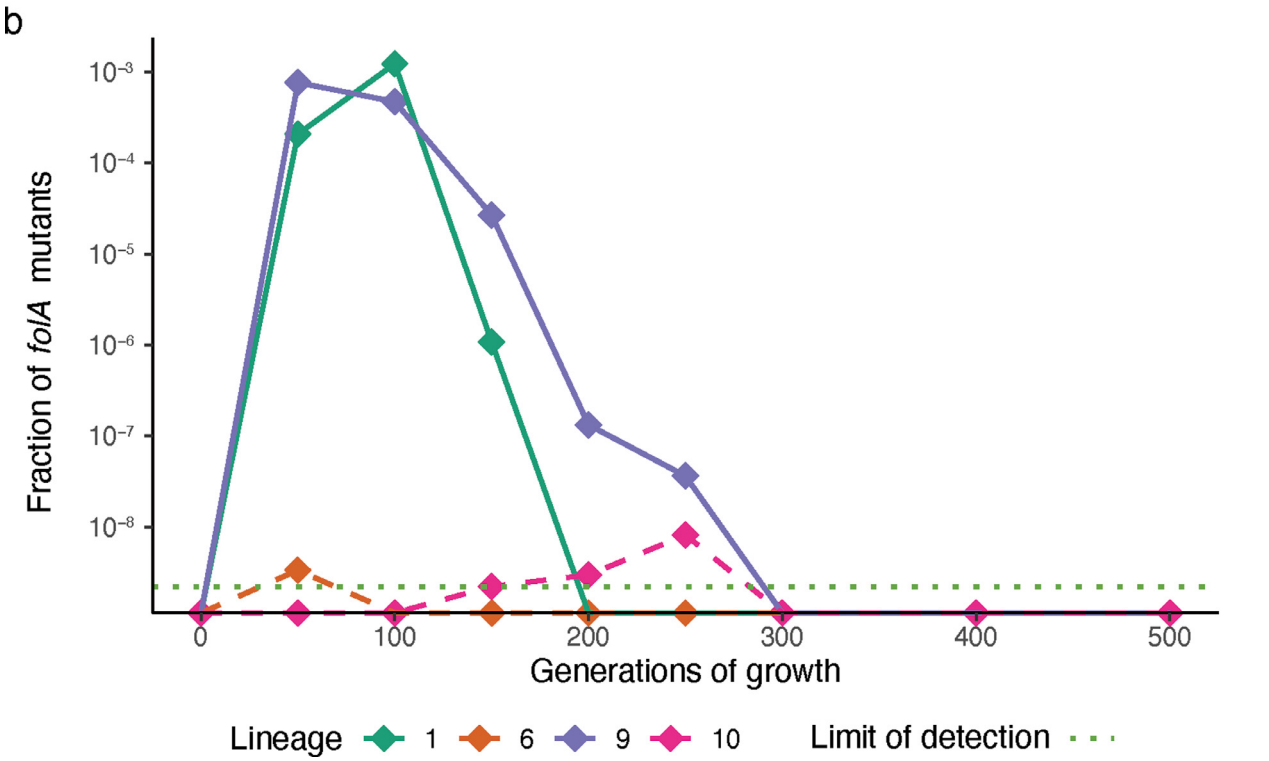


Fig. 4. Evolution of TMP^R during MTX exposure for 500 generations. (a) Sub-MIC evolution experimental set-up. Ten biological replicates of K56–2 (MP06–01) were evolved for ~500 generations with 400 μg/mL MTX and three biological replicates without drug. All lineages were screened for TMP^R every 50 generations. After 500 generations all end-point populations were plated on 32 mg/mL MTX. All populations were able to grow at 32 mg/mL MTX, but not a single clone isolated conferred TMP^R, strongly suggesting that reduced susceptibility to MTX with no cross-resistance to TMP evolved in the endpoint populations. (b) Fractions of TMP^R *folA* mutants isolated every 50 generations from the lineages where these were detected. The detection limit of the assay was $\sim 2 \times 10^{-9}$. Solid lines represent the two lineages where TMP^R emerged and ascended in frequency whereas dotted lines indicate spontaneous mutants.

This is particularly relevant for non-antibiotics for which bacteria display reduced susceptibility. In *E. coli,* MTX is a substrate for the AcrAB-TolC efflux pump [17] and selective effects as those demonstrated here would not have been detected in classical susceptibility and/or growth assays in bacterial monocultures. This was recently supported in an *E. coli* chemical genetic screen where clear growth inhibitory effects of MTX, as well as for a range of other non-antibiotics, were only demonstrated in a *tolC* knock-out mutant (i.e. in a mutant lacking the intrinsic mechanism of resistance) [2].

Given that many cytotoxic drugs are structurally similar to antibiotics (e.g. doxorubicin/tetracyclines), or target similar key processes as the major antibiotic groups (e.g. DNA/protein synthesis) it is possible that cancer chemotherapy may lead to increased levels of antibiotic resistance in a vulnerable patient group that very often rely on efficient antibiotic treatment for survival. To acquire a deeper understanding of the evolutionary potential of novel, non-antibiotic drivers of antibiotic resistance the approaches presented here are essential. These approaches need to be combined with an improved understanding of the intestinal pharmacokinetics of MTX and other cytotoxic drugs, possible effects of co-administered drugs such as leucovorin mediated MTX rescue [49], and their interactions with the human microbiome. Such knowledge could allow identification of antibiotic + non-antibiotic drug combinations that should be avoided to preempt resistance evolution. This would be particularly relevant when considering repurposing cytotoxic drugs as antibiotics [50].

Taken together with recent studies showing that non-antibiotics can increase mutation rates [11,12] and promote horizontal gene transfer [48,51], the data presented here strengthens the evidence that non-antibiotic drugs can affect the evolution, selection, and spread of antibiotic resistance determinants. Our study is however not without limitations. Despite our pharmacokinetic considerations, which suggest that MTX selects and co-selects for antibiotic resistance determinants at clinically relevant concentrations, the lack of clinical data does limit our ability to conclude on the clinical and physiological significance of the results. Carefully designed *in vivo* experiments and/or clinical patient studies are important next steps to verify how MTX affect evolution, selection and spread of TMP resistance. One such approach could be a case control study comparing antibiotic resistance levels in patients that receive MTX compared to a group that does not, followed by microbiological and molecular analyses of bacteria and resistance determinants.

In this study we present data suggesting that MTX, a widely used drug in the treatment of several cancers as well as inflammatory diseases, may affect the evolution, selection and spread of antibiotic resistance. Moreover, we present an experimental frame-work where the true selective windows of non-antibacterial drugs can be determined. We argue that these approaches are critical to improve our understanding of non-antibacterial drugs as potential drivers of antibiotic resistance.

5. Contributors

The study was designed by JSG, EGAF, ØS and PJJ. Strain constructions were done by PT and JSG. Experiments were conducted by JSG, EGAF and CIMK. WGS analysis was done using a bioinformatic pipeline designed by JH. WGS data was verified by JH and JSG. Underlying data was verified by JSG and PJJ. Formal data analysis and data visualization was done by JSG. The first draft of the manuscript was written by JSG and revised by PJJ. All authors revised subsequent version and provided key edits to the manuscript. All authors read and approved the final version of the manuscript. Funding acquisition and resources were provided by PJJ and DIA.

Declaration of Competing Interest

The authors declare no competing interests.

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Data sharing statement

All data used to draw the conclusions in this paper are provided in the paper and/or in the supplementary materials. WGS data are available at NCBI (BioProject PRJNA677979).

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2021.103742.

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Paper II

Paper III

Mischief managed!

- Fred Weasley