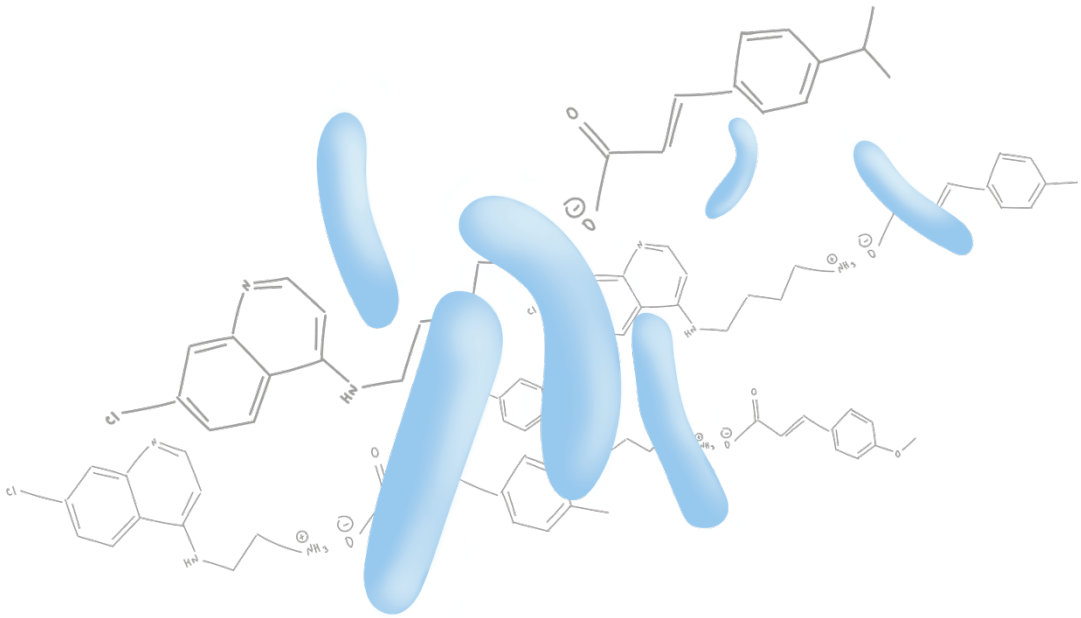

Evaluation of the effects of selected ionic liquids against *Mycobacterium avium*

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, 26 / 1 / 2019

The work described on this thesis was conducted at the Iron and Innate Immunity group at Instituto de Biologia Molecular e Celular/Instituto de Investigação e Inovação em Saúde (IBMC/i3S), in collaboration with Paula Gomes' group at Laboratório Associado para a Química Verde (LAQV/REQUIMTE), Faculdade de Ciências da Universidade do Porto, Portugal.

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Abstract

Mycobacteria from the *Mycobacterium avium* complex act as opportunistic pathogens and infect patients with a compromised immune system, namely those infected with HIV, with cancer or immunosuppressed for the purpose of organ transplant. In the host, mycobacteria proliferate inside phagocytic cells, such as macrophages. There, they control the intracellular vesicular trafficking by inhibiting the phagosome-lysosome fusion, which allows these bacteria to escape the lysosomal acidic environment and to have access to nutrients.

Different strains of *M. avium* have different susceptibilities to conventional antibiotics used in the clinic. It is then important to understand their behavior when trying to find new solutions for the treatments, including the re-purposing of old drugs. Recently, ionic liquids (ILs) have gained much attention in the area of drug development, as they can potentially be used to overcome unfavorable properties of some drugs, like solubility or toxicity. Chloroquine (CQ) is an antimalarial drug, which was shown to have an inhibitory effect in the viability of *M. avium*, suggesting it can be of use to treat infections by this agent.

The aim of this work was to evaluate the susceptibility of different *M. avium* laboratory strains to antibiotics currently used to treat non-tuberculous mycobacteria-related diseases, and, afterwards, to test the capacity of CQ-based ILs to inhibit the viability and growth of *M. avium* in axenic cultures and inside macrophages.

Our results indicate that *M. avium* 2447 SmT was susceptible to all the tested conventional antibiotics, in contrast to what happens with *M. avium* 25291 SmT, which seems to be resistant to the same antibiotics. The SmOp variant of *M. avium* 2-151 was slightly more susceptible than the SmT variant of the same strain.

ILs based on CQ-cinnamic acid conjugates were as effective against *M. avium,* extracellularly and inside macrophages, as its covalent equivalents. However, they were more soluble and less toxic for the host cells. The conjugation of CQ or primaquine (PQ) with fluoroquinolones, that are sometimes used to treat mycobacterial infections, resulted in ILs that have the same direct activity as the original antibiotics to *M. avium*, but are more active against the mycobacteria growing inside macrophages.

In the future we aim to test new ILs based in first-line drugs against mycobacteria, conjugated with active molecules against other pathogens that can occur concomitantly with non-tuberculous mycobacterial infections.

Keywords: antibiotics; chloroquine; ionic liquids; *Mycobacterium avium*.

Resumo

As micobactérias que pertencem ao complexo *Mycobacterium avium* atuam como patogénios oportunistas que infetam pacientes com o sistema imune comprometido, nomeadamente doentes infetados com VIH, com cancro ou que foram sujeitos a transplante de órgão. No hospedeiro, as micobactérias proliferam preferencialmente dentro de células fagocíticas, como os macrófagos. No seu interior, controlam o tráfico intracelular de vesículas inibindo a fusão fagossoma-lisossoma, o que permite a estas bactérias escaparem ao ambiente acídico do lisossoma e terem acesso a nutrientes.

Diferentes estirpes de *M. avium* têm diferentes suscetibilidades aos antibióticos usados convencionalmente na clínica. É, portanto, relevante perceber o seu comportamento quando se procuram novas soluções para os tratamentos atuais, incluindo o reposicionamento de fármacos previamente existentes. Recentemente, os líquidos iónicos (ILs) têm chamado muita atenção na área do desenvolvimento de novos fármacos, uma vez que têm o potencial de resolver problemas relacionados com a solubilidade e toxicidade de fármacos antigos. A cloroquina (CQ) é um fármaco anti-malárico que já demonstrou um efeito inibitório na viabilidade de *M. avium*, sugerindo que poderá ser útil para tratar infeções por este agente.

O objetivo deste trabalho foi avaliar a suscetibilidade de diferentes estirpes laboratoriais de *M. avium* a antibióticos atualmente usados no tratamento de doenças causadas por micobactérias não-tuberculosas. De seguida, testar a capacidade de ILs baseados na CQ em inibir a viabilidade e crescimento de *M. avium* em culturas axénicas e dentro de macrófagos.

Os resultados obtidos neste trabalho indicam que a estirpe *M. avium* 2447 SmT foi suscetível a todos os antibióticos testados, ao contrário da estirpe *M. avium* 25291 SmT, que parece ser resistente contra os mesmos. Em relação à estirpe 2-151, a variante SmOp foi ligeiramente mais suscetível do que a variante SmT.

Os ILs baseados em CQ conjugada com ácido cinâmico tiveram um efeito semelhante contra *M. avium* extracelular e intracelular aos seus equivalentes covalentes. No entanto, são mais solúveis e menos tóxicos para as células do hospedeiro. A conjugação da CQ ou da primaquina (PQ) com fluoroquinolonas, outra classe de antibióticos usados na clínica para tratar infeções causadas por micobactérias, resultou em ILs que têm a mesma atividade direta que os antibióticos originais, mas são mais ativos contra a micobactéria a crescer dentro de macrófagos.

No futuro pretendemos testar novos ILs baseados em fármacos de primeira-linha contra micobactérias, conjugados com moléculas ativas contra outros patogénios que podem ocorrer concomitantemente com micobacterioses não-tuberculosas.

Palavras-chave: antibióticos; cloroquina; líquidos iónicos; *Mycobacterium avium*.

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List of abbreviations

ADC – Albumin-dextrose-catalase

ATCC – American Type Culture Collection

BMM – Bone-marrow derived macrophages

CFUs – Colony Forming Units

clogP – Calculated logarithm of the partition coefficient

CQ – Chloroquine

DMEM – Dulbecco's Modified Eagle's Medium

FBS – Fetal Bovine Serum

HBSS – Hank's Balanced Salt Solution

HIV – Human immunodeficiency virus

IC_{50} – Concentration that inhibits by 50% the cellular viability

IFN – Interferon-gamma

ILs – Ionic liquids

LCCM – L929 cells-conditioned medium

M-CSF – Macrophage Colony Stimulating Factor

MAC – *Mycobacterium avium* complex

MIC – Minimal inhibitory concentration

Mtb – *Mycobacterium tuberculosis*

NTM – Nontuberculous mycobacteria

OADC – Oleic acid-albumin-dextrose-catalase

OD – Optical density

PBS – Phosphate-buffered saline

PQ – Primaquine

Rg – Rough variant

SmOp – Smooth-opaque variant

SmT – Smooth-transparent variant

TB – Tuberculosis

TNF – Tumor necrosis factor

WHO – World Health Organization

Introduction

Tuberculosis and NTM infection epidemiology

According to the World Health Organization (WHO) [1], about one-quarter of the world's population has latent tuberculosis (TB). In 2017, 10 million of those people fell ill with TB and 1.6 million died from it, including 230 000 children. Although it can affect any person from all age groups, external factors like smoking or concomitant conditions that impair the immune system, increase the susceptibility to TB. Actually, infection with the human immunodeficiency virus (HIV) raises about 20 to 30 times the predisposition of co-infection with *Mycobacterium tuberculosis* (Mtb). Although TB incidence is falling about 2% per year, the development of resistances to first-line antibiotics is delaying the WHO deadline to eradicate TB by 2030.

The incidence of infections by nontuberculous mycobacteria (NTM) is increasing significantly worldwide, in particular caused by *Mycobacterium avium* complex (MAC) species. This complex includes two species with very similar medical characteristics, *M. avium* and *M. intracellulare* [2]. This type of mycobacteria act as opportunists and infect patients with a compromised immune system, namely patients who are infected with HIV, with cancer or who were subjected to a transplant [3-6]. The fact that NTM are widely-distributed organisms found in soil or water, with a highly hydrophobic cell wall, which facilitates its aerosolization, may explain its high-infectious behavior. Besides that, NTM are able to survive in harsh environments, such as chlorinated water, and they can adhere to surfaces and form biofilms, allowing them to persist in an environment for long periods of time [3, 4]. Their resistance to antibiotics is growing, which urges the finding of new alternative therapies to combat mycobacterial infections.

Curiously, it has been reported that a local decline in TB incidence is coincidental with an increase in infections caused by NTM [6, 7]. There is not a single reason that justifies that phenomenon, but some explanations can be taken into account. For example, cases of cross-immunity between Mtb and NTM, in which each type of mycobacteria sensitizes the host to a second exposure of the other. Also, better public health conditions can be, in this case, a double-edge sword. While improved ventilation and plumbing was essential to reduce TB incidence, centralized water supply systems, disinfection of drinking water and the habit of showering instead of tub-bathing are associated with NTM colonization, leading to selection of these microorganisms due to their resistance to chlorination and higher exposure to mycobacteria through aerosolization. It must always be borne in mind that higher clinical awareness and better methods of diagnosis can explain an increased incidence

rate of NTM infections in the last years. However, every year studies are published worldwide reporting cases of NTM infection, mainly of patients fragilized by other lung-associated diseases, like bronchiectasis, or with an immune system compromised by conditions like rheumatoid arthritis or HIV-infection.

Mycobacterium avium

Mycobacteria cell wall structure

Mycobacteria are Gram-positive, aerobic bacilli (**Figure 2A**) with a characteristic dense cell wall enriched in lipids (**Figure 1**). Mycolic acids, complex fatty acids of 60 to 90 carbons, interact by esterification with arabinogalactan, a sugar polymer, forming an intermediate layer that confers high hydrophobicity and impermeability to several molecules, including antimicrobials [8, 9]. Below, a thick layer of peptidoglycan gives structural strength to the cell wall. The exterior layer of the wall is composed of glycolipids and lipoglycans that interact with the mycolic acids, which is covered by loose proteins, lipids and glycans [9]. All these lipid layers make mycobacteria acid-fast, so they are not stained by the Gram method, instead they require a harsh procedure like the Ziehl-Neelsen staining [8].

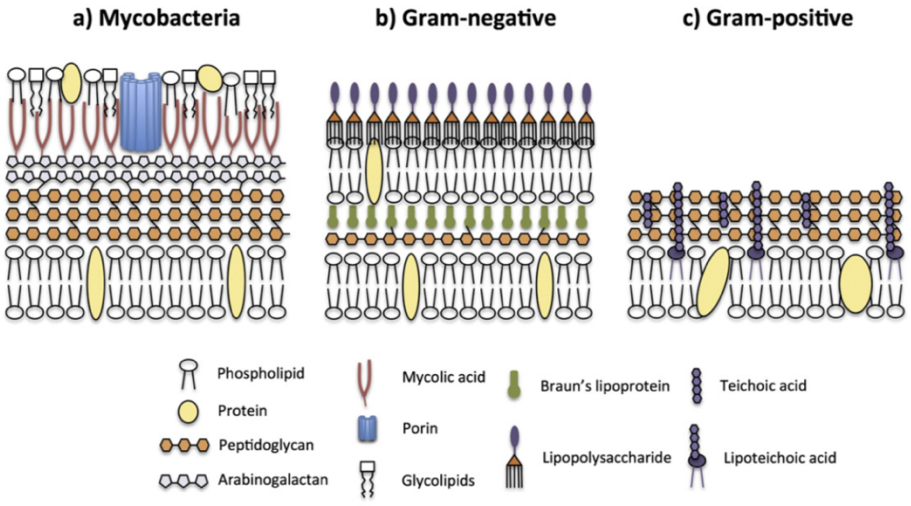


Figure 1 – Bacterial cell walls. Comparison between cell envelopes of mycobacteria and other bacteria (from [9]).

M. avium *infection*

Mycobacteria enter the body either by the respiratory or gastrointestinal tracts. The latter is more common in AIDS patients, the former is frequent in people with pre-existing pulmonary diseases [8, 10]. Among the more than 200 species of NTM known, MAC species are the mycobacteria most often isolated from respiratory samples of patients with lung disease, and from the four subspecies of MAC, *M. avium* subspecies *hominissuis* is the main responsible for the disease in humans [11-13]. The other subspecies of MAC are associated and have been isolated from cattle and poultry.

The analysis of MAC isolates allows the identification of several strains. The virulence of the different strains is highly variable and can be related to the origin of the isolate or by the morphotypic variant of each strain. Three variants of MAC, based on colony morphology, have been described: smooth-transparent (SmT), smooth-opaque (SmOp) and rough (Rg). The SmT variants are usually characterized for being highly virulent, while the SmOp variant are often not virulent. The virulence of the Rg variants is very variable. Isolates from animals are generally more virulent than human isolates, and isolates from the environment are usually not able to infect mice [14]. The *M. avium avium* strain ATCC 25291, isolated in the 1960s from a chicken, is a SmT variant. It is used worldwide as a reference strain and is one of the strains with the highest virulence described so far. The strain 2447, isolated in the 1980s from an AIDS patient in Belgium, is also a SmT variant. When compared with other strains, it is of intermediate virulence. The strain 2-151 was also isolated from an AIDS patient, but in the US. From that isolate, SmT and SmOp variants were cloned. The latter forms dome-shaped, opaque colonies on solid medium and is less virulent than the SmT variant [14].

Mycobacteria infect many types of cells, but their main and most studied host is the macrophage. When *M. avium* infects murine bone-marrow derived macrophages (BMM), it localizes in tight, individual vacuoles in the cell (**Figure 2B**), where the mycobacteria exponentially grow, multiplying about six fold in seven days [15]. It was also observed that the vacuole divides, accompanying the multiplication of the mycobacteria in order to maintain it in tight vacuoles. This strategy allows the mycobacteria to better control the fusion of their vacuoles with others present in the cell, like lysosomes. The arrest of maturation of early mycobacterium-containing phagosomes to phagolysosomes is a well-known protective mechanism of this kind of bacteria [15]. That maturation arrest is possible due to recruitment to the phagosome membrane of proteins and phosphoinositides required to intracellular trafficking. This mechanism prevents the exposure of the mycobacteria to the lysosome acidic

environment, hydrolytic enzymes and macrophage antigen-presenting organelles [9]. Besides that, this mechanism facilitates the access of mycobacteria to nutrients, like iron, located in cell membrane-derived vesicles with which the early mycobacterium-containing phagosomes are able to interact [15, 16].

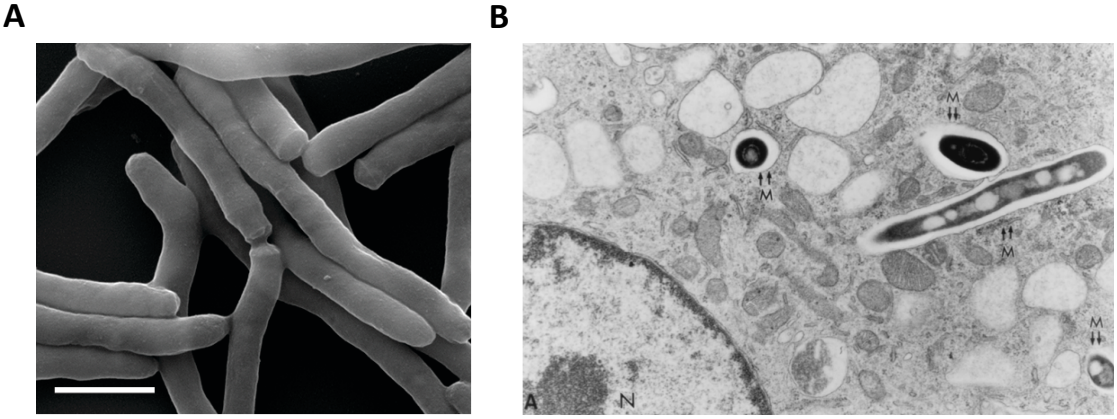


Figure 2 – *Mycobacterium avium*. (A) Scanning electron microscopy (SEM) image of *M. avium*. Scale: 1 μm (from [17]). **(B)** Electron micrograph of murine BMM infected with *M. avium* (M). The bacterium is contained in tight vacuoles (double arrows) (from [15]).

Control of M. avium *growth by macrophages*

When infected, the macrophage may inhibit the bacterial growth by mechanisms that involve oxidative damage. However, the production by the macrophage of nitric oxide [18], hydrogen peroxide [19] or superoxide [20] is not able to cause bacteriostasis or kill *M. avium*, while *M. tuberculosis* is not so resistant to these mechanisms. This can be explained by the ubiquitous presence of *M. avium* in environments, as water, that in certain conditions are enriched in oxygen and nitrogen reactive species, allowing these mycobacteria to develop resistance [16]. That does not happen to *M. tuberculosis*, as it can only survive inside mammalian hosts.

Thus, macrophages must have oxygen and nitrogen reactive species-independent mechanisms that can control and eliminate *M. avium* [20]. An immune response centered in $CD4^+$ T cells that produce IFN-γ plays an important role in controlling this kind of infection. The depletion of IFN-γ or of cytokines as IL-12 and IL-18 that promote the maturation of naïve $CD4^+$ T cells into IFN-γ-producing Th1 cells, exacerbates *M. avium* infection [16, 21]. TNF also induces anti-*M. avium* activity in vitro, and in vivo is involved in maintaining the structure and integrity of granulomas. A deregulation in TNF signaling is related to apoptosis of the cells of the granulomas [16].

Another way of restricting the growth of intracellular *M. avium* is by depriving the bacteria of nutrients essential for their survival. Signaling by cytokines such as IFN-γ can prevent the interaction of the *M. avium*-containing phagosomes with endosomes that carry nutrients [16, 22]. Playing an important role in the bacteriostasis of this type of mycobacteria are ion transporters present in the membrane of the infected phagosomes, such as NRAMP1, that pumps iron and other cations out, depriving the mycobacteria from it [20]. Also, enzymes that degrade important phagosome molecules and compounds that sequester other molecules, as is the case of iron chelators [22], are essential as well.

Macrophages undergo apoptosis as a mechanism to control the progression of infection. However, it was shown that some mycobacteria from MAC are able to escape the apoptotic bodies to the extra-cellular space, where they are phagocytized by healthy macrophages, infecting them [23].

NTM-related diseases and current treatments

Diseases caused by MAC infection

When a person is infected with *Mycobacterium tuberculosis* (Mtb), the preferable site for location of the bacteria is the lung. A patient with pulmonary tuberculosis exhibits symptoms such as chronic cough, sputum production, appetite and weight loss, fever, night sweats and hemoptysis [24]. Some of these clinical features are very similar to the ones caused by a NTM infection. It is then comprehensible that the first treatments used to combat this kind of infection were antituberculous drugs, which were successful in some cases but demonstrated lower activity than against Mtb [25]. However, with the dissemination of NTM infections, mainly of MAC species, and the emergence of AIDS, a new strategy had to be followed.

Besides lung disease, MAC infection can affect other regions of the body. Among the NTM, MAC is the main responsible for infections on the central nervous system (CNS) in patients either infected or non-infected with HIV, with mortality rounding the 80% of the cases [11]. The enlargement of one or more lymph nodes due to infection, lymphadenitis, is the most common NTM-associated disease in immunocompetent children between 1 and 5 years old [11, 26]. With clinical signs very similar to the ones presented by Mtb infection, lymphadenitis caused by slow growing

mycobacteria, mainly MAC, can persist for a long period of time even with a macrolide-based therapy regimen. Other diseases caused by NTM have also been described either in immunosuppressed or immunocompetent patients, as is the case of chronic osteomyelitis, arthritis, endobronchial disease and peritonitis [26].

Conventional antibiotics used against MAC infection

The discovery of antibiotics that have a better effect against MAC than the antituberculous drugs previously used, revolutionized the treatment of MAC lung disease. The treatment basis of slow-growing NTM, in which MAC is included, is a macrolide. Clarithromycin or azithromycin (**Table 1**) are the usual options, having no significant differences in response between the two [12], although, as clarithromycin has been more extensively studied, it is the preferable choice. Azithromycin is, however, often administered in order to prevent drug interaction, as clarithromycin works both as substrate and inhibitor of cytochrome P 3A enzymes [27]. Macrolides inhibit protein synthesis by binding to the 50S ribosomal subunit in bacteria and preventing the elongation of the nascent peptide chain [27, 28]. These antibiotics have the best correlation between in vitro susceptibility results and clinical (in vivo) response [11, 25]. The innate drug resistance of NTM to the antimicrobial agents influences the in vivo response in a way that often cannot be predicted by in vitro susceptibility results, such as minimum inhibitory concentration (MIC). These differences were observed in several species of rapid growth mycobacteria, such as *M. abscessus*, as well as in Mtb, and are related to the inducible macrolide resistance gene or *erm* gene. The activation of this gene reduces the binding of macrolides to the ribosome by methylation of an adenine in the 23S rRNA [11, 12, 27-29].

A regimen of monotherapy with macrolides is, thus, very dangerous as it will often lead to drug resistance and consequent treatment failure associated with increased levels of mortality. So, a three-drug macrolide-based regimen with ethambutol and a rifamycin, is the MAC recommended treatment, with favorable microbiologic results and avoidance of antibiotic resistances [12, 27, 29]. Ethambutol interferes with the mycobacterial cell wall synthesis by inhibition of arabinosyl transferases, which affects the synthesis of arabinogalactan (AG) and lipoarabinomannan (LAM). Its ability to alter the permeability of the cell wall, allowing the passage of other antimycobacterial drugs, is its major advantage. Besides that, there are no known drug interactions with ethambutol. This antibiotic has a favorable activity against slow growth mycobacteria, including MAC, but high levels of resistance

against rapid growth species excludes its use in the treatment of this type of bacterial infection [11, 27, 28]. Rifamycins like rifampicin (**Table 1**) and rifabutin complement the treatment. They bind and inhibit DNA-dependent RNA polymerases, interrupting protein synthesis early on the transcription process. Co-infection with HIV may decrease the absorption of rifamycins, and particularly rifampicin is known to reduce the concentrations of several antiretroviral drugs. Mtb has acquired resistance to this antimycobacterial agent due to mutations on the gene that encodes RNA polymerase β-subunit. Rifamycins are involved in multiple interactions with other drugs, and studies with patients infected with NTM show low tolerance to rifabutin, although it has better in vitro and in vivo activity against MAC than rifampicin [11, 12, 27, 28]. From the three types of drugs recommended to treat MAC infections, rifamycins contribute the most to suboptimal pharmacokinetics (PK) and pharmacodynamics (PD) parameters. In fact, poor antimycobacterial activities and several interactions with other agents, resulting in the reduction of the plasma concentration of these drugs, require the increase of the dosages to levels that become intolerable to the patients [12, 29].

New developments and challenges in the treatment of MAC infection

The addition of a fourth drug to the regimen, like the aminoglycosides amikacin or streptomycin (**Table 1**), can be important in specific cases, like severe disseminated MAC disease in patients with AIDS, and is essential in cases of macrolide-resistant MAC. Low tolerance to rifamycins can be overcome with clofazimine combined with a macrolide and ethambutol, however its use was related to an increase in mortality of HIV co-infected patients. Fluoroquinolones, like norfloxacin and ofloxacin (**Table 1**), have been included in the therapy of lung disease caused by macrolide-resistant MAC, however their use as first-line drugs is inadvisable as well as in a macrolide/fluoroquinolone regimen, in order to minimize drug resistance [11, 12, 27, 29, 30]. Therefore, new drugs with improved pharmacological indices are indispensable to maximize the success of the treatments and improve the life of the patients. Amikacin liposome inhalation suspension (ALIS) and bedaquiline are the two new promises, the former is still in phase III trials but phase II tests were encouraging, and the latter is very effective against drug-resistance tuberculosis [12].

At the core of the unsuccess of the MAC infection treatments is also the lack of adherence by the health professionals to the established guidelines [12, 29, 31]. That is, actually, one of the greatest issues behind the threat that is the resistance to antibiotics nowadays. The prescription of a multi-drug therapy is the cornerstone to

avoid macrolide resistance and to prevent unnecessary deaths, since surgical lung resection is many times the only solution for patients who fail drug therapy by developing antibiotic resistances [11]. Cases of re-infection with MAC after or during therapy are also a concern, affecting mainly patients with a severely immunocompromised system [25]. On the other hand, as the drugs utilized for MAC infection frequently have high toxicity, not compensated by high efficacy against MAC, health professionals sometimes opt for no treatment. Instead, the less severe cases are kept under clinical observation.

Alternative approaches to treat bacterial infections

The role of chloroquine in the viability of M. avium

Chloroquine (CQ) (**Figure 3**) was first synthetized during World War II, in consequence of a series of tests regarding the antimalarial potential of 4-aminoquinolines, in which CQ showed to be the most promising drug, due to its good efficacy, low toxicity, tolerable adverse effects and affordability [32-34]. Besides malaria, CQ and its analogues are also used as secondary drugs in the treatment of chronic diseases, like rheumatoid arthritis, lupus or sarcoidosis [32]. It was demonstrated that CQ has anti-HIV-1 activity in vitro, but also that it has anti-inflammatory properties, as it reduces the secretion of several pro-inflammatory cytokines. Furthermore, it was shown that CQ has an inhibitory effect in the viability of *M. avium* in mouse cells [35]. All these properties together suggested an urge in exploring CQ as a multi-versed drug in the treatment of AIDS-related opportunistic infections.

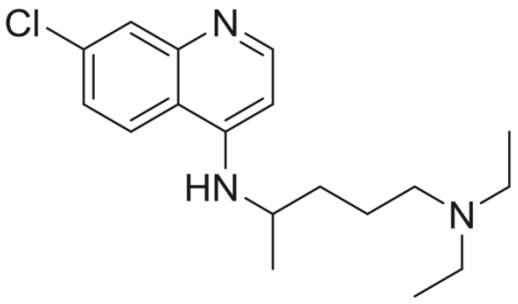


Figure 3 – Chloroquine. Chemical structure of chloroquine (adapted from: [36]).

Past experiments in our laboratory showed that CQ has a significant inhibitory effect in vitro against the axenic growth of *M. avium* and even when the bacteria are growing inside bone marrow derived macrophages (BMM). That inhibitory effect was also demonstrated in vivo: BALB/c and C.D 2 mice infected with *M. avium* 2447 SmT treated with 30 mg/kg of CQ every other day showed a significant decrease of bacterial loads in the liver (unpublished data).

In a more recent work, which tested the effect of CQ in a gram-positive bacteria, *Rhodococcus equi*, that, similarly to *M. avium*, infects preferentially macrophages, it was shown that the exposure to CQ, although it had no effect in the extracellular multiplication of *R. equi*, reduced significantly the viability of these bacteria inside murine and foal macrophages [37]. An explanation for these results relies on the fact that CQ is a weak base, which increases the pH of endocytic vesicles and lysosomes inside eukaryotic cells. Thus, it prevents the release of iron from those vesicles, which only happens when they have an acidic environment. Without iron, bacteria like *R. equi* or *M. avium* are deprived of one of their most essential nutrients and they are not able to survive [35, 37, 38].

Primaquine (PQ) is the most effective and less toxic 8-aminoquinoline used as an antimalarial since the 1950s [39]. More recently, it was reported that primaquine at 5 μM was able to inhibit the intracellular growth of Mtb [40], and some PQ-derivatives tested against Mtb, *M. paratuberculosis* and MAC showed strong antimycobacterial activity [41]. Therefore, PQ as well as CQ, are very interesting candidates for a new multi-targeted therapy.

The chloroquine-cinnamoyl conjugates

Paula Gomes' research group has been working for years in the chemical recycling of classical antimalarial drugs, including CQ, with the purpose of finding new and innovative compounds that can be applied to the therapy of malaria. Issues like the emergence of new resistant strains and the lack of multi-stage treatments have led their investigation on the way to a "covalent bitherapy" approach. That is the creation of a hybrid molecule that conjugates two individual molecules with intrinsic activity that can enhance the potential of both parent drugs. The covalent linkage of the heteroaromatic ring of CQ to differently substituted cinnamoyl groups showed very promising results against CQ-resistant *Plasmodium falciparum* [42] and as dual-stage antimalarial leads [43]. A study that evaluated the effect of the same N-cinnamoylated CQ conjugates against another parasite, *Leishmania infantum*, which infects

macrophages of mammalian hosts, obtained interesting results as well [44]. These conjugates were also recently tested against *Pneumocystis jirovecii*, an opportunistic fungus that infects patients with a compromised immune system [45].

Given the promising results of these covalent compounds in terms of activity in vitro and the fact that they have effect in certain conditions that CQ by itself never showed having, our group decided to test them against *M. avium* in axenic cultures and when growing inside macrophages, as it is their main mammalian host.

Characteristics and applications of ionic liquids

A new approach has been developed to rescue old and less utilized drugs, as is the case of CQ, due to its unfavorable pharmacological properties, such as low solubility, spontaneous crystallization, high dosage needed to achieve the desirable effects or toxicity to the host infected cells. These difficulties can be overcome by the non-crystalline forms of those drugs, the ionic liquids (ILs), which are organic salts made by the combination of the active pharmaceutical ingredient, in its cationic or anionic form, and an inert counterion, or a counterion with additional biological interest. The cost of these procedure can even be lowered by, for example, combining classical drugs of opposed polarities [36, 46, 47].

With remarkable physical and chemical properties, ILs were first used to improve the performance and safety of chemical procedures as green-solvents. Recent studies regarding the interaction between ILs and biomaterials have revealed their high potential to improve sensors and drug delivery systems [47]. It has been demonstrated that ILs work as well as antimicrobial agents, affecting several bacteria, including mycobacteria, and fungi [47]. The right combination of cations and anions can provide innovative compounds that help combat resistance issues.

The combination of anionic ampicillin with organic cations resulted in ILs with activity against Gram-negative bacteria resistant to antibiotics [48]. ILs derived from a classical antimalarial drug, primaquine, an 8-aminoquinoline, were found to exhibit improved in vitro performances in comparison to primaquine and better in vitro activities than their covalent analogues [46]. ILs derived from N-cinnamoylated CQ conjugates were reported to have similar activity against *Pneumocystis jirovecii* than their covalent equivalents, however showed to be less cytotoxic to two different cell lines than those covalent equivalents [45].

ILs mechanism of action is not yet fully understood. However, structural characteristics as the length of the cation side chain or the presence of polar functional

groups can alter properties such as lipophilicity and surface tension that are known to influence the activity of the compounds [47]. As active pharmaceutical ingredients, ILs emerge as a promising alternative for overcoming issues related to polymorphism while improving solubility and bioavailability in a cost-effective way.

ILs are very promising in terms of efficacy, affordability and as a form of bypassing problems of resistance. Its properties allow the combination of conventional drugs with different effects but with a joined purpose. For example, a bacterial infection in an AIDS patient could be treated in the future with only one medicine, an IL formed by an anti-retroviral ion and a counterion with antibacterial activity.

Objectives

The objectives of this work were to:

- Evaluate the susceptibility of different laboratory strains of *M. avium* to antibiotics conventionally used in the treatment of NTM infections;
- Evaluate the capacity of CQ-derived ionic liquids to inhibit the viability and growth of *M. avium* 2447 SmT growing extracellularly and inside mouse macrophages.

All the compounds tested were provided by Paula Gomes' laboratory at LAQV/REQUIMTE, Faculdade de Ciências da Universidade do Porto, Portugal, unless stated otherwise.

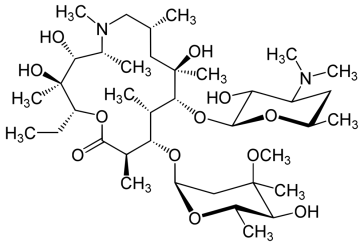
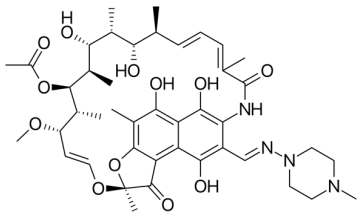
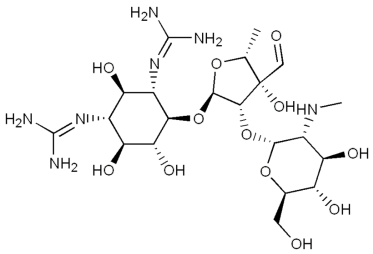
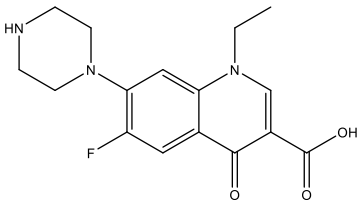
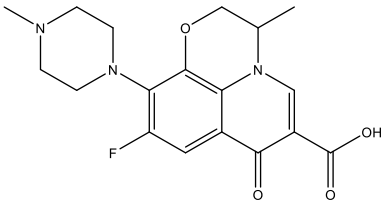
Materials and methods

Compounds

Conventional antibiotics

Table 1 shows the properties of the conventional antibiotics tested against different strains of *M. avium*.

Table 1 – Properties of the conventional antibiotics.

Name	Chemical structure	Molecular weight (g/mol)
Azithromycin [a]		785.02
Rifampicin [b]		822.94
Streptomycin [a]		728.69
Norfloxacin [a]		319.34
Ofloxacin [a]		361.37

[a] Acquired from Sigma-Aldrich. [b] Acquired from EDQM - European Directorate for the Quality of Medicines & Healthcare, Council of Europe.

CQ-cinnamic acid derivatives

Table 2 and **Table 3** show the properties of the CQ-cinnamic acid-derived ILs and covalent compounds, respectively, tested against *M. avium* 2447 SmT. CQ1 is the covalent equivalent of CQ1-IL, CQ2 is the covalent equivalent of CQ2-IL, CQ3 is the covalent equivalent of CQ3-IL and CQ4 is the covalent equivalent of CQ4-IL.

Table 2 – Properties of the CQ-cinnamic acid-derived ionic liquids.

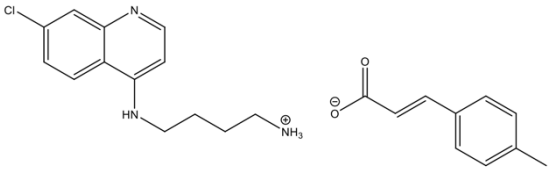
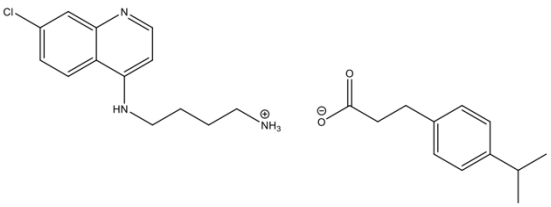
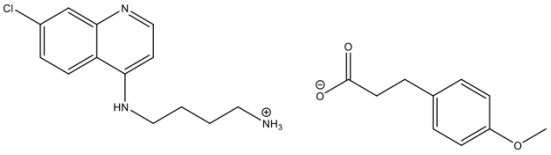
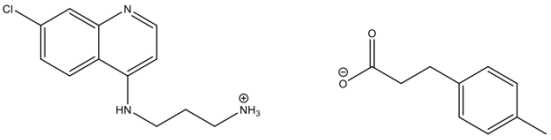
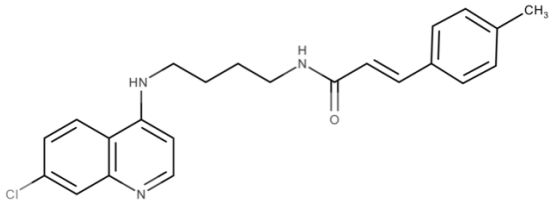
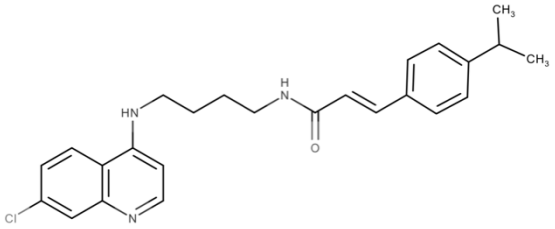
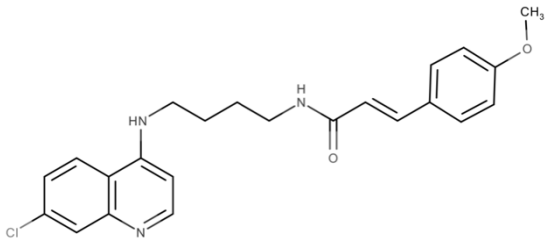
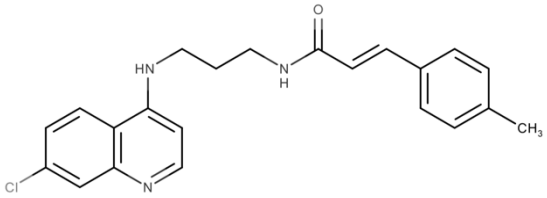
Designation	Chemical structure	Molecular weight (g/mol)
CQ1-IL		441.91
CQ2-IL		441.98
CQ3-IL		429.92
CQ4-IL		399.90

Table 3 – Properties of the CQ-cinnamic acid-derived covalent compounds.

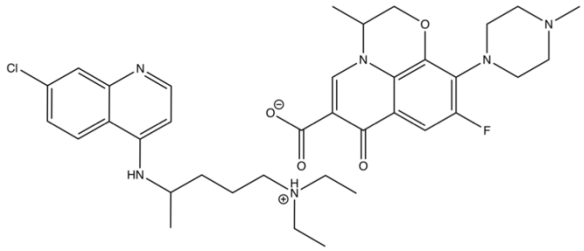
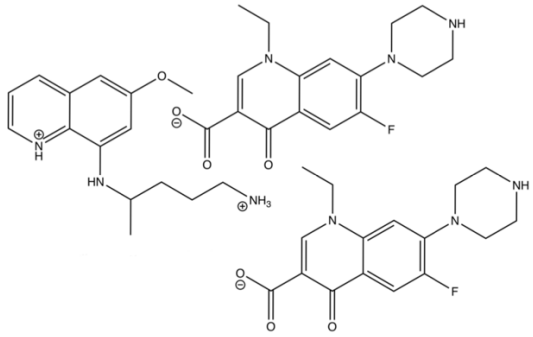
Designation	Chemical structure	Molecular weight (g/mol)	clogP [a]
CQ1		393.92	4.68
CQ2		421.97	5.41
CQ3		409.91	4.01
CQ4		379.89	4.16

[a] Calculated logarithm of the partition coefficient. Calculator Plugins were used for logP values prediction and calculation, Marvin 19.9.0, 2019, ChemAxon (http://www.chemaxon.com).

CQ/PQ-conventional antibiotic derivatives

Table 4 shows the properties of the CQ/PQ-conventional antibiotics-derived ILs tested against *M. avium* 2447 SmT.

Table 4 – Properties of the CQ/PQ-conventional antibiotics derivatives.

Designation	Chemical structure	Molecular weight (g/mol)
CQ5-IL		681.20
PQ1-IL		898.03

Bacteria

M. avium 2447 smooth-transparent variant (SmT) was isolated from the bone marrow of an AIDS patient and was provided by Dr. Françoise Portaels (Institute of Tropical Medicine, Antwerp, Belgium).

M. avium 25291 SmT was isolated from chicken and acquired from ATCC (American Type Culture Collection).

M. avium 2-151 variants SmT and SmOp (smooth-opaque) were isolated from an AIDS patient and were provided by Dr. John Belisle (Colorado State University, Colorado, USA).

Axenic assay

In order to assess the direct antimycobacterial activity of the compounds, the bacteria were grown in liquid culture medium and incubated with increasing concentrations of the different compounds. The viability of the mycobacteria was assessed through resazurin reduction and by Colony Forming Units (CFUs) assay.

An axenic culture of *M. avium* was expanded in Middlebrook 7H9 medium (BD Difco™, Sparks, USA) supplemented with 10% of ADC (albumin-dextrose-catalase). The inoculum was incubated at 37ºC and the bacterial growth was monitored by measuring daily the optical density (OD) at 600 nm of the culture diluted 1:2 in medium. When the exponential phase was reached, the culture was diluted 1:10 in 7H9 medium, with a final concentration of approximately 10^6 CFU/mL.

In a 96-well plate, each compound was diluted successively 1:2 in 7H9/10% ADC. Then, mycobacterial culture was added 1:1 to each well, with a final volume of 200 µL. Wells containing only medium and wells without treatment were also included. Each condition was tested in triplicates.

The plate was incubated at 37ºC for six days. The antimycobacterial activity of the compounds was assessed by resazurin reduction. A solution of resazurin phosphate-buffered saline (PBS) 1× (2.5 mM) was prepared and 10% (v/v) was added to each well. After 24 hours of incubation at 37ºC, the fluorescence of resorufin, resulting from the conversion of resazurin by metabolically active cells, was measured at λ_{ex} = 530 nm and λ_{em} = 590 nm in a Synergy™ Mx microplate reader using the software Gen5.

From the results obtained by resazurin reduction, the value of IC_{50} (concentration of the compound that inhibits by 50% the mycobacterial viability) was calculated for each condition. For that, GraphPad Prism 8 (GraphPad Software, LLC) software was used. The experimental values were subjected to a 4 parameters logistic sigmoidal regression and the values of IC_{50} were obtained by interpolation of the curve.

On the seventh day of incubation, the viability of *M. avium* was also evaluated by colony forming units (CFUs) assay, using the same wells as the resazurin assay. The amount of bacteria incubated with the compounds was also determined by this method on day 0. The bacterial suspension in each well was serially diluted (1:10) in water containing 0.05% of Tween-80 and plated in Middlebrook 7H10 agar medium (BD Difco™, Sparks, USA) supplemented with 10% of oleic acid-albumin-dextrose-catalase (OADC), for 7 days at 37ºC.

Bone marrow macrophages (BMM)

Macrophages were derived from the bone marrow of BALB/c or C57BL/6 mice bred at the i3S animal facility. Macrophages derived from the former strain of mice were used for experiments involving the CQ-cinnamic acid derivatives, while the macrophages derived from the latter strain of mice were used for experiments involving the CQ/PQ-conventional antibiotics derivatives and their parental antibiotics. Each strain was used according to momentary convenience; no differences are expected to result from the use of BMM from these two strains.

The animal was euthanized by CO_2 inhalation and its femurs and tibias removed. Each bone was flushed with Hank's Balanced Salt Solution (HBSS, Gibco, Paisley, U.K.). The resulting cell suspension was centrifuged for 10 minutes at 259 g, 4ºC, and re-suspended with Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Paisley, U.K.) supplemented with 10 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 10% Fetal Bovine Serum (FBS, Biowest, France), and 10% of L929 cell conditioned medium (LCCM) as a source of Macrophage Colony Stimulating Factor (M-CSF).

Cells were cultured for at least 4 hours at 37ºC in a 7% CO_2 atmosphere to remove fibroblasts. Non-adherent cells were collected and the plate was washed three times with cold HBSS. The cellular suspension was centrifuged at 259 g, 4ºC for 10 minutes, re-suspended with DMEM and the number of cells counted using a Neubauer chamber. The cell concentration was adjusted to 4×10^5 cells/mL with supplemented DMEM/10% LCCM and the cell suspension was plated on 24-well plates (1 mL per well) or on 96-well plates (200 µL per well).

The plates were incubated at 37ºC in a 7% CO_2 atmosphere and, four days after seeding, 10% of LCCM was added to the culture medium. On the seventh day, the culture medium was renewed.

On the day 10 of culture, the cells are considered fully differentiated into macrophages. The culture medium was removed and the adherent cells were incubated with a bacterial suspension of *M. avium* 2447 SmT at 10^6 CFU/mL, previously prepared in supplemented DMEM, for 4 hours at 37ºC in a 7% CO_2 atmosphere. After incubation, cells were washed four times with warm HBSS to remove non-internalized bacteria and re-incubated with new supplemented DMEM/10% LCCM with or without different concentrations of each compound. Each condition was tested in triplicates.

The viability of macrophages cultured in 96-well plates was determined by resazurin reduction. Four days after infection and treatment with compounds, a solution of resazurin in PBS 1× (1.25 mM) was prepared and 10% was added to each well. After 24 hours of incubation at 37ºC, the fluorescence of resorufin, resulting from the conversion of resazurin by metabolic active cells, was measured at λ_{ex} = 530 nm and λ_{em} = 590 nm in a Synergy™ Mx microplate reader using the software Gen5.

From the results obtained by resazurin reduction, the value of IC_{50} (concentration of the compound that inhibits by 50% the macrophage viability) was calculated to each condition. For that, GraphPad Prism 8 (GraphPad Software, LLC) software was used. The experimental values were subjected to a 4 parameters logistic sigmoidal regression and the values of IC_{50} were obtained by interpolation of the curve.

The intracellular growth of *M. avium* 2447 SmT was evaluated 5 days after infection, by CFUs assay. The macrophages in 24-well plates were lysed with 0.1% saponin. The bacterial suspension was serially diluted in water containing 0.05% of Tween-80 and plated in Middlebrook 7H10 agar medium supplemented with 10% of OADC. The number of colonies was counted after 7 days at 37ºC and compared with the number of colonies present at time zero. The difference, in terms of log10 CFU/mL between the last and the first day of incubation was designated “log increase”.

Optical microscopy

To evaluate the toxicity and the solubility of the compounds, 6 days after incubation, in the case of axenic assays, or after 4 days, in the case of BMM assays, images of bright field optical microscopy of each well were obtained using a Leica DMI6000 (Leica Microsystems, Germany) microscope and a Hamamatsu FLASH4.0 (Hamamatsu, Japan) camera. The software used was LAS X. The equipment and software belonged to the Advanced Light Microscopy scientific platform at i3S.

Results

Effect of conventional antibiotics against *M. avium*

In order to understand the susceptibility of the *M. avium* strains we have in the laboratory to antibiotics that are currently used to treat NTM infections, we performed axenic assays with four different strains: *M. avium* 2447 SmT, *M. avium* 25291 SmT and *M. avium* 2-151 (variants SmOp and SmT). Each strain was incubated with increasing concentrations of three antibiotics: azithromycin, rifampicin and streptomycin, and the mycobacterial viability was assessed by resazurin reduction. The antimycobacterial effect of azithromycin was more accentuated against *M. avium* 2447 SmT (**Figure 4A**) and 2-151 SmOp (**Figure 4C**) than against the other two strains.

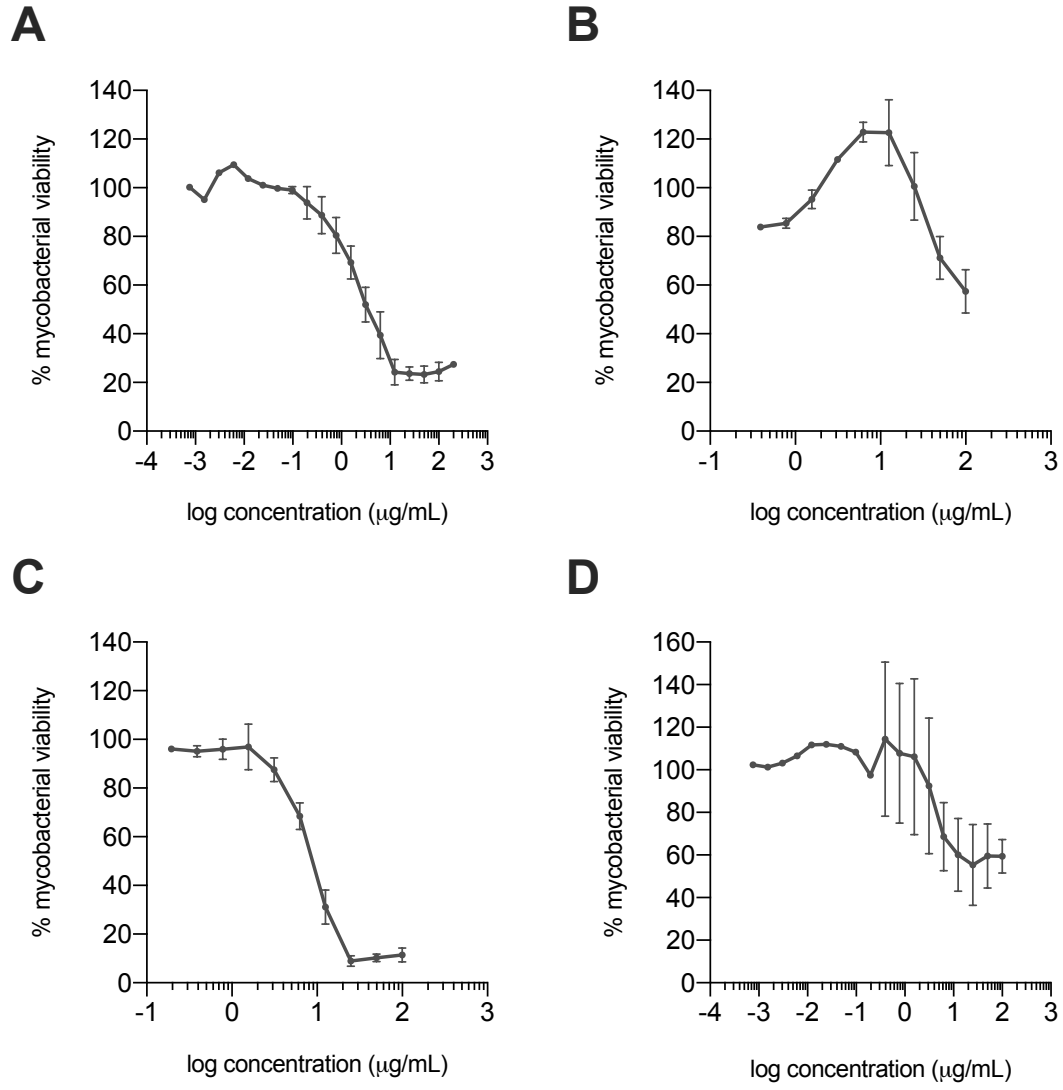


Figure 4 – Antimycobacterial activity of azithromycin. *M. avium* 2447 SmT **(A)**, *M. avium* 25291 SmT **(B)**, *M. avium* 2-151 SmOp **(C)** or *M. avium* 2-151 SmT **(D)** was incubated with increasing concentrations of the antibiotic azithromycin, for 7 days at 37 °C. Mycobacterial viability was assessed through resazurin reduction. The graph shows the averages ± standard deviations of three **(A)** or two **(B-D)** independent experiments, presented as percentages of viable mycobacteria relative to the number of corresponding non-treated mycobacteria.

Rifampicin caused a significant decrease in the viability of *M. avium* 2447 SmT (**Figure 5A**) and the two variants of *M. avium* 2-151 (**Figure 5C-D**). However, against *M. avium* 25291 SmT (**Figure 5B**), this antibiotic does not seem to have an inhibitory effect.

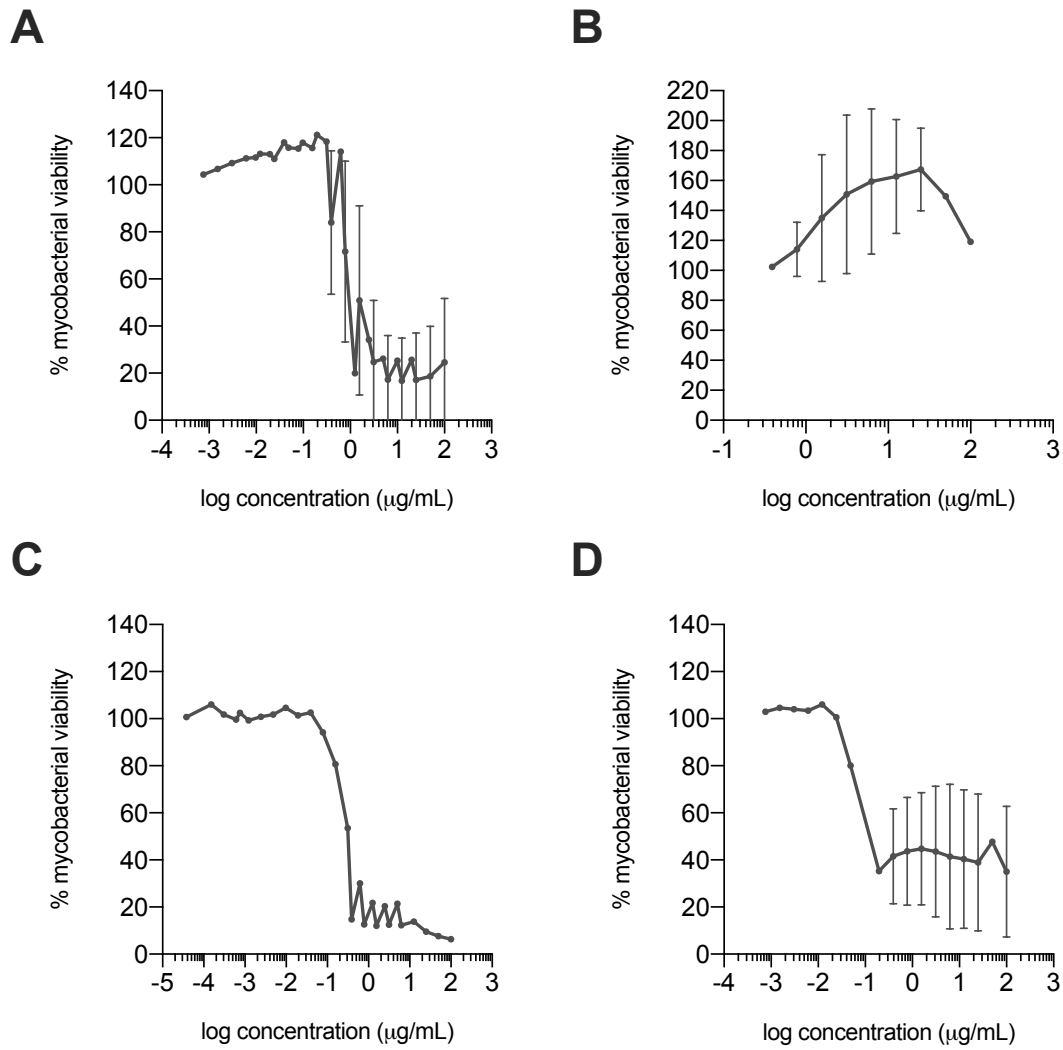


Figure 5 – Antimycobacterial activity of rifampicin. *M. avium* 2447 SmT **(A)**, *M. avium* 25291 SmT **(B)**, *M. avium* 2-151 SmOp **(C)** or *M. avium* 2-151 SmT **(D)** was incubated with increasing concentrations of the antibiotic rifampicin, for 7 days at 37 °C. Mycobacterial viability was assessed through resazurin reduction. The graph shows the averages ± standard deviations of three **(A)** or two **(B-D)** independent experiments, presented as percentages of viable mycobacteria relative to the number of corresponding non-treated mycobacteria.

Streptomycin was more effective against the strains 2447 SmT (**Figure 6A**) and 2-151 SmOp (**Figure 6C**). *M. avium* 25291 SmT was the strain less susceptible to this antibiotic, with its viability not decreasing below 60% at the higher concentration of the drug (100 μg/mL) (**Figure 6B**).

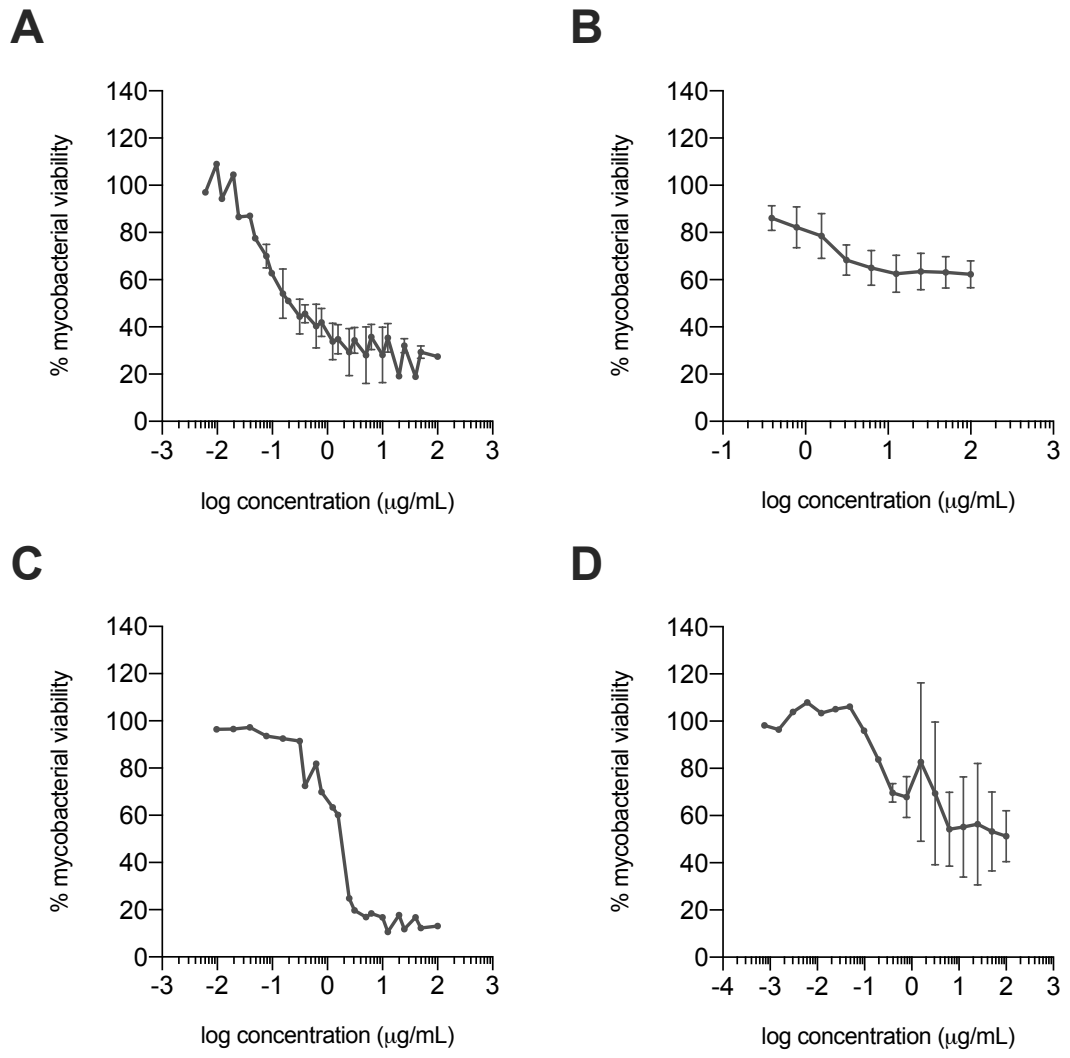


Figure 6 – Antimycobacterial activity of streptomycin. *M. avium* 2447 SmT **(A)**, *M. avium* 25291 SmT **(B)**, *M. avium* 2-151 SmOp **(C)** or *M. avium* 2-151 SmT **(D)** were incubated with increasing concentrations of the antibiotic streptomycin, for 7 days at 37 °C. Mycobacterial viability was assessed through resazurin reduction. The graph shows the averages ± standard deviations of three **(A)** or two **(B-D)** independent experiments, presented as percentages of viable mycobacteria relative to the number of corresponding non-treated mycobacteria.

Two additional conventional antibiotics, norfloxacin and ofloxacin, were tested against *M. avium* 2447 SmT (**Figure 7**). This strain was susceptible to both antibiotics, reducing the viability of the mycobacteria around 80% at the highest concentration tested.

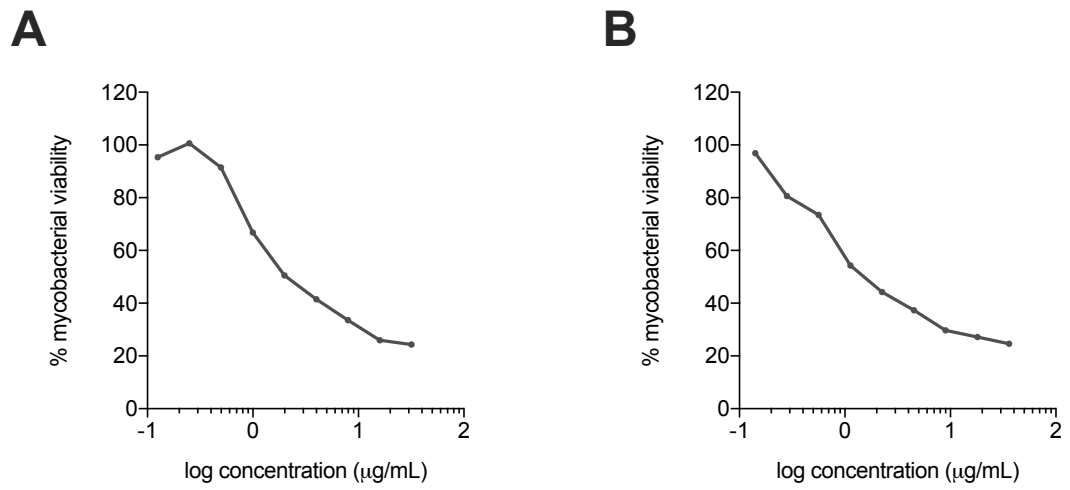


Figure 7 – Antimycobacterial activity of norfloxacin and ofloxacin. *M. avium* 2447 SmT was incubated with increasing concentrations of the antibiotics norfloxacin **(A)** and ofloxacin **(B)** for 7 days at 37 °C. Mycobacterial viability was assessed through resazurin reduction. The graph shows the results of one experiment, presented as percentages of viable mycobacteria relative to the number of corresponding non-treated mycobacteria.

Table 5 summarizes the activity of the antibiotics tested against the different strains of *M. avium*. For some cases it was not possible to calculate the IC_{50}, as the inhibitory effect of the antibiotics was not significant at the highest concentration tested.

Table 5 – Antimycobacterial activity of the antibiotics azithromycin, rifampicin, streptomycin, norfloxacin and ofloxacin against different strains of *M. avium*.

Antibiotic	**Activity [a] against different strains *of M. avium***			
	2447 SmT	25291 SmT	2-151 SmOp	2-151 SmT
Azithromycin	4.17 (3.27)	>127 (100)	10.92 (8.57)	>127 (100)
Rifampicin	1.50 (1.23)[b]	>122 (100)	0.33 (0.27)	0.10 (0.08)[b]
Streptomycin	0.31 (0.24)	>137 (100)	2.10 (1.53)	>137 (100)
Norfloxacin	4.10 (1.31)	-	-	-
Ofloxacin	2.42 (0.87)	-	-	-

[a] IC_{50} (concentration of the compound that inhibits by 50% the mycobacterial viability) in μM (in parenthesis μg/mL); [b] $R^2<0.9$

All together, these results reveal a high variability in the susceptibility to conventional antibiotics amongst the various strains of *M. avium*. Strains like *M. avium* 2447 SmT seem to be susceptible to all the antibiotics tested, but the same drugs do not look effective against *M. avium* 25291 SmT. Even between the same strain, as is the case of *M. avium* 2-151, different variants have different susceptibilities to the tested antibiotics.

It is important to understand the behavior of these strains when looking for new alternatives to the established treatments to NTM infections. However, for the purpose of this thesis, we proceeded with the testing of new compounds on strain 2447 SmT.

Effect of CQ-based ionic liquids on *M. avium* 2447 SmT

Effect of CQ-cinnamic acid derivatives against axenic cultures of M. avium

The fact that with the technology of ILs it is possible to conjugate CQ with other active molecules in the same drug, conserving both activities and at the same time bypassing pharmacological problems, caught our attention. Our first approach was to test ILs based on CQ, conjugated with differently substituted cinnamoyl groups.

To test the direct antimycobacterial activity of the CQ-cinnamic acid-derived ILs and their respective covalent compounds we performed axenic assays. *M. avium* 2447 SmT was incubated with increasing concentrations of each compound and the mycobacterial viability was assessed by resazurin reduction. Our results show that for most pairs of covalent-IL compounds, the covalent compound had higher direct inhibitory effect against *M. avium* than its equivalent ionic liquid (**Figure 8A,B,C**). The erratic behavior of the covalent compounds could be explained by their precipitation in contact with the culture medium, visible by optical microscopy (**Figure 9**). Nevertheless, we can conclude that all compounds, either covalent or ILs, do not show a significant activity against *M. avium* 2447 SmT, with viability not decreasing below 60% for most of the cases. These results were confirmed by colony forming units (CFUs) assay (data not shown).

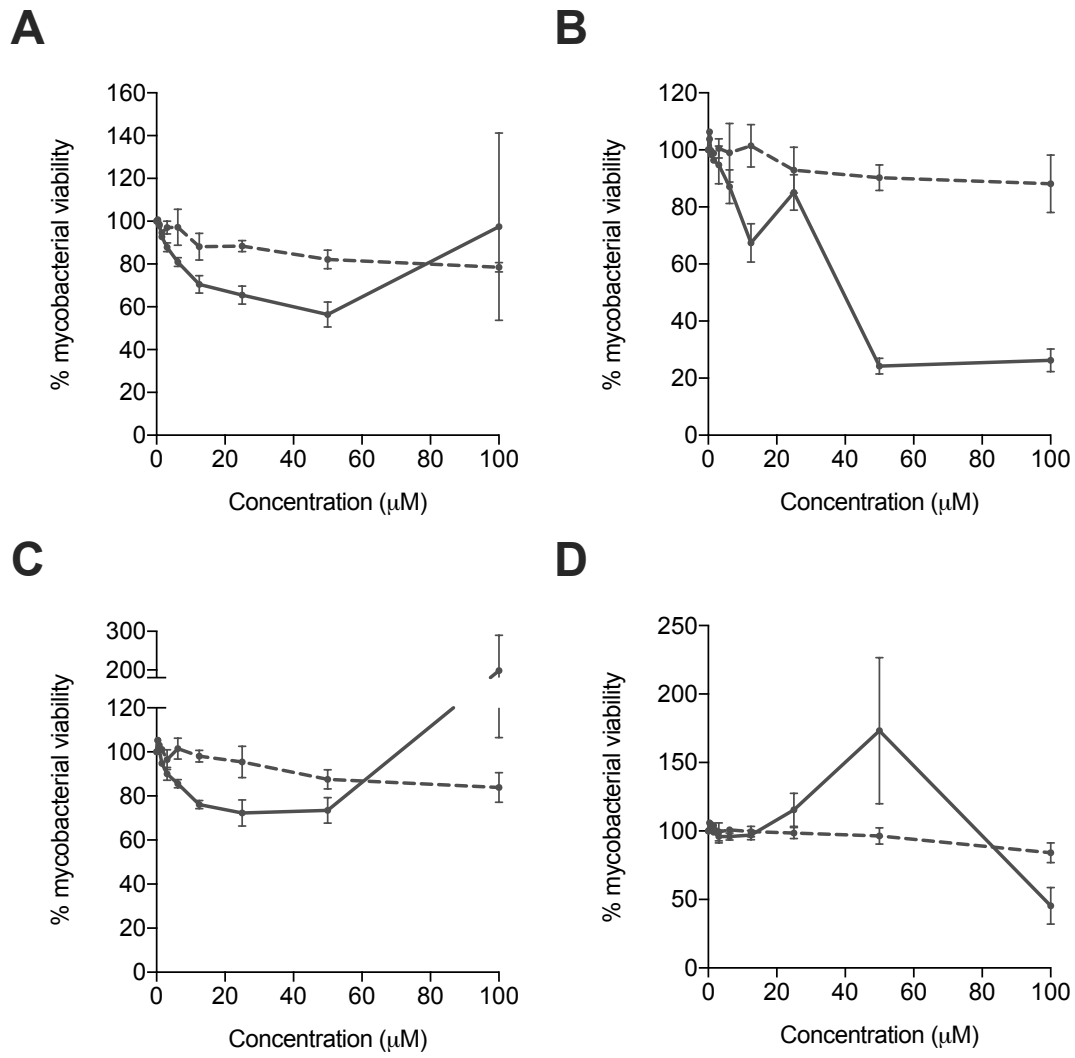


Figure 8 – Direct antimycobacterial activity of CQ-cinnamic acid derivatives. *M. avium* 2447 SmT was incubated with increasing concentrations of the covalent compounds (full line) CQ1 **(A)**, CQ2 **(B)**, CQ3 **(C)** or CQ4 **(D)** or its correspondent ionic liquids (dashed line) CQ1-IL **(A)**, CQ2-IL **(B)**, CQ3-IL **(C)** or CQ4-IL **(D),** for 7 days at 37 °C. Mycobacterial viability was assessed through resazurin reduction. The graph shows the averages ± standard deviations of four independent experiments, presented as percentages of viable mycobacteria relative to the number of corresponding non-treated mycobacteria.

The images obtained by bright field optical microscopy show the precipitation of the covalent compounds in contact with the culture medium in contrast with what happens to the ILs (**Figure 9**). Over a layer of mycobacteria, the covalent compounds form different types of precipitates. In the images of the wells incubated with ILs, only the mycobacterial film is visible, even at concentrations four times higher than the covalent compounds.

These results suggest that the CQ-cinnamic acid-derived ILs do not have an improved antimycobacterial activity, however are more soluble than the covalent compounds, which may be an important advantage.

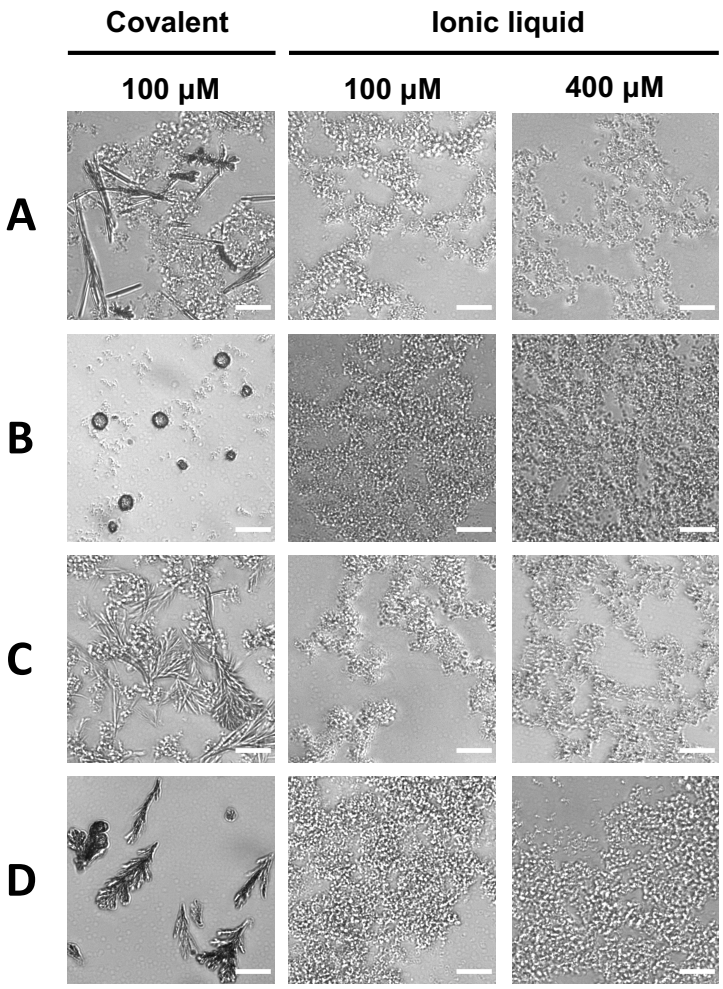


Figure 9 – Covalent compounds are less soluble than their equivalent ionic liquids. *M. avium* 2447 SmT was incubated with the covalent compounds CQ1 **(A)**, CQ2 **(B)**, CQ3 **(C)** or CQ4 **(D)** at 100 µM or its equivalent ionic liquids CQ1-IL **(A)**, CQ2-IL **(B)**, CQ3-IL **(C)** or CQ4-IL **(D),** at 100 or 400 µM. Representative brightfield images were acquired using a Leica DMI6000 (Leica Microsystems, Germany) microscope. Scale bar: 30 µm.

Effect of CQ-cinnamic acid derivatives on M. avium*-infected macrophages*

Since the macrophage is one of the main host cells for *M. avium* upon infection, the next step was to test the CQ-cinnamic acid-derived compounds in a more relevant model. We performed in vitro assays with murine bone marrow derived macrophages (BMM) as a way to study the intramacrophagic antimycobacterial activity of the compounds. But, in first place, to evaluate the toxicity of the compounds to the host cells, BMM infected with *M. avium* 2447 SmT were incubated with increasing concentrations of each compound, and the toxicity was assessed by resazurin reduction (**Figure 10**). The macrophages in contact with the ILs are viable at higher concentrations than the macrophages in contact with each respective covalent

compound. The pair CQ2/CQ2-IL (**Figure 10B**) presents the biggest difference in terms of toxicity to the macrophages between the two types of compounds.

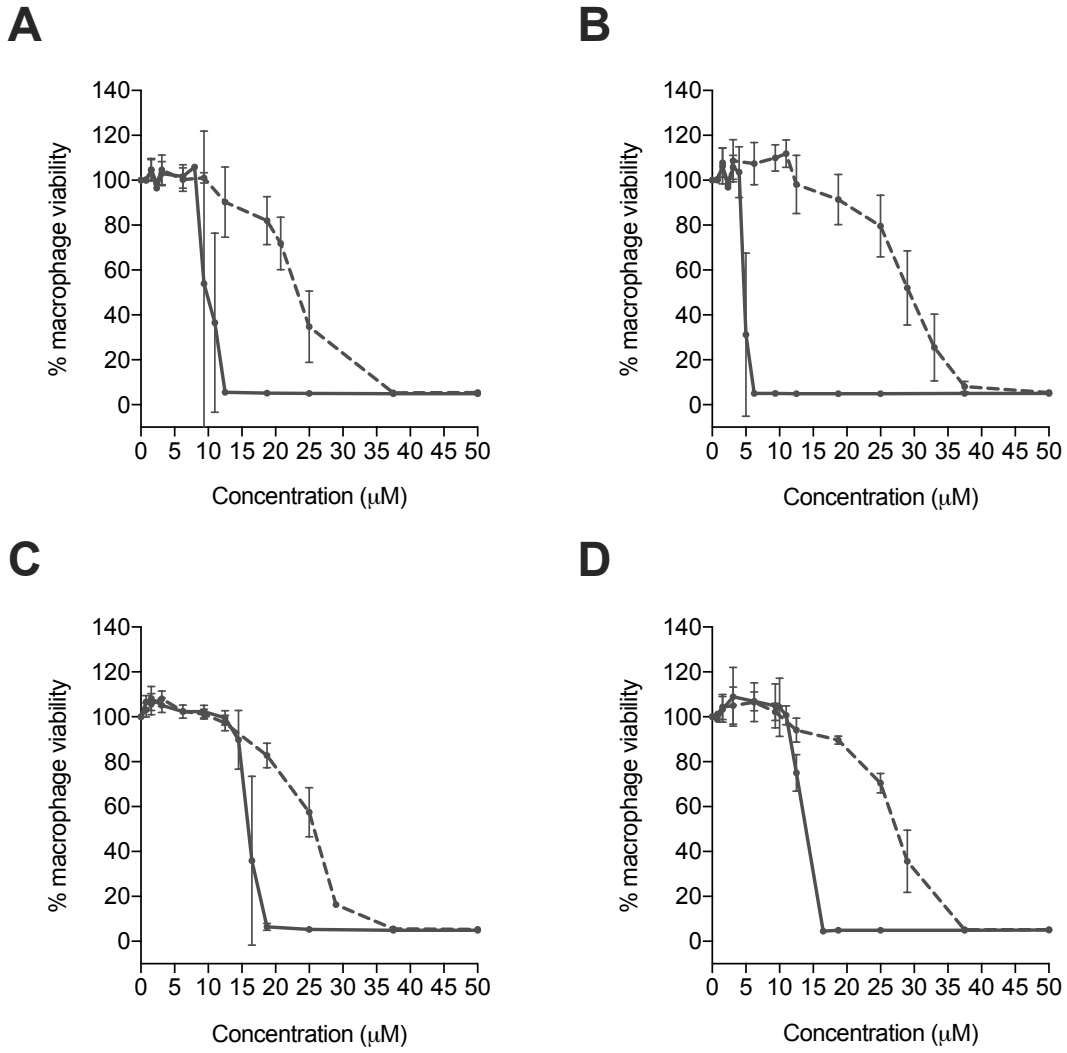


Figure 10 – Toxicity of CQ-cinnamic acid derivatives to host cells. BALB/c BMM infected with *M. avium* 2447 SmT were treated with increasing concentrations of the covalent compound (full line) CQ1 **(A)**, CQ2 **(B)**, CQ3 **(C)** or CQ4 **(D)** or its correspondent ionic liquid (dashed line) CQ1-IL **(A)**, CQ2-IL **(B)**, CQ3-IL **(C)** or CQ4-IL **(D),** for 5 days. Macrophage viability was assessed through resazurin reduction. The graph shows the averages ± standard deviations of three independent experiments, presented as percentages of viable macrophages relative to the number of corresponding non-treated macrophages.

Similar assays were performed with non-infected BMM. **Table 6** shows the calculated IC_{50} of each compound when in contact with infected or non-infected BMM. The non-infected macrophages seem to be more resistant to both ILs and covalent compounds than the infected macrophages.

Table 6 – Toxicity of the covalent compounds CQ1, CQ2, CQ3 and CQ4 and of the ionic liquids CQ1-IL, CQ2-IL, CQ3-IL and CQ4-IL against bone marrow derived macrophages (BMM).

Condition	Toxicity [a] of the compound to BMM							
	CQ1	CQ1-IL	CQ2	CQ2-IL	CQ3	CQ3-IL	CQ4	CQ4-IL
Infected	9.88 (3.89)	23.07 (10.19)	4.84 (2.04)	28.90 (12.77)	16.00 (6.56)	24.83 (10.67)	13.24 (5.03)	27.23 (10.89)
Non-infected	13.96 (5.50)	27.10 (11.98)	7.02 (2.96)	35.16 (15.54)	22.44 (9.20)	25.88 (10.70)	16.63 (6.32)	37.67 (15.06)

[a] IC_{50} (concentration of the compound that inhibits by 50% the macrophage viability) in μM (in parenthesis μg/mL).

Images obtained by bright field optical microscopy show that the covalent compounds precipitate in contact with the culture medium at 100 μM, which does not happen with the ILs. **Figure 11** shows representative images of infected BMM treated either with the covalent compound CQ2 or its equivalent ionic liquid CQ2-IL. At the higher concentration (100 μM) (**Figure 11A**), both compounds are toxic for the host cells, but the precipitate of the covalent compound is visible over the layer of dead macrophages, which does not happen with the IL. These images also confirm that the macrophages are viable at higher concentrations of the IL (18.75 μM) than of covalent compound (3.23 μM) (**Figure 11B**). This pattern is similar for the other 3 pairs of compounds (data not shown).

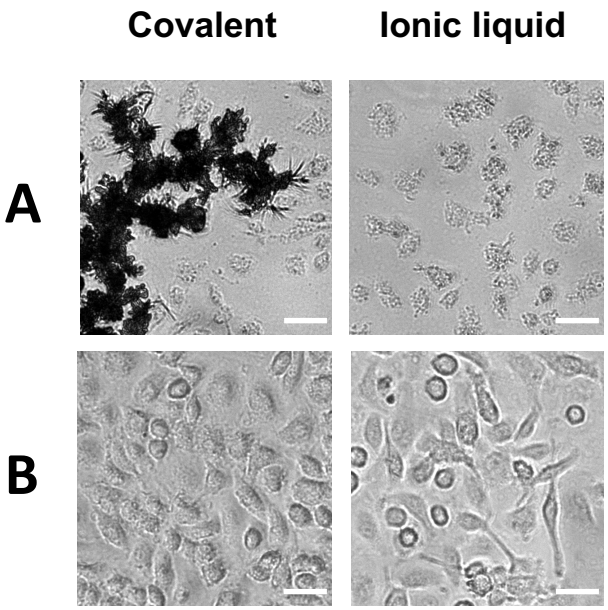


Figure 11 – Ionic liquid CQ2-IL is more soluble and less toxic to BMM than the covalent compound CQ2. BALB/c BMM infected with *M. avium* 2447 SmT were incubated with the covalent compound CQ2 or its equivalent ionic liquid CQ2-IL at the highest concentration (100 μM) **(A)** or at the highest concentration where cells are viable, 3.23 μM for the covalent compound and 18.75 μM for the ionic liquid **(B)**. Representative brightfield images were acquired using a Leica DMI6000 (Leica Microsystems, Germany) microscope. Scale bar: 30 μm.

To test the activity of these compounds against the bacteria inside the macrophages we performed CFUs assays. We evaluated the effect of the compounds at two concentrations that showed to be non-toxic to the macrophages. At the higher concentration the compounds were only administrated once, and at the lower concentration they were added every other day in a total of three administrations. We did not observe a significant inhibition of the mycobacterial growth by any of the compounds tested, IL or covalent (data not shown).

These results suggest that the CQ-cinnamic acid-derived ILs have no activity against intramacrophagic *M. avium*, however they are less toxic and more soluble than their covalent equivalents.

Effect of CQ/PQ-conventional antibiotics derivatives on M. avium *viability in axenic culture*

Since the CQ-cinnamic acid derivatives did not show the desired activity against *M. avium*, we decided to focus on ILs based on conventional molecules used to treat infections by NTM. We saw that depending on the strain, *M. avium* behaves differently to different antibiotics. *M. avium* 2447 SmT is a strain of intermediate virulence that showed to be susceptible to all the antibiotics tested (**Figure 4-7**).

As a first approach in this new phase, we tested the direct antimycobacterial activity of a CQ-conventional antibiotic-derived IL. This IL, CQ5-IL, is based on the molecule of ofloxacin. We also compared its activity with an IL based on the conjugation of another antimalarial, primaquine (PQ), and the antibiotic norfloxacin, denominated PQ1-IL.

We performed axenic assays in which *M. avium* 2447 SmT was incubated with increasing concentrations of each compound and the mycobacterial viability was assessed by resazurin reduction. Both PQ1-IL (**Figure 12A**) and CQ5-IL (**Figure 12B**) have a similar inhibitory effect on the viability of *M. avium* 2447 SmT. The IC_{50} calculated for each IL was 2.08 μM (1.87 μg/mL) for PQ1-IL and 2.70 μM (1.84 μg/mL) for CQ5-IL. These results were confirmed by colony formation units (CFUs) assay (data not shown).

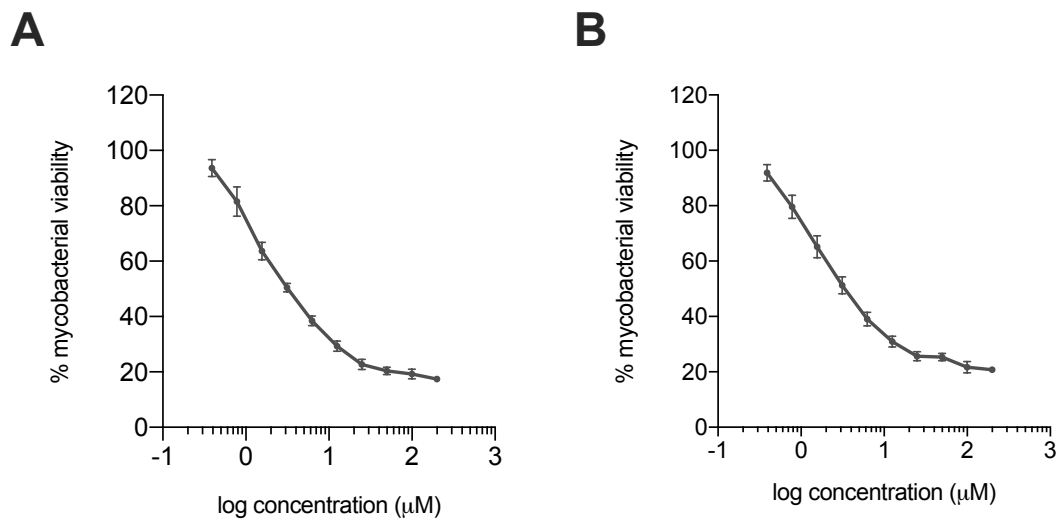


Figure 12 – Antimycobacterial activity of CQ/PQ-conventional antibiotics derivatives. *M. avium* 2447 SmT was incubated with increasing concentrations of the ionic liquids PQ1-IL **(A)** and CQ5-IL **(B)** for 7 days at 37 °C. Mycobacterial viability was assessed through resazurin reduction. The graph shows the averages ± standard deviations of four independent experiments, presented as percentages of viable mycobacteria relative to the number of corresponding non-treated mycobacteria.

The ILs based on conventional antibiotics present a better direct activity against *M. avium* than the CQ-cinnamic acid-derived ILs (**Figure 8**). Comparing with their parental antibiotics (**Figure 7 and Table 5**), the activity of CQ5-IL (IC_{50} = 2.70 μM) is very similar to the activity of ofloxacin (IC_{50} = 2.42 μM). PQ1-IL is slightly more active (IC_{50} = 2.08 μM) than norfloxacin (IC_{50} = 4.10 μM). Overall, in terms of extracellular action against *M. avium*, these ILs do not present an advantage compared to the already clinically approved compounds.

Effect of CQ-conventional antibiotics derivatives on M. avium*-infected macrophages*

To test the toxicity of the CQ/PQ-conventional antibiotic-derived ILs for the host cell and evaluate the intramacrophagic antimycobacterial activity of the compounds, we performed in vitro assays with infection of BMM with *M. avium* 2447 SmT. In order to compare the ILs with their parental drugs we also tested the toxicity and intramacrophagic activity of the antibiotics norfloxacin and ofloxacin. **Figure 13** presents the toxicity of PQ1-IL (IC_{50} = 15.74 μM) (**Figure 13A**) and CQ5-IL (IC_{50} = 20.65 μM) (**Figure 13B**) to the host cells. Both parental antibiotics were not toxic to the macrophages at any concentration tested (1 to 100 μM) (data not shown).

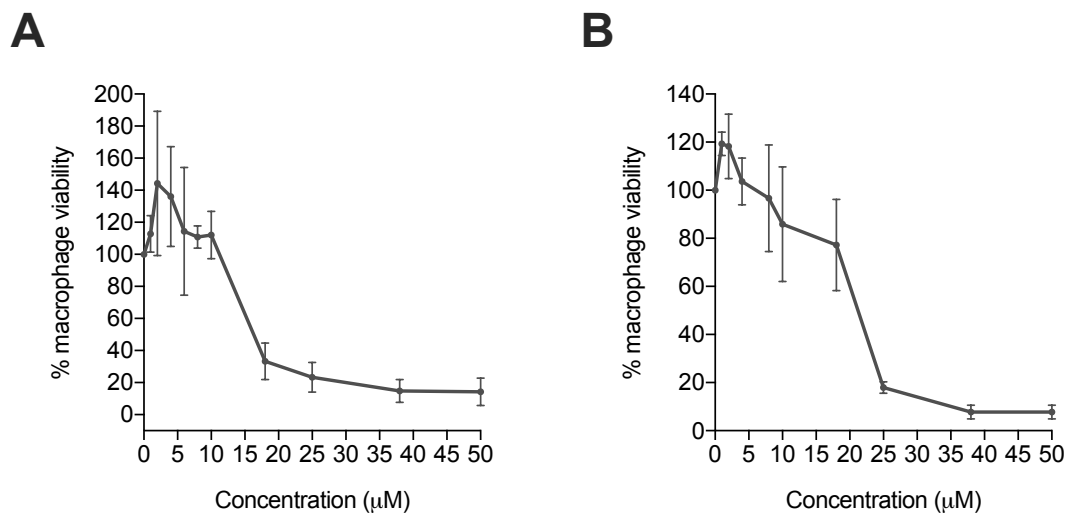


Figure 13 – Toxicity of CQ/PQ-conventional antibiotic-derived ILs to host cells. C57BL/6 BMM infected with *M. avium* 2447 SmT were treated with increasing concentrations of the ionic liquids PQ1-IL **(A)** and CQ5-IL **(B)**, for 5 days. Macrophage viability was assessed through resazurin reduction. The graph shows the averages ± standard deviations of three independent experiments, presented as percentages of viable macrophages relative to the number of corresponding non-treated macrophages.

To test the activity of these compounds against the bacteria inside the macrophages we performed CFUs assays. We evaluated the effect of the compounds at three concentrations that showed to be non-toxic to the macrophages (**Figure 14**). The ILs seem to be more active than the parental antibiotics. At the concentrations tested, the fluoroquinolones do not show significant activity against the intracellular growth of *M. avium*. However, as they are not cytotoxic, higher concentrations may be more effective without causing damage to the host cells. At the same time, these results suggest that by opting for the ILs instead of the conventional antibiotics, it can be possible to reduce the concentration administered.

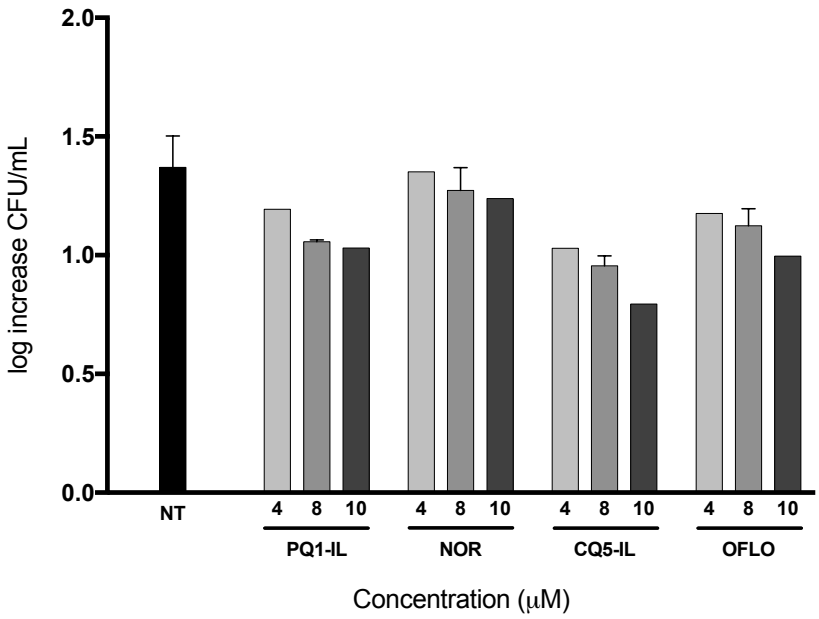


Figure 14 – Intramacrophagic activity of CQ/PQ-conventional antibiotic-derived ILs and their parental antibiotics. C57BL/6 BMM infected with *M. avium* 2447 SmT were treated with 4, 8 and 10 μM of PQ1-IL, CQ5-IL, norfloxacin (NOR) or ofloxacin (OFLO), for 5 days. The intramacrophagic bacterial loads were quantified by CFUs assay. The graph shows the averages ± standard deviations of two independent experiments, presented as the difference, in terms of log10 CFU/mL between day 5 and day 0 of incubation (log increase).

Discussion

One of the main issues of trying to find new solutions to treat *M. avium* related diseases is the great variability of susceptibilities that each strain has to the treatments. We tested five antibiotics that are currently used in the clinic against three different strains of *M. avium* and the results could not be more diverse. Azithromycin, a macrolide that is the base of the treatment of infections by slow-growing NTM [12], was able to significantly inhibit the viability of two strains of intermediate virulence, *M. avium* 2447 SmT and *M. avium* 2-151 SmOp [14]. However, its effect against *M. avium* 25291 SmT, a strain of higher virulence [14], was much more modest. The IC_{50} of azithromycin, the concentration that inhibits 50% of the mycobacterial viability, was 3.27 µg/mL for *M. avium* 2447 SmT and 8.57 µg/mL for *M. avium* 2-151 SmOp, which are consistent with reported values for MAC isolates [49].

Rifampicin is an antibiotic that was added to the regimen when the problem of macrolide-resistance emerged [27]. Together with a macrolide and ethambutol, the three constitute the recommended MAC therapy [29]. We observed that, like what happened with the macrolide, the *M. avium* strains of intermediate virulence, 2447 SmT and the two variants of 2-151, were susceptible to rifampicin, but the same was not visible for *M. avium* 25291 SmT. This strain seemed even to increase its viability in the presence of the antibiotic. However, what seems an increase in viability could only mean that the bacteria were experiencing a faster metabolism, as cells metabolically more active reduce more resazurin. Heifets [50] tested the susceptibility of several *M. avium* strains and found that the majority (70.9%) were moderately susceptible to rifampicin, with a MIC varying between 1 and 4 µg/mL. Our IC_{50} value for *M. avium* 2447 SmT (1.23 µg/mL) fits in that interval. The IC_{50} for *M. avium* 2-151 SmOp (0.27 µg/mL) fits in the “susceptible” category of that study (MIC $\leq$ 0.5 µg/mL), like 19.4% of the MAC strains studied.

In more severe or specific cases of MAC disease, a fourth antibiotic is often added to the treatment, as is the case of aminoglycosides like streptomycin [30]. In this work, this antibiotic seems to be more active against *M. avium* 2447 SmT and *M. avium* 2-151 SmOp than against *M. avium* 25291 SmT and *M. avium* 2-151 SmT. Heifets later assessed the susceptibility of *M. avium* strains, isolated from patients with or without AIDS, to injectable antibiotics like streptomycin [51], concluding that the majority of isolates from both groups of patients fitted between a MIC range of 4 to 6 µg/mL. Our *M. avium* 2447 SmT and *M. avium* 2-151 SmOp seem to be more susceptible to streptomycin, as the IC_{50} for both strains is lower than 2 µg/mL.

We additionally tested two fluoroquinolones, norfloxacin and ofloxacin, against *M. avium* 2447 SmT. Like the aminoglycosides, fluoroquinolones are sometimes joined to the treatment in cases of severe and resistant MAC disease [27]. It was not possible

to test these fluoroquinolones against the other strains of *M. avium*, however we can observe that *M. avium* 2447 SmT was susceptible to these antibiotics in a concentration-depending way, similarly to what happened with the other antibiotics. The IC_{50} that we obtained for ofloxacin was 1.61 μg/mL, which is lower than the IC_{50} determined by Vacher *et al.* against 41 MAC isolates (8 μg/mL) [52]. In our work, norfloxacin also seems to be more active (IC_{50} = 2.17 μg/mL) than Gay *et al.* reported against 20 MAC isolates (IC_{50} = 16 μg/mL) [53].

Altogether, these results confirm the different behavior of each strain of *M. avium* in the presence of the same antibiotic. This work must be optimized, testing more clinically approved antibiotics and evaluating the strain's susceptibility using more accurate methods, like CFUs assays. The great variability of susceptibilities inside the same species is a factor that challenges the research with *M. avium*, as all the different strains must be taken into account to have a better representation of the population. We can infer from our results that a regimen of mono-therapy would not be the most effective, and thus, not recommended. A combined therapy of antibiotics with different mechanisms of action, besides avoiding the development of resistances, increases the probability of success of the treatment, mainly when the strain is not previously identified. Establishing a method of diagnosis including the identification of the strain before the patient starts the therapy would be very useful and important to ensure the success of the treatment.

It was important for us to have a general idea of the susceptibility of *M. avium* to conventional antibiotics, to help identify interesting and relevant structures that can be applied in new compounds with potential action against *M. avium*. The strategy of using ionic liquids to overcome problems of solubility or toxicity to the host cells and, at the same time, improve the activity of the original drugs [47] is very promising and tempting.

We started by testing ILs that were synthetized having as base the molecule of CQ. The multi-versed properties of CQ, an antimalarial with activity against HIV, that has also an inhibitory effect in the viability of *M. avium* [35], brought interest to this molecule when considering a new strategy to combinatory therapies. The covalent conjugation of CQ with differently substituted cinnamoyl groups enhanced the activity of CQ against different parasites [43-45]. Therefore, it was interesting to test these compounds against *M. avium* and, at the same time, evaluate their activity in the form of IL. It was visible that *M. avium* 2447 SmT was a strain very consistent in its susceptibility to all the antibiotics tested. We decided, then, to test the new compounds against this strain of *M. avium*.

In terms of direct activity against *M. avium*, we observed that the ILs present a low inhibitory effect, which did not allow us to calculate their IC_{50}. The covalent compounds present a very erratic behavior. We confirmed by optical microscopy that the covalent compounds precipitate in contact with the culture medium, which could interfere with the fluorescence measure. Thus, for the covalent compounds, this quantification may not translate the real viability of the bacteria. The ILs had a much more constant behavior, as they do not precipitate in contact with the culture medium even when four times more concentrated than the covalent compounds. Thus, we can conclude that the CQ-cinnamic acid-derived ILs are not more active against *M. avium* than their covalent equivalents, however they are more soluble, which confirms the ability of these compounds to overcome pharmacological issues of their drugs of origin.

Next, it was important to evaluate the effect of the CQ-cinnamic acid derivatives against *M. avium* inside their host cells, the macrophages. One important factor in the development of new compounds is the selectivity towards the pathogen of interest, meaning, they should have a high antimicrobial activity, while at the same time not being toxic for the host. Comparing the toxicity of the ILs with the covalent compounds, we can see that all the ILs are cytotoxic at higher concentrations than the respective covalent compound. Again, by optical microscopy it is possible to observe that the covalent compounds precipitate, which does not happen with the ILs. Regarding their intramacrophagic antimicrobial activity, none of the compounds, covalent or ionic liquid, showed a significant inhibition of the mycobacterial growth, even when administered more than once. Thus, the ILs are more soluble and less toxic to the macrophages, however they are not more active than the equivalent covalent compounds.

These CQ-cinnamic acid-derived ILs have not yet been tested against any microorganism, so it is not possible to compare our results with results previously described in literature. Some of their covalent equivalents have been tested against two parasites, *Plasmodium falciparum* [42, 43], that causes malaria, and *Leishmania infantum* [44], and the fungus *Pneumocystis jirovecii* [45]. The life cycle of the malaria parasite inside a human host comprises two different stages: a silent liver stage with no manifestation of disease and a clinically symptomatic blood stage with invasion of erythrocytes [54]. CQ is only active against the blood-stage malaria. However, when studying these compounds against *P. falciparum*, it was visible that CQ-cinnamic acid conjugates display high in vitro potency against both blood and liver stage parasites and showed actually higher activity in vitro against erythrocytic parasites than CQ itself [42, 43]. The performance of these covalent compounds against the malaria parasite in vivo was more modest and some of them were highly toxic to the infected mice [43]. The high lipophilicity (logP values around 5, see Table 3) of these compounds can

explain those issues, as this factor is related to extensive binding to plasma proteins and to accumulation in cellular membranes with concomitant damage. Actually, from the compounds that we tested, CQ2 was the most toxic to mice in that study. In our work, CQ2 was also the covalent compound with higher toxicity to BMM. Contributing to the toxicity of CQ2 are a great number of hydrophobic chemical groups (Table 3), which makes this the compound with highest logP (5.41). On the other hand, CQ3 was considered the compound with better "druglikeness" of the study, as it had a high activity and low toxicity in vivo. Again, CQ3 was also the covalent compound with lower toxicity to BMM, although it does not show activity against *M. avium* in vitro.

Against *Leishmania infantum*, although none of the CQ-cinnamic acid-derived covalent compounds showed better activity than CQ against promastigotes (the form that infects the insect), they all performed better than CQ against amastigotes, which is the intramacrophagic form of the parasite [44]. The authors speculate that CQ is more easily expelled from the amastigote than the cinnamic acid conjugates through species-specific transporters; however, they confirmed by resazurin reduction assay that these conjugates are more cytotoxic to the host cells, and in consequence to the parasite, than CQ, explaining their higher activity. CQ2 was again the most toxic compound to BMM and CQ3 was one of the most active and less cytotoxic compounds [44].

A recent study evaluated the effect of some of the CQ-cinnamic acid-derived covalent compounds against the fungus *Pneumocystis jirovecii*. The compounds showed similar or improved activity than CQ (CQ1 and CQ3 had the higher activities) and most of them did not show cytotoxicity against the mammalian cell lines A549 and L2, with the exception of CQ2 [45]. They also tested the activity of two CQ-cinnamic acid-derived ILs against the fungus, however they were not the same ones as we tested. Nevertheless, those ILs had a similar activity than their covalent counterparts but were much less cytotoxic. Our results also suggest the same relation between ILs and covalent compounds.

The fact that these CQ-cinnamic acid derivatives can effectively inhibit the growth of two parasites with a similar life cycle and an intramacrophagic fungus, but are not active against intracellular bacteria like *M. avium*, given that all of these microorganisms were more or less susceptible to CQ, suggests that a chemical alteration in the synthesis of these conjugates could have changed the mechanism of action of CQ. Also, the addition of a cinnamoyl group that turned the CQ-based compound in a drug with effect against both stages of *P. falciparum*, might have "hidden" a chemical group that was essential for the activity of CQ against *M. avium*. In fact, it has been reported [55] that the tertiary amine in the aliphatic chain of CQ

(Figure 3) is a key factor for the activity of CQ, given the basicity that this chemical group confers. Although afterwards it has been proved that that tertiary amine is not so important, because the CQ-cinnamic acid conjugates have effect against the malaria parasite without it [42, 43], that group could still be important for the activity against mycobacteria. Gressler *et al.* [37] suggested that the weak basicity of CQ is essential for altering the pH of iron containing vesicles, preventing the release of this nutrient into the cell and depriving the bacteria from it, which could be crucial for the antimycobacterial activity of CQ.

The CQ-cinnamic acid-derived compounds did not show direct or intramacrophagic activity against *M. avium*. But the results we obtained with the ILs were promising in terms of bypassing pharmacologic issues such as solubility or toxicity to the host cells. So, we kept the research in the line of the ILs but focusing in new combinations that could be more favorable. In the first part of the work we saw that there is a great variability in the susceptibility of different strains of *M. avium* to conventional antibiotics. Norfloxacin and ofloxacin have a simpler structure than the other conventional antibiotics, which makes them more appealing in a first phase for the synthesis of new ILs. They were conjugated with CQ and PQ having in mind their described activity against *M. avium* [35, 41] and because of the ambition of finding a compound that could work in a combinatory therapy for concomitant diseases. CQ was thus combined with ofloxacin and PQ was combined with norfloxacin. Both CQ/PQ-conventional-derived ILs had a direct inhibitory effect against *M. avium*. While these results are very encouraging, as these new ILs have a much more accentuated effect than the previously tested ILs, we must consider the effect of their parental drugs. The IC_{50} of ofloxacin was 2.42 μM, whereas the IC_{50} of CQ5-IL, the IL based on the molecule of ofloxacin, was 2.70 μM. The fluoroquinolones were only tested once, thus no straightforward conclusions can be taken, but all indicates that the activity of the two compounds is very similar. PQ1-IL, the IL based on the molecule of norfloxacin, had an IC_{50} of 2.08 μM and norfloxacin had an IC_{50} of 4.10 μM. At first glance, the IL seems to be twice as active as the conventional antibiotic. But, as can be seen in Table 4, PQ1-IL was not synthetized equimolarly, as it has 2 molecules of norfloxacin to 1 one of PQ, which could explain the doubled activity of the IL in relation to the parental drug.

The direct activity of these CQ/PQ-conventional-derived ILs in comparison to the CQ-cinnamic acid-derived ILs against *M. avium* is, on the other hand, very significant. If we do not consider that the strength of the compound is all placed in the fluoroquinolone, we can speculate that the former follow a much more advantageous mechanism of action, interacting with targets linked to a better antimycobacterial effect.

It was, however, important to test the CQ/PQ-conventional-derived ILs in a more relevant model. In contact with infected macrophages, the ILs showed to be more toxic than norfloxacin and ofloxacin. Neither of the fluoroquinolones caused macrophage death at the higher concentration tested (100 μM), but the ILs were toxic above 15 to 20 μM. The fact that these ILs are heavier molecules constituted by several heterocyclic aromatic groups from both the anionic and cationic parts could explain the higher cytotoxicity comparing with the parental antibiotics. Nevertheless, the intramacrophagic activity of the ILs is higher than the activity of the fluoroquinolones. This is a very important factor, as it indicates that this new formulation could be of interest for future developments.

The conjugation of fluoroquinolones with CQ in the form of IL is just the first step towards the development of new drugs with the potential to be used against more than one pathogen and, at the same time, be a solution to the emergent problem that is the resistance to antibiotics. In the future, our aim is to optimize the syntheses in order to conjugate more complex molecules, like first line NTM antibiotics, with other active pharmacophores. With those ILs we expect to enhance or at least maintain the activity of their parental drugs, and to improve pharmacological issues that excluded them from the current accepted therapies. Rescuing cheap and safe old drugs is one of the solutions to overcome the scarcity of effective antibiotics and combat the increase in the incidence of some diseases that affect millions of people per year.

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