Trabajo Fin de Grado

Biological characterization of a new series of chemical compounds with antimicrobial activity

Caracterización biológica de una nueva serie de compuestos químicos con actividad antimicrobiana

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D²AMR DISCOVERY & DEVELOPMENT ANTIMICROBIALS MECHANISMS OF RESISTANCE

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1. Abstract/resumen

1.1 Abstract

Antimicrobial resistance has become a serious global health problem, not only because resistant bacteria cause millions of deaths worldwide, but also because fewer antibiotics are approved to treat these infections. Currently, there is a great need to find new antimicrobial agents effective against multi-drug resistant strains.

In this project, we have characterized the antimicrobial activity of a library containing 47 chemical compounds with related structural features never tested before in biological processes. To this aim, a screening strategy was followed to select those compounds showing *in vitro* activity and without cytotoxicity.

First, a single concentration assay (single shot) was performed against 17 bacterial strains, including Gram-positives, Gram-negatives, and mycobacteria. Thirteen compounds were selected with activity at 50 µM against at least one of the strains. Second, dose-response and time-kill kinetics assays were also performed, selecting 5 compounds with bactericidal activity against Gram-positive bacteria at concentrations ranging from 0.78 to 50 µM. Third, cytotoxicity assays in human hepatic cells were performed to determine selectivity indexes (S.I); these ranged from 3.4 to 70.5. Fourth, concentrations of 5 µM were found not mutagenic by the Ames' Test. Finally, structure-activity relationship (SAR) studies defined chemical structural features determining the compounds' antimicrobial activity.

In summary, we have identified active compounds against Gram-positive bacteria with good S.I., which opens a new research line with the potential to develop new antimicrobial agents. Future assays may include more detailed studies of *in vitro* and *in vivo* activity, toxicity, determination of the molecular mechanism of action and medicinal chemistry efforts in order to optimize these series of compounds.

1.2 Resumen

La resistencia a los antimicrobianos supone un gran problema de salud global, no solo porque las bacterias resistentes causan millones de muertes en todo el mundo, sino también porque cada vez se aprueban menos antibióticos para tratar las infecciones que producen. Es por esto que actualmente existe una gran necesidad de encontrar nuevos agentes antimicrobianos eficaces contra cepas bacterianas multirresistentes.

En este proyecto hemos caracterizado la actividad antimicrobiana de una librería de 47 compuestos químicos con características estructurales relacionadas, nunca antes ensayados en procesos biológicos. Para ello se siguió una estrategia de cribado de compuestos químicos, con el objetivo de seleccionar aquellos que mostraran actividad *in vitro* y no fueran cito-tóxicos.

Primero se realizó un ensayo a concentración única (*single shot*) contra 17 cepas bacterianas (incluyendo Gram-positivas, Gram-negativas y micobacterias), seleccionando 13 compuestos que mostraron actividad a 50 µM en al menos una de las cepas. A continuación, se llevaron a cabo experimentos de dosis-respuesta y cinéticas de muerte, seleccionando 5 con actividad bactericida contra bacterias Gram-positivas a concentraciones entre 0,78 y 50 µM. Posteriormente, se realizaron ensayos de citotoxicidad en células hepáticas, determinando índices de selectividad entre 3,4 y 70,5. Además, se determinó mediante el Test de Ames que a la concentración máxima ensayada de 5 µM los compuestos no eran mutagénicos. Finalmente se llevaron a cabo estudios de relación estructura-actividad (SAR) definiendo características estructurales químicas determinantes para la actividad de los compuestos.

En resumen, se han seleccionado compuestos activos contra bacterias Gram-positivas con buenos índices de selectividad que abren una nueva línea de investigación con potencial de desarrollar nuevos agentes antimicrobianos. Ensayos futuros pueden incluir estudios más en detalle de la actividad *in vitro* e *in vivo*, toxicidad, de determinación del mecanismo molecular de acción y de química médica para optimización de los compuestos.

2. Introduction

2.1 History of antimicrobials

Since the 1940s, the decade when antibiotics started to be broadly available to the public, they have mostly brought good news. Thanks to the use of antibiotics, mortality and morbidity due to bacterial infections were reduced, doctors could perform surgeries safely and many illnesses that used to mean a sure death became a problem that could be overcome with a proper treatment.

Starting with the discovery of penicillin in 1928 and during the following decades, dozens of antibiotics were discovered from environmental sources, many of them produced by fungi or even by bacteria. Some others were chemically synthesized in laboratories, and their properties improved by making them less toxic, more effective or able to kill a broader spectrum of bacteria. Due to their ability to cure diseases that some years before were mostly lethal, they were known as the "miracle drugs"; in fact, they are reported to have saved hundreds of millions of lives (1,2).

However, at the same time that we were learning how to kill bacteria more effectively, bacteria also evolved and were able to counteract their activity. Soon, clinical treatment failure due to bacterial resistance to antibiotics was detected. Strains previously killed by standard treatment remained unaffected, causing a public health emergence, as it was more difficult to treat the infections they caused.

An approach to fight bacterial resistance is the development of new antimicrobial agents. During the "Golden Age of Antibacterials", many different families of antibiotics were discovered, such as beta-lactams, macrolides, aminoglycosides or tetracyclines. This Age lasted until the 1960s, when after decades of obtaining new antimicrobial agents from nature, no new antibiotics could be found.

Soon after followed the "Golden Age of Medicinal Chemistry", when the strategy consisted in chemically modifying specific parts of the structure of previous antibiotics to avoid bacterial resistance mechanisms. For example, modifications of the structure of the original cephalosporins discovered were able to counteract beta-lactamase resistance. This had a partial success, because for every new antibiotic developed soon after arose resistance to such compound resulting in first, second, third and fourth generation cephalosporins (3).

At the end of the 20th century, it became clear that humankind was losing the war on antibiotics; not only have we detected resistant bacteria to every single one of our antibiotics (1), but also the number of new systemic antibacterials approved by FDA has been decreasing since the 1980s (4,5), as shown in Figure 1. Even though efforts made in recent years have resulted in new antimicrobials, it is clear that they are still insufficient. The number of multiresistant bacteria keeps increasing alarmingly fast, so their resistance mechanisms need to be confronted with new molecules that are yet to be developed. (5).

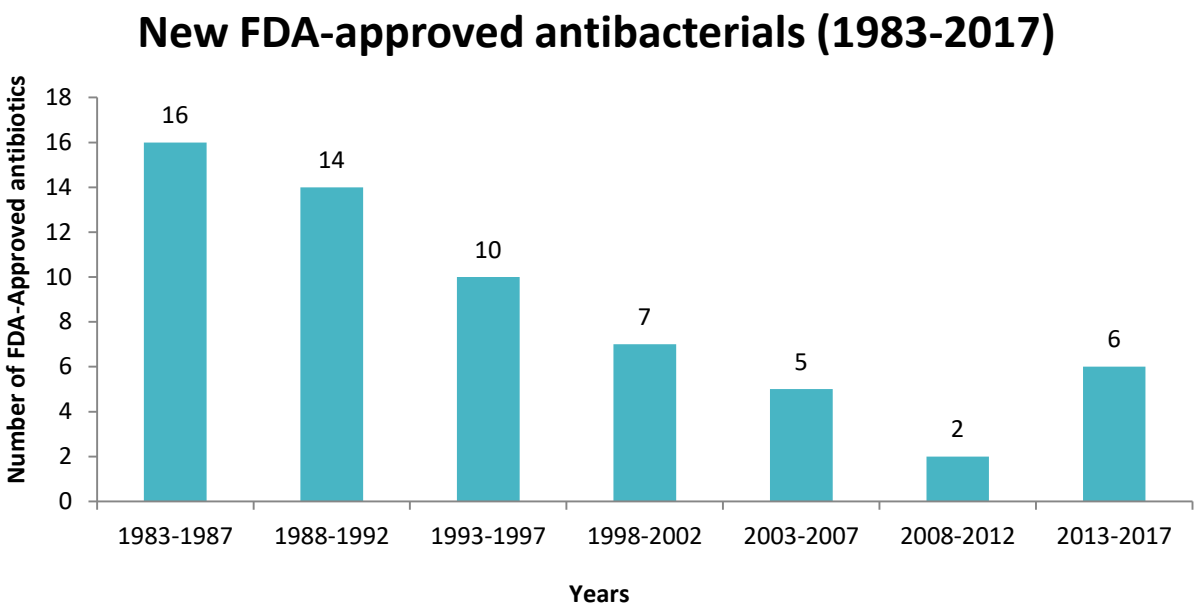

Figure 1: New systemic antibacterial agents approved by the FDA of the United States of America per five-year period. Modified from Boucher et al. Clin Infect Dis. (2013) (4) and according to FDA and CenterWatch databases (6,7).

2.2 The problem of bacterial resistance

To fight bacteria, first we need to know the enemy; so to design new more efficient drugs we need to understand their resistance mechanisms and bacterial targets. These bacterial resistance mechanisms are very diverse, but they can be grouped into four main strategies:

1. Inactivation of the antibiotic. For example, beta-lactamase enzymes can break penicillin, so it becomes unable to bind its target.

2. Prevention of antibiotic entry into the cell. For example, the outer membrane of Gram negative bacteria provides a permeability barrier that antibiotics must overcome.

3. Extrusion of the antibiotic once it has entered but before it can reach its target. For example, *Mycobacterium tuberculosis* has efflux pumps that expel most antibiotics, making it very difficult to treat.

4. Alteration of the target, so the antibiotic does not affect it. For example, PBP2A protein in *Staphylococcus aureus* is a mutated version of PBP2 that is unaffected by most beta-lactams.

Even though new antimicrobials have been developed over the last decades, antibiotic resistance has still increased (8). This is because bacteria are organisms with high division rates (around one division in 30 minutes in optimal conditions), and mutation rates of around 10^{-9} mutations per nucleotide per generation (9). This implies that in short periods of time we could potentially find mutant bacteria resistant to an antibiotic. If these mutants managed to survive and proliferate (for example when exposed to the antibiotic they are resistant to), resistance would spread. Moreover, bacteria have also the capacity to transmit genetic

information horizontally, so they can make resistant other cells around them, not necessarily from the same strain.

This situation was aggravated by our misuse and overuse of antibiotics. For examples, antibiotics to treat human illnesses such as bacitracin or neomycin penicillin are usually given to animals in farms to promote growth and as prophylaxis (10). This turns livestock farms in a high selective pressure ambient, allowing resistant bacteria to grow and proliferate, spreading resistance.

Furthermore, many people take antibiotics without prescription, which is useless and dangerous in absence of a bacterial infection (but only 48% of Spanish population know that antibiotics aren't effective against cold or the flu) (11). To worsen this situation, in primary assistance and hospitals the same antibiotics are used over and over, without change or rotation, increasing the selective pressure and favouring resistant strains (5).

All this together made antibiotics resistance a global health problem recognized even by the WHO (12). Resistant strains such as penicillin-resistant *Neisseria gonorrhoeae* have been found in every country (1), and deaths caused by them keep increasing. In a report released on May 2018, more than 35.000 deaths were reported in Spain due to multiresistant bacteria, which is 30 times more than deaths caused by traffic accidents (13).

It is obvious that we need to find new antibiotics, different from those currently available, with different bacterial targets and molecular mechanisms of action that enable us to defeat resistant strains.

2.3 Drug discovery process

There are different approaches to drug discovery: assays can be performed with (i) the whole-cell screening method, which consists in testing compounds directly against bacteria; or (ii) using a target-based approach, where compounds are tested against the purified target. Also, rational design can be performed if the structure of the target is known, i.e., inhibitors can be optimised *in silico* to make them more effective. All methods have advantages and disadvantages and the use of one or the others depends on the aim and resources of the project, among other factors (14).

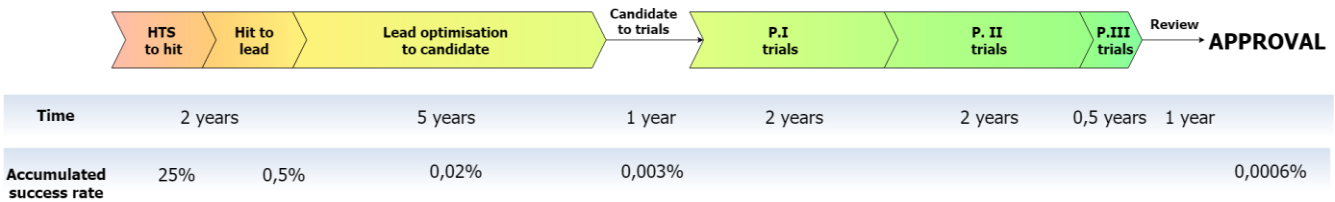

Figure 2: Phases from the start of the drug discovery project until a new antimicrobial drug reaches the market. It includes the time each phase takes on average (according to GSK data) and accumulated success rate, this is, percentage of initial molecules that reach the next stage. Modified from Payne et al, 2007 (15).

As Figure 2 shows, in the drug discovery process there are three stages prior to candidate selection, i.e., a potential drug to be tested in humans. First, hits are identified; these are molecules that show primary activity, selectivity and basic safety. Second, more assays (described below) are performed, so compounds with the best features are selected and called leads. Finally, these leads are optimised to improve their performance as drugs, with the aim of finding a candidate molecule which could start clinical trials. In the end, a drug would only reach the market after successful clinical trials and extensive reviews.

2.3.1 High Throughput Screening (HTS) to hit

Nowadays, drug discovery process is done mostly by HTS and with a target-based approach, this is, purifying the desired targets previously validated and testing the compounds that bind them.

In this first stage, different assays have to be performed to finally select the hits, molecules that meet the initial requirements (14):

- HTS: it is the first assay performed and consists in testing the compounds against the target and selecting those with the best binding activities.
- HTS confirmation: intends to pull apart active compounds from false positives, so it is a stricter assay.
- Dose-response assays: where compounds are tested at different serial concentrations to determine potency.
- Selectivity assays: are performed in order to select only those compounds that bind specifically to the desired target. HTS are carried out against other targets and compounds with cross activities will be removed from the library due their non-specificity.
- Phenotypic assays: where compounds are tested against whole cells. In antimicrobial discovery programs this is essential given that not all compounds that bind the target are able to kill the bacteria for various reasons: usually due to inability to penetrate the cell, but also because cross-resistance mechanisms, instability in the cytoplasm, etc.
- Cytotoxicity assays: consist in testing the compounds against human cell lines to determine at which concentrations they would be toxic. This is crucial because a large percentage of compounds tested end up being toxic to humans at small concentrations, therefore making them useless as a therapeutic drug.

Only around 25% of the initial compounds will meet the requirements in all this assays, so they progress through the development pipeline and be called hits (15). Before going to the next stage, they should be validated by resynthesizing them and repeating the assays.

2.3.2 Hit-to-lead

The next step is refining each hit in order to produce more potent and selective compounds. Many of the hits usually belong to the same chemical series, which enables us to detect a structure-activity relationship (SAR). Through medicinal chemistry, different motives or groups of the molecule can be modified and more assays performed. With the data gathered, a pharmacophore is created: the minimum molecular features and structure with activity. Starting from a pharmacophore, and through medicinal chemistry, more groups could be added to obtain a compound with improved characteristics.

Hits undergo different tests in order to determine their physicochemical properties (for example, optimal solubility and permeability are crucial in a drug), mechanisms of action, and metabolism (e.g. measuring CYP450 inhibition). They are also assessed for their pharmacokinetics in rats to define half-life and absorption, among other parameters. In addition, its synthesis is optimized to use the compound at a larger scale. The selected compounds, with the best features, will be called leads. The next phase will focus on lead optimization, to generate candidate drugs to be administered in human.

2.3.3 Lead optimisation to candidate

In this stage, the aim is to maintain favourable properties while improving on the deficiencies previously seen. Here, many more tests are performed to determine more accurately its safety, genotoxicity, pharmacokinetics, and pharmacodynamics, both *in vitro* and *in vivo*. Finally, a candidate molecule would be selected to start human trials after review from regulation agencies, such as FDA (14–16).

Only a small number of leads move forward to become candidates, as many small molecules programs in the pharmaceutical industry fail before reaching this stage. Later, during clinical human trials, many compounds might not demonstrate acceptable levels of safety and effectiveness in humans, and therefore would be withdrawn from further development.

2.3.4 Drawbacks of current drug discovery process

All this shows that the problem is not only that bacteria are becoming resistant, but also the difficulties that developing new antibiotics involves. First, it requires a huge economic inversion: around 1,600 million USD and around 15 years from bench to clinic (15). Second, drug regulation affairs are now stricter than in earlier times, focusing on customer protection, to the point that many drugs approved last century, such as aspirin, probably would not be approved under current safety standards. In addition, pharmaceutical companies often use defined libraries containing always the same structures and motifs, so chemical diversity is limited even though having millions of compounds.

Other reasons might include lack of profitability of antibiotics compared to other drugs, such as those for diabetes, neurodegenerative diseases, obesity, etc., that are targeted to wealthier population and chronic diseases. In addition, they could have a shorter life due to the development of bacterial resistance. So, even if a company manages to bring a new drug into the market, they may not be able to fully retrieve the investment.

Despite these difficulties and given the urgent need for new antimicrobials, there are still efforts to bring new antimicrobial drugs to the market. New models usually start from public-private partnerships between academia and the pharmaceutical sector to increase the odds of success.

2.4 TFG brief summary

In this TFG, we initiated an academic drug discovery program in collaboration with the "Electro and Photoactive Molecular Materials (EPMM) Group", from the Organic Chemistry Department (University of Zaragoza). EPMM owns libraries of chemical compounds synthesized for industrial purposes unrelated to therapeutic clinical applications. These libraries contained new chemical matter, thus offering diversity from conventional libraries, and were considered as a novel start point for antimicrobial discovery.

Briefly, compounds were tested against a selection of bacterial strains in phenotypic whole cell and those more potent evaluated in cytotoxicity assays. We identified compounds active against Gram-positive bacteria in the low micromolar range, with good selectivity indexes, lack of genotoxicity and chemical features compatible with an SAR.

3. Background and objectives

The compounds of this series had similar structures to two compounds that showed activity against *Mycobacterium tuberculosis* in previous experiments performed between EPMM and the Group of Mycobacterial Genetics (GGM). In these scouting assays, 20 compounds with different chemical scaffolds were selected from libraries synthesized by EPMM and one series showed activity against *M. tuberculosis.* Compounds structurally related to these active ones were then selected for further analysis, together with other structures not previously assessed.

Thus, the objective of this TFG project is to perform the initial biological characterization of this new series of compounds as potential new antimicrobial activities.

4. Materials and methods

4.1 Compound management

A library comprising 47 compounds with related chemical structures was tested for antimicrobial properties. Compounds were received in powder, dissolved in DMSO at 4 mM and stored at -20˚C (primary stock). In order to minimize freeze-thaw cycles and preserve compounds' activity, secondary stocks were prepared in 96-well plates at 40-fold the final concentration in the assay plate (40x) for every experiment. A new secondary stock plate was used for each assay.

Experiments were performed under sterile conditions in a laminar flow BSL2 hood in the dark, as compounds were photoactive. To protect them from light they were also stored in opaque black boxes.

4.2 Minimal inhibitory concentration (MIC) and Minimal Bactericidal concentration (MBC) determination

Bacterial strains and culture conditions used in this study are described in Table 1 below. Details of culture medium used are included in Annex I.

Species	Strain	Type	Incubation time	Culture medium
Acinetobacter baumanii	ATCC 19606	Gram-negative	24h	Mueller-Hinton II
Corynebacterium diphteriae	ATCC 39255	Gram-positive	24h	BHI
Corynebacterium glutamicum	ATCC 13032	Gram-positive	48h	Mueller-Hinton II
Enterobacter cloacae	ATCC 23355	Gram-negative	24h	Mueller-Hinton II
Enterococcus faecalis	ATCC 19433	Gram-positive	24h	Mueller-Hinton II
Klebsiella pneumoniae	ATCC 13883	Gram-negative	24h	Mueller-Hinton II
Mycobacterium abscessus sp abscessus	ATCC 19977	Mycobacteria	72h	7H9
Mycobacterium abscessus sp bolletii	CCUG 50184	Mycobacteria	72h	7H9
Mycobacterium abscessus sp massiliense	CCUG 48898	Mycobacteria	72h	7H9
Mycobacterium avium	ATCC 25291	Mycobacteria	72h	7H9
Mycobacterium smegmatis	mc^2155	Mycobacteria	72h	7H9
Pseudomonas aeruginosa	ATCC 15442	Gram negative	24h	Mueller-Hinton II
Salmonella typhimurium	ATCC 14028	Gram negative	24h	Mueller-Hinton II
Staphylococcus aureus	ATCC 6538	Gram positive	24h	Mueller-Hinton II
Staphylococcus epidermidis	CECT 231	Gram positive	24h	Mueller-Hinton II
Streptococcus agalactiae	ATCC 13813	Gram positive	48h	Mueller-Hinton II

Table 1: strains tested and culture conditions used in this study.

Strains were stored in frozen glycerol aliquots and the number of cells enumerated. Every experiment was started from a new frozen aliquot in order to minimize inter experiment variations. Assays were performed in the standard mediums for each strain, described in Table 1, and with a starting bacterial inoculum of 10^5 colony forming units (CFU)/ml.

To perform each assay a mother plate was prepared with the compounds to be tested at 40x, and then 5 µl of each compound were transferred from this plate to the assay plate, in duplicate. Then, 195 µl of culture medium seeded with bacteria at the concentration required was added to each well. Positive controls (free of compounds and DMSO) and negative controls (only culture medium) were also included, as well as a DMSO control with 5 µl of DMSO (2.5%).

Plates were incubated at 37ºC during 24-72h depending on the bacterial strain (see Table 1). The read out was made with an MTT assay: 30 µl of yellow tetrazolium salt with 20% Tween were added to each well. After one hour of incubation, viable cells can convert MTT into a purple formazan salt precipitate, which is solubilised by Tween. Absorbance is then read at 580 nm in a spectrophotometer (17).

The percentage of viable cells was calculated basing on absorbance of the sample, blanks, and positive controls, and then calculating the mean of both duplicates.

$$\%\ of\ viable\ bacteria = \left(\frac{Sample\ absorbance\ \cdot\ 100}{Positive\ control\ mean}\right) -\ Blanks\ mean$$

Before the addition of the MTT, a parallel measure was also performed in solid culture medium, where 5 µl of each well were transferred to a 96-well LB-agar plate and incubated for further 24h. The readout for this plate was made using a resazurin assay, a blue salt which metabolically active cells are able to turn into pink. Thirty µl of resazurin are added in each well and, after 1h of incubation, the colour change was assessed visually (18). All tests were made with technical duplicates.

The activity of the compounds was evaluated in a two-step process. First, a single shot assay was performed, where compounds 1 to 39 were tested at 50 µM against the 17 strains described in Table 1.

Second, dose-response assays were performed. Sixteen compounds were tested (2,4, 5, 7, 11, 13, 15, 18, 19, 27, 28 36 and 39 selected according to results obtained in *single shot* and 1, 20 and 34 as negative confirmation controls) against 7 bacterial strains: 6 Gram-positive strains mentioned in Table 1 plus *Pseudomonas aeruginosa*.

The highest concentration tested in dose-response assays was 50 µM and then, 1:2 serial dilutions were made in DMSO to test 25, 12.5, 6.25, 3.12, 1.56 and 0.78 µM. Experiments were performed twice.

4.3 Time-kill kinetics

Two strains of *Staphylococcus aureus*: methicilin-sensitive ATCC 6538 (MSSA) and methicillin-resistant NTC 12493 (MRSA), were tested against compound 18. Concentrations of 50, 25, 10 and 5 µM were assessed, and a control with 25 µl DMSO (highest volume added in this experiment was included too. Five ml of both strains were seeded at 10^5 CFU/ml.

The first sample was taken at the moment of seeding and before adding the compounds, (0 hours, t=0). Flasks were then incubated at 37ºC for 24 h further. Time points were taken at 1h, 3h, 6h, and 24h. Serial 1:10 dilutions were performed in PBS of each flask and 100 µl of 4 of these dilutions (according to the turbidity of the culture) were then seeded in LB agar plates, in duplicate, and then incubated for 24 h.

Finally, the readout was made by counting the colonies grown on each plate for each dilution and calculating the CFU/ml of the compound, with the following formula:

$$Concentration\ (CFU/ml) = \frac{Mean\ of\ colonies\ counted}{dillution\ blank \cdot volume\ transferred\ (ml)}$$

4.4 Ames' Test

Prior to the Ames' Test, a MIC/MBC assay was performed with the strain used, *Escherichia coli WP2 (uvrAB, trpE(AT), pKM101)* (19) with the selected compounds, as it was a strain not previously used.

Bacteria were inoculated in minimal agar medium (with only glucose and minerals, see Annex I) with the 5 previously selected compounds (2, 11, 18, 19, and 27) at concentrations of 5, 2.5, 0.5 and 0.05 µM. Negative controls were also included with water, DMSO (as it is the dissolvent of the compounds) and positive controls with methyl methanesulfonate, a well-known mutagenic compound (with 1, 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} dilutions) (17).

Plates were incubated for 72h at 37ºC and colonies on each plate counted. All measures were performed in duplicate.

4.5 Cytotoxicity assays

Hepatic human cells (HepG2 ECACC 85011430) were tested against compounds 2, 11, 19, 18 and 27 at concentrations of 250, 100, 50, and successive 1:2 serial dilutions until 1.56 µM. A secondary stock and assay plate was prepared using a new primary stock with compounds diluted at 10 mM in DMSO, thus enabling higher concentrations of compound without exposing cells to more DMSO, which can be toxic for them.

To determine toxicity, a Neutral Red Uptake (NRU) assay was performed as described in ISO 10993-5:2009(E) (20). Cells were first seeded in a 96 plate well at a concentration of $2.5 \cdot 10^4$ cells per well and then incubated for 24h in DMEM medium. Culture medium was then removed and 100 µl of fresh culture medium added together with the compounds to be tested. 2.5 µl of each compound from the assay plate were added in each well, reaching less than a 2.5% of DMSO in each well.

After incubation with the compounds for 24h, cells were inspected under an optical microscope, culture medium removed and new culture medium with neutral red added. Cells were incubated with the red dye for 3h, so that viable cells take it through the lysosomal pathway, and then a desorb solution (composed by ethanol and acetic acid) is used to dissolve the red dye. The readout was done by measuring absorbance at 540 nm.

The assay was performed in triplicate and five different controls were included:

- Positive control: cells and DMEM medium;
- Negative control: DMEM medium;
- DMSO 2.5%: the same concentration to which cells with compounds are exposed; served to discern cell damage caused by compounds that potentially caused by DMSO;
- DMSO 10%: control for dead cells, as that concentration is very toxic for HepG2;
- Rifampicin control: antibiotic rifampicin at 1 µg/ml is not harmful to HepG2, so it was used as a positive control.

A plate with the compounds and culture medium but without cells was also tested, with the aim of making sure that the strong colours of the compounds didn't interfere with the data obtained.

Data analysis was made in the same way than previously described.

4.6 Cheminformatics

The SMILES (Simplified Molecular Input Line Entry Specification) code of the compounds was obtained and used to gather cheminformatic data. Molinspiration Server was used to predict drug-likeness and molecular properties (21) of compounds 2, 11, 18, 19 and 27.

Swiss Similarity Server was used in order to find coincidences between the structures of these compounds and molecules contained in Sigma Aldrich, ZINC15, ChEMBL or drug databases, as well as every other database included in Swiss Similarity server (22).

5. Results

5.1 Single shot

This first assay intended to be a preliminary test to pull apart active and inactive compounds. A whole-cell screening approach was used, i.e., molecules were tested directly against bacteria instead of against purified targets, which enabled to select only those compounds truly acting as antimicrobial agent.

Fifty micromolar was considered the highest concentration to be acceptable (given that high concentrations usually mean high toxicity) and thus all compounds were tested at that concentration. A cut-off of 90% was set, i.e., we considered a positive if remaining bacteria were less than 10% of the positive control.

Those that only showed activity in one of the duplicates were taken as positive and further analysed in dose-response assays. This way we try to minimize false negatives, while false positives can be removed in subsequent trials.

Results are shown in the Table 1 in Annex II. Of the 39 compounds tested in single shot, 13 of them were selected for further analysis, as they showed activity in at least one bacterial strain at 50 µM: 2, 4, 5, 7, 11, 13, 15, 18, 19, 27, 28, 36 and 39.

These results showed that compounds are only active against Gram-positive bacteria, while mycobacteria and Gram-negative bacteria remained unaffected. The only exception was *P. aeruginosa*, which seemed partially inhibited by compounds 2 and 11. Because of this, we also included this strain in dose-response assays, excluding the rest of Gram-negatives and mycobacteria.

The aim of the parallel measurement in solid culture medium performed prior to adding the MTT was to determine if compounds were able to kill bacteria at that concentration or just inhibit growth (i.e., if they are bactericidal or bacteriostatic compounds). Here, it was observed that compounds killed bacteria at 50 µM, so this assay was also repeated in dose-response experiments in order to determine the exact MBC. This is because, sometimes compounds are bactericide at certain concentrations and at lower ones they act as bacteriostatic, so MBC and MIC are different.

This second measurement also helped to reduce false negatives, as the compounds of these series were strongly coloured. Those with darkest colours (such as blues and purples) could give a distorted absorbance measure at 580 nm. In the case of the resazurine assay, blue wells after adding the reactive meant that there had not been any bacterial growth, i.e., compounds had killed bacteria, regardless of the absorbance signal.

5.2 Dose-response assays

This assay had the aim of determining the lowest concentration of compound that inhibits the visible growth of bacteria (MIC) and also the lowest concentration that kills the bacteria (MBC). This assay included 16 compounds: the 13 that had shown activity in single shot, and other 3 included as a confirmation of inactivity (1, 20 and 34), tested against the Gram-positive bacteria and against *P. aeruginosa*. Data analysis was done in the same way that in single shot and cut-offs of 90% were also used (MIC_{90}); this is, the minimal concentration that inhibits 90% of growth.

Compounds 1, 20 and 34 remained inactive, while MICs and MBCs of the other 13 are shown Table 2 of Annex II. Here, false positives in single shot were detected, given that compounds 4, 15, 28 and 36 showed no activity.

Based on this, the 9 active compounds remaining were divided in those that affect Gram-positive in general and compounds affecting only 1 or 2 strains. Thus, we selected compounds 2, 11, 18, 19 and 27 for further analysis, given that they are active against a wider range of bacteria (Table 2 of Annex II).

MIC and MBC values matched in most cases, suggesting bactericidal activity. The only exception was compound 5, which seemed to have bactericidal activity. This could be seen because, while cells don't appear to grow in the MTT liquid assay, they form colonies when transferred to solid medium, so that they haven't been killed, but inhibited.

5.3 Time-kill kinetics

After dose-response experiments, kill kinetics assays were performed to confirm the bactericidal activity of the compounds and to determine whether they could act as a sterilizers (meaning that they kill every cell, while a bactericide would kill 99.9% of them).

Compound 18 was selected as the most active compound against *S. aureus* MSSA in dose response assays and evaluated in kill-kinetic assays, (figure 3). Methicillin-resistant *S. aureus* (MRSA) was also chosen for these assays since it is one of the most concerning pathogens, causing important trouble in hospitals (23).

Results of time-kill kinetics are shown in Figure 3:

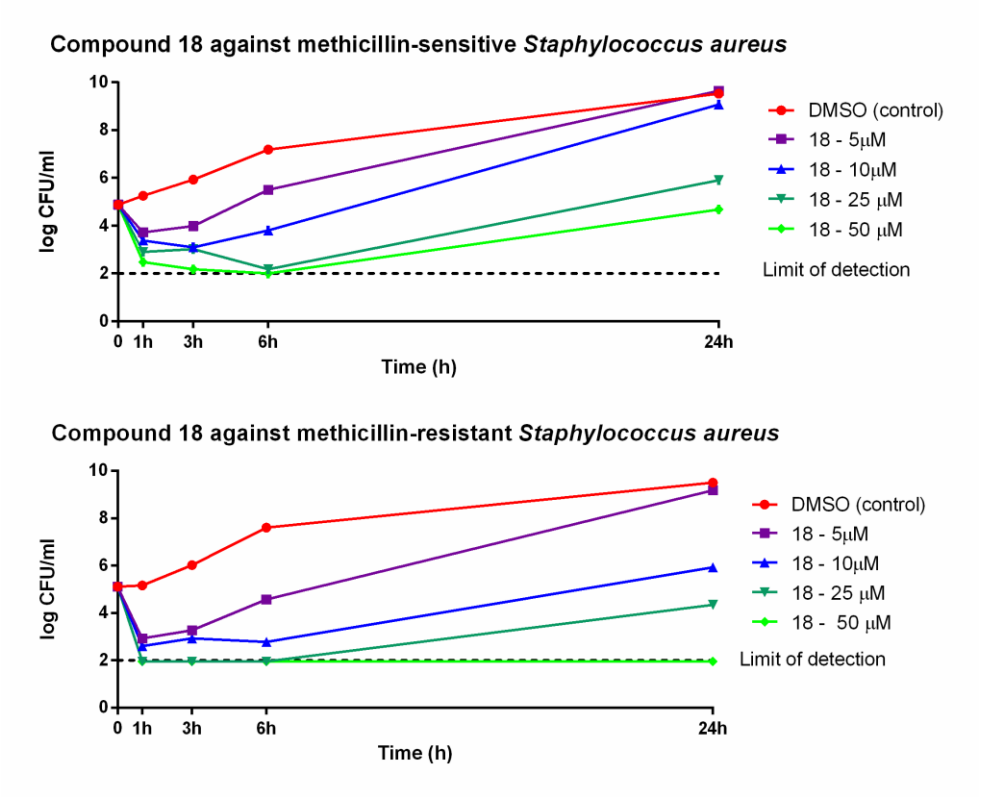

Figure 3: Time-kill kinetics of both *S. aureus* strains tested against compound 18. Samples taken only at the hours marked. Some data were under the limit of detection, i.e., no colonies could be counted, so the exact concentration is unknown.

Compound 18 showed activity against both MSSA and MRSA; however, it seemed slightly more potent against MRSA. These data confirm that the compounds were bactericidal, given that they kill more than 99.9% of the bacteria.

We could also observe that, even when bacteria were killed in presence of the compound, some hours later they were able to resume growth. This could be due to mutations that cause resistance as previously explained or to phenotypic resistance (tolerance), where no genetic changes are involved.

These bacteria were isolated and kept in frozen stocks. As phenotypic resistance disappears once it is no longer needed, they should be cultivated in absence of compound and then, MIC assays performed again to determine their kind of resistance. If the MIC rises, would indicate that resistance is caused by genetic stable mutations; otherwise it would mean that it's phenotypic.

Given that culture flask with MRSA against compound 18 at 50 µM didn't show an increase of bacterial concentration, it was incubated a week further. As no growth was observed, this compound seems to act as sterilizing at higher concentrations, killing all the bacteria, instead of killing 99.9%. However, further analysis should be done to confirm this finding.

5.4 Ames' Test

The aim of Ames' Test is to determine mutagenicity of a chemical compound. Some molecules interfere with DNA or replication systems and promote a higher rate of mutations, and at higher concentrations can end up causing the cell's death.

For that, compounds were tested against a bacterial strain whose auxotrophy for tryptophan has been inactivated due to a single base mutation, making them useful for testing mutagenicity (19).

Due to bacterial spontaneous mutation rate (9), we expect to find colonies that grow in minimal medium because their single-base mutation has reverted, making them auxotroph to tryptophan. However, if bacteria have been incubated with a mutagenic compound, their mutation rate would be higher, and the number that has become auxotroph to tryptophan would be higher too. A compound can be considered mutagenic if the number of colonies that appear in the plates is twice higher than in the negative controls treated with water.

Colonies counted after incubation with selected compounds are shown in Figure 4, compared to controls.

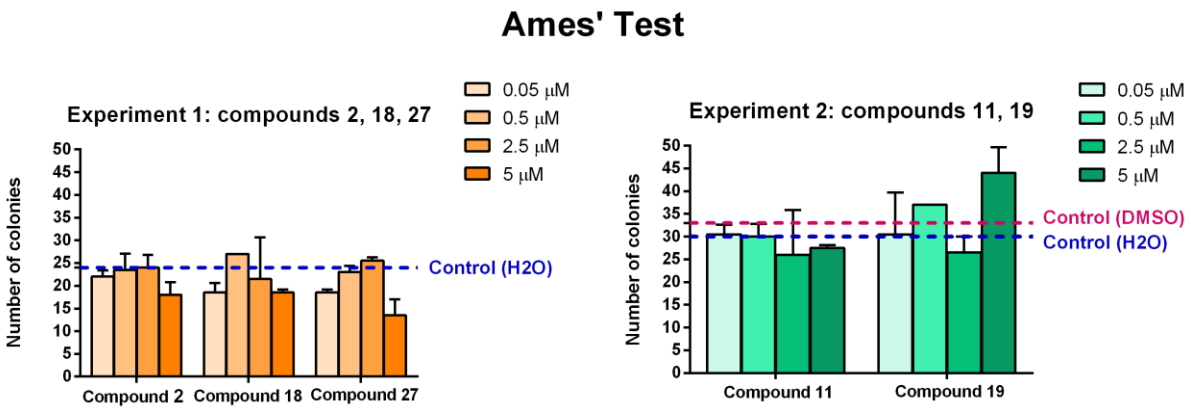

Figure 4: Ames' Test. Two graphs are shown because experiments took place in 2 different days, one with compounds 2, 18 and 27 and the other with 11 and 19.

Compounds don't seem to be mutagenic at concentrations under 5 µM, as in no case can we see twice as colonies as in controls. However, as *E. coli* WP2 is a Gram-negative strain which remained unaffected by our compounds at concentrations as high as 100 µM, this data might not reflect the actual mutagenicity of these compounds due to limited penetration through the cell permeability barriers.

5.5 Cytotoxicity assays

The five most active compounds were tested against a representative human cell line, HepG2, in order to determine toxicity in human cells. These cells are derived from human liver tissue with a hepatocellular carcinoma, widely used in drug cytotoxicity assays, given that, even transformed, they retain many of the specialized functions of the hepatic cells *in vivo* (24).

The NRU assay was chosen due to its good reproducibility, sensitivity, and the use of more stable and cheaper reagents than other assays such as MTT or lactate dehydrogenase. This makes it one of the tests more widely used in cytotoxicity with biomedical applications (25).

As shown in Table 2, cytotoxicity assays showed that toxicity in this cell line occurs at much higher concentrations than in Gram-positive bacteria. Toxicity appears in different concentrations depending on the compound. Compound 27 seems toxic at 50 µM, 18 and 19 at 100 µM, 2 at 250 µM and 11 seems not toxic at these concentrations.

	2	11	18	19	27
CC_{50} (µM)	170	250	55	55	25
CC_{90} (µM)	250	>250	100	100	50

Table 2: Cytotoxicity in HepG2 expressed in half maximal cytotoxic concentration (CC50), which is the concentration that kills 50% of the cells; and CC90, concentration that kills 90% of the cells; expressed in µM.

With these data and dose-response results, we can calculate selectivity index (SI), which is the relation between the half maximal inhibitory concentration (IC_{50}) against bacterial cells and the half maximal cytotoxic concentration (CC_{50}) against HepG2 cells. S.I. varies according to the strain, as compounds have different MICs for each of them. SI for these compounds and HepG2 cells ranged from 3.4 to 70 and are shown in Table 3.

	2	11	18	19	27
S. aureus	13.6	3.4	4.4		
S. epidermidis	13.6	7.2	17.6	17.6	
S. agalactiae	27.2	28.8	8.8	17.6	16.0
E. faecalis	13.6	3.6	17.6	70.5	7.7
C. glutamicum	27.2	7.2	8.8	35.3	4.0
C. diphteriae	27.2	14.4	35.3	70.5	7.7

Table 3: Selectivity indexes for selected compounds, calculated with CC50/IC50. Blank means that compounds showed no activity in those strains.

Selectivity Indexes give information about the selectivity of the compound, and high values are positive, given that they mean that concentrations of compounds that inhibit bacteria are much lower than concentrations that kill human cells.

5.6 Cheminformatics

Compounds tested could be considered not drug-like (21) based on their structure, for example, they have molecular masses near to 500 Da and low solubility (octanol-water partition coefficient over 6.7).

Searches in Swiss Similarity revealed no coincidences with any approved, experimental, investigational, withdrawn or illicit drugs, as well as with any similar structure in any of the databases it comprises (data not shown) (22).

5.7 Structure-activity relationship

After comparing the data gathered in single shot and dose-response experiments with the information about compounds' structure, some chemical features that seemed to be related to the antimicrobial activity were observed. To confirm these relations, 8 more compounds specifically chosen were tested in a dose-response assay against the 6 Gram-positive bacteria and *P. aeruginosa*, in the same conditions than the previous experiment.

First, it had been observed that all active compounds present a carboxyl group at the end. As many other inactive compounds had this group too, 2 compounds that only differed from 18 and 19 in their COOH group, which was replaced by a cyano (compounds 40 and 41), were tested.

Second, it had been observed that compound 2 had the simplest structure, while the rest of the compounds were designed by structural modifications, such as introducing different substituents in selected positions, modifying a part of the carbon skeleton, or substituting the carboxyl group for other more inert units. In order to determine whether a simpler structure could be active too, a compound which lacked a thiophene group (compound 42) was tested.

Third, compound 27 had shown activity at lower concentrations and its main difference with compound 2 was the presence of a bulky silicon group. In order to determine whether the size of the substituents determine the compound's activity, a compound that featured an even bulkier group (compound 46) was tested.

Fourth, all active compounds included a thiophene group, present also in many inactive compounds. To find out the relevance of this heterocycle two new compounds in which the thiophene ring was replaced by a thiazol (compounds 43 and 44), a furan (compound 45) or a N-methyl pyrrole (compound 47), respectively were tested.

The results of MIC and MBC (in µM) obtained are shown Table 4:

			40	41	42	43	44	45	46	47
Gram +	*S. aureus*	MIC	>50	>50	>50	50	50	25	>50	50
		MBC	>50	>50	>50	>50	50	50-25	>50	50
	S. epidermidis	MIC	>50	>50	>50	12.5	50-25	12.5-6.25	>50	12.5
		MBC	>50	>50	>50	25	50-25	12.5-6.25	>50	25
	C. diphteriae	MIC	>50	>50	12.5	12.5-6.25	6.25	12.5	50	12.5
		MBC	>50	>50	25-12.5	12.5-6.25	6.25-12.5	12.5	50	12.5
	C. glutamicum	MIC	>50	>50	>50	12.5	12.5	25	>50	12.5
		MBC	>50	>50	>50	12.5	12.5	25	>50	12.5
	E. faecalis	MIC	>50	>50	>50	25	50-25	50-12.5	>50	25
		MBC	>50	>50	>50	50	50	50-12.5	>50	50
	S. agalactiae	MIC	>50	>50	>50	12.5	25	12.5	50-12.5	25-12.5
		MBC	>50	>50	>50	12.5	25	12.5	50-12.5	25-12.5
Gram -	*P. aeruginosa*	MIC	>50	>50	>50	>50	>50	>50	>50	>50
		MBC	>50	>50	>50	>50	>50	>50	>50	>50

Table 4: Minimal Inhibitory and Minimal Bactericide Concentrations of compounds 40-47 in µM. These compounds were selected based upon characteristics of active compounds.

A structure-activity relationship summary table can be found in Annex III. All together, it has enabled to describe the molecular features that had a significant relation with activity:

- *tert*-Butyl groups at one end of the molecules appear to be necessary for the antimicrobial activity, as when they were replaced by a phenyl group, compounds became inactive.
- A carboxyl group (COOH) was essential, given that when it was replaced by a cyano (CN) group, compounds became inactive.
- The presence of a thiophene ring seemed to be essential because when removed, activity was almost completely lost. However, when it was replaced by other heterocycles, like a thiazol or an N-methyl pyrrole group, activity was retained. This suggests that the presence of a 5-member heterocycle might be a truly important feature.
- Big groups as substituents did not determine the activity. Even when compound 27 was very active, 46 was not. This could be because too big groups hinder antimicrobial activity or because they make it more difficult for the molecule to penetrate the cell.

These findings would need to be validated with more experiments, and compounds with different chemical features should be included in order to describe a pharmacophore, which could be modified in search of the optimal performance as an antimicrobial agent.

6. Discussion

In this project, a novel approach to the drug discovery process has been taken, with the aim of exploring a new chemical series and finding potential new hits. Testing the compounds against whole cells has enabled to study the compounds' antimicrobial activity, instead of studying their ability of binding to a target. Bacteria have many possible targets to inhibit in

order to kill them, such as ribosomes, DNA, metabolic enzymes or cell wall; and by performing whole-cell screenings we were able to test them all at the same time, as well as observe if compounds were affected by resistance mechanisms.

The process followed went from the most general to the most specific assays with the aim of finally selecting new active and non-toxic compounds. As the inconvenience of working with a library increases with the number of molecules it contains, after each assay compounds that had given negative results were removed from the library, thus making the process simpler.

After performing all the experiments, 5 compounds that met the requirements were selected. They have shown bactericidal activity against Gram-positive bacteria while being non-mutagenic and having acceptable selectivity indexes when compared the IC_{50} against the bacterial strains to the CC_{50} against human hepatic lines (Table 3). This is a very promising result, given that we have found new structures that are interesting as antimicrobial agents and that have a great potential to be improved and made more active or selective. However, many more assays have to be performed before calling it a hit.

In the first place, mechanisms of action have to be elucidated, because understanding which target these compounds bind to and how it is inhibited is a way of being able to improve the microbial activity. This can be studied by isolating mutant bacteria resistant to the compound. As previously explained, when the time-kill kinetic was performed it was observed that bacteria were able to grow after some hours in contact with the compound that was killing them (Figure 3). Bacteria isolated there could be analysed with the aim of finding a mutation in a target that is determinant for resistance, thus enabling a better improvement of compounds' mechanisms of action.

The fact that those more active compounds were also the more toxic (Table 2 and Table 2 of Annex II) could imply that the target is present both in bacteria and in eukaryotic cells. This, together with the flat structure of these molecules could indicate the ability to interact with DNA, which was tested through the Ames' Test (Figure 4). But given the limitations of this assay, further analysis is required to confirm their non-mutagenicity. This is because the strain used, *E. coli* WP2, is a Gram-negative which remained unaffected by compounds at 100 µM. Our data suggest that it is possible that compounds were unable to penetrate Gram-negative bacteria, so that, even if they were mutagenic, they could not reach the DNA, appearing then as false non-mutagenic negative. In this case, carcinogenicity assays in human cell lines could be an alternative (26).

Also, resistance mechanisms to these compounds present in mycobacteria and in Gram-negative would be very interesting to analyse. Resistant Gram-negative bacteria are usually more concerning in terms of global health and, in fact, they account for 7 of the 10 pathogens listed as the priority for the research and development of new antimicrobials (27), including the 3 considered as critical.

One of the reasons of their resistance is the structure of their outer membrane, which makes it more difficult for antimicrobials to reach the cytoplasm. Also, they usually have more efflux

pumps and enzymes that modify the drugs' structure in order to inactivate them. To determine whether these compounds are affected by efflux pumps or modifying enzymes, it would be useful to perform dose-response experiments combining the compounds with other molecules, such as efflux pumps inhibitors (28).

Understanding why Gram-negative and mycobacteria are unaffected by these compounds may allow modifying the compounds or using them in combination with other drugs, such as efflux pumps inhibitors or membrane-permeabilizers (29). This could enable to achieve sensitivity in these other strains, making the spectrum even broader.

Referring to cytotoxicity, the fact that compounds are harmful to hepatic cell lines at concentrations much higher than to bacteria is very positive (table 2). During drug discovery programs, many active molecules are removed due to their toxicity, which is a serious problem. However, these compounds have shown acceptable selectivity indexes, meaning an interesting starting point to further analyze this series.

First, safety also needs to be proven true in cell lines from other tissues. Apart from that, metabolism of the compounds needs to be studied in later stages, because sometimes drugs are not toxic but their metabolites are, which is dangerous *in vivo* too. For the metabolic studies, assays targeting Cytochrome P450 (CYP450) can be performed too, as it's one of the key enzymes of drug metabolism. CYP450 inhibition experiments are performed usually in hit-to-lead phase, as molecules inhibiting this enzyme can cause toxicity (16).

6.1 Structure-activity relationship

With the structure-activity relationship studies, it has been possible to define some chemical features that determine microbial activity. Assays in which some functional groups were removed and substituted one by one were performed and data gathered provided information about which functional groups were essential for the activity of the molecules (Table 1 of Annex III).

These data, together with results from future experiments analysing other chemical features and stereochemical orientations, would be very useful in order to design a pharmacophore. Three of its components have been described: the tert-butyl and carboxyl groups and a 5-member heterocycle, while additional features are yet to be defined.

This means a starting point towards the creation, through medicinal chemistry, of a candidate molecule with the best possible balance between activity and toxicity, also taking into account spectrum of action and susceptibility to resistance mechanisms. In many cases, a change in a single group makes the compound more resistant to modifying enzymes; for example, kanamycin can be modified in some of its amino groups in order to make it resistant to aminoglycoside acetyl- and nucleotidyl-transferase enzymes, which inactivate them. This

modification of antibiotics is key in current drug development, as many of the antimicrobials in use are the result of modifications of previous ones (30).

The most important characteristic of these compounds was that their structures were novel. The fact that we found no coincidences with any molecule contained in the most important databases means that, with high probability, structures similar to these compounds are absent from any chemical library with therapeutic purposes, so they have never been tested in biological assays.

This is interesting because, given the serious antimicrobial resistance problem we are currently facing, we need to find new bacterial targets, especially targets that can't be easily modified, such as the cellular membrane, instead of proteins, that can mutate and become unaffected in just one division. Also, to avoid cross-resistance, we need to find families and structures, different from the classical ones that are usually tested, such as the featured in this series.

In this scenario, incorporating molecules generated for industrial indications into the biomedical field could contain the key to find antimicrobial agents that are effective against multidrug resistant strains. Hence, this new series provides us with the opportunity of opening a research line that could influence the development of future antimicrobial agents.

7. Conclusions/conclusiones

7.1 Conclusions

In this project, the following conclusions have been extracted:

1. Five compounds of this series have demonstrated bactericidal activity against Gram-positive bacteria (including methicillin-resistant *Staphylococcus aureus*) at concentrations in the micromolar range and with acceptable selectivity indexes when tested against human hepatic cells.
2. The screening method performed is valid, given that it has enabled to separate in each stage those compounds that met the requirements set.
3. Carboxyl and tert-butyl groups, as well as a 5-member heterocycle are essential for the antimicrobial activity of the compounds.
4. Many more detailed studies have to be performed before finding a molecule that has the potential to become a candidate effective against resistant bacteria. However, these data open a new line of research with new structures absent from chemical libraries used by pharmaceutical companies in drug discovery campaigns.

7.2 Conclusiones

En este proyecto se han extraído las siguientes conclusiones:

1. Cinco compuestos de esta serie han demostrado actividad bactericida contra bacterias Gram-positivas (incluyendo *Staphylococcus aureus* resistente a meticilina) a concentraciones en el rango de micromolar y con índices de selectividad aceptables al ensayarlos en células hepáticas humanas.
2. El método de cribado es válido, dado que ha permitido separar en cada etapa aquellos compuestos que cumplían los requisitos establecidos.
3. Los grupos carboxilo y terc-butilo, así como un heterociclo de 5 átomos, han demostrado ser esenciales para la actividad antimicrobiana de los compuestos.
4. Se deben realizar muchos más estudios detallados antes de encontrar una molécula con el potencial de convertirse en un candidato efectivo contra bacterias resistentes. Sin embargo, estos datos abren una nueva línea de investigación con nuevas estructuras ausentes de las librerías químicas utilizadas por las compañías farmacéuticas en el descubrimiento de fármacos.

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