Universidad Zaragoza
1542

Instituto Universitario de Investigación Biocomputación y Física de Sistemas Complejos
Universidad Zaragoza

D²AMR
DISCOVERY & DEVELOPMENT
ANTIMICROBIALS
MECHANISMS OF RESISTANCE

Master's Final Project

Mode of action elucidation studies of new antimicrobial compounds

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Contents

List of Figures

List of Tables

List of Abbreviations

ADC: Albumin-Dextrose-Catalase

AMR: Antimicrobial resistance

ATCC: American Type Culture Collection

BCG: Bacille Calmette Guerin

C18: Compound 18

CFU: Colony forming unit

CLSI: Clinical & Laboratory Standards Institute

CNB: Centro Nacional de Biotecnología

DPA: Decaprenyl-phospho-arabinose

FAS: Fatty Acid Synthase

FDA: Food and Drug Administration

HIV: Human Immunodeficiency Virus

HTS: High-throughput screening

INH: Isoniazid

MBC: Minimal Bactericidal Concentration

MDR: Multidrug-resistant

MIC: Minimal Inhibitory Concentration

MRSA: Methicillin-resistant *S. aureus*

Msm: *Mycobacterium smegmatis*

OADC: Oleic acid-Albumin-Dextrose-Catalase

PBP: Penicillin-binding protein

PBS: Phosphate buffered saline

rRNA: Ribosomal RNA

SAR: Structure-activity relationship

Sau: Staphylococcus aureus

SEIMC: Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica

SEL: Selamectin

TB: Tuberculosis

VRE: Vancomycin-resistant *Enterococcus spp.*

WHO: World Health Organization

XDR: Extensively drug-resistant

Abstract

The incidence of drug-resistant bacteria is soaring worldwide in the last decades and becoming increasingly difficult to treat. This emergence has been accompanied by a failure to deliver new antimicrobials into the market. Drug discovery approaches include target-based and phenotypic screening, but most current antimicrobials have been identified using the latter. However, this strategy does not provide any information on how the identified compounds inhibit bacterial growth, being the elucidation of the mode of action a major bottleneck in the discovery process.

The antimicrobial activity of two series of compounds, the avermectins and the EPMM compounds, was recently characterised at the D²AMR Group in the Department of Microbiology. Based upon these studies, the main aim of this Master's Final Project (TFM) was to isolate mutants with changes in their susceptibility profile to the compounds. Subsequent genomic analysis, beyond the scope of this TFM, will aid to elucidate their mode of action.

Two complementary approaches were used to isolate mutants with changes in their susceptibility to avermectins: first, standard mutant isolation assays with the *Mycobacterium smegmatis* Δ*nucS* hypermutator strain using inhibitory concentrations of selamectin; and second, screening of a *Mycobacterium bovis* BCG transposition library to identify either resistant or hypersusceptible mutants. Susceptibility changes were evaluated by testing the candidates' susceptibility in broth, agar plates and by time-kill kinetics. Using these strategies, eight *M. smegmatis* mutants resistant to selamectin were validated.

Similar methodologies were used to initiate mode of action studies for EPMM Compound 18 (C18). *Staphylococcus aureus* mutants were isolated by two different strategies: first, from liquid cultures exposed to inhibitory concentrations of C18; and second, from standard mutant isolation assays. Seventeen *S. aureus* mutants resistant to C18 were confirmed.

1.- Introduction

1.1.- The history of antimicrobials

The discovery of antimicrobials was one of the milestones of medicine in the 20th century. Their introduction into clinical practice dramatically reduced the incidence and mortality of infectious diseases. But the impact of antimicrobials on medicine goes far beyond: they have been key to the development of other areas of medicine, such as invasive surgery, organ transplantations or cancer chemotherapy, in which antimicrobials are used to prevent bacterial infections associated with these procedures.

Modern antimicrobial chemotherapy is based upon the principle of **selective toxicity**. Antimicrobials work as *magic bullets*, being toxic to pathogens, yet innocuous to the host. Following this principle, Paul Ehrlich and Sahachiro Hata developed Salvarsan, an anti-syphilis drug, in the first decade of the 20th century. This drug is considered the first modern antimicrobial (1).

In the succeeding decades, novel antimicrobial agents were successfully discovered from both natural and synthetic sources. The introduction of the novel sulphonamides in 1936, isolated from dye libraries, and penicillin in 1938, discovered by Alexander Fleming, resulted in a huge decrease of the mortality rates from infectious diseases in the subsequent years (2). Penicillin, for instance, proved to be an invaluable resource in World War II, saving thousands of Allied lives.

These early discoveries were soon followed by the discovery of the major classes of antibiotics currently available (aminoglycosides in 1944, tetracyclines in 1945, cephalosporins in 1948, macrolides in 1949, glycopeptides in 1956 and rifamycins in 1957). The period from 1940 to 1970 is known as the **Golden Era of Antibiotic Discovery** (3,4) (**Fig. 1**).

Novel antibiotics were identified thanks to the discovery platform introduced by Selman Waksman, co-discoverer of streptomycin together with Albert Schatz. The **Waksman platform** involved screening soil microorganisms, in particular Actinomycetes, for the production of metabolites that inhibit the growth of pathogenic bacteria (2). It remained effective until the mid-1960s. By that time, the discovery of novel antibiotics had become harder. Those new metabolites identified with antimicrobial activity were already known antibiotics or had significant pharmacological or toxicological issues. At the same time, bacteria resistant to the early antibiotics were arising (5).

All these issues ushered in the **Medicinal Chemistry Era,** which spanned from the 1970s to the end of the century**.** The strategy during this Era broadly consisted in the modification of the chemical structure of molecules discovered during the Golden Era of Antibiotic Discovery. Semi-synthetic derivatives of natural antibiotics were introduced with **improved properties** such as better activity and selectivity, reduced toxicity to the host, evasion of the mechanisms of resistance and broader activity spectrum. Among these antibiotics, the fluoroquinolones, a novel class of synthetic antimicrobials, were introduced in the late 1960s. The perceived success in the development of such new molecules during these decades seemed enough to outpace the emergence and dissemination of drug-resistant strains (4). However, the strategy followed during this period resulted in lack of innovation and a subsequent decrease in the discovery of molecules with novel modes of action.

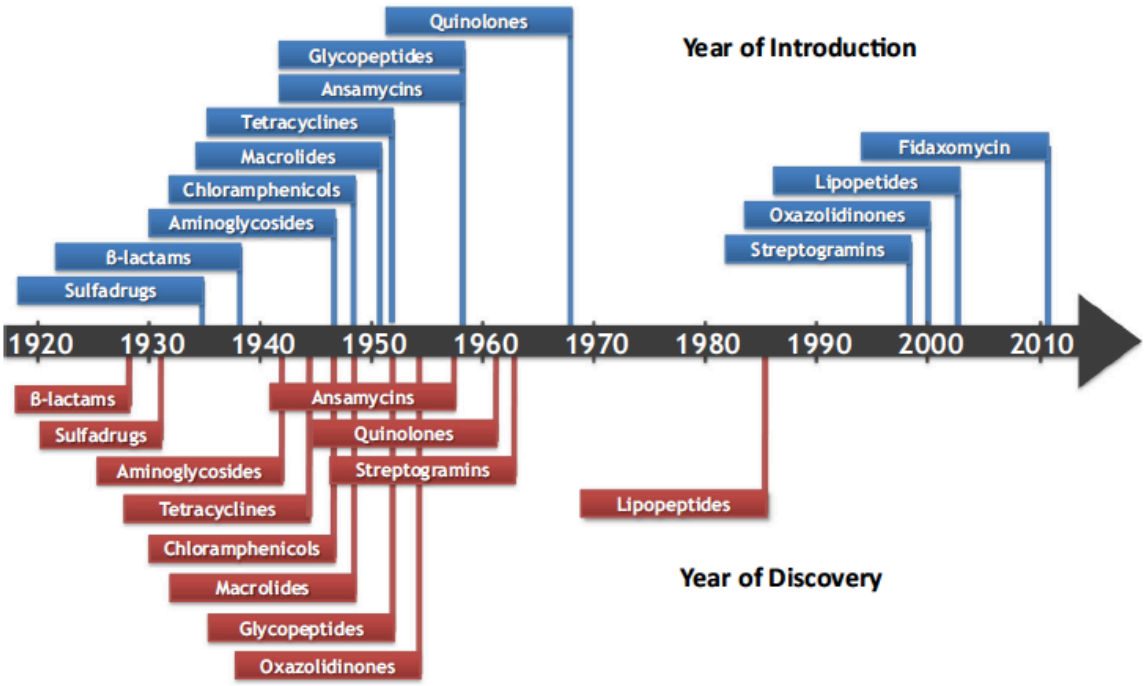

Fig. 1. The timeline of antimicrobial discovery (bottom, red) and introduction into clinical practice (top, blue). Most of the currently used antimicrobial classes were discovered during the Golden Era of antibiotic discovery (1940-1970). All classes except the synthetic sulpha-drugs, oxazolidinones and fluoroquinolones are antibiotics. In the last 50 years, only the class of lipopeptides was discovered. Reproduced from Lewis 2017 (3).

Technical breakthroughs in genome sequencing, genetic engineering, large-scale protein expression, high-throughput chemical synthesis, robot liquid handling and computer science paved the way for the development of **high-throughput screening (HTS) assays**. The sequencing of the first bacterial genomes at the end of the last century brought a new paradigm for the discovery of new antimicrobials. Genomic information allowed the identification of

highly conserved targets which were essential for the survival of bacteria. Big pharmaceutical companies embarked in costly HTS campaigns to identify inhibitors of these conserved protein targets. Nonetheless, these approaches have failed to provide compounds with new modes of action and, as a result, the number of new approved antimicrobials has decreased over the last decades. (2,6).

Nowadays, the discovery paradigm has shifted again towards phenotypic screenings. Current efforts focus on improving the chemical diversity of compound libraries and using natural products as a source of new compounds. Most antimicrobials are secondary metabolites produced by bacteria (or their derivatives). Accessing the chemical diversity that has been hidden might provide novel compounds with antimicrobial activity. Approaches to increase the diversity of secondary metabolisms might include the prospection of new ecosystems (e.g. the bottom of the oceans), growing uncultured bacteria in their natural environment or turning on silent operons, among others (4).

Antibacterial agents have been the most effective of all medicines. Their introduction into clinical practice brought an unprecedented revolution in medicine. However, the emergence of multidrug-resistant strains in the past decades, together with the failure to develop antimicrobials with novel modes of action, jeopardises the breakthroughs that have improved our quality of life and life expectancy.

1.2.- Antimicrobial resistance

Antimicrobial resistance (AMR) arises when microorganisms survive the exposure to clinically achievable inhibitory concentrations of antimicrobials, which allows them to grow and spread in their presence. AMR implies a **loss of therapeutic efficacy** in the control and treatment of bacterial infections and has become a global problem; the misuse and overuse of antimicrobials over the past decades has increased the rate at which drug-resistant bacteria are selected and spread.

The Review on Antimicrobial Resistance (7), published in 2016, estimated that **700,000 people** die every year from infectious diseases caused by drug-resistant bacteria. The Review also states that if the current trend is not reverted, the number of deaths attributable to AMR would reach 10 million per year by 2050, becoming the first cause of death worldwide. In Spain, a report recently published by the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC), estimated the number of nosocomial infections caused by multidrug-resistant (MDR) strains in 180,000 cases, with 35,000 estimated deaths in 2018 (8). Drug-

resistant bacteria are relevant in hospitals; nosocomial infections are mainly caused by a group of bacteria known as ESKAPE (***E**nterococcus spp.*, ***S**taphylococcus aureus*, ***K**lebsiella pneumoniae*, ***A**cinetobacter baumannii*, ***P**seudomonas aeruginosa* and ***E**nterobacter spp.*) (9). In addition, drug-resistant *M. tuberculosis* is currently the first cause of death from AMR, accounting for more than 200,000 deaths per year (10).

Treatment of infections caused by drug-resistant strains often requires the use of antimicrobials with less efficacy or more frequent side effects, leading to reduced success rates and increased mortality. These second-line drugs are usually more expensive; for example, the treatment of methicillin-resistant *S. aureus* (MRSA) infections is more than twice as expensive as the treatment of methicillin-susceptible *S. aureus* (11).

In order to tackle this global health emergency, antimicrobials with novel modes of action are urgently needed. The World Health Organization (WHO) published guidelines to be followed in the research of new antimicrobials (12), establishing *M. tuberculosis* (including M/XDR strains) and M/XDR Gram-negative strains as the top priorities for the development of new antimicrobials.

The incidence of **Gram-negative** strains resistant to (almost) all antibiotics available, such as carbapenem-resistant *A. baumannii*, *P. aeruginosa* and Enterobacteriaceae is increasing worldwide, making them another critical priority for the development of novel antimicrobials. The emergence of resistant strains has left few therapeutic options available, in many cases antimicrobials that were once discarded for systemic use because of their side effects (e.g. colistin) are now been used as a last resource therapy (13).

The development of new antimicrobials with activity against Gram-negative pathogens is especially challenging because of their intrinsic antimicrobial resistance due to permeability barriers and efflux pump systems that hamper drug access to their target. In fact, most antimicrobials designed against Gram-negative bacteria that are currently in clinical testing are later generations of already used antibiotic classes (14,18,19).

Gram-positive bacteria are in general more susceptible to antimicrobials, due to the lack of an outer membrane. Among MDR Gram-positive pathogens, the most relevant are MRSA and vancomycin-resistant *Enterococcus spp.* (VRE). Vancomycin is the frontline treatment for MRSA, but vancomycin-intermediate and resistant strains have emerged in the past decades (16). Some recently approved compounds, such as lipopeptides (daptomycin) and oxazolidinones (linezolid) remain active against these strains but have severe side effects.

Hence, it is important to broaden the pipeline, not only to forestall the emergence of resistance to these compounds, but also to have alternatives with reduced toxicity.

1.3.- *Mycobacterium tuberculosis*: a global priority for antimicrobial discovery

Mycobacterium tuberculosis is the causative agent of **tuberculosis (TB),** the ninth cause of death worldwide and the leading cause from a single infectious agent. In 2017, TB took an estimated 1.6 million lives, and 10 million people developed the disease (10). In addition to the high incidence and mortality figures, an estimated 1.7 billion people are infected with latent tubercle bacilli and are at lifelong risk of developing the active form of the disease, especially if their immune system is compromised by certain risk factors such as HIV co-infection, malnutrition, smoking, diabetes, etc. (17). For this reason, the incidence is much higher in areas where many of those conditions coincide, such as the south of Africa or Southeast Asia.

The **treatment** of TB is based upon combinations of many anti-TB drugs in order to decrease the emergence of drug-resistant strains. The current frontline treatment for **drug-susceptible TB**, implemented in the 1990s, consists of a two-month intensive phase of rifampicin, isoniazid, pyrazinamide and ethambutol, followed by a continuation phase of four months of rifampicin and isoniazid. The cost of this treatment is about 35 euros per patient, and the global success rate is over 85% under optimal conditions (10).

Drug-resistant TB is the main cause of death from drug-resistant infections. An estimated 460,000 people developed MDR-TB (i.e. TB resistant to the cornerstone frontline drugs, rifampicin and isoniazid) in 2017. The therapy for MDR-TB is much longer and requires the use of second-line drugs with **more frequent and severe side effects**, which complicates the adherence to the treatment. Second-line therapy is individual for each patient, including at least a fluoroquinolone and an injectable (kanamycin, amikacin or capreomycin), and can last up to 18-24 months. Treatment success rate for MDR-TB is 55%. XDR-TB (MDR-TB with additional resistance to second-line fluoroquinolones and injectables) is even more difficult to treat and survival even lower than for MDR-TB, with only one person of every three surviving XDR-TB (10,18) with currently available therapeutic options.

Bedaquiline (2012) and delamanid (2014) are the first drugs with novel mechanisms of action against *M. tuberculosis* in more than four decades and are the last resort in many cases. Although, resistance to bedaquiline and delamanid has already been reported in clinical isolates and this could limit their use as anti-TB drugs (14,19,20), several regimes for MDR and XDR-TB including them are undergoing clinical evaluation, such as the Nix-TB trial. This

regime, recently approved by the FDA, is a combination of bedaquiline, pretomanid and linezolid for the treatment of XDR-TB, with potential application to MDR and drug-susceptible TB (21,22).

1.4.- Mechanisms of antimicrobial resistance

AMR is an **unavoidable consequence of bacterial evolution**. Bacteria have been fighting each other with toxic metabolites for thousands of years in order to keep a given ecological niche. Along this warfare, they have developed a repertoire of mechanisms to get over the actions of these molecules (e.g. efflux systems, which protect bacteria from toxic agents present in their environment). The development of such mechanisms as a physiological response to environmental threats had the collateral effect of giving rise to resistance to antibiotics. The collection of genes responsible for resistance to antibiotics is called the **resistome** (2).

Resistance to antimicrobials (and antiseptics) can be intrinsic or acquired. **Intrinsic resistance** is owed to **structural or functional features** of bacteria. The mycobacterial cell wall, which hampers the access of many different antimicrobials due to the presence of a layer of lipids (including mycolic acids) covalently linked to arabinogalactan and a low number of porins, is a good case in point (23). **Acquired resistance,** on the other hand, is due to stable chromosomal mutations or horizontal gene transfer and makes bacteria resistant to antimicrobials which were previously effective against them. For example, mutations in the *katG* locus confer resistance to isoniazid in *M. tuberculosis*, as the mutant forms of the enzyme lack the capacity to transform the prodrug isoniazid into the active compound.

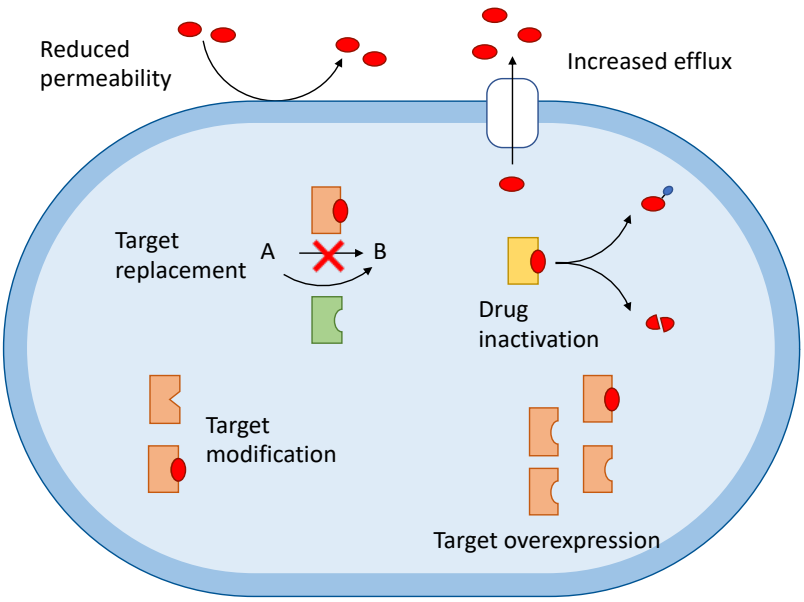

Fig. 2. Overview of the mechanisms of antimicrobial resistance.

Overall, mechanisms of antimicrobial resistance can fall into several categories: drug inactivation, target overexpression, target modification, target replacement and reduced antimicrobial uptake (**Fig. 2**).

1.4.1.- Drug inactivation

One of the most common mechanisms of antimicrobial resistance involves **enzymatic modification of the antimicrobial**, which alters its structure to a molecule incapable of binding to its target. This result can be achieved by destruction of the essential reactive centre of the molecule or by modifications of the drug resulting in a reduction of binding affinity.

Cleavage of β-lactams by β-lactamase enzymes is the best-known example of resistance by antimicrobial inactivation. β-lactams kill bacteria by covalently binding to penicillin-binding proteins (PBP), which are involved in peptidoglycan biosynthesis and essential for cell wall structure. The inhibitory effects of β-lactams depend on the β-lactam ring, which is the reactive group involved in the covalent binding to PBPs. β-lactamase enzymes hydrolyse the β-lactam ring, thereby conferring resistance to β-lactam antibiotics (24).

1.4.2.- Target overexpression

Increased expression of the drug target confers resistance because higher concentrations of antimicrobial are needed to reduce its activity to levels that actually compromise bacterial growth. Overexpression of the dihydrofolate reductase has been associated with resistance to trimethoprim in *Escherichia coli* (24).

1.4.3.- Target modification

Most antimicrobials bind to their targets, typically proteins or nucleic acids, in a fairly specific way. In such circumstances, modifications in the target structure can result in a **loss of affinity**, which can lead to resistance if the mutation does not impair the biological function of the target. For example, single point mutations in the *rpoB* locus (β subunit of the RNA polymerase) can confer high-level resistance to rifampicin.

Target alteration can also be due to **enzymatic modifications** that reduce the binding affinity of the antibiotic. The most common example of this mechanism is the inducible macrolide resistance. Macrolides bind to the P-site of the 50S subunit of the ribosome, blocking translation. Ribosomal methylation at the 23S rRNA by *erm* genes inhibits macrolide binding, thus conferring resistance to them (24).

1.4.4.- Target replacement

High-level resistance can be achieved by the acquisition of genes with the **same function** as the target gene, but with **much lower affinity** for the antibiotic. An example of such acquisition is the *mecA* gene of *S. aureus*, which encodes the PBP2a, a PBP variant with low affinity to β-lactams, resulting in high-level resistance.

A more complex example is vancomycin resistance in *Enterococcus spp*. and *S. aureus*. Vancomycin binds to the terminal D-Ala-D-Ala of the peptidoglycan pentapeptide precursor, preventing the access of PBPs to these peptides and the transpeptidation reaction. Vancomycin resistance is encoded in transferable operons, which produce a series of enzymes whose activity results in the substitution of the normal pentapeptide by alternative D-Ala-D-lactate precursors. The affinity for the modified precursor is 1,000-fold lower than for the D-Ala-D-Ala, which results in high-level resistance (24).

1.4.5.- Reduced antimicrobial uptake

Bacteria can become also resistant to antimicrobials due to a **reduction in the uptake**, an **increase in efflux** or a combination of both. In any case, the result is a reduced effective availability of the drug to the target site.

The outer membrane of Gram-negative bacteria and the mycobacterial cell wall form an antimicrobial barrier that hinders the diffusion of antimicrobials towards the cytoplasm. Many hydrophilic antimicrobials penetrate these barriers through porins, membrane proteins permitting the diffusion of compounds into the periplasm. Porin depletion can thus lead to reduced uptake of the drug (24).

Multi-drug efflux pumps protect bacteria from a broad range of toxic molecules, including some antibiotics (and thus conferring intrinsic resistance to them). Furthermore, mutations resulting in a loss of the regulatory mechanisms can lead to increased expression and resistance. An example of this mechanism of resistance is the overexpression of the MmpL5-MmpS5 efflux pump in *M. tuberculosis*, which confers resistance to bedaquiline and clofazimine (19).

1.5.- Discovery of new antimicrobials

One of the long-term solutions to the current scarcity of antimicrobials is the continuous development of new drugs with novel modes of action, since resistance to virtually any

compound will eventually emerge. The development of new antimicrobials starts with the identification of a *hit* compound, i.e. a molecule that is active *in vitro*, which is subsequently modified and optimised along the process until a candidate is selected and enters the pre-clinical and finally clinical phase studies.

1.5.1.- Hit identification

In the past decades, the first step of the drug discovery process was done mostly by **HTS of large compound libraries**. Compound libraries used for HTS usually might contain a large number of compounds (ca. 0.5-2 million), which can come from different sources: natural products, combinatorial synthesis, etc.

Hits can also be identified by ***in silico*** **approaches,** such as fragment-based drug discovery (FBDD) or high-throughput virtual screening. These approaches require *a priori* knowledge of the three-dimensional structure of the target in order to dock a ligand or fragment library and subsequent experimental validation.

Two different approaches have been used for HTS in antimicrobial discovery: whole-cell and target-based screening. The former is a phenotypic screening of compounds against live bacteria. Whole-cell screening is conceptually and methodologically simple, allowing the identification of **compounds that inhibit bacterial growth** and it has been very useful in the identification of antimicrobials coming from microbes such as those from the genus *Streptomyces*. In fact, the majority of antibiotics discovered during the period between 1940 and 1970 were identified following this approach (6). However, no information on the molecular mechanism of action is provided. Hence, the development of hits following this strategy requires first the identification of their target and elucidation of their antimicrobial mode of action.

The **target-based approach** consists in searching compounds that inhibit a purified protein target previously identified as essential for bacterial survival under specific growth conditions. This approach requires the production of large amounts of the target (typically by heterologous protein expression) and the development of a robust assay that allows the identification of **inhibitors** from the chemical library (e.g. by specific enzymatic assays or by assays based on protein stabilisation by binding). The main advantage of this approach is that the target is readily known (25). Although this approach has been very effective in the development of drugs for other clinical applications, it has failed to deliver antimicrobials with novel modes of action (5,6). Target-based approaches have an additional limitation when

applied to the development of antimicrobials, i.e. the impermeable bacterial cell wall barrier might prevent the inhibitor to reach its target. For this reason, there is not a straightforward correlation between target inhibition and antimicrobial activity.

Target-based whole-cell screening combines both previous strategies. The implementation of novel genetic tools allowed combining the advantages of the existing approaches, as well as cancelling their drawbacks. One of these approaches is the use of conditional knock-down strains, which is based upon the assumption that a reduction in the concentration of the target may render the cell hypersensitive (compared to a wild-type strain) to compounds inhibiting that target (26). Another possibility is to overexpress genes conferring resistant phenotypes (such as efflux pumps) and screen for compounds that specifically reverse the resistant phenotype.

One of the possible reasons for the failure of HTS might be inherent to the composition of the chemical libraries used for screening. Generation of these libraries was usually guided by filters such as the **Lipinski's rules** on desirable physicochemical properties to improve the likelihood of having an oral drug (27). Even Lipinski stated that his rules were not intended as a decision-making mainstay in drug discovery (28). In addition, most of the currently used antimicrobials fall out of the Lipinski's rules, so the application of such criteria may complicate the identification of leads, since some compounds that could penetrate into the cells may be excluded (6,15,27).

In summary, several hits are usually discovered in screening campaigns, but not all of them have the same potential to become new antimicrobials. For this reason, it is essential to evaluate the different hits before settling them to the next stage in order to select a lead compound. This evaluation typically assesses the physicochemical properties of the compounds, preliminary activity assays against relevant strains, preliminary safety assays in mammalian cell lines and assays of spontaneous resistance (29).

1.5.2.- Hit to lead

This stage aims to refine each hit series to produce more potent and selective compounds by means of structure-activity relationship (SAR) investigations to provide information on activity and potential liabilities. Once a lead compound is identified, minimal inhibitory concentrations (MIC) assays against a panel of relevant pathogens and cytotoxicity assays against eukaryotic cell lines are performed (29). The most promising compounds are also screened against panel of contemporary clinical isolates, including MDR strains. Time-kill

kinetic assays against the target pathogen provide information on whether the compound is bactericidal (i.e. it kills the pathogen) or bacteriostatic (i.e. it blocks bacterial growth but does not kill bacteria).

Subsequent experiments assess hit liabilities, providing information on potential interferences with human anti-targets (e.g. cytochrome P450s and hERG potassium channel) and metabolic stability in human microsomes. Mutagenic potential of the compounds is determined by the Ames' test.

Finally, frequency of resistance studies are made to measure the rate at which spontaneous mutants emerge, and its associated fitness cost. Resistance mechanism studies, by whole-genome sequencing, transcriptomics or proteomics, provides a better understanding on the mode of action of the compounds. Knowing the mode of action and evasion of a compound is critical for further refinement and development of the molecule (29). At the end of this stage a **lead** compound is declared to enter the lead optimisation process.

1.6.- Experimental approaches to elucidate the mode of action of antimicrobials

The intrinsic difficulties of the target-based approaches have led to a return to the whole-cell phenotypic screenings. However, the outcome of whole-cell screenings are molecules with ability to prevent bacterial growth *in vitro*, but no information on the mode of action is provided, being this a **bottleneck in the drug discovery process**. Understanding the mode of action of the new molecule is a key point in the subsequent development process, since it allows the establishment of structure-activity relationships to improve the activity and selectivity of the compound.

Approaches to identify the mode of action of newly discovered inhibitors might include the (i) selection of mutations conferring resistance *in vitro*, (ii) the identification of mutants with increased susceptibility in mutant libraries and (iii) the use of omics to identify changes in gene expression or metabolites.

(i) The most straightforward approach to elucidate the mode of action consists in the **isolation and characterisation of spontaneous mutants** resistant to the compound. These approaches rely on the natural variability of microbial populations: if the number of bacteria screened is large enough to exceed the frequency of mutations conferring resistance to the compound resistant mutants can be isolated. In some cases, it may not be possible to isolate spontaneous mutants with permanent resistance to the compound of interest. A common

approach to overcome this problem is to increase the frequency of mutation, increasing the variability of the population as a result. This can be achieved by different means, such as the use of bacterial strains defective in the DNA repair systems (30) and chemical mutagenesis. Another strategy to isolate resistant mutants is using antimicrobials as a selective pressure. Sub-inhibitory concentrations of antimicrobials can favour the emergence of resistant individuals. Combination with a later exposure to lethal concentrations of the compound may increase the chances to isolate resistant mutants (31). In all three cases, the subsequent characterisation of the mutants can be done at the phenotypic (e.g. changes in the growth rate, colony and cell morphology or biochemistry) or genotypic level. The development of next-generation sequencing allows the quick identification of the mutations underlying the resistant phenotype (32).

(ii) **Mutant libraries** can be an invaluable shortcut to identify the mode of action of novel compounds. With enough coverage, it is possible to search for individuals with increased or decreased susceptibility among others carrying an unrelated mutation at any other point of the genome. Besides, the identification of genes responsible for the resistant or hyper-susceptible phenotypes can be reduced to finding the location of a foreign sequence in the genome.

(iii) An alternative approach is the use of **omics** (transcriptomics, proteomics, metabolomics, etc.), which can provide insights on the mode of action of novel antimicrobials by revealing changes in gene expression, proteomic or metabolic profiles (33,34). Once candidate genes have been identified, it is necessary to confirm that they are actually involved in the resistant phenotype. Different approaches can be followed depending on the outcomes of the previous assays. Introduction of mutations by genetic engineering to reproduce the resistant phenotype or characterisation of the target at the biochemical and structural levels are some examples of such approaches (35).

1.7.- Drug repurposing

Lead compounds must undergo a process of optimisation, similar to the hit to lead scheme. The suite of assays, in addition to the aforementioned ones, includes pharmacokinetics and efficacy assays in rodent models. The most promising compounds at this stage are re-synthesised at large scale and enter preclinical assays. These assays are aimed at ensuring safety before testing them in the clinic and allow dosage adjustment for the first

trials in humans. Finally, successful compounds can enter clinical trials (29). The full process can take up to 15-20 years to be completed from bench discovery to the patient (Fig. 3).

An alternative to the long process described above is drug **repurposing**, i.e. finding new applications for drugs already approved for clinical use, although for other indications, and with known pharmacological properties. Repurposing efforts bypass much of the discovery and preclinical stages, since pharmacokinetic, pharmacodynamic and toxicity information is already known. Because the candidate has already been used in humans, the preclinical phase only requires a demonstration of activity in a model system. Besides, since phase I information has been collected previously, it can enter phase II, saving time and money.

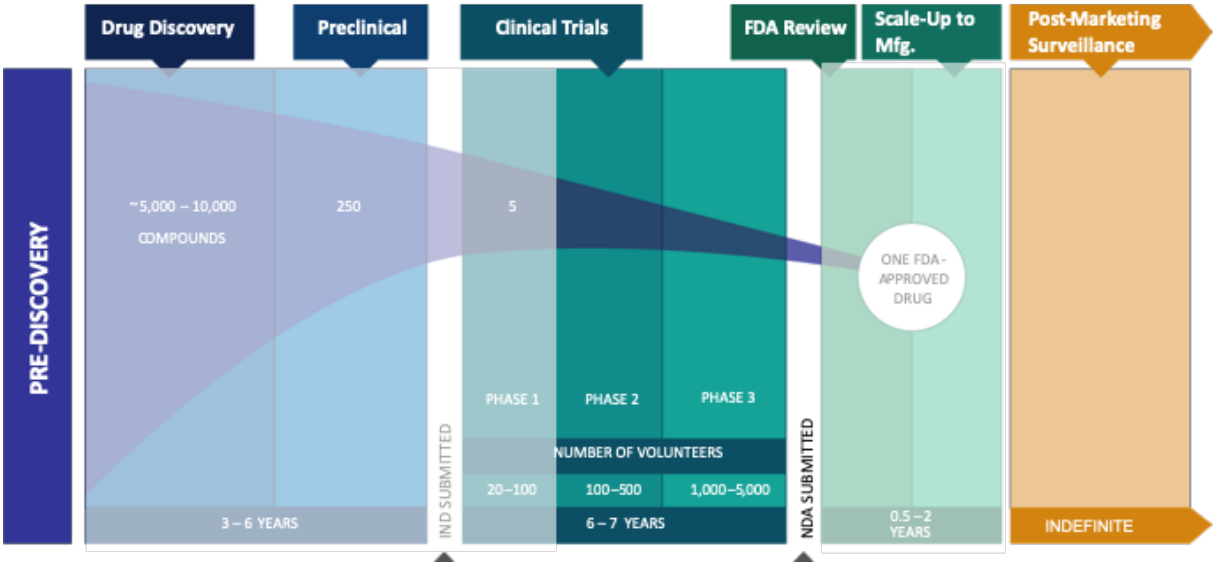

Fig. 3. The timeline of the drug discovery process. It includes the time required to complete each phase and the number of molecules that reach the end of each stage. Drug repurposing bypasses much of the discovery and preclinical stages, as well as the scale-up of the manufacturing process (shaded areas) Adapted from Drug Discovery and Development: Understanding the R&D Process, www.innovation.org. CBO, *Research and Development in the Pharmaceutical Industry*, 2006.

However, the repurposing process also comes with a number of limitations that must be considered. First, it is necessary to define the new mode of action of the compound against the target pathogen. It can be expected that the compound shows a different mode of action and protein target than in its original application. Additional limitations of the process are toxicology, dosage and pharmacokinetics. Since antimicrobials require higher concentrations than other drugs to be active (due to permeability issues), activity is often observed at much higher doses, which may result in toxicity and side effects. In such cases, repurposing may provide new lead scaffolds that would need to undergo the whole development process (4,36).

The best-known example of repurposing is sildenafil (Viagra®), which was designed to treat angina pectoris and hypertension. Sildenafil did not fulfil the superiority criteria in clinical trials, and after a re-evaluation of its properties, it was marketed to treat erectile dysfunction (37).

1.8.- Avermectins

Avermectins are a family of broad-spectrum antiparasitic agents produced by *Streptomyces avermitilis*. They were discovered in the 1970s in a campaign carried out by the Kitasato Institute and Merck, Sharpe & Dohme. Avermectins are active against ectoparasites and endoparasites, at very low doses (38).

Ivermectin (**Fig. 4**), a semi-synthetic derivative of two of the avermectins produced by *S. avermitilis*, is one of the most commonly used drugs since its approval in 1987. Only in the campaigns aimed at preventing the onchocerciasis (river blindness) and lymphatic filariasis (elephantiasis), more than 2 billion doses have been used.

Their anti-parasite mechanism of action consists in the selective binding to glutamate-gated chloride channels in nerve and muscle cells, which increases permeability to chloride ions, cell hyperpolarisation and, eventually, paralysis and parasite death. Despite their use in monotherapy for decades, no resistance has been found in human parasites (38).

Avermectins are safe compounds, with a wide therapeutic margin. In mammals, the glutamate-gated chloride channels are only present in the central nervous system, to which avermectins cannot access at the usual dosages, since they are P-glycoprotein substrates. However, they can be neurotoxic at higher dosages.

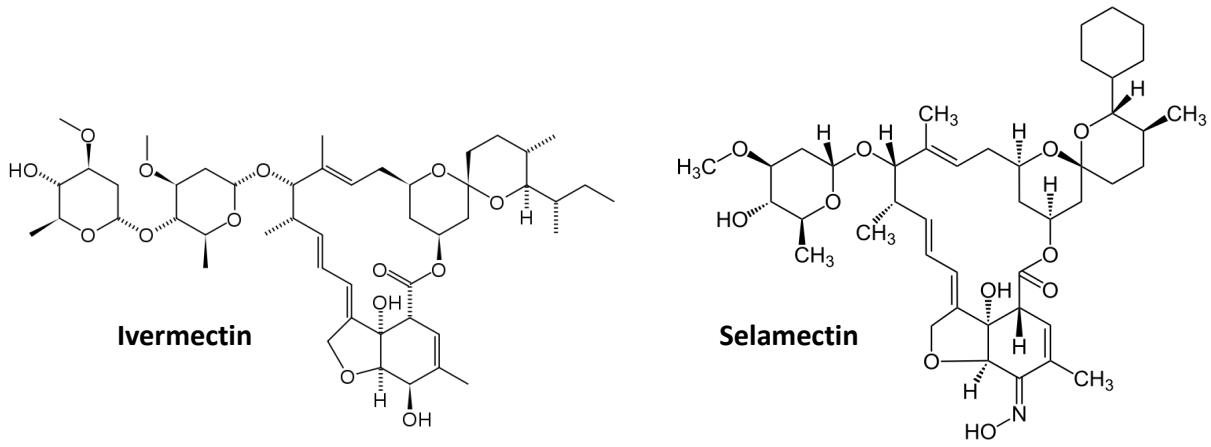

Fig. 4. Chemical structures of ivermectin B1a and selamectin. Ivermectin, the most widely used avermectin in humans, is a semi-synthetic derivative of two of the avermectins naturally produced by *S. avermitilis*. Selamectin is an avermectin exclusively used in veterinary medicine and displays better *in vitro* activity against mycobacteria.

It has been recently found that avermectins kill *Mycobacterium tuberculosis*, including MDR and XDR strains, and other mycobacteria (39). This was surprising since avermectins were traditionally considered ineffective against bacterial pathogens. In fact, they have no activity against Gram-positive and Gram-negative bacteria but are potent anti-mycobacterial agents, suggesting that avermectins could be repurposed for TB therapy. However, there is a lack of knowledge in their mode of action against mycobacteria, knowledge that is essential in order to advance to later stages of pre-clinical development.

1.9.- EPMM compounds

Increasing the chemical diversity of compound libraries is key to increase the likelihood of discovering new compounds with antimicrobial activity. The EPMM compounds come from chemical libraries synthesised for purposes unrelated to clinical applications. EPMM compounds with activity against Gram-positive bacteria in the low micromolar range, with good selectivity indexes and lack of genotoxicity have been identified (Raquel Alonso and Santiago Ramón García, unpublished).

2.- Objectives

Mode of action elucidation of new active molecules is one of the bottlenecks in the antimicrobial discovery process. At the Discovery and Development of Antimicrobials and Mechanisms of Resistance Team (D²AMR), two families of compounds were recently discovered with antimicrobial activity: i) avermectins, with activity against mycobacteria (including *M. tuberculosis*); and ii) EPMM compounds, active against Gram-positive bacteria.

One of the next steps for their progression through the drug discovery pipeline is the elucidation of their mode of action. This Master's Final Project thus focuses on the main objective of isolating mutants with genotypic resistance or hyper susceptibility to these compounds, which eventually will provide insights into their mode of action.

In order to achieve this main objective, several partial objectives were proposed:

1. To optimise experimental conditions for the isolation of mutants with genotypic resistance to selamectin (the avermectin chosen as model for this study) by classical mutant isolation assays with the wild-type *Mycobacterium smegmatis* mc²155 and a hypermutator strain (*M. smegmatis* Δ*nucS*).
2. To screen a *Mycobacterium bovis* BCG mutant library for the isolation of mutants with changes in the susceptibility profile to selamectin.
3. To isolate *S. aureus* mutants with resistance to EPMM Compound 18 by means of different mutant isolation assays.
4. To establish a methodology to confirm that the mutants isolated by the different assays display genotypic resistance.

3.- Materials and Methods

3.1.- Bacterial strains, general growth conditions and preservation

3.1.1.- Bacterial strains

Bacterial strains used in this study are listed in **Table 1**.

Table 1. Bacterial strains used in this study and their relevant features

Strain	Relevant features	References
Mycobacterium bovis BCG Pasteur	Slow growing, non-pathogenic mycobacteria, tuberculosis vaccine. 99% genome identity to *Mycobacterium tuberculosis*	-
M. bovis BCG TnSPAZ library	BCG Pasteur mutant library generated by random insertion of a transposon in the chromosome	(40)
Mycobacterium smegmatis mc²155	Fast growing, non-pathogenic mycobacterial strain widely used as *M. tuberculosis* surrogate	-
M. smegmatis mc²155 Δ*nucS*	*M. smegmatis* mutant lacking the DNA mismatch repair systems, with a 100-fold higher frequency of mutation	(41)
Staphylococcus aureus ATCC 29213	Methicillin-susceptible *S. aureus*. One of the most commonly used strains in drug discovery	-

Mycobacterium smegmatis and ***Mycobacterium bovis*** **BCG** strains were used as surrogates of the pathogenic *M. tuberculosis*, a pathogen that requires specialised training and Biosafety Level 3 facilities for handling. Besides, *M. tuberculosis* grows slowly, doubling approximately each 22 hours, which makes each experiment time consuming (42).

Mycobacterium smegmatis is a soil dwelling mycobacterium, non-pathogenic to humans and with a doubling time of ca. 4 hours. *M. smegmatis* is thus a convenient model, making experiments less time consuming (colony formation takes 2-3 days, while for *M. tuberculosis* requires 2-3 weeks).

M. bovis BCG is an attenuated strain derived from *M. bovis*, a member of the *M. tuberculosis* complex which causes TB in cattle. While it grows as slowly as *M. tuberculosis*, its handling can be done in Biosafety Level 2 laboratories.

Staphylococcus aureus was selected as experimental model for the EPMM compounds because of its clinical relevance and its use as model for Gram-positive bacteria. *S. aureus* is a widely distributed species, which is estimated to be colonising skin and nostrils in 20-30% of

the world population. It is a fast-growing species, which doubles in ca. 30 minutes and forms colonies after overnight incubation.

3.1.2.- Growth conditions

Mycobacterial strains were grown in Middlebrook 7H9 broth (Difco) supplemented with 10% (v/v) Middlebrook ADC (0.085% NaCl, 0.5% bovine albumin fraction V, 0.2% dextrose, 0.0003% bovine catalase, final concentrations) (Difco) and 0.2% glycerol (Sigma-Aldrich), 0.05% Tween 80 (Scharlau) or 0.05% Tyloxapol (Sigma-Aldrich) and Middlebrook 7H10 agar (Difco) supplemented with 10% (v/v) Middlebrook OADC (0.006% oleic acid, 0.085% NaCl 0.5% bovine albumin fraction V, 0.2% dextrose, 0.0003% bovine catalase, final concentrations) (Difco).

LB broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) with 0.05% Tyloxapol and LB agar (LB broth with 17 g/L bacteriological agar) were used for the propagation of *M. smegmatis* strains and colony counts, respectively.

Staphylococcus aureus was cultivated in Müller-Hinton broth (Panreac AppliChem), supplemented with 22 mg/L Ca^{2+} and 12 mg/L Mg^{2+} (hereinafter referred to as Müller-Hinton II), which is the recommended by the Clinical & Laboratory Standards Institute (CLSI) for such assays (43). Müller-Hinton agar (Panreac AppliChem) supplemented with 22 mg/L Ca^{2+} and 12 mg/L Mg^{2+} (hereinafter referred to as Müller-Hinton II agar) was used in antimicrobial susceptibility testing in solid medium. LB agar was used for colony counts.

All strains were cultivated under aerobic conditions at 37 ˚C, which are the optimal growth conditions for all strains.

3.1.3.- Strain preservation

For their long-term preservation, aliquots of cultures (24-hour incubation for *S. aureus*, 72-hour for *M. smegmatis* and 7-day for *M. bovis* BCG) were mixed with glycerol to a final concentration of 15% (v/v) and stored at -80 ˚C.

3.2.- Compounds

Compounds used in this project are listed in **Table 2**. Stocks were prepared at 10 mg/mL (ethambutol, amikacin, isoniazid, rifampicin and selamectin) or 10 mM (Compound 18). For their long-term preservation, stocks were stored frozen at -20 ˚C, except selamectin, which was stored at -80 ˚C to minimise ethanol evaporation.

Table 2. **Antimicrobials used in this project**

Antimicrobial	Reference	Solvent
Compound 18	This study	DMSO
Amikacin	A1774 (Sigma-Aldrich)	Water
Ethambutol	E4630 (Sigma-Aldrich)	Water
Isoniazid	I3377 (Sigma-Aldrich)	Water
Rifampicin	R3501 (Sigma-Aldrich)	DMSO
Selamectin	Y0000814 (European Pharmacopoeia)	Ethanol

3.3.- Antimicrobial susceptibility testing

3.3.1.- Broth microdilution susceptibility testing

The Minimal Inhibitory Concentration (MIC) of the different antimicrobial compounds was determined following CLSI standards (43). Briefly, assays were carried out in technical duplicates in 96-well flat-bottom polystyrene plates (TPP), in a final volume of 150 µL. Growth controls (i.e. bacteria in the absence of any compound) and blanks (150 µL of broth without bacteria) were also included in each plate. An additional susceptibility control, namely a compound with a well-defined MIC (ethambutol for mycobacteria, rifampicin for *S. aureus*), was included when the activity of the compound was tested against a specific strain for the first time.

Antimicrobial stocks at twice the maximal test concentration were prepared in Müller-Hinton II broth or 7H9-ADC with 0.2% glycerol. From these stocks, two-fold serial dilutions with fresh medium were done in a final volume of 75 µL. Subsequently, 75 µL of bacterial suspension were added to each well, at a final density of approximately 10^5 bacteria/mL. Bacterial density, in colony forming units (CFU)/mL, was adjusted by using previously determined correlations with optical density measures at 600 nm (Table 3).

Table 3. **Correlations between optical density and CFU/mL**

	OD (600 nm)	CFU/mL
M. smegmatis, M. bovis BCG	0.13	10^7
S. aureus	0.44	10^8

Plates were incubated at 37 ˚C for 20 hours (*S. aureus*), 3 days (*M. smegmatis*) or 6 days (*M. bovis* BCG). After incubation, 30 µL of a 2.5 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) with 10% (v/v) Tween 80 were added. MTT is a redox reporter of metabolic activity and, therefore, bacterial growth. Only metabolically

active bacteria are able to reduce MTT (yellow) to the corresponding formazan product (deep purple).

Following MTT addition, plates were incubated for 1 hour (*S. aureus*), 3 hours (*M. smegmatis*) or overnight (*M. bovis* BCG) before reading the optical density at 580 nm in a microtiter plate reader. Readouts were used to determine the MIC, which was defined as the minimal concentration of compound that inhibited MTT conversion by 90% compared to the untreated growth control (MIC_{90}).

3.3.2.- Minimal Bactericidal Concentration determination

Minimal Bactericidal Concentration (MBC) was determined following MIC assay. Before MTT addition, 10 µL were transferred from wells used to determine the MIC to 96-well LB agar plates. These plates were incubated overnight (*S. aureus*) or for 3 days (*M. smegmatis*). The readout was done after the addition of 30 µL of a 0.1 mg/mL resazurin solution (Sigma-Aldrich). The resazurin assay is another redox assay in which bacterial growth is evaluated by the reduction of resazurin (blue) to resorufin (pink). Colour changes were visually evaluated after 1-hour incubation at 37 ˚C. MBC was defined as the minimal concentration of compound for which no resazurin conversion was visually observed.

3.3.3.- Time-kill kinetics

Mycobacterium smegmatis strains were tested against different concentrations of selamectin. 25 cm^2 culture flasks containing 10 mL of 7H9-ADC with 0.2% glycerol were inoculated with bacteria to a final density of 10^5 CFU/mL from liquid cultures in late exponential phase. Bacteria were grown in the presence of 0, 1, 5 and 20 µg/mL of selamectin and incubated at 37 ˚C and aliquots were taken at 0, 24, 48, 72 and 96 hours to evaluate bacterial density by CFU counting. Cultures were shaken vigorously at every timepoint. Then, 10-fold serial dilutions in PBS + 0.1% Tyloxapol, in order to break clumps. Finally, 100 µL of the dilutions were seeded in LB agar quad plates. The colony forming units were determined after three days of incubation at 37 ˚C using the following formula:

$$Bacterial\ density\ (CFU/mL) = \frac{Number\ of\ colonies\ counted}{dilution \cdot volume\ seeded\ (mL)}$$

3.4.- *Mycobacterium smegmatis* mutant isolation

3.4.1.- Mutant isolation in agar plates

Mycobacterium smegmatis mc²155 and Δ*nucS* strains were incubated in agar plates with inhibitory concentrations of selamectin and isoniazid (a first-line anti-TB drug with a well-known frequency of mutation *in vitro* (44)).

Three different inocula, 10^5, 10^6 and 10^7 total CFU (in a total volume of 200 µL) were seeded in 7H10-OADC agar plates containing 4x, 10x or 20x the MIC of selamectin, taking as reference the MIC values determined in 7H10-OADC agar for different inocula of *M. smegmatis* mc²155 (**Supplementary material S2**). Plates were incubated for up to 7 days, checking colony formation every day starting from day 3. In parallel, 10-fold serial dilutions of the inoculum were seeded in LB agar plates to determine the actual number of bacteria plated, which is necessary to determine the frequency of mutation.

3.4.2.- Validation of the resistant phenotype

Colonies of potentially resistant mutants were transferred to LB broth with 0.05% Tyloxapol and incubated for 3 days, then a primary validation of the resistant phenotype was done by MIC and MBC determination as described in **Sections 3.3.1** and **3.3.2**.

Secondary validation of the resistant phenotype was carried out by seeding 5 µL spots of four 10-fold serial dilutions of the mutants (starting from adjusted suspensions of ca. 10^7 CFU/mL) in 7H10-OADC agar plates containing selamectin concentrations of 0.25x, 1x, 4x or 10x its MIC. The growth of each potential mutant strain in the presence of selamectin was assessed visually after 72 hours of incubation. The readout was done by comparing the minimum dilution at which mutants displayed growth at each concentration of compound with the Δ*nucS* strain.

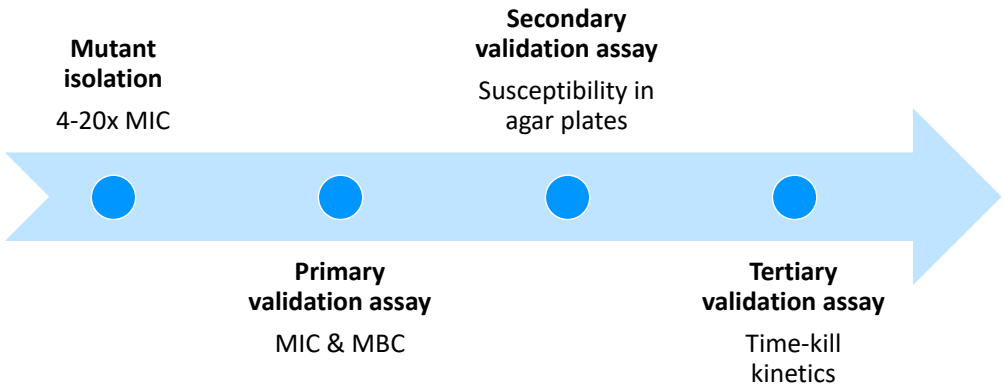

Fig. 5. Workflow of the mutant isolation assay and the subsequent validation of isolated mutants.

The resistant phenotype of selected mutants was finally confirmed by time-kill kinetics, as described in **Section 3.3.3**. Confirmed mutants were propagated and their DNA isolated for whole-genome sequencing.

3.5.- Screening of the *Mycobacterium bovis* BCG TnSPAZ transposition library

3.5.1.- The *M. bovis* BCG TnSPAZ transposition library

The *M. bovis* BCG TnSPAZ library contains 2,880 mutants (individually isolated in thirty 96-well plates) with the TnSPAZ transposon randomly inserted in their chromosome. This transposon consists of the following elements (**Fig. 6**): the transposase (*tnpA*), the inverted repetitions of the *M. smegmatis* IS1096 transposon, a kanamycin resistance cassette (*aph*) and a strong and constitutive promoter derived from the *Mycobacterium fortuitum* β-lactamase (pBlaF*) (40).

Fig. 6. Schematic representation of the TnSPAZ transposon.

The transposon library was screened to identify those mutants with an increased susceptibility to selamectin. Mutants were also available in 3 pool plates, with each column containing the mutants of a whole plate and each well containing the 12 mutants of a row (40). These pools were used to screen the library for resistant mutants to selamectin.

3.5.2.- Screening conditions

Mutants were transferred from glycerol stock plates (15% glycerol (v/v), stored at -80 ˚C) to fresh 7H9-ADC with 0.025% Tyloxapol (200 µL/well) and incubated at 37 ˚C until growth was observed (8-10 days). Cultures were then diluted 10-fold in 7H9-ADC with 0.2% glycerol and a 96-pin replicator (with a pin diameter of 3.2 mm transferring ca. 3 µL) was used to transfer bacteria to test plates containing 0.25x MIC of selamectin. Control plates without compound were inoculated in parallel. Test and control plates were incubated for 6 days before assessing bacterial growth. The readout was done by adding 30 µL of resazurin to each well. After 48 hours, colour changes were visually evaluated.

Those wells with no growth observed in the test plate but grown in the control plate were selected for further phenotypic validation. The validation was done by testing the susceptibility to selamectin of mutant candidates, as described in **Section 3.3.1**. Serial dilutions of 1.4-fold were done instead of 2-fold dilutions. In this way, every 3rd well in the dilution series contains a 2-fold dilution of compound, allowing subtle changes in susceptibility to the compound to fall out the 1-dilution range, which is considered to be within the experimental error of the microdilution susceptibility testing assay.

To select for resistant mutants, pool plates were screened following the aforementioned procedure but using an inhibitory concentration of selamectin (4x MIC).

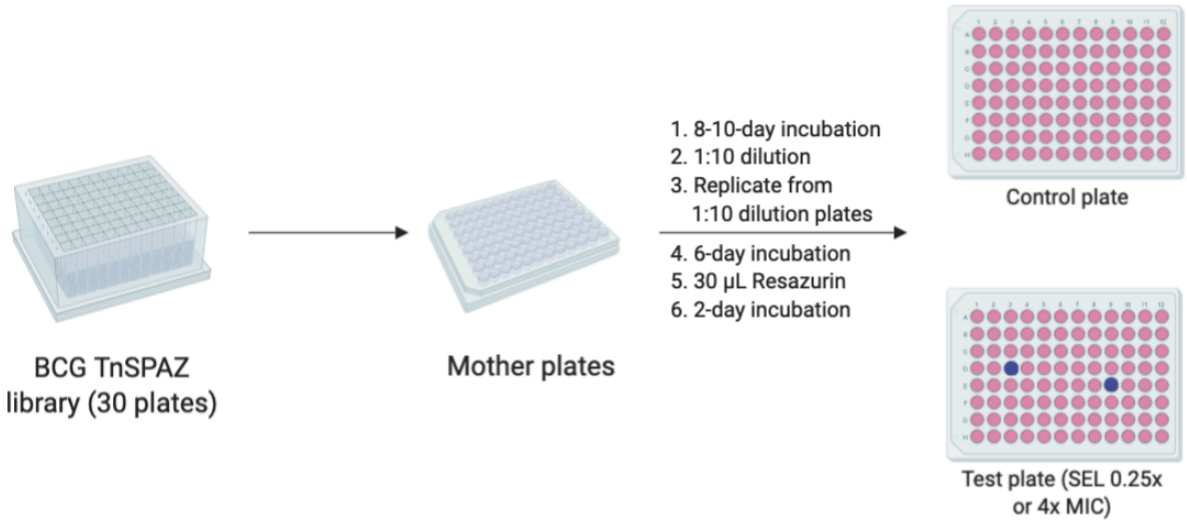

Fig. 7. Screening strategy for the *M. bovis* BCG TnSPAZ library

3.6.- *Staphylococcus aureus* mutant isolation

3.6.1.- Mutant isolation from liquid cultures

S. aureus ATCC 29123 glycerol stocks from cultures previously treated with 25 µM (1x MIC) Compound 18 (C18) were screened for the presence of resistant mutants. The stock was propagated in fresh Müller-Hinton II broth and incubated overnight at 37 ˚C. From this culture, 5 µL of the bacterial suspension were transferred to 1 mL of Müller-Hinton II broth with 0, 1x, 2x or 4x the MIC of C18 and incubated for an additional 24 hours.

In order to isolate individual clones resistant to C18, 100 µL droplets of 10-fold serial dilutions (ranging from 10^{-1} to 10^{-7}) of each culture were seeded in LB agar plates. Following incubation, colonies isolated in plates corresponding to the highest concentration of compound were selected for phenotype validation.

3.6.2.- Mutant isolation in agar plates

Three different inocula of *S. aureus* ATCC 29123 (10^7, 10^8 and 10^9 CFU, all in a final volume of 200 µL) were seeded in Müller-Hinton II agar plates containing 4x MIC of C18. Plates were incubated for 48 hours, checking colony formation every 24 hours.

10-fold serial dilutions of the suspensions used as inoculum were seeded onto LB agar plates to determine the actual number of CFU in the mutant isolation assay. Colony counts in both the test plates and the 10-fold serial dilutions provided an estimate of the frequency of mutation, which was calculated as the ratio of colonies growing in the plates with compound to the total number of CFU seeded, as described in **Section 3.3.3**.

3.6.3.- Validation of the resistant phenotype

As a primary assay, colonies of potential resistant mutants were transferred to Müller-Hinton II broth and incubated overnight. Grown cultures were used to test their susceptibility to the compound used in the mutant isolation assay, following the methodology described in **Section 3.3.1.** Mutants with a 4-fold or greater increase in the MIC value compared to the reference strain were defined as resistant and selected for subsequent analyses.

The secondary analysis consisted in the determination of the MIC (in the cases of mutants in which the MIC was higher than the highest concentration tested in the previous screening) and the MBC (as described in **Section 3.3.2**).

Finally, the resistant phenotype was validated by seeding 5 µL of 10-fold serial dilutions (starting from 10^5 CFU/mL) in Müller-Hinton agar plates containing 1x, 2x or 4x MIC. After overnight incubation, mutants were defined as resistant if growth was observed at lower inoculum densities (i.e. at greater dilutions) than the wild-type strain at the different concentrations tested.

4.- Results

During my TFG (45), the activity of selamectin against *M. smegmatis* mc²155 was characterised in order to establish the experimental conditions for subsequent mode of action studies. This characterisation involved testing the activity of selamectin against *M. smegmatis* wild-type in different broths, determining the activity against different inocula (**Supplementary material S1**) and studying the time-kill kinetics of the selamectin. Once the characterisation was completed, the isolation of resistant mutants was attempted seeding either the wild-type strain or a pool of *M. smegmatis* HS42 transposition mutants containing the TnSPAZ transposon (**Fig. 6**) in agar plates containing inhibitory concentrations of selamectin. Colonies were isolated with both approaches, but none of the tested colonies retained the resistant phenotype after a passage in the absence of selamectin, suggesting the activation of tolerance mechanisms instead of selection of stable genetic mutations.

Since these approaches proved fruitless, two alternative strategies were assayed: the isolation of resistant mutants using a hypermutator strain following the aforementioned strategy (**Section 4.1**); and the screening of an *M. bovis* BCG transposition library with individual clones for both hypersusceptible and resistant mutants (**Section 4.3**). Furthermore, this accumulated knowhow has been used to start mode of action studies of the EPMM compounds, which display activity against Gram-positive bacteria (Raquel Alonso and Santiago Ramón García, unpublished) (**Section 4.4**).

4.1.- *Mycobacterium smegmatis* mutant isolation

Mutant isolation assays in agar plates were performed with the hypermutator *M. smegmatis* Δ*nucS* strain, kindly provided by Dr Jesús Blázquez (CNB). This strain was generated from *M. smegmatis* mc²155 and its frequency of mutation is estimated to be around 100-fold higher due to the inactivation of the DNA mismatch repair system (41).

To set up the mutant isolation assay, the MIC of selamectin was determined against the hypermutator strain, which was the same as for the wild-type strain. The hypermutator strain did not show either any difference in the susceptibility to ethambutol and amikacin compared to the wild-type strain, suggesting that the mutation in the DNA repair system does not affect its susceptibility profile (**Table 4**).

Table 4. Susceptibility of *M. smegmatis* mc²155 and *M. smegmatis* Δ*nucS* to different antimicrobials. MIC_{90} values given in μg/mL.

	Amikacin	Ethambutol	Selamectin
***M. smegmatis* mc²155**	2	1	4
M. smegmatis* Δ*nucS	2	1	4

Experimental conditions for the mutant isolation assay were established based upon results of previous characterisation assays performed with *M. smegmatis* mc²155 (45). The *in vitro* activity of selamectin against mycobacteria displays several particularities that must be taken into account in the experimental setup. First, its activity is inoculum-dependent, i.e. higher concentrations of compound are needed to kill a greater density of bacteria; and second selamectin activity against mycobacteria is inhibited by detergents, such as Tween 80 or Tyloxapol, which are usually added to culture media and PBS to avoid bacterial clumping.

A broad range of inocula and selamectin concentrations were tested, namely 1x up to 50x MIC of selamectin and 10^6 to 10^8 CFUs per plate. Within this range, the number of colonies isolated did not show a linear correlation with the initial inoculum. The lowest inoculum (10^6 CFUs) did not allow the isolation of potentially resistant mutants, whereas bacteria grew over the entire surface of the agar plate at the highest inoculum (10^8 CFUs).

In the light of these results, it seemed reasonable to assume that the likelihood of finding mutants with genotypic resistance to selamectin would be maximised under those conditions in which no colonies were isolated against the wild-type strain with the previous approaches (**Table 5**). In order to ensure that the conditions tested using the *M. smegmatis* Δ*nucS* did not allow the isolation of colonies with the wild-type strain, the assay was also carried out in parallel with the wild-type strain.

Isoniazid, a first-line anti-TB drug with a well-known frequency of mutation (44), was used as an internal control. The frequency of mutation of each strain was determined by seeding 10^6 CFU in 7H10-OADC plates with 160 μg/mL isoniazid (10x MIC). For the wild-type strain, the frequency of mutation was $1.8 \cdot 10^{-5}$ (slightly higher to the one described in ref. (44), in which the isolation assay is performed at higher concentrations of compound), while the hypermutator strain displayed a much higher frequency of mutation ($5.2 \cdot 10^{-4}$, 29-fold increase), thus confirming the validity of the methodology used.

As expected, the frequency of mutation was much lower for selamectin ($1.4 \cdot 10^{-6}$) than for isoniazid. Moreover, the frequency of mutation of the hypermutator strain was estimated to be two orders of magnitude higher than the wild-type strain (41). On the basis of this, which

roughly applies to isoniazid, the frequency of mutation in the wild-type strain could be calculated in the order of 10^{-8}, ca. 1000-fold lower than isoniazid.

This low frequency of mutation can be very favourable from the standpoint of an eventual introduction into clinical practice. Since high *in vitro* frequencies of mutations conferring resistance to a candidate compound cast shadows on its potential as antimicrobial, having low frequencies of mutation *in vitro* is thus highly desirable.

Table 5. Summary of the experimental conditions used for the isolation of *M. smegmatis* mc²155 and *M. smegmatis* Δ*nucS* mutants resistant to selamectin (SEL) and isoniazid (INH). Frequency of mutation is calculated as the ratio of number of colonies isolated in the presence of the drug over the total colony forming units seeded.

Strain	Inoculum	Compound concentration (x MIC)	Number of colonies isolated	Frequency of mutation
M. smegmatis Δ*nuc*S	10^5 CFU	SEL 16 μg/mL (4x)	0	-
		SEL 40 μg/mL (10x)	0	-
		SEL 80 μg/mL (20x)	0	-
	10^6 CFU	SEL 16 μg/mL (4x)	1	$1.4 \cdot 10^{-6}$
		SEL 40 μg/mL (10x)	0	-
		SEL 80 μg/mL (20x)	0	-
		INH 160 μg/mL (10x)	386	$5.2 \cdot 10^{-4}$
	10^7 CFU	SEL 40 μg/mL (10x)	35	$1.4 \cdot 10^{-6}$
		SEL 80 μg/mL (20x)	2	$2.7 \cdot 10^{-7}$
M. smegmatis mc²155	10^6-10^8 CFU	SEL 16-80 μg/mL (4x-20x)	0	-
	10^6 CFU	INH 160 μg/mL (10x)	18	$1.8 \cdot 10^{-5}$

4.2.- Validation of the resistant phenotype

A total of 38 colonies were selected for phenotypic validation. Before testing their susceptibility to selamectin, mutants were cultured in drug-free medium, in order to reverse any transient phenotypic resistance. As a primary validation, the MIC and MBC of selamectin were determined against each potential mutant. Twenty-five of them (66%) displayed a 2-fold increase in their MIC and MBC compared to the Δ*nucS* parental strain. This difference is within the experimental error of the technique and was, therefore, considered not significant.

However, given the consistency of these results, the assay was repeated using smaller dilution steps. Serial dilutions of 1.4-fold, instead of 2-fold, were done so that a 2-fold increase

in the MIC would result in a two-dilution difference. Again, most clones displayed a consistent one-dilution increase in their MIC to selamectin (**Table S3**).

Subsequently, a secondary validation assay was carried out by seeding 10-fold serial dilutions of the mutants in agar plates containing increasing concentrations of selamectin (**Fig. 8** and **Supplementary material S2**), confirming that those mutants with slightly increased MIC values were indeed less susceptible to selamectin. Mutants with higher MIC values were able to grow at higher concentrations of selamectin (16 µg/mL and 40 µg/mL), at which the growth of both *M. smegmatis* mc²155 and Δ*nucS* was severely impaired. The resistant phenotype was also evident in plates with 4 µg/mL, a concentration at which many mutants were able to grow normally for all four dilutions tested, while the parental strain growth was notably reduced at the two lowest inocula. These results confirmed the presence of potentially resistant mutants among the 38 colonies isolated.

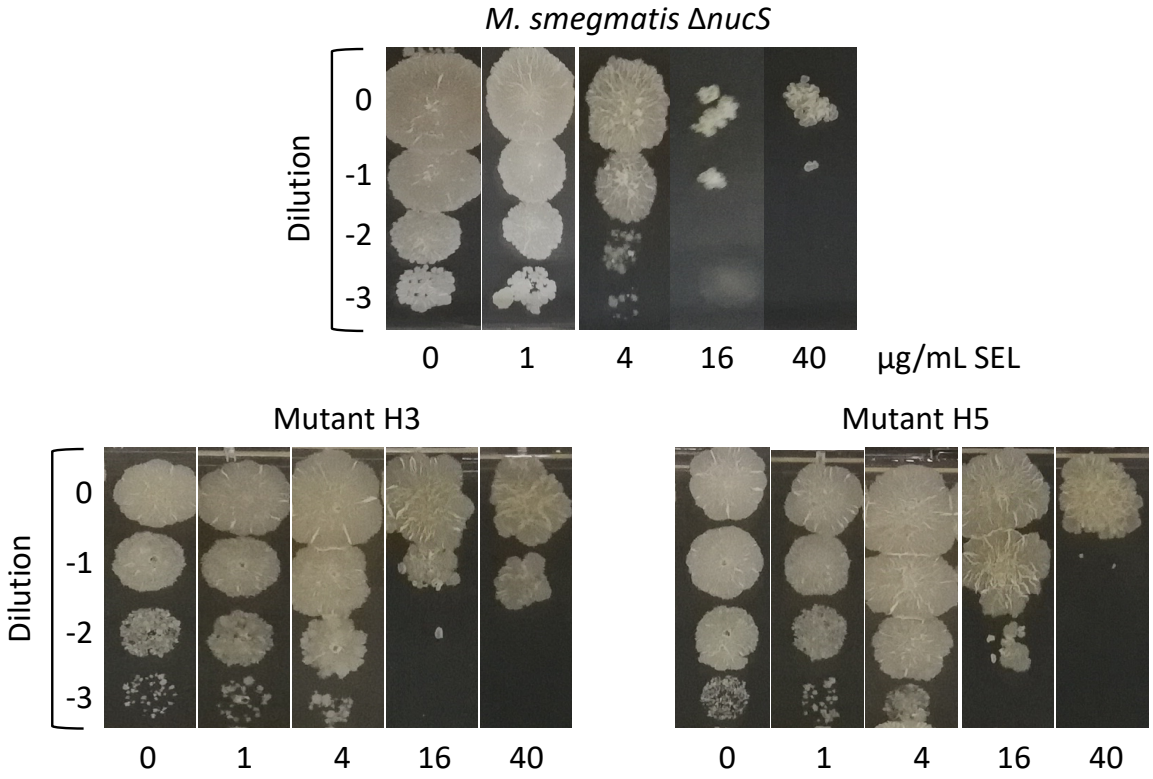

Fig. 8. Susceptibility testing of representative *M. smegmatis* candidate mutants in agar plates. 10-fold serial dilutions of 0.12 $OD_{600\ nm}$ cultures were seeded in agar plates containing 0, 1, 4, 16 or 40 µg/mL of selamectin. Candidate mutants H3 and H5 were able to proliferate at inhibitory concentrations of selamectin, while the parent strain growth was impaired at such concentrations. The remaining mutants can be found in **Fig. S1**.

As a tertiary validation assay, 12 clones were selected to evaluate their susceptibility by time-kill kinetics. (Fig. 9). Although this assay is much more time-consuming than the previous ones, it provides more information than liquid MIC determinations, which are usually considered the rule of thumb to evaluate resistance to compounds. Time-kill kinetics has a lower detection threshold, distinguishing between bactericidal and bacteriostatic activities, as well as providing information on adaptative responses to the compound tested.

Time-kill kinetics confirmed that 8 of the clones showed changes in their growth patterns in the presence of inhibitory concentrations of selamectin. As it is shown in Fig. 9, candidate mutants H3 and H5 were able to grow in the presence of 5 µg/mL of selamectin, a concentration bacteriostatic to the wild-type strain. Moreover, 20 µg/mL selamectin was only bacteriostatic to mutant H3, while for the wild-type strain the viability was reduced 2 $\log_{10}$ after 96-hour incubation. Candidate mutant H1 did not show differences in the growth pattern with the parent strain and was discarded.

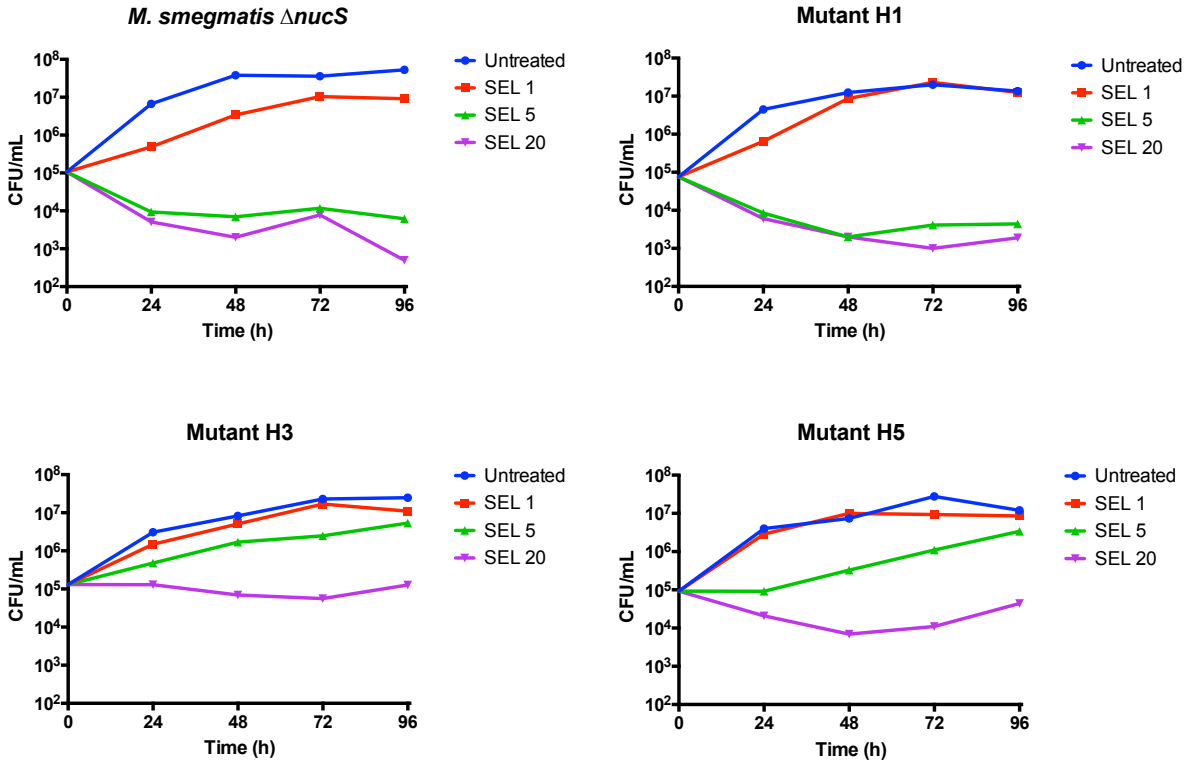

Fig. 9. Time-kill kinetics of selamectin (SEL) against representative *M. smegmatis* candidate mutants. Candidate mutant H1 shows a growth pattern similar to the parental strain (*M. smegmatis* Δ*nucS*) in the presence of inhibitory concentrations of selamectin, while candidates H3 and H5 show a marked decrease in susceptibility to selamectin and were selected for whole-genome sequencing. The time-kill kinetics for the 9 remaining candidates and the wild-type strain can be found in Fig. S2. SEL concentrations are given in µg/mL.

Regarding their phenotype, mutants grew at the same rate as the parental strain, forming colonies after 48-72 hours. However, they were more prone to grow in aggregates, even in detergent-containing medium (Fig. 10). The differences are especially significant in media without detergent, in which mutants grow at the liquid surface forming colony-like structures.

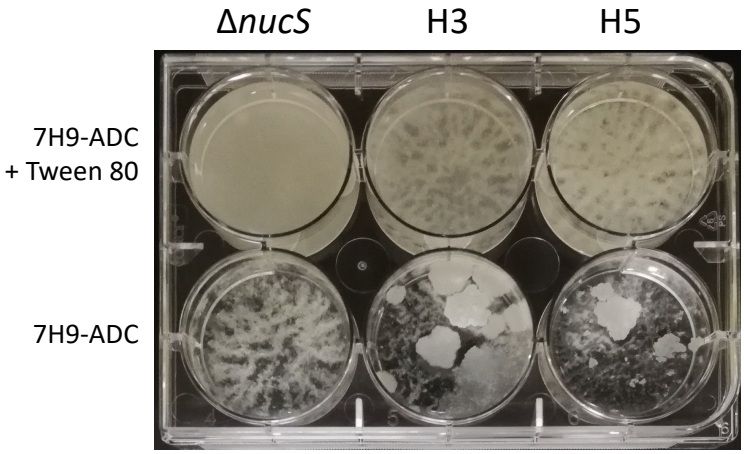

Fig. 10. Liquid cultures of *M. smegmatis* Δ*nucS* and candidate mutants H3 and H5. The parental *M. smegmatis* Δ*nucS* displays the typical growth pattern in 7H9-ADC with 0.05% Tween 80, growing in a homogeneous suspension. In the absence of detergent, *M. smegmatis*, and mycobacteria in general, tend to form cord-like structures. Candidate mutants show an increased tendency to aggregation, forming cords even in the presence of Tween 80. In free-detergent medium, they tend to form aggregates in the medium surface.

4.3.- *Mycobacterium bovis* BCG TnSPAZ library screening

Growth dynamics of *M. bovis* BCG are markedly different from those of *M. smegmatis*. As such, screening conditions were optimised to establish inoculum, incubation times, compound concentrations and the readout method. First, the activity of selamectin was determined in 7H9-ADC broth with 0.2% glycerol against four different inocula. As with *M. smegmatis*, the activity of selamectin was notably inoculum-dependent (Fig. 11).

Since the insertion of the transposon in the chromosome may result in fitness costs and slower growth rates, mutants were incubated for a period enabling most of them to reach stationary phase. We tested three different intermediate dilutions (1:10, 1:100, 1:1000), from which bacteria were transferred to control and test plates containing 0.0625x, 0.25x, 4x and 16x the MIC of selamectin (MIC = 2 μg/mL), ethambutol (MIC = 1 μg/mL) and rifampicin (MIC = 0.004 μg/mL). After 6-day incubation, resazurin conversion (at 48 hours) was only observed in plates inoculated from the 1:10 intermediate dilution, suggesting that the other densities were too low for the screening.

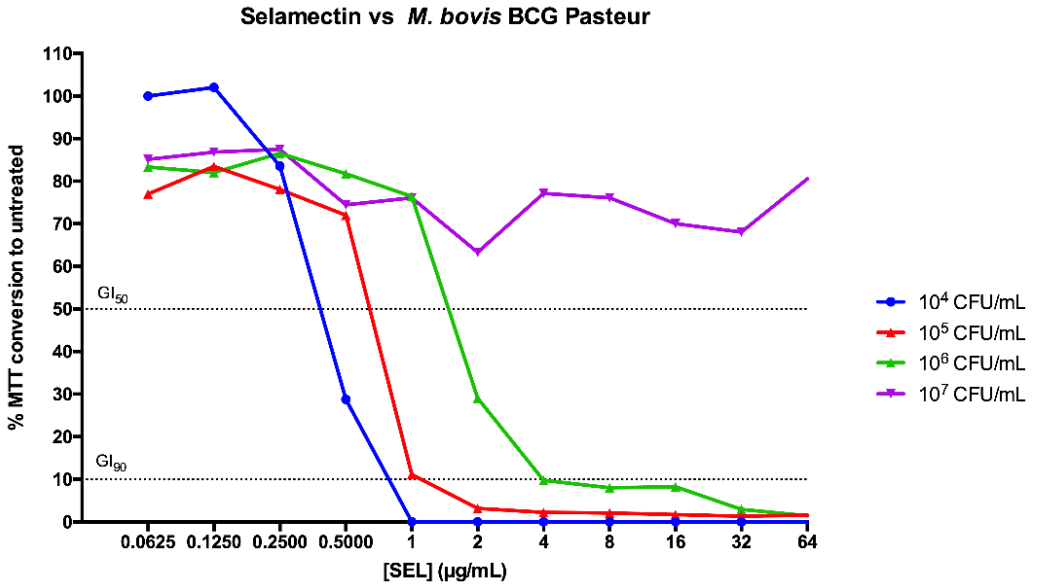

Fig. 11. Dose-response of selamectin against different inocula of M. bovis BCG Pasteur. Values are the mean of duplicates. GI_{50} and GI_{90} represent the 50% and 90% of MTT colour conversion. MIC_{90} is 0.5-1 µg/mL for 10^4 CFU/mL; 1 for 10^5 CFU/mL; 2 for 10^6 CFU/mL and >64 µg/mL for 10^7 CFU/mL.

Regarding compound concentrations, both 4x and 16x MIC inhibited bacterial growth, while the two sub-MIC had a similar effect on bacterial growth. With these results, the screening assay was set up as described in **Section 3.5.2.**

For the screen under the resistance selection approach, no hits were retrieved from the pool plates. For the susceptibility screening approach, 28 potentially hypersensitive mutants (0.97% of the library) that did not grow in the presence of subinhibitory concentrations of selamectin (but did grow in the control plate) were identified. Their susceptibility to selamectin was tested using the 1.41-fold serial dilutions strategy, but none of them displayed changes in their MIC.

The lack of resistant mutants in the *M. bovis* BCG library could be due to the essentiality of the avermectins' target in mycobacteria. Insertion of the transposon can only affect target expression, but not its sequence, and as such knock-out mutants will not be viable; thus, the only potential option by which target expression could be affected would be disruption of a non-essential transcription activator (or repressor of the repressor) of the selamectin resistant determinant.

Concerning the susceptibility screen, the lack of confirmed hypersusceptible mutants might be due to several factors intrinsic to the screening process. First, it is difficult to control the inoculum density of thousands of mutants to the same extent as for a single mutant. As a result, it is likely that a few mutants were screened at lower than optimal inoculum densities.

Those mutants would display increased susceptibility for the sole reason of the number of bacteria tested. Thus, in the subsequent validation analysis with better inoculum control, MIC values matched those of the wild-type strain.

The sensitiveness of the chosen validation method could also explain these results. As it has been demonstrated for the *M. smegmatis* validation procedure, microdilution susceptibility testing is not sensitive enough to detect subtle differences in susceptibility profiles. For this reason, variations in drug sensitivity could be also assessed by susceptibility assays in agar plates and by time-kill kinetics.

4.4.- *Staphylococcus aureus* mutant isolation

4.4.1.- Mutant isolation from liquid cultures

Two approaches were used to isolate *S. aureus* mutants resistant to C18. First, *S. aureus* ATCC 29213 cultures coming from a previous time-kill kinetics with 25 μM (1x MIC) of C18 were used to isolate potentially resistant clones. Under those conditions, C18 killed *S. aureus*, reducing its density below the limit of detection (100 CFU/mL), but later growth was restarted, suggesting that a resistant subpopulation had been isolated (Raquel Alonso and Santiago Ramón García, unpublished). After a passage without compound to reverse possible transient phenotypes, bacteria were transferred to fresh medium with different inhibitory concentrations of C18. Colonies were isolated at up to 100 μM C18 (**Table 6**).

Table 6. Growth of *S. aureus* in the presence of different concentrations of Compound 18. n.d.: not detected; the density of the culture treated with 200 μM was below the limit of detection of the technique (100 CFU/mL).

C18 concentration (x MIC)	CFU/mL after 24-hour incubation
Untreated	10^8
25 μM (1x)	10^8
50 μM (2x)	10^3
100 μM (4x)	10^3
200 μM (8x)	n.d.

Twenty-three colonies isolated from the culture exposed to 100 μM C18 were selected for phenotypic validation. Of them, 15 (65%) displayed a 4-fold or higher MIC compared to the parental *S. aureus* ATCC 29213. The increase in their MIC was confirmed by streaking them in agar plates containing 100 μM of C18 (**Fig. 12**).

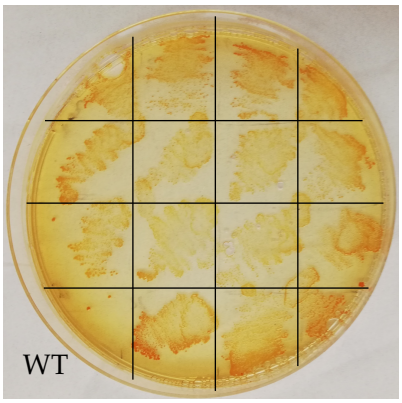

Fig. 12. Streaking of *S. aureus* ATCC 29213 clones in Müller-Hinton II agar with 100 µM C18. The wild-type strain (bottom left) did not show significant growth under these conditions, while the clones displayed normal growth. WT, wild-type *S. aureus*.

4.3.2.- Mutant isolation in agar plates

In order to estimate the frequency of mutation of *S. aureus* ATCC 29213 to C18, a classical mutation assay in agar plates was carried out. First, C18 was tested against different inocula (10^5, 10^6 and 10^7 CFU/mL), showing inoculum-dependent activity (Table 7).

Table 7. MIC of C18 against different inocula of *S. aureus* ATCC 29213. The activity against three different inocula was determined in Müller-Hinton II broth. MTT was added after 20-hour incubation. The three inocula reached the saturation level of the plate reader ($OD_{580} = 4$)

Inoculum (CFU/mL)	MIC_{90} (µM)
10^5	25
10^6	50
10^7	100

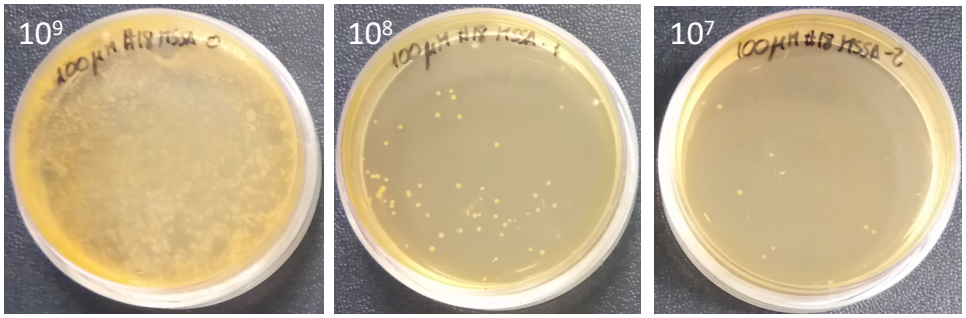

Fig. 13. Isolation of *S. aureus* ATCC 29213 clones in plates containing 100 µM C18. The number of CFUs seeded in each plate is shown at the top left corner. It can be seen that the growth of potentially resistant mutants is proportional to the inoculum seeded.

Subsequently, 10^7-10^9 CFU of *S. aureus* ATCC 29213 were seeded in plates containing 4x MIC (100 µM) C18 to calculate the frequency of mutation (Fig. 13 and Table 8). This was in the same range of the frequency of mutations conferring rifampicin resistance in *S. aureus* clinical isolates (46).

Table 8. Summary of the experimental conditions used for the isolation of *S. aureus* ATCC 29213 mutants resistant to C18. The frequency of mutation was calculated as the ratio of number of colonies isolated over the total colony forming units. The term *Lawn* indicates that discrete colonies could not be distinguished due to confluent growth.

Inoculum	Number of colonies isolated	Frequency of mutation
10^9 CFU	Lawn	-
10^8 CFU	100	$7.46 \cdot 10^{-7}$
10^7 CFU	9	$6.72 \cdot 10^{-7}$

A total 19 *S. aureus* ATCC 29213 clones (15 isolated from liquid cultures and 4 from agar plates) were selected for whole genome sequencing. Their resistant phenotype was validated by seeding 10-fold serial dilutions in agar plates containing 50, 100 or 200 μM (2x, 4x or 8x MIC) of C18 (**Fig. 14** and **Fig. S3**). As expected, the wild-type *S. aureus* ATCC 29213 did not grow under those conditions, whereas some of the mutants were able to grow in up to 200 μM C18.

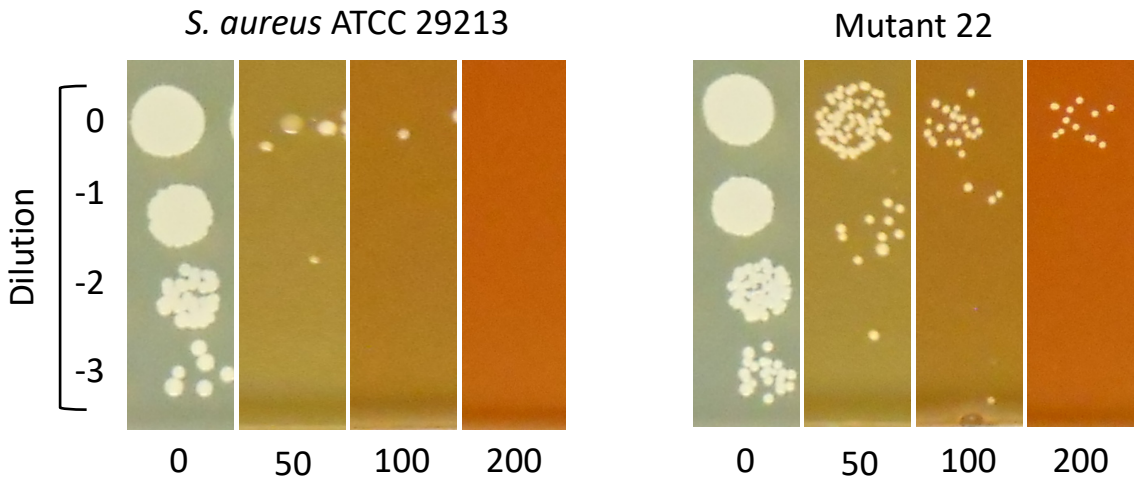

Fig. 14. Susceptibility testing of *S. aureus* candidates in agar plates. Dilution 0 was a ca. 10^5 CFU/mL suspension; dilutions -1, -2 and -3 are 10-fold serial dilutions of the initial suspension. Mutant 22 was able to grow under inhibitory concentrations of C18, while the growth of wild-type strain was severely impaired. C18 concentrations are given in μM. The results for the remaining mutants can be found in **Fig. S3**.

In summary, the mutant isolation and validation strategies tested allowed the isolation of *M. smegmatis* Δ*nucS* mutants with genotypic resistance to selamectin and *S. aureus* ATCC 29213 mutants resistant to C18. It is expected subsequent whole-genome of the selected clones will reveal the genetic basis of resistance and will provide some insights into the mode of action of the compounds.

5.- Discussion

The discovery and development of antimicrobials at the beginning of the 20th century allowed humankind to successfully treat infectious diseases for the first time in history. Management of bacterial infections opened the door to the transformation of medicine with the development of techniques relying on the ability of antimicrobials to prevent bacterial infections such as pneumonia and skin and soft tissue infections All these breakthroughs are currently challenged by the emergence of bacterial strains resistant to most, and in some cases, all drugs clinically available.

The AMR crisis has been sharpened by the difficulties of finding new targets and candidate compounds, which has led to a decrease in the number of approved compounds over the past decades. Target-based approaches, which have successfully brought a plethora of drugs in other areas, have proven to be ineffective in the discovery of novel antimicrobials. Phenotypic screening remains the lone strategy that has provided molecules with novel modes of action.

However, this approach does not provide any information on how such molecules inhibit bacterial growth. Thus, the elucidation of the mode of action is key for the development of new antimicrobials. The knowledge gathered from these studies allows the identification of their molecular target and to rationally develop the subsequent steps of the process.

In this Master's Final Project, I performed mode of action elucidation studies with two families of compounds previously characterised in the D²AMR Group: the avermectins and EPMM compounds

5.1.- Avermectins

Avermectins are a family of anthelmintic compounds traditionally thought to be inactive against bacteria. The antimycobacterial activity of avermectins was found in a screening campaign to identify new molecules with activity against *M. tuberculosis*. For this reason, they were proposed as candidates to be repurposed for the treatment of TB (39).

The mode of action of avermectins against mycobacteria remains elusive. During my TFG, I characterised the activity of selamectin against *M. smegmatis* and attempted mutant isolation (45). Following this previous research, two different strategies were attempted in this TFM to isolate mutants with changes in their susceptibility to selamectin: (i) mutant isolation

in agar plates with a hypermutator *M. smegmatis* strain; and (ii) screening of an *M. bovis* BCG transposition library.

The former allowed the isolation of 38 clones, most of which displayed consistent 1-dilution increases in their MIC. Secondary validation confirmed that there were less susceptible mutants among the 38 candidates. Finally, time-kill kinetics corroborated these results for 8 of the clones, which displayed a marked reduced susceptibility to inhibitory concentrations of selamectin. The *M. bovis* BCG mutant library, however, did not yield resistant or hypersusceptible mutants to selamectin.

Avermectins are only active against mycolic acid containing bacteria (39), which suggests that they can be targeting the mycobacterial envelope, a structure that is only present in the suborder *Corynebacterineae*. This envelope is a highly organised structure which comprises the inner membrane, a core of covalently linked peptidoglycan, arabinogalactan and mycolic acids; a leaflet of extractable lipids and a capsule (47). The metabolic pathways involved in the biosynthesis of the mycobacterial envelope are indeed the target of several of the current anti-TB drugs, such as isoniazid, ethambutol, pyrazinamide and ethionamide (48,49). Moreover, these pathways could represent good targets for the development of novel anti-TB drugs.

M. smegmatis mutants resistant to selamectin isolated in this study were more prone to aggregate in liquid cultures, even in detergent-containing medium (**Fig. 10**). Mycobacterial clumping is associated to the hydrophobicity of their envelope. Thus, the observed changes in the growth pattern suggest that resistant mutants have undergone changes in cell wall physiology and that this structure is involved in the resistance to selamectin.

Another evidence that supports this hypothesis is the fact that selamectin activity is enhanced in the presence of sub-inhibitory concentrations of the first-line anti-TB drug ethambutol (Santiago Ramón García, unpublished). Ethambutol targets the mycobacterial envelope biosynthesis, in particular inhibits arabinosyl transferases involved in arabinogalactan and lipoarabinomannan synthesis by blocking the enzymes EmbA (Rv3794), EmbB (Rv3795) and EmbC (Rv3793) (49,50). Fluorescence recovery after photobleaching (FRAP) assays have demonstrated that the exposure to sub-inhibitory concentrations of ethambutol increases the fluidity of the mycobacterial envelope (51), probably associated to a reduction in the number of anchorage sites for mycolic acids in arabinogalactan.

Also related to the arabinogalactan biosynthetic pathway, it has been observed that the MIC of an *M. tuberculosis* strain harbouring a mutation in *dprE1* (*Rv3790*) is between 2- and 4-fold lower than the wild-type strain. This increased susceptibility was later confirmed by time-kill kinetics (collaboration with Marilina Pasca and Camilla José Sammartino, University of Pavia, Italy, unpublished). DprE1 is an enzyme involved in the synthesis of the decaprenyl-phospho-arabinose (DPA) precursors, which are indeed the substrate of the Emb arabinosyl transferases. The mutated DprE1 displays a reduction in its catalytic efficacy (52), which would presumably result in a reduction in the production of DPA. This would eventually lead to a situation similar to the exposure to sub-MIC concentrations of ethambutol.

Avermectins also change the fatty acid composition of *M. tuberculosis* (collaboration with Catherine Vilchèze, Howard Hughes Medical Institute, Albert Einstein College of Medicine, USA, unpublished). Treatment with different avermectins results in a reduction in oleic acid (C18:1 Δ9) and tuberculostearic acid (C18:10Me) and an increase in stearic acid (C18:0) and C26:0. The latter two are the products of the mycobacterial "eukaryotic-like" fatty acid synthase (FAS-I) (48). Under normal physiological conditions, these fatty acids are the substrates of a multi-enzymatic "prokaryotic-like" FAS complex (FAS-II), which transforms them in long-chain fatty acids that are incorporated to mycolic acids. The accumulation of precursors suggests then that the activity of the FAS-II complex may be inhibited in the presence of avermectins. The enzymes belonging to this complex are exclusive of the *Corynebacterineae* suborder and most of them are essential for *M. tuberculosis*, thus suggesting that selamectin could be targeting any enzyme belonging to this complex. However, selamectin is active against *Corynebacterium glutamicum* (JM Ezquerra and Santiago Ramón García, unpublished) but FAS-II is absent in *Corynebacterium*, suggesting that either selamectin has more than one target or that FAS-II is not the actual target of avermectins.

Interestingly, many genes involved in the mycobacterial envelope biosynthesis are not essential for *C. glutamicum*, although knock-out mutants of these genes grow considerably more slowly than the wild-type strain (53). If selamectin impairs mycolic acid biosynthesis enough to lessen growth of *C. glutamicum*, this would result in a lack of detectable growth at the endpoint of the microdilution assay, thus having a MIC. This hypothesis could be easily confirmed by assessing *C. glutamicum* growth in the presence of different concentrations of selamectin.

Finally, the activity of avermectins is inhibited by the presence of detergents, such as Tween 80 or Tyloxapol. This could be explained either by changes in cell wall physiology

induced by the detergent, by the formation of complexes of detergent and selamectin, or a combination of both effects. Several assays can be used to better understand the interaction between selamectin, detergents and mycobacteria. Thin layer chromatography of total lipids can provide information on how the lipid composition of *M. smegmatis* changes when exposed to detergents, selamectin or both simultaneously. Fluorescence correlation spectroscopy could also be useful in order to determine if selamectin binds preferentially to detergent micelles or to mycobacteria when both are present.

Selamectin is a strongly hydrophobic compound, with a predicted octanol-water partition coefficient (logP) of 6.83 (54). This hydrophobicity could result in a high affinity towards the hydrophobic mycobacterial envelope in an aqueous environment, thus generating an increase in the effective concentration in the surroundings of mycobacteria. This could provide an alternative explanation to the differences in activity observed for *C. glutamicum*, whose envelope is less hydrophobic and in fact its culture does not require the use of detergents. Moreover, the water-induced binding of selamectin to mycobacteria would explain the dependency between initial inoculum and selamectin activity, since a greater number of bacteria would result in a higher binding substrate for selamectin and a reduced effective concentration.

The latter assumption seems to be incompatible with the phenotypic changes observed in some of the mutants with increased resistance to selamectin, given that they seem to have a more hydrophobic envelope. It has been proposed, however, that the tightly packed structure of the mycobacterial wall is indeed the responsible for the observed low permeability to lipophilic compounds (55). The enrichment of the mycobacterial envelope in hydrophobic compounds would result then in a reinforcement of the permeability barrier, decreasing susceptibility to selamectin.

On the basis of the above, it seems reasonable to propose that avermectins act at the mycobacterial envelope level, either targeting a single protein or having pleiotropic effects. The low-level resistance observed in the confirmed mutants suggests that the mechanism of resistance is more likely to be associated to a complex change in the phenotype rather than a mutation in a single target. For this reason, it is expected to find mutations in genes involved in the mycobacterial envelope biosynthesis in the mutants with confirmed resistance to selamectin.

In addition to the genomic analysis, several assays evaluating the integrity of the envelope, such as ethidium bromide accumulation assays (56) could be done in order to provide further information on the mode of action of selamectin against mycobacteria. Finally, differential gene expression analysis in the presence of selamectin could reveal how mycobacteria respond to the presence of selamectin.

5.2.- EPMM Compound 18

The knowledge generated and expertise gained along the previous mutant isolation and validation assays, together with previous information on the activity of EPMM compounds (Raquel Alonso and Santiago Ramón García, unpublished), was used to begin mode of action elucidation studies on this novel chemical series. EPMM compounds are active against various Gram-positive pathogens such as *S. aureus* (including MRSA), *Enterococcus faecalis*, *E. faecium* and *Corynebacterium diphtheriae.*

As a first approach to elucidate their mode of action, mutant isolation was attempted with C18 and *S. aureus* as representative compound of the series and Gram-positive species, respectively. Mutants were isolated using two different approaches: passages in liquid medium containing inhibitory concentrations of compound and classical mutant isolation assays in agar plates. Validation of the resistant phenotype was done following the same steps as for *M. smegmatis*.

First, susceptibility of *S. aureus* clones was evaluated by MIC and MBC. It is remarkable the fact that the proportion of resistant mutants was notably higher among the clones isolated in broth (15/23 displayed 4-fold or higher increases in their MIC) than in clones coming from agar plates (of the 11 colonies tested, only a single colony showed a 4-fold increase in the MIC and three displayed a 2-fold increase). Secondary validation was done by seeding 10-fold serial dilutions of the 19 selected mutants in agar plates with different concentrations of C18, validating the resistant phenotype of 17 isolates.

Given the evident susceptibility differences between the mutants and the parental *S. aureus* ATCC 29213, the tertiary validation by time-kill kinetics was not assessed. Such big increases in the MIC suggest that resistance may be caused by mutations in the C18 target.

Ongoing genomic analysis of the seventeen mutants will allow the identification of the target and/or mechanisms of resistance to C18. Then, candidate mutations should be validated by genetic engineering *S. aureus* and determination of the MIC of each mutant. Finally, once

the mutations are confirmed, characterization of the interaction between C18 and its target will provide a robust biochemical assay needed to lead optimization for better activity and selectivity.

The novelty of EPMM compounds chemical structure strongly suggests that their target is not common to any of the ones exploited by currently used antimicrobials. This is especially important given the serious AMR crisis, which needs to be resolved with the development of antimicrobials with novel modes of action and activity against MDR and XDR strains. In this scenario, this series could be a starting point for the development of novel antimicrobials for Gram-positive pathogens.

6.- Conclusions

1. The methodology developed to isolate and validate *M. smegmatis* mutants resistant to selamectin is valid. It has been possible to isolate colonies under inhibitory concentrations of selamectin and distinguish between genotypic and transient resistant phenotypes.

2. Time-kill kinetic assays are more suitable to confirm changes in the susceptibility profile to selamectin than microdilution susceptibility testing.

3. The eight *M. smegmatis* confirmed mutants display low-level resistance, with 2-fold or lower increases in their selamectin MIC.

4. Mutants display phenotypic changes, being more prone to grow in clumps. These changes, together with other experimental findings, suggest a potential involvement of the mycobacterial envelope in the activity of selamectin and the mechanisms of resistance.

5. None of the twenty-eight potentially hypersusceptible mutants isolated from the *M. bovis* BCG TnSPAZ library had a lower confirmed MIC to selamectin. Secondary and tertiary validation assays will be needed to confirm or dismiss these results.

6. The mutant isolation and validation strategy established for *M. smegmatis* can be applied to other compounds and targets, demonstrated by the isolation of *S. aureus* mutants resistant to EPMM C18.

7. C18 resistant phenotype was confirmed for seventeen mutants, most of which display high-level resistance with 8-fold or higher increases in their C18 MIC, suggesting stable genetic mutations.

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Supplementary material

S1.- Characterisation of selamectin activity against *M. smegmatis* mc^{2}155

Several assays were performed in *Mycobacterium smegmatis* mc^{2}155 in order to set up optimal conditions for classical mutant isolation assays. The MIC of selamectin was the same in the different liquid media (**Table S1**), while the addition of Tyloxapol resulted in a 16-fold increase in the MIC.

Table S1. MIC of selamectin against *M. smegmatis* mc^{2}155 in different broths. MIC was determined following the same methodology described in Section 3.3.1.

Medium	MIC_{90} (μg/mL)
7H9-ADC + 0.2% Glycerol	4
7H9-ADC + 0.2% Glycerol + 0.05% Tyloxapol	64
LB broth	4
Müller-Hinton II broth	4
NE broth	4

MIC of selamectin was also determined in 7H10-OADC agar plates, showing a strong dependence between selamectin activity and the initial inoculum (**Table S2**). MIC was determined in 24-well plates filled with 2.5 mL of agar containing 2-fold serial dilutions of selamectin, which was added to tempered agar. Plates were inoculated with 10 μL of bacterial suspension containing 10^3 to 10^7 total CFU.

Table S2. MIC of selamectin in 7H10-OADC agar against different inocula of *M. smegmatis* mc^{2}155.

Inoculum (total CFU)	MIC (μg/mL)
10^3	4
10^5	8
10^7	16-32

As the activity of selamectin shows a strong dependence with the initial inoculum, the bacterial density per surface unit at which plates are seeded should be taken into account. 90 mm Petri dishes have a surface which is ca. 100 times the one of a 10 μL droplet. Thus, the bacterial density per surface unit reached was about 100-fold as if the same number of bacteria were spread over a 90 mm diameter Petri dish (i.e. seeding 10^5 CFU in a Petri dish is equivalent to seeding 10^3 in a well of a 24-well plate) and therefore, the reference MIC for mutant isolation assays was the one corresponding to a 100-fold smaller inoculum.

S2.- Characterisation of mutants potentially resistant to selamectin

Table S3. MIC of selamectin against *M. smegmatis* Δ*nucS*, mc²155 and candidate mutants.

Strain	MIC (μg/mL)	Strain	MIC (μg/mL)
M. smegmatis Δ*nuc*S	2.83	*Msm* Mutant H20	4
Msm Mutant H1	4	*Msm* Mutant H21	2.83
Msm Mutant H2	4	*Msm* Mutant H22	4
Msm Mutant H3	5.66	*Msm* Mutant H23	2.83-4
Msm Mutant H4	4	*Msm* Mutant H24	2.83
Msm Mutant H5	5.66	*Msm* Mutant H25	2.83
Msm Mutant H6	4	*Msm* Mutant H26	4
Msm Mutant H7	4	*Msm* Mutant H27	2.83
Msm Mutant H8	4	*Msm* Mutant H28	4
Msm Mutant H9	4	*Msm* Mutant H29	2.83
Msm Mutant H10	4	*Msm* Mutant H30	2.83
Msm Mutant H11	4	*Msm* Mutant H31	2.83
Msm Mutant H12	4	*Msm* Mutant H32	4
Msm Mutant H13	4	*Msm* Mutant H33	2.83
Msm Mutant H14	2.83	*Msm* Mutant H34	2.83
Msm Mutant H15	4	*Msm* Mutant H35	2.83
Msm Mutant H16	4	*Msm* Mutant H36	2.83
Msm Mutant H17	4	*Msm* Mutant H37	4
Msm Mutant H18	4-5.66	*Msm* Mutant H38	2.83
Msm Mutant H19	4	*M. smegmatis* mc²155	4

Fig. S1. Susceptibility testing in agar plates of the 38 *M. smegmatis* candidate mutants. Concentrations of selamectin (SEL) are indicated in µg/mL. Dilution 0 was a 0.12 OD_{600} (ca. 10^7 CFU/mL) suspension; dilutions 1, 2 and 3 are 10-fold serial dilutions of the initial suspension.

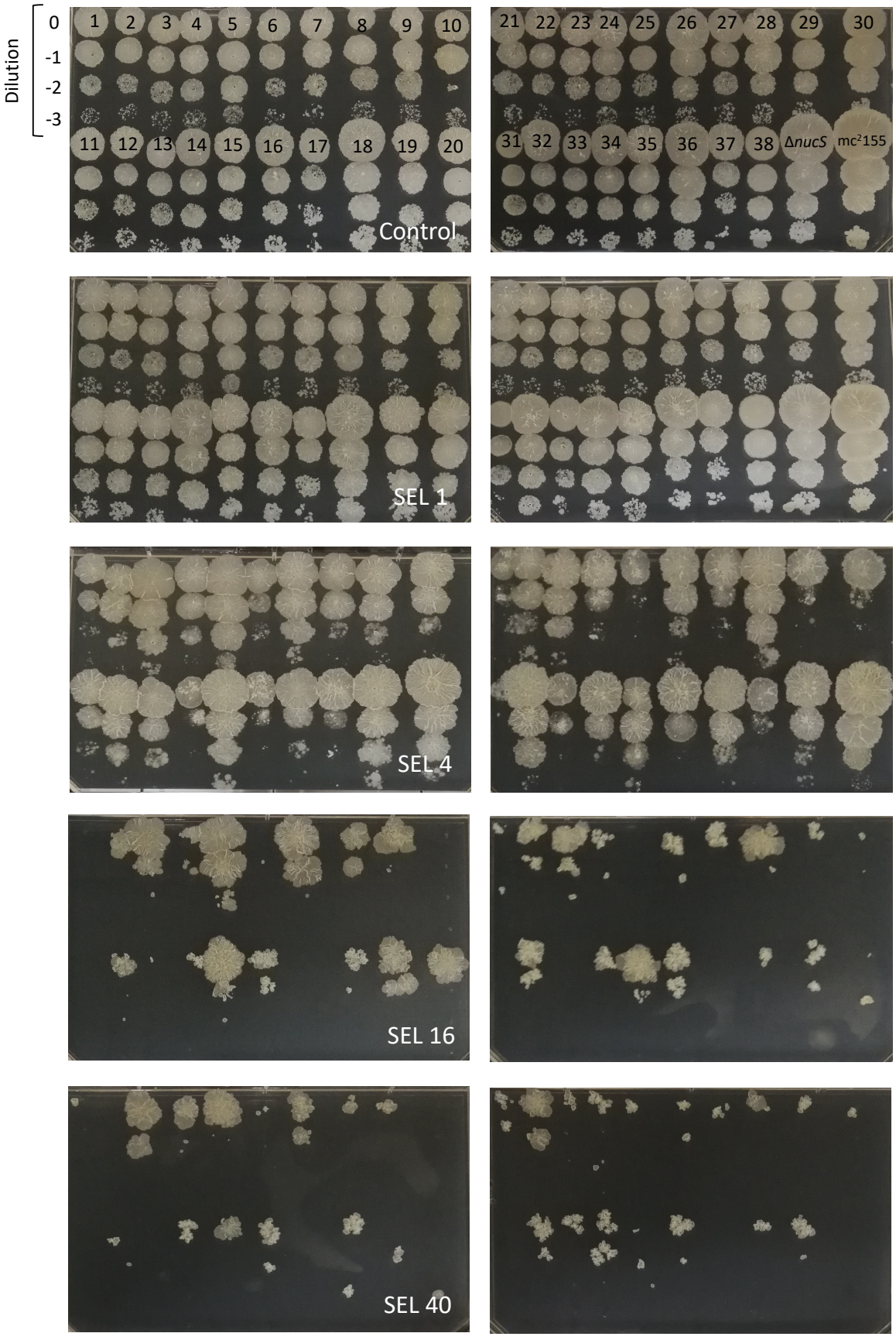
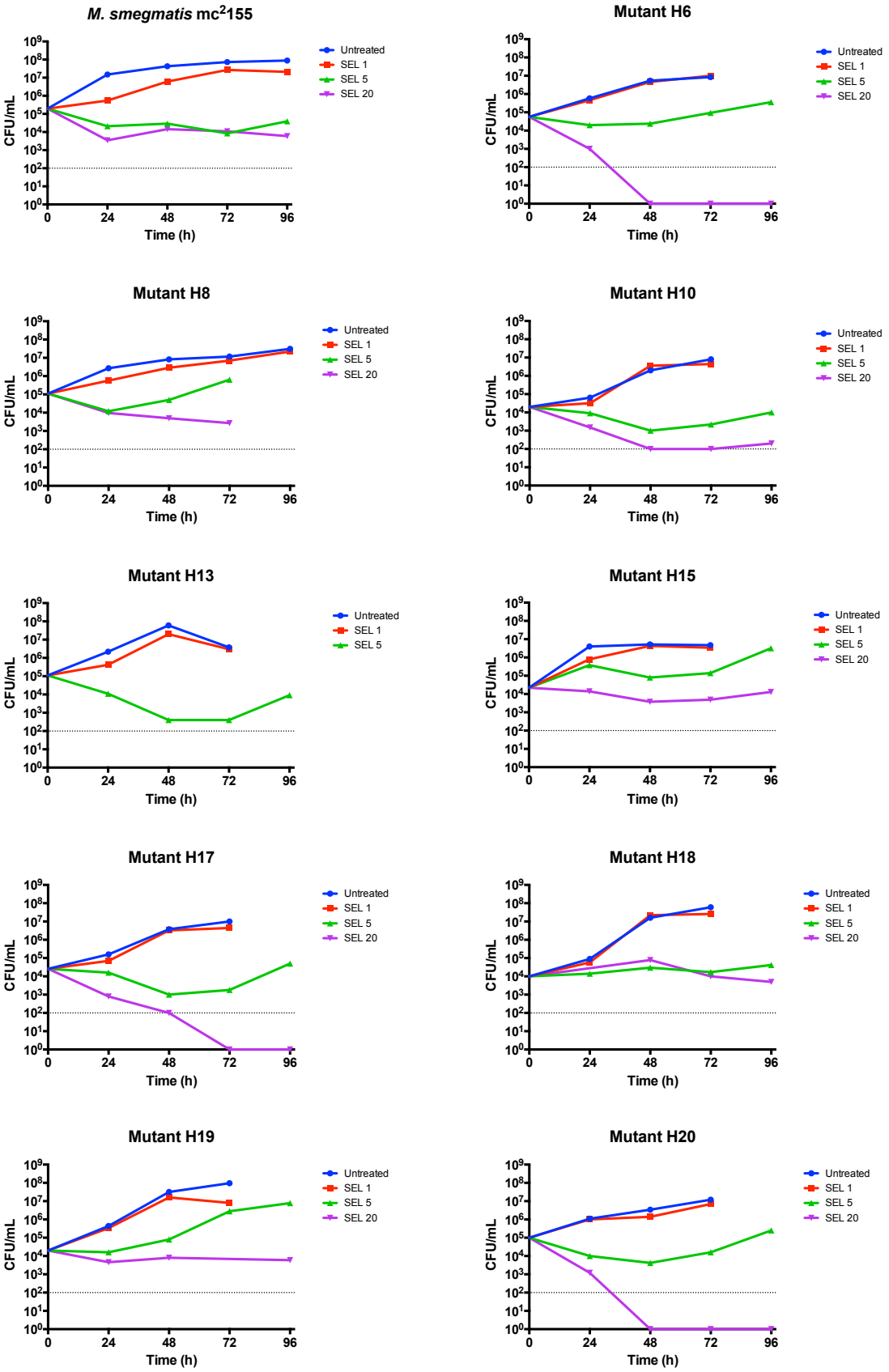


Fig. S2. Time-kill kinetics of *M. smegmatis* mc²155 and nine potentially resistant mutants. Mutants H10, H13 and H17 remain susceptible to selamectin. Mutants H6, H8, H15, H18, H19 and H20 show changes in growth patterns in the presence of selamectin. Concentrations are in μg/mL.



S3.- Characterisation of mutants potentially resistant to Compound 18

Table S4. MIC of C18 against *S. aureus* ATCC 29213 and candidate mutants.

Strain	MIC (µM)	Strain	MIC (µM)
S. aureus ATCC 29213	25	*Sau* Mutant 12	>200
Sau Mutant 1	>200	*Sau* Mutant 13	>200
Sau Mutant 2	200	*Sau* Mutant 14	200
Sau Mutant 5	>200	*Sau* Mutant 15	>200
Sau Mutant 6	>200	*Sau* Mutant 19	200
Sau Mutant 7	>200	*Sau* Mutant 21	>200
Sau Mutant 8	>200	*Sau* Mutant 22	>200
Sau Mutant 9	>200	*Sau* Mutant 24	100
Sau Mutant 10	>200	*Sau* Mutant 25	50
Sau Mutant 11	>200	*Sau* Mutant 26	50
Sau Mutant 12	>200	*Sau* Mutant 27	50

Fig. S3. Susceptibility testing of 19 *S. aureus* candidate mutants in agar plates with Compound 18. Dilution 0 was a ca. 10^5 CFU/mL suspension; dilutions 1, 2 and 3 are 10-fold serial dilutions of the initial suspension. Mutants 25 and 26 were discarded for whole-genome sequencing, since they were not able to grow in the presence of the compound.