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LIFE AND ENVIRONMENTAL SCIENCES
***curr*. Biomolecular Sciences**

Investigating the effectiveness of innovative antimicrobial molecules against bacterial species of clinical interest

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Chapter 1 – INTRODUCTION

1.1 Antimicrobial resistance: a long-standing issue

Since their clinical introduction in the 1930s, antibiotics have greatly influenced life on Earth (Rice, 2008). Ironically, antibiotics themselves have facilitated and contributed to the rapid dissemination of antimicrobial resistance (Pendelton *et al*., 2013). Actually, resistance to antimicrobials is a natural process that has been observed since the first antibiotics were discovered. The emergence of antibiotic resistance in bacterial pathogens is an inevitable consequence of antibiotic use and abuse and it has a significant negative impact on the outcome of therapies (French, 2005; Kollef, 2000). The propensity of antibiotic usage to favour the emergence of resistant pathogens is called antibiotic pressure revealing that their misuse promotes the emergence of resistance (Rolain *et al*., 2013). Antimicrobial resistance among the human pathogens arises when microorganisms, which cause infection, survive exposure to drug normally kill or inhibit the growth of bacteria. In absence of competition from other strains, resistant strains can spread, leading to the emergence of the so-called "superbugs", bacteria untreatable with existing antimicrobials (O'Neill, 2016). Antibiotic resistance has increasingly become a serious threat to human health worldwide, with highly resistant pathogens of many difficult to treat species (Walker *et al*., 2009; Redgrave *et al*., 2014). Moreover, during the last few years, there has been a significantly increasing trend of bacterial multidrug resistance in many European countries. Data from European Antimicrobial Resistance Surveillance Network (EARS-net) have highlighted a wide variation in the occurrence of antimicrobial resistance associated with microorganism,

drug and geographical region (EARS-net, 2013). One of the major concern about antimicrobial resistance, consists in the reduced chances of successfully treating such infections and in the resulting increase of morbidity and mortality by common bacterial diseases (Klevens *et al*., 2007, Akova, 2016a). The global spread of antibiotic resistance genes and their acquisition by clinically relevant microorganisms represents a serious problem for the health and welfare of both humans and animals (WHO, 2014). Multiple attempts have been made to quantify the local and global impact of antimicrobial resistance. Infections by resistant bacteria result in greater mortality, morbidity and costs of treatment compared to infections caused by their antimicrobial susceptible counterparts (Howard *et al*., 2003; Tang *et al*., 2014). The mounting inability to prevent infections with antimicrobial prophylaxis will massively and negatively affect the risk equation in performing more complex medical procedures (such as major surgery, cancer chemotherapy or organ/stem cell transplantation) (Tang *et al*., 2014). The growing numbers of antimicrobial-resistant pathogens, which are increasingly associated with nosocomial infections, place a significant burden on healthcare systems and have important global economic costs (Santajit & Indrawattana, 2016). It has been estimated that by 2050, 10 million lives per year and an economic output of 100 trillion of dollars are at risk due to the rise of infections caused by multidrug resistant bacteria. To date, 700,000 people die every year as consequence of bacterial infections (figure 1.1) (O'Neill, 2016). Political agendas, legislation, development of therapies and educational initiatives are essential to mitigate this increasing rate of antibiotic resistance (Frieri *et al*., 2016).

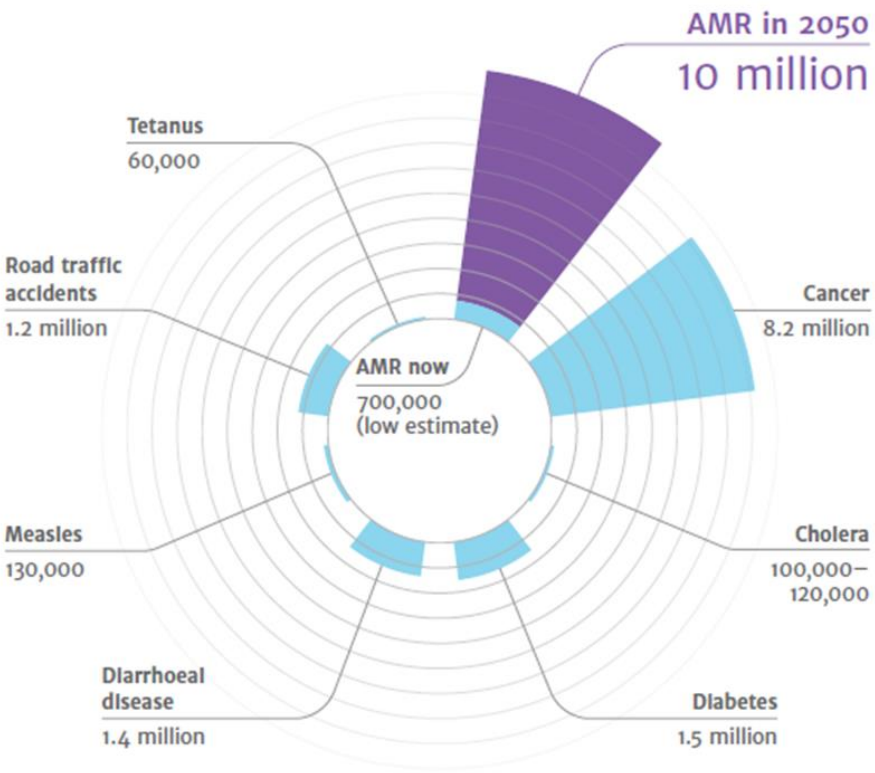

Figure 1.1 Deaths attributable to antimicrobial resistance every year (O'Neill, 2016).

Although most antibiotic therapy is applied within the community, the greatest concentration of use per patient occurs in hospitals, hence nosocomial pathogens are likely to be the most resistant (French, 2010; O'Neill, 2016). However, the increasing prevalence of hospital and community-acquired infections caused by multidrug resistant bacterial pathogens is limiting the options for effective antibiotic therapy (Cassir *et al*., 2014). Multidrug resistant Gram-positive and -negative bacterial infections have resulted as difficult-to-treat or even untreatable with conventional antimicrobials (Frieri *et al*., 2016). Moreover, hospital infections are now microbiologically heterogeneous, being caused by many different species of multidrug resistant bacteria. Antibiotic usage selects, constantly, pathogens resistant also to last line antimicrobials (French, 2010). Data from the Centers for Disease Control and Prevention

(CDC) have showed a rapidly increasing rates of infection due to methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE), and fluoroquinolone-resistant *Pseudomonas aeruginosa* strains (Boucher *et al*., 2009). Furthermore, nosocomial infections caused by pan-drug resistant bacteria are now occurring. A lot of people die in hospitals for MRSA infection (Boucher & Corey, 2008) and in this setting several highly resistant Gram-negative pathogens such as *Acinetobacter* spp., multidrug resistant *P. aeruginosa*, carbapenem resistant *Klebsiella* spp. and *Escherichia coli* are emerging as significant pathogens worldwide (Falagas & Kasiakou, 2005; Falagas & Bliziotis, 2007; Urban *et al*., 2008). Recent report (Rice, 2008) basing on data from the Infectious Diseases Society of America has begun to refer to a clique of nosocomial pathogens, acronymically termed as "***ESKAPE****"* pathogens. *ESKAPE* is an acronym for a group of bacteria, encompassing both Gram-positive and Gram-negative species: ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa* and ***E****nterobacter* spp. These microorganisms are capable of "escaping" the biocidal action of antibiotics and collectively represent new paradigms in pathogenesis, transmission and resistance (Rice, 2008). They are responsible of the bulk of antimicrobial resistant infections (Tang *et al*., 2014) amongst critically ill and immunocompromised individuals and are characterized by potential drug resistance mechanisms (Rice, 2010).

1.1.1 A multidrug resistant Gram-negative bacterium: *Acinetobacter baumannii*

The incidence of infections caused by multidrug resistant Gram-negative bacteria has increased worldwide over the last decade. EARS-net (EARS-net, 2014) reported that the majority of *Escherichia coli* and *Klebsiella*

pneumoniae isolates was resistant to at least one of the antimicrobial tested, and many had combined resistance. To date, *A. baumannii* is also one of the most successful pathogens in the modern health care system (WHO, 2010). Multidrug resistant *A. baumannii* is a rapidly emerging pathogen in the hospital setting, where it causes severe infections (Jaward *et al*., 1996; Fourier & Richet 2006).

The genus *Acinetobacter* consists of strictly aerobic, Gram-negative coccobacillary rods, oxidase negative, non-motile, usually nitrate negative and non-fermentative (Murray *et al*., 2003). Since 1986, the taxonomy of the genus *Acinetobacter* has been modified several times. Currently, at least 34 species can be distinguished within the genus, 23 of which have been assigned species name (Visca *et al*. 2011). *Acinetobacter* spp. are widely distributed in the environment and readily contaminate the hospital environment (Lin & Lan, 2014). They are the second non-fermenters most commonly isolated in human specimens. *Acinetobacter* spp. are able to survive on moist and dry surfaces, in foodstuff and on healthy human skin, especially in health care settings (Murray *et al*., 2003). Since they have a relatively long survival time on human hands, they can lead to high rates of cross contamination in nosocomial infections (Houang *et al*., 1998). *Acinetobacter* spp. may also colonize or live in a patient without causing infection or symptoms. Despite there are several species within genus, able to cause human disease, *A. baumannii* remains the most frequently isolated species and accounts for about 80% of reported infections (WHO, 2010). Moreover, hospital acquired infections, caused by *A. baumannii*, are most likely to involve the respiratory tract (related to endotracheal tubes or tracheostomies), urinary tract and wounds (including catheter sites) and many of them progress to septicaemia (Murray *et al*., 2003; Maragakis & Perl, 2008; Lin & Lan, 2014). These infections often occur

in older patients, affected by chronic diseases and previously treated with antimicrobial therapies (Rodriguez-Baño *et al*., 2004; Lin *et al*., 2009). The mortality of patients with *A. baumannii* infections in hospitals and in the intensive care units has ranged from 7.8% to 23% and from 10% to 43%, respectively (Lin & Lan, 2014). *A. baumannii* rarely occur outside of health care settings; however, community-acquired *A. baumannii* has been reported in China and tropical Australia (WHO, 2010), and they have even affected patients with co-morbidities in the community (Falagas & Karveli, 2007). Previous studies on patients in the intensive care unit affected by bloodstream infections and burn infections due to carbapenem resistant *Acinetobacter* species, demonstrate an increased mortality rate (26% - 68%), as well as increased morbidity and length of stay in the intensive care unit (Maragakis & Perl, 2008). Antimicrobial resistance among *Acinetobacter* species has increased substantially in the past decade (Lockhart *et al*., 2007). Their extensive antimicrobial resistance may be due to the selective ability to prevent various molecules from penetrating their relatively impermeable outer membrane and to a large reservoir of resistance genes (EARS-net, 2014). Indeed, *A. baumannii* appears to be particularly effective at acquiring genetic material from other organisms and thus rapidly developing drug resistance (WHO, 2010). The antimicrobials still active against this pathogen include some fluoroquinolones (e.g. ciprofloxacin and levofloxacin), aminoglycosides (e.g. gentamicin, tobramycin and amikacin), carbapenems (imipenem, doripenem and meropenem), polymyxins (polymyxin B and colistin), sulbactam and tigecycline (EARS-net, 2014). However, data from EARS-net (2014) on 28 countries reporting antimicrobial susceptibility information revealed that in Europe the percentages of fluoroquinolones (ciprofloxacin or levofloxacin) resistant isolates ranged from 2.9 % to 95.3

%. The highest percentages (≥ 50%) of resistant strains was encountered in many countries (e.g. Italy, Spain, Portugal, Greece, Cyprus, Hungary and Bulgaria) as showed in figure 1.2. The high levels of antimicrobial resistance in *A. baumannii*, reported from several countries in Europe, are of great concern, especially when resistance to the last-line treatment alternatives such as carbapenems occurs (EARS-net, 2014).

Moreover, the presence of multi drug resistant *Acinetobacter* spp. in the healthcare environment is of further concern since this pathogen can persist in the environment for long periods and is notoriously difficult to eradicate once established (Maragakis & Perl, 2008). This pathogen is emblematic of the mismatch between medical needs and the current antimicrobial research and development pipeline (Boucher *et al*., 2009).

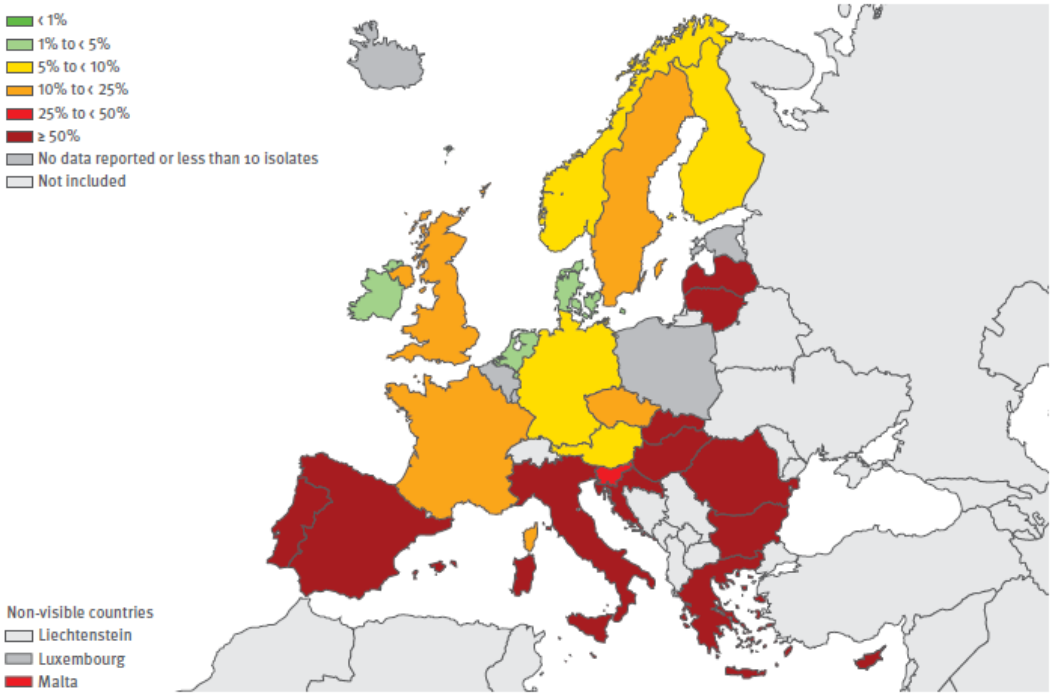

Figure 1.2 *Acinetobacter* spp. Percentage (%) of invasive isolates with resistance to fluoroquinolones, by country (EARS-net, 2014).

1.1.2 A multidrug resistant Gram-positive bacterium: the methicillin resistant *Staphylococcus aureus* (MRSA)

Staphylococci are Gram-positive coccal bacteria (0.5 to 1.5 µm in diameter), that occur singly and in pairs, tetrads, short chains (three or four cells), and irregular "grape-like" clusters. They are non-motile, non-spore-forming and catalase positive (Murray *et al*., 2003).

Staphylococci are widespread in nature and the role of *S. aureus* as one of the most important human pathogens is largely due to its virulence potential and ubiquitous occurrence as coloniser of humans, domestic animals, and livestock (Morgan, 2008). With non-fastidious growth requirements, staphylococci are mainly found living on the skin, skin glands, and mucous membranes of mammals and birds. They can be found in the mouth, blood, mammary glands, and intestinal, genitourinary and upper respiratory tracts of hosts (Murray *et al*., 2003; Gordon & Lowy, 2008). Staphylococcal infections represent a serious challenge for clinicians, particularly for serious infections such as bacteraemia, severe pneumonia and skin and soft-tissue infections. Staphylococci represent the second most frequent cause of hospital acquired infections (12.3%) in Europe (Campanile *et al*., 2015). *S. aureus* is both a commensal and pathogenic microorganism and the anterior nares are its main ecological niche (Wertheim *et al*., 2005) with approximately 20% of individuals permanently colonized, and 30% occasionally (Gordon & Lowy, 2008). Indeed, *S. aureus* is an opportunistic microorganism being responsible of severe infections. This "persistent pathogen" *S. aureus*, is even more challenging since has developed the resistance to methicillin and to other semi-synthetic penicillins, giving rise to the acronym "MRSA" (methicillin resistant *S. aureus*) (Sabath & Finland, 1962). A year after the methicillin introduction in clinical use (1959–1960), methicillin-resistant

isolates were reported (Gordon & Lowy, 2008) and in the following ten years, it became widespread in Europe, Australia, and United States (Barber, 1961; Elek, 1965). This methicillin-resistant form has been the most important cause of antimicrobial resistant and healthcare-associated infections worldwide (EARS-net, 2014). Presently, and perhaps in a more virulent fashion, due to its association with Panton-Valentine leucocidin (PVL, an exotoxin active against neutrophils), MRSA has been also implicated in a surge of community-acquired infections that is reaching epidemic proportions (Vandenesch *et al* 2003; Labandeira-Rey *et al* 2007). Resistance to methicillin primarily derives from acquisition of the *mecA* gene, not native in this species, which encodes the penicillin-binding protein 2A (PBP2A) with a low binding affinity for β-lactam antibiotics (Wu *et al*., 1996) thus preventing beta-lactams to inhibit cell wall synthesis. *S. aureus* is a versatile pathogen capable of causing a wide range of human diseases (Gordon & Lowy, 2008): the worldwide diffusion of MRSA increases morbidity and mortality rates in healthcare-associated infections, adding a further burden to *S. aureus* diseases (Köck *et al*., 2010; Stefani *et al*., 2012). The occurrence of MRSA in Europe is variable: MRSA percentages were generally lower in northern Europe and higher in the southern and south-eastern parts (figure 1.3).

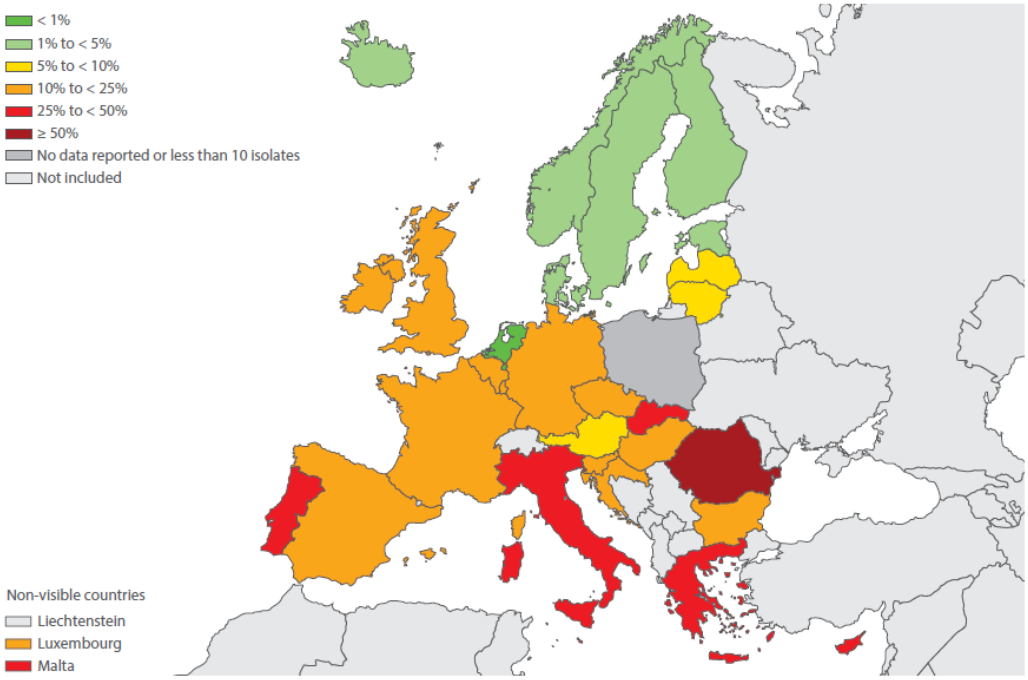

Figure 1.3 *Staphylococcus aureus*. Percentage (%) of invasive isolates with resistance to methicillin (MRSA), by country (EARS-net, 2014).

A recent epidemiological Italian survey (Campanile *et al*., 2015), aiming to determine the prevalence and antibiotic resistance of *S. aureus* in nosocomial infections, disclosed that *S. aureus* infections accounted for 11.6% and multidrug resistant MRSA accounted for approximately one-third of all isolates causing diseases. In this work, it has been found that, among clinical MRSA, percentages of isolates resistant to several antimicrobials (*i.e.* tetracycline, rifampicin, clindamycin and gentamicin) ranged from 12.6% to >39%. Higher percentages of resistant strains were found to erythromycin (65.4%) and fluoroquinolones (72.3 - 85.8%).

Tackling the problem of MRSA infections is a top priority for public health systems worldwide. In most cases, glycopeptides (vancomycin and teicoplanin) are used as first-line antibiotics for treatment of specific MRSA infections. However, the selective pressure of these antibiotics has reduced susceptibility of some strains to glycopeptide with reports of

clinical vancomycin-intermediate (VISA) and vancomycin-resistant (VRSA) *S. aureus* (Chambers & DeLeo, 2009).

Despite, a decreasing trend in MRSA infections has been reported, it remains a public health priority in Europe. Therefore, comprehensive strategies targeting all healthcare divisions (acute care, long-term care and ambulatory care) remain essential to continue reducing the spread of MRSA throughout the Europe (EARS-net, 2014).

1.2 Quinolones and fluoroquinolones

Unlike the first antibiotics discovered during the past century, the quinolone class of antimicrobial agents was not isolated from living organisms but, rather, was synthesized by chemists. Indeed, the quinolone class is structurally based on the fully synthetic naphthyridone (Andriole, 2005). Nalidixic acid, a related naphthyridone structure and the first member of the quinolone class, was accidentally discovered by George Lesher as synthesis by-product of the antimalarial compound chloroquine in 1962 (Lesher *et al*., 1962). This discovery led to the development of a series of quinolone compounds (Andriole, 1994) but its usage was strictly limited to the treatment of urinary tract infections caused by Gram-negative bacteria and resistance emerged quickly. As consequence of nalidixic acid discovery, other compounds were developed but only few were of signal importance: pharmaceutical chemists, modified the core of quinolone and the related chemical scaffolds (Domagala & Hagen 2003), generating compounds with greater potency, broader spectra of activity, improved pharmacokinetics and pharmacodynamics properties, and lower frequency of development of resistance (Andriole, 1999; Andriole, 2000). Therefore, changes have resulted in second-, third-, and fourth-generation fluoroquinolones, each defined by structural modifications and type of bacterial infection target including those by Gram-positive bacteria (Ball, 2000; King *et al*., 2000; Oliphant & Green, 2002).

Currently, there are four generations of quinolone/fluoroquinolone antibiotics as showed in table 1.1 (Redgrave *et al*., 2014).

Table 1.1 Quinolones licensed for clinical use (Redgrave *et al*., 2014).

Generation	Drug	Use in clinical practice
First generation	Nalidixic acid	Generic form available
	Cinoxacin	Discontinued
Second generation	Norfloxacin	Available as Noroxin
	Ciprofloxacin	Available as Cipro and generic form
	Lomefloxacin	Discontinued
	Ofloxacin	Available as Floxin and generic form
	Levofloxacin	Available as Levaquin and generic form
Third generation	Sparfloxacin	Discontinued
	Gatifloxacin	Discontinued
	Grepafloxacin	Discontinued
Fourth generation	Trovafloxacin	Discontinued
	Moxifloxacin	Available as Avelox
	Gemifloxacin	Available as Factive

Fluoroquinolones (figure 1.4) differ from quinolones by the replacement of eight carbon atom of the backbone with a nitrogen atom and the addition of a fluorine substituent at position six (Ball, 2000). This addition became the common feature of the fluoroquinolone class with the introductions of norfloxacin in 1986 and ciprofloxacin in 1987 that showed substantially greater effectiveness against Gram-negative bacteria (Hooper & Jacoby, 2016). Actually, fluoroquinolone antibiotics are extensively used in a wide range of clinical applications because of their potency, spectrum of activity, oral bioavailability, and generally good safety profile (table 1.2; Owens & Ambrose 2000; Kim & Hooper 2014; Redgrave *et al*., 2014; Hooper & Jacoby, 2016). The most common fluoroquinolones prescribed are ciprofloxacin, levofloxacin, and moxifloxacin (Redgrave *et al*., 2014).

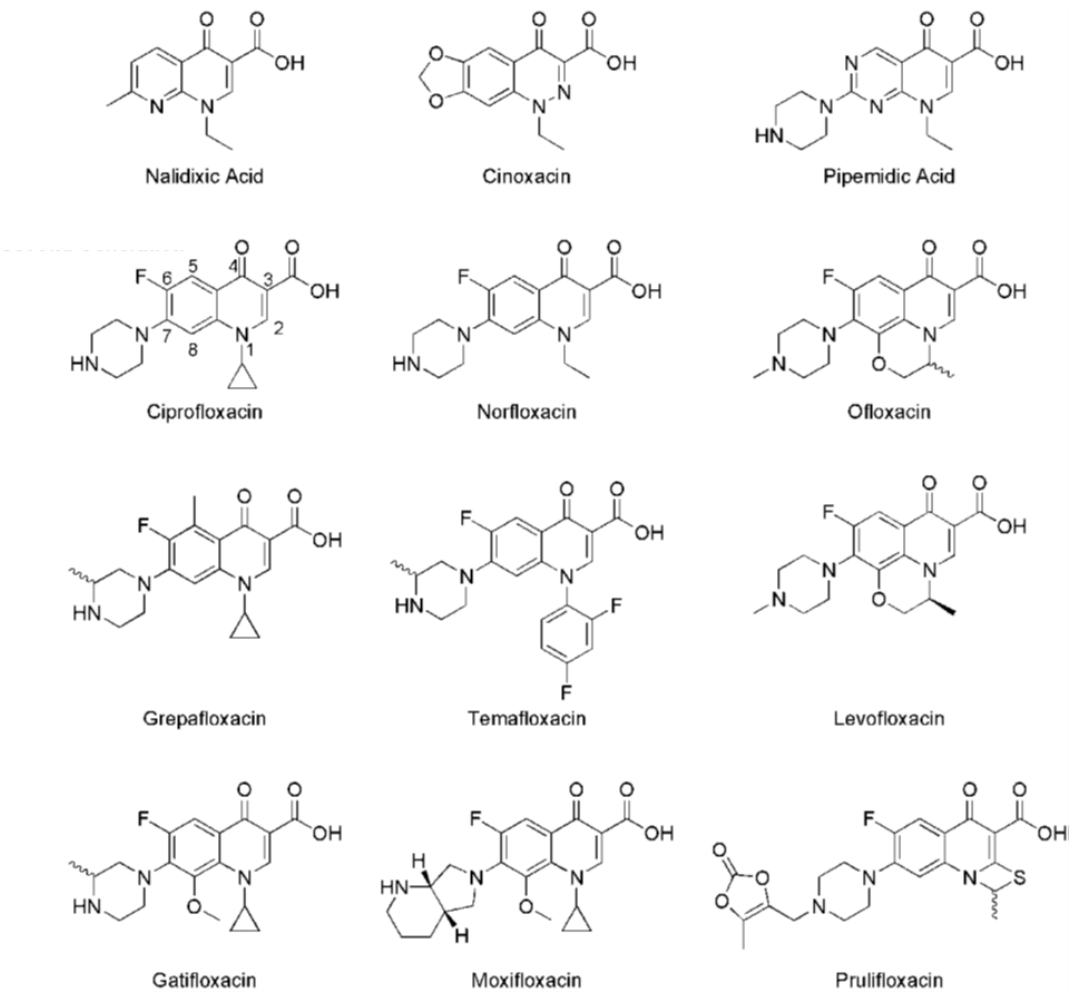

Figure 1.4 Chemical structures of most relevant quinolones.

First generation quinolone antibiotics have been used for the treatment of uncomplicated urinary tract infections. The first generation agents were typically effective against a narrow range of Gram-negative bacteria, excluding *Pseudomonas* spp. (Oliphant & Green, 2002). Second generation quinolones were the first in class containing fluorine atom and they have expanded Gram-negative activity but limited efficacy against Gram-positive. Clinical infections treated using these second generation fluoroquinolones included urinary tract infections (both complicated and uncomplicated), pyelonephritis, sexually transmitted diseases, prostatitis, and localized skin and soft tissue infections (Oliphant & Green, 2002). Third generation fluoroquinolones were used also against respiratory

infections. Broad-spectrum activity was further increased since they retained expanded Gram-negative efficacy and have improved Gram-positive coverage. Differences between third- and fourth generation fluoroquinolone were poorly distinguished. Fourth generation fluoroquinolones were used to treat similar infections as third generation drugs, but also have activity against anaerobic targets (Oliphant & Green, 2002).

Table 1.2 Approved clinical uses for selected fluoroquinolones (Andriole, 2005)

Agent	Approved indications
Ciprofloxacin	Acute uncomplicated cystitis in females (oral use only)
	Urinary tract infections
	Chronic bacterial prostatitis
	Uncomplicated cervical and urethral gonorrhea
	Skin and skin-structure infections
	Bone and joint infections
	Infectious diarrhea (oral use only)
	Typhoid fever (oral use only)
	Complicated intra-abdominal infections, in combination with metronidazole
	Acute sinusitis
	Lower respiratory tract infections
	Nosocomial pneumonia (iv use only)
	Empirical therapy for patients with febrile neutropenia, in combination with piperacillin sodium (iv use only)
	Inhalational anthrax (after exposure)
	Complicated urinary tract infections and pyelonephritis in pediatric patients (1–17 years old)
Levofloxacin	Uncomplicated urinary tract infections (mild to moderate)
	Complicated urinary tract infections (mild to moderate)
	Acute pyelonephritis (mild to moderate)
	Chronic bacterial prostatitis
	Uncomplicated skin and skin-structure infections (mild to moderate)
	Complicated skin and skin-structure infections
	Acute maxillary sinusitis
	Acute bacterial exacerbation of chronic bronchitis
	Community-acquired pneumonia[a]
	Nosocomial pneumonia
Moxifloxacin	Acute bacterial sinusitis
	Acute bacterial exacerbation of chronic bronchitis
	Community-acquired pneumonia[a]
	Uncomplicated skin and skin-structure infections
Gatifloxacin	Uncomplicated urinary tract infections
	Complicated urinary tract infections
	Pyelonephritis
	Uncomplicated urethral and cervical gonorrhea
	Acute uncomplicated gonococcal rectal infections in women
	Uncomplicated skin and skin-structure infections
	Acute sinusitis
	Acute bacterial exacerbation of chronic bronchitis
	Community-acquired pneumonia[a]
Gemifloxacin	Acute bacterial exacerbation of chronic bronchitis
	Community-acquired pneumonia (mild to moderate)[a]

[a] Includes pneumonia due to multidrug-resistant *Streptococcus pneumoniae*.

1.2.1 Bacterial topoisomerase type II and fluoroquinolones action mechanism

In both prokaryotes and eukaryotes, DNA exists as double strands forming a double-helix structure. Considering that each bacterium contains a chromosome composed of ~1300 µm long double-stranded DNA and bacteria are only ~2 µm in lenght and ~1 µm wide, they have to face a major topological problem (Andriole, 2005). Further twisting of this double-strand can occur throughout torsional force leading double helix to cross over on itself (Baranello *et al*., 2012). This process is called supercoiling and allows the chromosome to be highly condensed, hence can be placed into a bacterial cell. Supercoiling can be either positive or negative and this describes the direction in which twisting has occurred (Redgrave *et al.*, 2014). The maintenance of supercoiling is essential in genetic processes that control gene expression and thereby determine the phenotype of a cell (Dorman *et al*., 1988; Dorman & Corcoran, 2009; Cameron *et al*., 2011; Webber *et al*., 2013). Changes in the global degree of supercoiling alter the expression of multiple genes including those involved in responses to stress and in pathogenesis (Peter, 2004; Baranello *et al*., 2012; Webber *et al*., 2013). The degree of supercoiling of DNA is not fixed and there is continuous remodelling of DNA topology in bacteria in response to environmental stress, growth stage, and cellular processes (Corbett *et al*., 2005; Barnarello *et al*., 2012; Arnoldi *et al*., 2013; Webber *et al*., 2013). Topoisomerase I and topoisomerase II enzymes work in opposition to control the level of twisting within DNA. Topoisomerase I reduces the number of negative supercoils (Barnarello *et al*., 2012). By contrast, topoisomerase II introduces negative supercoils, which unwind over-twisted DNA into a relaxed state and can further change the DNA topology (Barnarello *et al*., 2012). In bacteria, DNA supercoiling is

controlled by type II topoisomerase enzymes, DNA gyrase and topoisomerase IV. Both are large heterodimeric enzymes composed of 2 pairs of subunits. The subunits of DNA gyrase are GyrA, and GyrB, 97-kDa and 90-kDa, respectively. The corresponding subunits of topoisomerase IV are ParC and ParE, 75 and 70 kDa, respectively (Jacoby, 2005). In *S. aureus*, topoisomerase IV subunits historically have also been referred as GrlA and GrlB (Hooper & Jacoby, 2016). The enzymes have homologous action but with subtle differences; although both DNA gyrase and topoisomerase IV relax positively supercoiled DNA, only DNA gyrase can introduce negative supercoils into relaxed DNA (Corbett *et al*., 2005). Topoisomerase IV has decatenating (unlinking) activity, allowing the segregation of catenated daughter chromosomes during cell division (figure 1.5; Corbett *et al*., 2005; Arnoldi *et al*., 2013; Drilica *et al*., 1999).

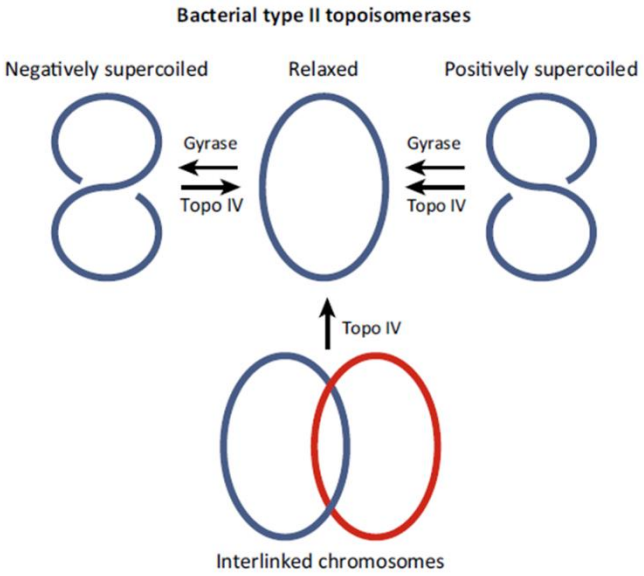

Figure 1.5 Action of type II topoisomerase on DNA (Redgrave *et al*., 2014).

The two enzymes work together in the replication, transcription, recombination and repair of bacterial DNA. The enzymes break both strands of double-stranded DNA, and, in an ATP-dependent reaction, pass

a second DNA double helix through the break, which is then resealed (Kampranis *et al*., 1999).

Fluoroquinolones are potent inhibitors of bacterial type II topoisomerases: DNA gyrase and topoisomerase IV (Drlica, 1990; Drlica & Zhao, 1997; Hooper, 2001). They inhibit bacterial control of DNA supercoiling: at lower concentrations impaired DNA replication and at high concentrations determine cell death (Drlica, 1999; Drlica *et al.*, 2009). Indeed, quinolones entrap DNA gyrase or topoisomerase IV in a drug-enzyme-DNA complex known as ternary complex (Drlica *et al*., 2008), with subsequent release of lethal, double-stranded DNA breaks (Hiasa & Shea, 2000). When a fluoroquinolone binds to the DNA-topoisomerase complex, the resulting ternary complex inhibits DNA replication and cell growth (Drlica & Zhao, 1997; Maxwell, 1997). In addition, this process stabilizes a catalytic intermediate covalent complex of enzyme and DNA. This can serve as a barrier to movement of the DNA replication fork (Wentzell & Maxwell 2000), to transcription complexes (Willmott *et al*. 1994) and finally to determine permanent double-strand DNA breaks (Drlica *et al*., 2008). Indeed, the binding of fluoroquinolones occurs within the enzyme at the target site of helix-4 in either GyrA or ParC, preventing the enzyme (DNA gyrase or topoisomerase IV) to re-ligate the DNA (Drlica *et al*., 2008). Quinolone interactions with DNA gyrase appear to result in more rapid inhibition of DNA replication than quinolone interactions with topoisomerase IV (Hooper & Jacoby, 2016). It could be possibly related to the overall proximity to the DNA replication complex of enzyme-binding sites on chromosomal DNA, with gyrase positioned ahead of the complex and topoisomerase IV behind it (Khodursky & Cozzarelli 1998). The targeting of either DNA gyrase or topoisomerase IV as the primary target by fluoroquinolones varies with

bacterial species: quinolones can differ in their potency for the two enzymes. There is greater activity against DNA gyrase in Gram-negative bacteria and greater activity against topoisomerase IV in Gram-positive bacteria (Blanche *et al*. 1996; Pan & Fisher 1997; Strahilevitz & Hooper 2005). Therefore, in Gram-negative bacteria, DNA gyrase is more susceptible to inhibition by quinolones than topoisomerase IV, whereas, in Gram-positive bacteria, topoisomerase IV is usually the prime target, and DNA gyrase is intrinsically less susceptible (Drlica *et al*., 2008).

1.2.2 Fluoroquinolone-resistance mechanisms

Resistance to quinolones has been a problem ever since nalidixic acid was introduced into clinical practice more than 50 years ago (Jacoby, 2005). Despite prescribing guidelines now recommending reserving fluoroquinolone use, resistance continues to rise and constitute a relevant problem in clinical setting (Redgrave *et al*., 2014). Indeed, fluoroquinolone resistance has a significant clinical impact and can be mediated by multiple mechanisms (Oliphant & Green, 2002). Several mechanisms of resistance to fluoroquinolones (table 1.3) are currently recognized (Hooper, 2003).

Quinolone-resistance can be mediated by a transmissible route: genes encoding different resistance mechanisms and located on mobile genetic elements can decrease susceptibility to quinolone or fluoroquinolone antibiotics. This is known as plasmid-mediated quinolone resistance (PMQR) and the plasmid mediated resistance gene has been named *qnr* (Martinez *et al*, 1998). Data from a structural analysis of a Qnr protein suggest that fluoroquinolones resistance is achieved by the binding of the Qnr protein to the topoisomerase, physically preventing the intercalation of the antibiotic with the target enzyme (Tran & Jacoby, 2002; Xiong *et*

al., 2011). The *qnr* genes generally confer to bacteria a modest protection against fluoroquinolones (Martinez *et al*., 1998).

Table 1.3 Impact on susceptibility to ciprofloxacin of different resistance mechanisms (Redgrave *et al*., 2014)

Resistance mechanism	Fold change in ciprofloxacin MIC
Gram-negative species[a]	
Topoisomerase substitutions	
gyrA	10–16
parC	0
gyrA (× 2) + *parC*	60
Permeability changes	
Efflux upregulation	4–8
Porin loss	4
PMQRs	
Carriage of *qnr* alleles	>30
Carriage of *qepA*	32
Carriage of *oxqAB*	16
Carriage of *aac(6′)Ib-cr*	4
Gram-positive species[b]	
Topoisomerase substitutions	
grlA	4–8
grlB	4–8
gyrA	0
grlA + *gyrB*	64–128
Permeability changes	
Efflux upregulation	4

[a] Based on data from *E. coli*
[b] Based on data from *S. aureus*

To reach their targets, quinolones must cross the cell wall and cytoplasmic membrane of Gram-positive bacteria; in Gram-negative bacteria, quinolones must traverse an additional outer membrane barrier. Multidrug efflux pumps are capable of actively removing fluoroquinolones and other drugs from the bacterial cell. Both Gram-negative and Gram-positive bacteria have nonspecific, energy-dependent efflux systems, some of which are constitutively expressed and others are controlled by regulatory

systems (Jacoby, 2005). These include transporters of various classes: in *S. aureus*, for example, resistance to quinolones can be mediated by the increased expression of *norA*, a gene that encodes a broad spectrum transporter for fluoroquinolones and other agents (Kaatz & Seo, 1995).

Finally, the most common mechanism conferring high-level of fluoroquinolone resistance is the occurrence of point mutations in at least one of type II topoisomerases encoding genes, *gyrA*, *gyrB*, *parC/grlA*, and *parE/grlB*. These mutations map within a DNA region defined as the quinolone resistance-determining region (QRDR; Redgrave *et al*., 2014) corresponding to amino acid residues 67 to 106 of GyrA protein (Yoshida *et al*., 1990) and to amino acid residues 63 to 102 of GrlA/ParC in *E. coli* (Hooper & Jacoby, 2016). In *S. aureus*, the QRDR is represented by amino acid residues 68 to 107 of GyrA (Margerrison *et al*., 1992) and 64 to 103 of the GrlA (Ferrero *et al*., 1994). Mutations in the QRDR of topoisomerase type II encoding genes, result in amino acid substitutions that alter the target protein structure and subsequently the fluoroquinolone binding affinity of the enzyme (Piddock, 1999; Hooper, 2000). Although the primary target of fluoroquinolones differs in Gram-positive and Gram-negative, they will bind also to the secondary target and exert an antibacterial effect even if point mutations in the primary target have already occurred. Alteration of the primary target site can be followed by secondary mutations in lower affinity binding sites and highly resistant organisms will typically carry a combination of mutations within *gyrA* and *grlA* (Redgrave *et al*., 2014). Indeed, once a first mutation arises in one of the gene encoding DNA gyrase or topoisomerase IV, a reduction in the susceptibility of the target enzyme occur and additional mutations can further enhance resistance level (Ince & Hooper, 2003).

1.3 The challenge of new antimicrobials: "the discovery void"

Antibiotics have been declared as one of the greatest medical advances in the modern age. The accelerated growth, diversity and burden of antimicrobial resistance became increasingly evident past the mid-1970s, although this was initially tackled by the development of new antimicrobial compounds that overcame the acquired resistance mechanisms (Tang *et al*., 2014). The revolutionary discovery of penicillin propelled the golden era of antibiotics, in which natural scaffolds and alternative versions of existing drugs were discovered. Currently the ever-increasing resistance (figure 1.6) and the management of bacterial infection could no longer be taken for granted (Brown & Wright, 2016).

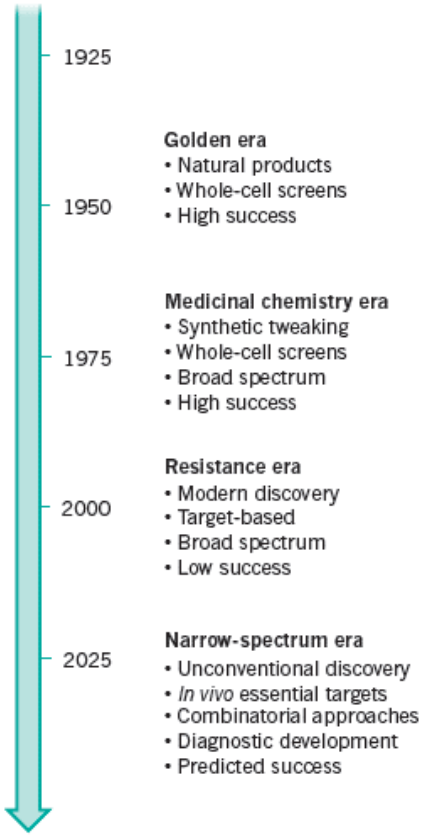

Figure 1.6 Models of antibiotic drug discovery and development (Brown & Wright, 2016).

Emphasis, in the resistance era, has fallen on target-based drug discovery to find broad-spectrum agents. In future a focus on innovative methods and unconventional targets should help to create narrow-spectrum agents and associated diagnostics (Brown & Wright, 2016).

Over the past 25 years, the challenge of antibacterial discovery has retained the release of novel drug classes to extraordinarily low levels, even though discovery programs took place at large and small pharmaceutical companies as well as academic laboratories over this period (Silver, 2011). Since the 1990s there has been a "discovery void" (figure 1.7) of unknown extent in the research of new classes of antimicrobial compounds.

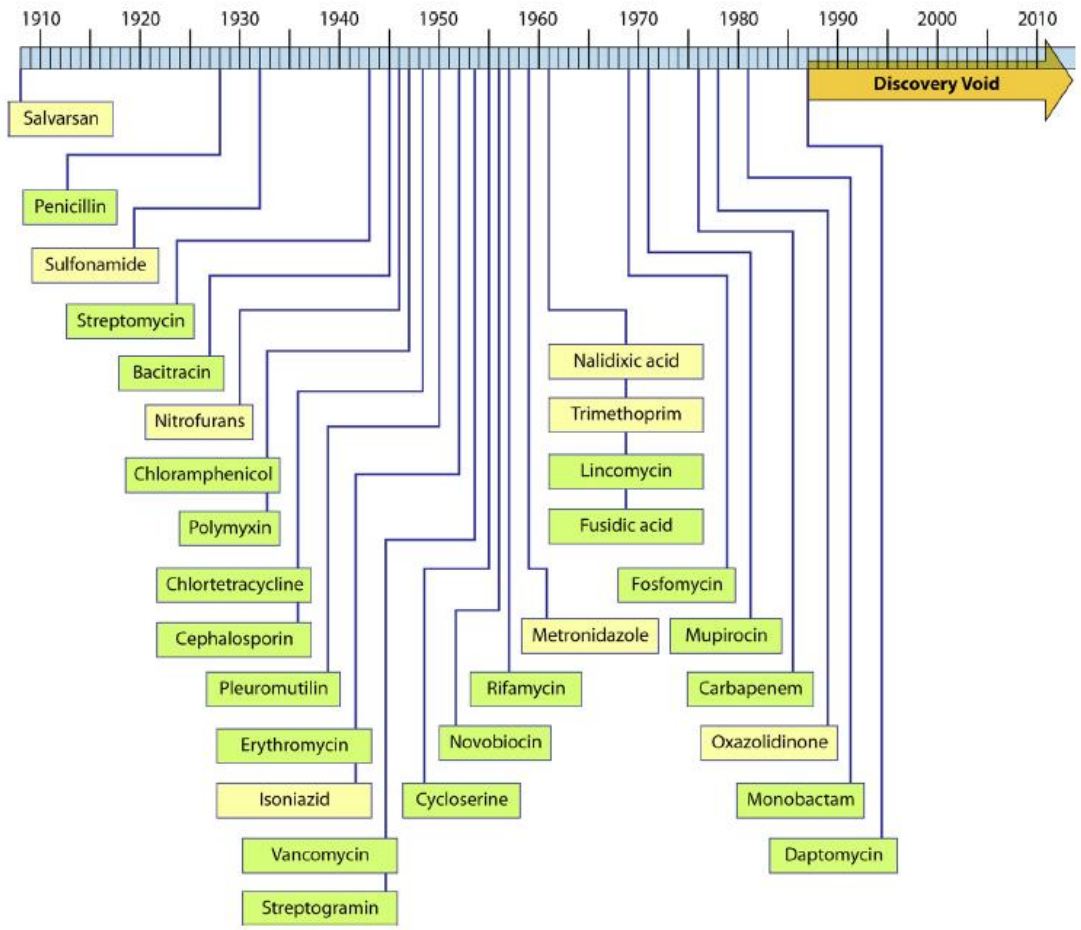

Figure 1.7 Timeline of antimicrobial drug discovery and illustration of the "discovery void." Years indicated are those of reported initial discovery or patent (Silver, 2011).

This problem was compounded by the persistent increase in new and modified mechanisms of antimicrobial resistance in disease-causing microbes (Talbot *et al*., 2006; Laxminarayan *et al*., 2013). Researchers have questioned if this "void" may be due to a lack of innovation or not: the simple definition of innovation is the act of introducing something new. However, it is noteworthy that almost all the drug discoveries (with few exceptions) have been unexpected, following to the screening of fermentation or chemical products for inhibition of bacterial growth (Silver, 2011).

Short-course dosing, impressive efficacy, limited side effects set the level whereby future antimicrobial compounds will be measured. However, this profile is unsustainable from both a practical and economic point of views. Most of large-pharma has severely reduced or eliminated infectious disease research and development, instead of pursuing more profitable therapeutics (Tomaras & Dunman, 2015) hence the route of exploiting novel antibacterial targets has met with significant failure over the last decade (Silver, 2011). It is alarming that although bacterial resistance continues to emerge, the rate at which antibiotics are being developed is decreasing (ECDC/EMEA, 2009). Although, there are a small number of novel compounds in the early clinical phase that might indicate the end of this "void", in most cases their eventual developmental success is unclear. Indeed, new antibiotics discovery deals with several developing barriers: *i) scientific barrier*, because it's hard to find new classes of antibiotics and trials are not feasible; *ii) regulatory barrier*, because it's difficult to get a licence from the Food and Drug Administration (FDA) and rules repeatedly change; *iii) financial barrier*, because antibiotics are not very profitable (Akova, 2016b). In 1983–1987 the FDA approved 16 new

systemic antibiotics, but, since that time, antibiotic approvals have progressively been declined (see figure 1.8).

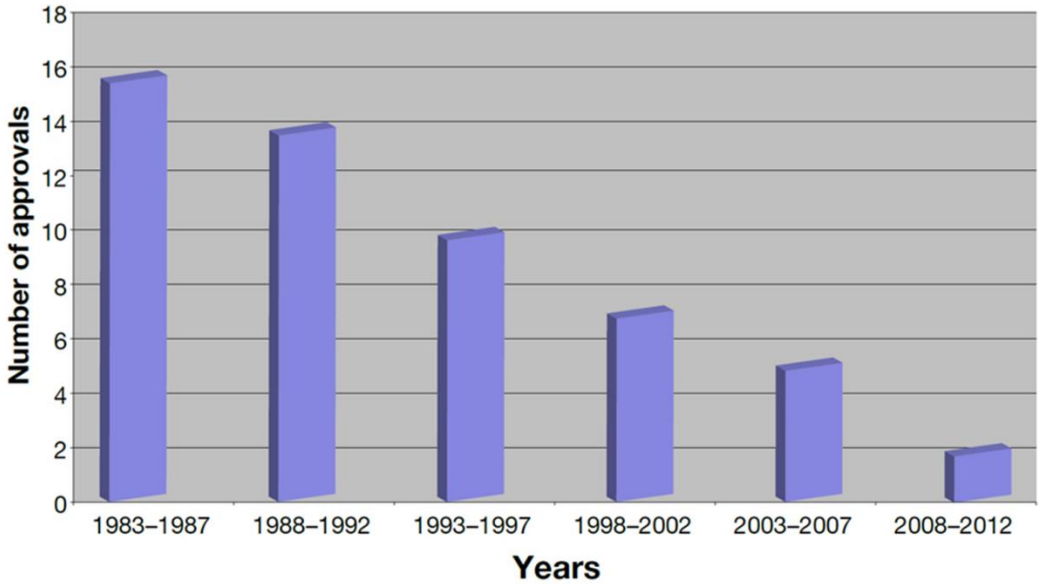

Figure 1.8 Antibiotic approvals by FDA over the last three decades in five-year increments (Cain, 2012).

Since 2008, only two systemic antibiotics have been approved. Moreover, just two new classes of broad-spectrum antibiotics have been validated in the last 40 years: the oxazolidinone (linezolid) and the lipopeptide (daptomycin) both effective against Gram-positive bacteria (Cain, 2012). Nowadays, agencies have provided incentives for large-pharma reinvestment in the antimicrobial research field but the damage has already been done due to the long-term consequences (Tomaras & Dunman, 2015). Moreover, reports of emerging multidrug-resistant Gram-negative bacteria, such as *Acinetobacter* spp., have stoked further concerns that there is not a robust pipeline addressing the issue posed by a highly diverse and variable spectrum of resistant pathogens (Cain, 2012). The resistance challenge with existing antibiotics needs of discovery and development of novel compounds with well differentiated modes of action to overcome the existing resistance mechanisms of

bacteria spread in clinical settings (Perez *et al*., 2008; Karaiskos & Giamarellou, 2014).

Some efforts in the discovery of novel antibacterial agents have resulted in compounds with potent antimicrobial activity against multidrug resistant pathogens (Lahiri *et al*., 2015).

A wise strategy may be to identify novel scaffolds able to bind novel pockets on clinically well validated targets that are conserved among bacteria and distinct from human, with a different type of inhibition (Lahiri *et al*., 2015). Because of the attractive possibility of dual targeting, an area of active research has been the pursuit of inhibitors of type II topoisomerase: bacterial DNA type II topoisomerases are clinically validated as antibacterial drug targets by the development and use of fluoroquinolone (Silver, 2011). One of the promising class among these inhibitors are the Novel Bacterial Topoisomerase Inhibitors (NBTIs) reported by many groups (Bax *et al*., 2010; Miles *et al*., 2013; Dougherty *et al*., 2014).

NBTIs inhibit both bacterial DNA gyrase and topoisomerase IV with minimal cross reactivity to human topoisomerase (Wiener *et al*., 2007; Black *et al*., 2008). Biochemical data (Bax *et al*., 2010), together with the co-crystal structure, have helped to understand the binding mode and the mechanism of inhibition of this class of inhibitors (Lahiri *et al*., 2015). Indeed, mechanistic and structural studies of NBTIs have revealed a binding site distinct from that of fluoroquinolones. Additionally, a novel mode of action of these inhibitors could be suggested by the absence of DNA-cleavage complex formation unlike the fluoroquinolones which stabilize a cleaved complex intermediate (Bax *et al*., 2010; Shapiro & Andrews, 2012). While the general mechanistic differences have been well established, the specific pathways of conformational changes remain

poorly defined. Recently, structural advances in the field of bacterial topoisomerases (Bax *et al*., 2010; Vos *et al*., 2011; Laponogov *et al*., 2013) have significantly improved the understanding of this complex machinery, where association and dissociation of various subunits result in DNA cleavage/religation and DNA strand passage. This novel binding mode provides a structural basis explaining why NBTIs are able to overcome target-mediated fluoroquinolone resistance (Bax *et al*., 2010).

Inhibitors of bacterial topoisomerases continue to expand beyond the quinolones but it remains to be verified that these agents can demonstrate clinical efficacy with minimal toxicity concerns.

These new topoisomerase inhibitors make advanced to the clinic and may be on track to be the first new class of clinically relevant DNA-targeted antibacterial agents (Bradbury & Pucci, 2008). Actually, novel compounds with dual target activity would have the advantage to reduce developmental frequency of antimicrobial resistance, due to the unlikely occurrence of two simultaneous mutations in both essential targets (Azam *et al*., 2015). These agents could offer coverage against quinolone-resistant strains, but further demonstrations of clinical usage are strictly required.

STATEMENT OF PURPOSE

The increasing rate of bacterial resistance to clinical antimicrobial agents and its impact on treatment of infectious diseases has begun a unique problem throughout the world. Many antibacterial agents can induce resistance by different mechanisms hence many last-resort compounds are becoming increasingly ineffective. Treatment of bacterial infections caused by drug resistant strains relies on development of new agents that are able to overcome current resistance mechanisms. Therefore, there is a continuing need to develop newer and more potent antibiotics. Among the targets for the development of new antibacterial agents, bacterial topoisomerases remain a vibrant area of discovery. Novel Bacterial Topoisomerase Inhibitors (NBTIs) are a new class of compounds able to inhibit both bacterial DNA gyrase and topoisomerase IV.

A series of NBTIs have been developed and provided by the Italian pharmaceutical company Angelini S.p.A. (ACRAF). These molecules, still subject to confidentiality, should have a mechanism of action similar to that of fluoroquinolones, acting simultaneously on two microbial target.

This Ph.D. research is focused on the study of the effectiveness of these NBTIs against bacterial species of clinical interest.

To this aim molecular and microbiological methods have been used:

- to assess the susceptibility of Gram-positive and -negative reference strains by MIC/MBC determination, time-kill assays and post antibiotic effect;

- to evaluate the occurrence in *S. aureus* of antimicrobial resistance to these NBTIs, investigating the ability of new compounds to select *in vitro* resistant mutants;
- to assess their effectiveness against multidrug resistant *A. baumannii* and MRSA strains of clinical origin.

Chapter 2 - MATERIALS AND METHODS

2.1 Antimicrobial agents

Ciprofloxacin and levofloxacin, were all purchased from Sigma Aldrich (Sigma-Aldrich Co., St. Louis, Mo, USA). Twelve NBTIs were supplied by the pharmaceutical company Angelini ACRAF as Dimethyl sulfoxide (DMSO)-soluble powder. The novel compounds tested in the present study are named as follow 73005, 73006, 73007, 73008, 73009, 73010, 73011, 73012, 73013, 73014, 73015, 73016.

2.2 Bacterial strains

Reference strains *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and methicillin-resistant *S. aureus* ATCC BAA 1720 were used to test the activity of all compounds. *Pseudomonas aeruginosa* ATCC 27853 was used in susceptibility assays with 73009, 73015 and 73016 molecules.

A total of 38 *Acinetobacter baumanii* strains (Table 2.2.1) and 24 methicillin-resistant *S. aureus* (MRSA, Table 2.2.2) strains were evaluated for their susceptibility to molecules 73015, 73016 and ciprofloxacin. All strains were isolated and identified by Clinical Microbiology Laboratory, Ospedali Riuniti of Ancona. Both *A. baumanii* and MRSA strains were selected for their multidrug-resistance, including the fluoroquinolone resistance.

Table 2.2.1: List of multidrug-resistant *A. baumannii*

Strains	**Clinical sample**	**Isolation period**
A. baumannii 1	cerebrospinal fluid	August 2015
A. baumannii 2	blood culture	September 2015
A. baumannii 3	tracheal suctioning	October 2015
A. baumannii 4	sputum	October 2015
A. baumannii 5	bronchoaspirate (BAS)	October 2015
A. baumannii 7	bronchoalveolar lavage (BAL)	October 2015
A. baumannii 8	protected specimen brush (PSB)	October 2015
A. baumannii 9	PSB	October 2015
A. baumannii 10	blood culture	October 2015
A. baumannii 11	BAS	November 2015
A. baumannii 12	PSB	November 2015
A. baumannii 13	PSB	November 2015
A. baumannii 14	PSB	November 2015
A. baumannii 15	BAL	November 2015
A. baumannii 16	tracheal suctioning	December 2015
A. baumannii 17	wound swabbing	December 2015
A. baumannii 18	BAS	December 2015
A. baumannii 20	PSB	January 2016
A. baumannii 21	BAS	January 2016
A. baumannii 22	PSB	January 2016
A. baumannii 23	PSB	January 2016
A. baumannii 24	tracheal suctioning	January 2016
A. baumannii 25	BAS	January 2016
A. baumannii 26	PSB	January 2016
A. baumannii 27	wound swabbing	January 2016
A. baumannii 28	PSB	January 2016
A. baumannii 29	PSB	February 2016
A. baumannii 30	tracheal suctioning	February 2016
A. baumannii 31	sputum	March 2016
A. baumannii 32	sputum	March 2016
A. baumannii 33	wound swabbing	March 2016
A. baumannii 34	wound swabbing	March 2016
A. baumannii 35	urine	March 2016
A. baumannii 36	urine	March 2016
A. baumannii 37	wound swabbing	March 2016
A. baumannii 39	rectal swab	April 2016
A. baumannii 40	tracheal suctioning	April 2016
A. baumannii 41	PBS	April 2016

Table 2.2.2: List of multidrug-resistant MRSA

Strains	**Clinical sample**	**Isolation period**
MRSA LU1	nasal swab	April 2012
MRSA G	auricular swab	April 2012
MRSA 1	BAS	September 2015
MRSA 2	sputum	September 2015
MRSA 3	sputum	September 2015
MRSA 5	BAS	September 2015
MRSA 7	sputum	September 2015
MRSA 15	blood culture	September 2015
MRSA 16	blood culture	October 2015
MRSA 17	BAS	October 2015
MRSA 22	pharyngeal swab	October 2015
MRSA 28	blood culture	October 2015
MRSA 30	blood culture	October 2015
MRSA 32	tracheal suctioning	October 2015
MRSA 43	sputum	November 2015
MRSA 49	BAL	November 2015
MRSA 50	BAS	November 2015
MRSA 52	sputum	November 2015
MRSA 55	blood culture	November 2015
MRSA 58	BAS	November 2015
MRSA 59	blood culture	January 2016
MRSA 60	blood culture	January 2016
MRSA 61	blood culture	March 2016
MRSA 62	blood culture	March 2016

2.3 Minimum inhibitory concentration (MIC) tests

MICs of ciprofloxacin and levofloxacin were determined by standard microdilution procedures as recommended by Clinical and Laboratory Standard Institute (M07- A9, CLSI 2012). MICs of all novel compounds supplied as DMSO-soluble powders, were determined following the scheme suggested by the CLSI for preparing dilutions of water-insoluble antimicrobial agents to be used in broth dilution susceptibility tests (Table 8B, M100-S25, CLSI, 2015).

The novel antibiotics were tested at final concentration ranging from 0.001 to 8 µg/ml, prepared from serial twofold dilutions in Cation-adjusted Mueller-Hinton broth (CAMHB, Sigma-Aldrich). Ciprofloxacin and levofloxacin were tested at final concentration ranging from 0.001 to 256 µg/ml. The overnight broth cultures, grown at 37°C in CAMHB (Sigma-Aldrich) were standardized to 0.1 optical density (λ=625 nm) and further diluted 1:100 in CAMHB in order to obtain 1×10^6 CFU/ml as final *inoculum*. Aliquots (50 µl) of standardized bacterial suspension were distributed into the wells of a microtiter plate containing the different concentrations of the antibiotic to obtain a final bacterial concentration of 5×10^5 CFU/ml in each well. The inoculated microtiter plates were incubated at 37°C for 18-24 h.

S. aureus ATCC 29213 and *E. coli* ATCC 25922 were used as quality control strains for MIC determination of clinical MRSA and *A. baumannii* strains, respectively.

Ciprofloxacin and levofloxacin MIC results for the reference strains *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 were assessed to be consistent with the quality control ranges (M100-S25, CLSI, 2015). Ciprofloxacin MIC values for clinical strains were interpreted according

to the susceptibility interpretive criteria reported in the appropriate CLSI Tables (M100-S25, CLSI, 2015). Since no breakpoints were available for novel compounds, their efficacy was established arbitrarily (MIC ≤2 µg/ml).

The MIC was defined as the lowest antibiotic concentration which yielded no visible growth.

2.4 Minimal bactericidal concentration (MBC) tests

Following performance of the MIC test by the broth microdilution method, Minimal bactericidal concentrations (MBCs) were determined to the evaluation of antibiotic bactericidal activity (Isenberg 1992, Clinical Microbiology Procedures Handbook, 1st Edition). After reading and recording the MIC values, aliquots (10 µl) from all the wells showing no growth were spotted onto Mueller Hinton agar (MHA, Oxoid, Basingstoke, UK) plates. Viable counts were performed after plates incubation at 37°C for 24 h.

The MBC was defined as the lowest antibiotic concentration that reduces the viability of the initial bacterial *inoculum* by ≥99.9%.

2.5 Time-kill assay

After determination of MICs, these concentrations were used as starting point for *in vitro* time-kill studies. Ciprofloxacin, levofloxacin and the molecules proven to be effective (MIC ≤2 µg/ml) were investigated for their killing kinetics against the reference strains. Time-kill experiments were performed following the procedure reported by Isenberg (1992). Three doubling concentrations of each antibiotic (1, 2, and 4 times the MIC value) were tested. Drug free control tube were included in each

experiment. Overnight bacterial cultures were standardized in order to obtain an initial *inoculum* ranged from 5×10^5 to 5×10^6 CFU/ml in CAMHB (Sigma-Aldrich). Cultures were incubated at 37°C in a shaking bath. At 0-, 2-, 4-, 8-, and 24-h intervals (here and after T_0, T_2, T_4, T_8, T_{24} respectively), viable counts were performed in triplicate by spotting aliquots of 10 µl of the suitable dilutions onto MHA (Oxoid) plates. Plates were incubated at 37°C for 24 h. Compounds were considered bactericidal at the lowest drug concentration that reduced viable organism count by ≥3 Log_{10} CFU/ml. The bactericidal activity, was assessed for each molecule as function of both time and concentration.

2.6 Post antibiotic effect (PAE)

Post antibiotic effect is the term used to describe the suppression of bacterial growth that persist after short exposure of organisms to antimicrobials (Lorian 4th edition, 1996).

PAE testing of ciprofloxacin, 73015 and 73016 was carried out as reported by Huband *et. al* (2015) with some modifications. Briefly, logarithmically growing bacterial cultures of *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA 1720, at a starting *inoculum* of 1×10^6 CFU/ml, were exposed to different concentrations of drugs (*i.e.* 1×, 4× and 16×MIC) for 1h at 37°C. Drug free control tube was included in each experiment (unexposed tube). After 1h exposure, viable counts were carried out in triplicate by spotting aliquots (10 µl) of the suitable dilution onto MHA (Oxoid) plates; the drug removal were performed by bacterial cultures dilution (1:1000) in CAMHB (Sigma-Aldrich). Diluted cultures were incubated in shaking bath at 37°C. In order to monitor over time (for 5 hours) the ongoing suppression of bacterial growth, aliquots from each

broth culture were periodically collected, diluted and plated to perform viable counts (in triplicate).

The following equation had been used for quantifying the PAE:

$$PAE = T - C$$

where *T* is the time required for the count of CFU in the test culture to increase 1 Log_{10} (10-fold) above the count observed immediately after drug removal, and *C* is the time required for count of CFU in an untreated control culture to increase 1 Log_{10} above the count observed immediately after completion of the same procedure used on the test culture for drug removal (Lorian 4th edition, 1996).

Indeed, PAE was defined as the difference between the times required for the cultures in the compound exposed and unexposed tubes to increase by 1 Log_{10} CFU/ml above the number present immediately after dilution (1:1000) of the compound.

2.7 Selection of resistant *S. aureus* mutants

In vitro selection of *S. aureus* ATCC 29213 and *S. aureus* ATCC BAA 1720 mutants was carried out using single-step mutation method as reported by Drago *et al.* (2010) with some modifications. *S. aureus* ATCC 29213 mutants have been selected using the molecules 73009, 73011, 73012, 73013, 73015, 73016 and ciprofloxacin. *S. aureus* ATCC BAA 1720 mutants have been selected using the molecules 73015 and 73016. A bacterial culture in Mueller Hinton broth (MHB, Oxoid) of both *S. aureus* strains were standardized to achieve a turbidity equivalent to a suspension containing approximately 10^9 CFU/ml. Aliquots (100 µl) of standardized bacterial cultures were spread onto MHA (Oxoid) plates containing antibiotic concentrations equal to 1, 2, 4, 8, 16, 32 times the

MIC value. In addition, aliquots (100 µl in triplicate) of appropriate dilutions of the same standardized bacterial culture were also plated on antibiotic-free MHA (Oxoid) for the viable count of *inoculum*. After incubation at 37° C for 24-48 h, counts were performed for all plates. The frequency of mutation was calculated as the ratio between the number of colonies (CFU/100 µl) grown on antibiotic-containing plates and the number (CFU/100 µl) of the colonies of the initial *inoculum.* Putative resistant colonies were confirmed by 5 passages on MHA (Oxoid) containing the same concentration of antibiotic used for selection: this was followed by MIC tests. The isolates showing increments in MICs with respect to the MIC value of wild-type strain were considered mutants. All mutants were then plated on drug-free MHA for 5 more passages and the MICs were determined once more to confirm the stability of the developed resistance by mutants.

2.8 Characterization of acquired resistance

S. aureus resistant mutants, (one for each molecule tested), were analysed for the presence of point mutations in genes encoding DNA gyrase (*gyrA, gyrB*) and topoisomerase IV (*grlA, grlB*).

2.8.1 Total bacterial DNA extraction

Bacterial DNA of both mutants and wild-type strains (*S. aureus* ATCC 29213 and *S. aureus* ATCC BAA 1720), were obtained according to the method suggested by Hynes *et al*. (1992) in which crude cell lysates are used as target DNA. Bacterial overnight cultures (1 ml) in Brain Heart Infusion Broth (BHIB, Oxoid) were centrifuged (13000 rpm) at room temperature for 10 minutes in order to harvest bacterial cells. Pellets were resuspended in 1 ml of sodium chloride-Tris-EDTA buffer (10 mM Tris-

HCl pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0) supplemented with 20% sucrose (w/v, Sigma-Aldrich), 2,5 mg/ml lysozyme (Sigma-Aldrich) and 100 µg/ml lysostaphin (Sigma-Aldrich) and incubated at 37°C for 1h and 30 min. Samples were centrifuged (13000 rpm) at room temperature for 3 minutes and pellets were resuspended in 1ml of lysis buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, 0.45% Tween 20) supplemented with proteinase K (Sigma-Aldrich) at final concentration of 5 µg/ml. After incubation at 60°C for 1h, the samples were heated at 95°C for 10 minutes in order to inactivate proteinase K and residual cellular proteases or nucleases and to denature the extracted DNA.

Bacterial DNA was analysed by agarose gel (0.8% in TAE buffer: 40 mM Tris-acetate; 1 mM EDTA pH 8.0) electrophoresis (80V for 1h). Green gel (at 0.7X final concentration, Fisher Molecular Biology), was used as intercalating agent and gel was observed using an ultraviolet transilluminator. Marker II (M-Medical srl, Milano, Italia) was used as standard molecular weight.

2.8.2 Polymerase Chain Reaction (PCR) assays

Bacterial DNA, obtained as previously described, was used in PCR assays to amplify *gyrA, gyrB, grlA* and *grlB* genes. The PCR reactions were performed in a 50 µl final reaction volume containing: 1X buffer, 200 µM deoxynucleoside triphosphate (Sigma-Aldrich), 0.6 mM of each primer pair (synthesised by Sigma-Aldrich), 1.5 U Taq DNA-Polymerase (DreamTaq DNA Polymerase, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 5 µl of bacterial DNA. The amplification program was as follows: 1 cycle of 4 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 60°C, 3 min at 72°C; and a final extension step of 5 min at 72°C. After the PCR reaction, all amplicons were loaded on

agarose gel (1 %) in TAE 1X, 80V. Green gel (at 0.7X final concentration, Fisher Molecular Biology), was used as intercalating agent and gel was observed using an ultraviolet transilluminator. GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific) was used as standard molecular weight. Primer pairs used in PCR assays are listed in Table 2.8.2.

Table 2.8.2: Primer pairs used in this study

Target	Primer	Sequence (5'- 3')	Reference
gyrA	gyrA-F1	AACTTGAAGATGCGATTGAAGCGGACC	Huband *et al*., 2014
	gyrA-R1	CACCATCAAGACTTATCAATGAAATACC	
	gyrA2_F	AGTGAATAAGGCTCGTATGA	This study
gyrB	gyrB-F1	TAGATGATTCGCGTCAAACG	Huband *et al*., 2014
	gyrB-R1	AGTATACGACGATGTACTGG	
grlA	parC_grlA-F1	TGACAAAGTACAACCTAGACGTGAATGG	Huband *et al*., 2014
	parC_grlA-R1	AATTTAACGATAAGTACTTGGTCAGC	
	grlA-parC2_F	CGTGATGAAACTGATAGAACT	This study
grlB	parE_grlB-F1	AACGAACGTACGTTTGCAGG	Huband *et al*., 2014
	parE_grlB-R1.1	TATTGAATTCACTAGATTTCCTCCTCATC	

2.8.3 Purification of PCR products

The PCR products were purified using the Genelute PCR Clean-up kit (Sigma-Aldrich), that allows rapid purification of single-stranded or double-stranded PCR products (ranged from 100 bp to 10 kb) from other components, such as excess primers, nucleotides, DNA polymerase, oil and salts. The fluorometric quantitation of DNA (ng/µl) contained in the eluate, was determined using Quant-it™ dsDNA HS Assay Kit (Invitrogen kit, Milan, Italy). An aliquot (3 µl) of the purified DNA was added to a 200 µl final volume of the Quant-it™ dsDNA HS buffer

containing the fluorophore (Quant-it™ reagent). The sample was incubated for 2 minutes at room temperature and then quantified.

2.8.4 Sequences analysis

Purified PCR products were subjected to sequencing by an external sequencing service, GATC Biotech (Cologne, Germany), that offers sequencing and bioinformatics solutions from single samples up to large scale projects (https://www.gatc-biotech.com/en/home.html). The method chosen to sequence amplicons was the Sanger sequencing approach: a simple and rapid method allowing to determine nucleotide sequences in single-stranded DNA. This technique, first commercialized by Applied Biosystems, is based on the incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication (Sanger and Coulson, 1975).

The DNA was prepared according to the specification required by GATC Biotech: the DNA concentration of sample had to be ranged from 20 ng/µl to 80 ng/µl. Aliquot, 5 µl, of purified PCR product was mixed with 5 µl of each primer (5 pmol/µl) used in PCR assays. A total sample amount of 10 µl in 1,5 ml tube was sent to GATC Biotech to perform sequencing.

The sequencing results were download from GATC web-site and the electropherograms were analysed using the Chromas software, available at http://www.technelysium.com.au/chromas.

The sequences of the four genes of mutant strains were compared with the sequences of the same genes of the wild-type strain to detect the occurrence of point mutations.

Analysis and comparison of the nucleotide sequences were carried out using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Functional domain of

Topisomerase IV and DNA gyrase affected by point mutations, were identified using Conserved Domains search tool of NCBI available at https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.

Chapter 3 - RESULTS

3.1 Susceptibility of control strains: MIC and MBC results

The antibacterial activity of twelve novel NBTIs was first evaluated by MIC and MBC determination against three reference strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, the methicillin resistant *S. aureus* ATCC BAA 1720) belonging to bacterial species of clinical interest. The susceptibility of the strains to the NBTIs compared to that to ciprofloxacin and levofloxacin is presented in table 3.1.1.

Table 3.1.1 MIC and MBC results

Antimicrobial molecule	**Strain**	**MIC µg/ml**	**MBC µg/ml**
ciprofloxacin	*E. coli* ATCC 25922	0.008	0.016
	S. aureus ATCC 29213	0.5	1
	S. aureus ATCC BAA 1720	128	256
levofloxacin	*E. coli* ATCC 25922	0.016	0.03
	S. aureus ATCC 29213	0.25	0.5
	S. aureus ATCC BAA 1720	16	32
73005	*E. coli* ATCC 25922	8	ND*
	S. aureus ATCC 29213	2	2
	S. aureus ATCC BAA 1720	4	4
73006	*E. coli* ATCC 25922	>8	ND
	S. aureus ATCC 29213	8	>8
	S. aureus ATCC BAA 1720	8	>8
73007	*E. coli* ATCC 25922	1	2
	S. aureus ATCC 29213	0.5	1
	S. aureus ATCC BAA 1720	1	2
73008	*E. coli* ATCC 25922	8	>8
	S. aureus ATCC 29213	0.5	0.5
	S. aureus ATCC BAA 1720	0.5	0.5
73009	*E. coli* ATCC 25922	0.5	0.5
	S. aureus ATCC 29213	0.5	0.5
	S. aureus ATCC BAA 1720	0.5	>8
	P. aeruginosa ATCC 27853	2	2

73010	*E. coli* ATCC 25922	8	>8
	S. aureus ATCC 29213	8	8
	S. aureus ATCC BAA 1720	8	>8
73011	*E. coli* ATCC 25922	2	2
	S. aureus ATCC 29213	0.125	0.125
	S. aureus ATCC BAA 1720	0.25	0.25
73012	*E. coli* ATCC 25922	8	>8
	S. aureus ATCC 29213	0.125	0.25
	S. aureus ATCC BAA 1720	0.125	0.25
73013	*E. coli* ATCC 25922	0.5	1
	S. aureus ATCC 29213	0.125	0.125
	S. aureus ATCC BAA 1720	0.25	0.25
73014	*E. coli* ATCC 25922	>8	ND
	S. aureus ATCC 29213	4	8
	S. aureus ATCC BAA 1720	4	8
73015	*E. coli* ATCC 25922	0.5	1
	S. aureus ATCC 29213	0.25	4
	S. aureus ATCC BAA 1720	0.5	1
	P. aeruginosa ATCC 27853	2	4
73016	*E. coli* ATCC 25922	0.5	1
	S. aureus ATCC 29213	0.03	0.5
	S. aureus ATCC BAA 1720	0.016	2
	P. aeruginosa ATCC 27853	>8	ND

*ND means not determined

Since no breakpoints were available for the novel NBTIs, efficacy for the compounds was arbitrarily established (MIC ≤ 2 µg/ml). Basing on MIC ranges, a fully effectiveness, intended as efficacy simultaneously against *E. coli* and both *S. aureus* control strains, was observed only for molecules 73007 (MIC 0.5 to 1 µg/ml), 73009 (MIC 0.5 to 2 µg/ml), 73011 (MIC 0.25 to 2 µg/ml), 73013 (MIC 0.125 to 0.5 µg/ml), 73015 (MIC 0.25 to 2 µg/ml), 73016 (MIC 0.016 to 0.5 µg/ml). Molecule 73008 and 73012 were active only against staphylococci. *P. aeruginosa* ATCC 27853 was slightly susceptible to 73009 and 73015 compounds (MIC equal to 2 µg/ml) and resistant to 73016 molecule (MIC > 8 µg/ml).

The molecules 73005, 73006, 73010 and 73014 were ineffective (MIC equal to 4, 8 and >8 µg/ml) in bacterial growth inhibition of all control strains.

MBCs mostly corresponded to a concentration of one or two times MIC value. The MBC determination of compounds for which the MIC value exceeded the maximum concentration tested (MIC > 8 µg/ml) was not performed (ND in table 3.1.1). In few cases, MBC values were much greater than the related MIC: these concentrations, able to reduce the viability of the initial *inoculum* by ≥99.9%, correspond to 16× MIC (73009 against *S. aureus* ATCC BAA 1720, 73015 against *S. aureus* ATCC 29213 and 73016 against *S. aureus* ATCC 29213) and 125× MIC (73016 against *S. aureus* ATCC BAA 1720).

3.2 Time-kill assays

Time-kill assays were first carried out with ciprofloxacin and levofloxacin, against all the three reference strains. Among the novel NBTIs, seven (73007, 73009, 73011, 73013, 73015, 73016 and the molecule 73012 effective only against staphylococci) were also investigated for their killing kinetics.

3.2.1 Time-kill performance of ciprofloxacin and levofloxacin

The two antibiotics were more effective against *E. coli* ATCC 25922 than against both *S. aureus* strains. No bactericidal effect was observed at the MIC value for both antibiotics, according to the results obtained from MBC tests. Ciprofloxacin was bactericidal only against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 at concentration equal to 0.016 and 1 µg/ml respectively (2×MIC) despite the time required was different for each strain (figure 3.2.1). Ciprofloxacin had no bactericidal effect against the *S. aureus* ATCC BAA 1720. Bactericidal effect of levofloxacin was observed against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 at concentration equal to 0.03 and 0.5 µg/ml respectively (2×MIC) after 4 and 8 h incubation; a concentration of 64 µg/ml (4×MIC) was bactericidal against the MRSA strain only after 24 h incubation (figure 3.2.2).

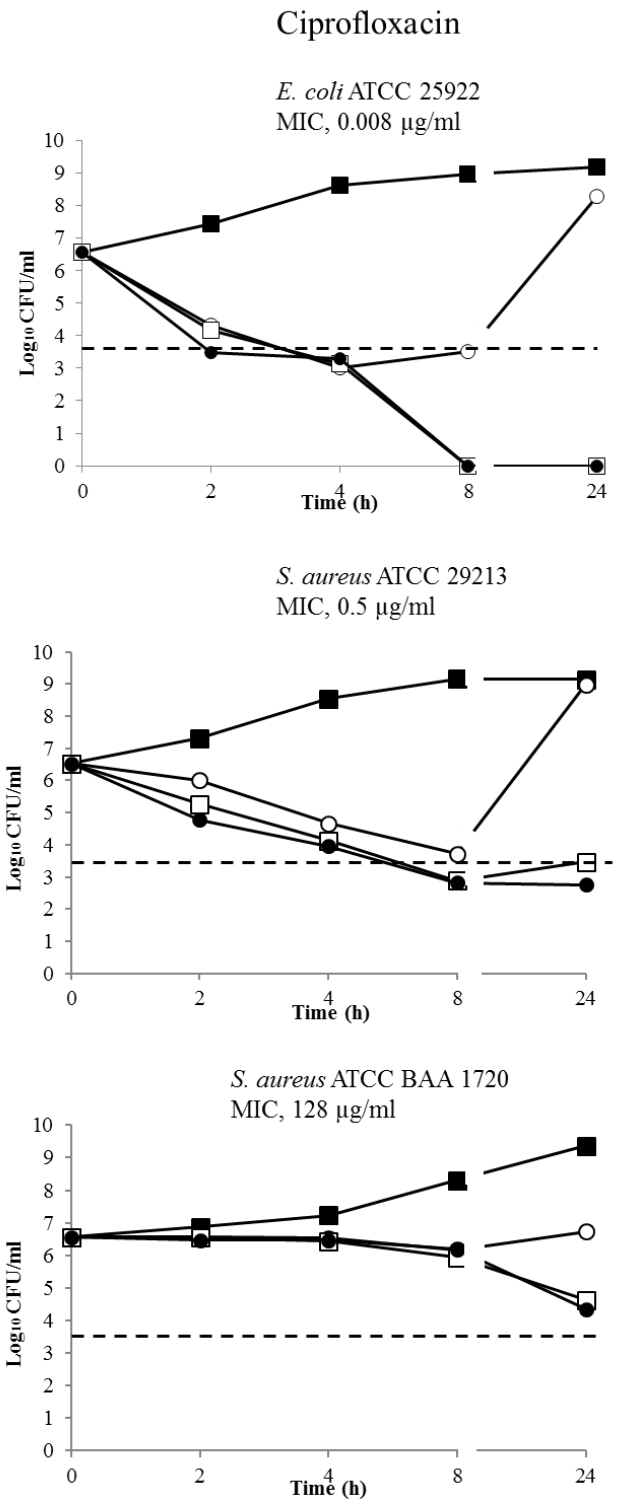

Figure 3.2.1 Killing kinetics of ciprofloxacin against three control strains: *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA 1720. All experiments were performed in triplicate. The Threshold corresponding to the 3 Log_{10} CFU/ml reduction was identified by black dashed line. Standard deviations of the reported values were always below 0.5 Log_{10}. ■, growth control; ○, 1×MIC; □ 2×MIC; ●, 4×MIC.

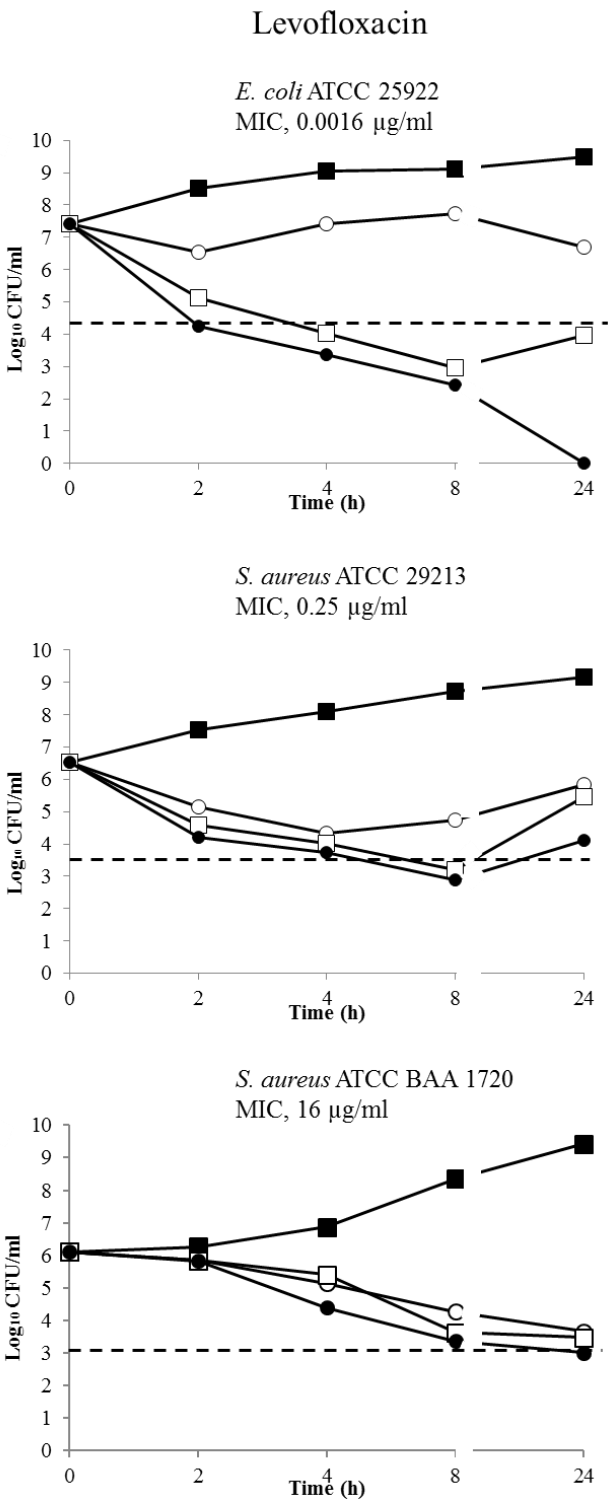

Figure 3.2.2 Killing kinetics of levofloxacin against three control strains: *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA 1720. All experiments were performed in triplicate. The Threshold corresponding to the 3 Log_{10} CFU/ml reduction was identified by black dashed line. Standard deviations of the reported values were always below 0.5 Log_{10}. ■, growth control; ○, 1×MIC; □ 2×MIC; ●, 4×MIC.

3.2.2 Time-kill performance of 73007

This molecule had a good efficacy against *E. coli* ATCC 25922 since all tested concentrations (1 µg/ml, 2 µg/ml and 4 µg/ml) reduced the initial *inoculum* by 3 Log_{10} CFU/ml after 4 and 8 hours incubation time. Moreover, concentration equal to 2 µg/ml and 4 µg/ml (2×MIC and 4×MIC, respectively) maintained their efficacy even after 24 hours. A bactericidal effect at a concentration of 2 µg/ml (4×MIC) was also detectable against *S. aureus* ATCC 29213 after 8 hours. No bactericidal activity was recorded against *S. aureus* ATCC BAA 1720 at tested concentration during 24 h incubation time (see figure 3.2.3).

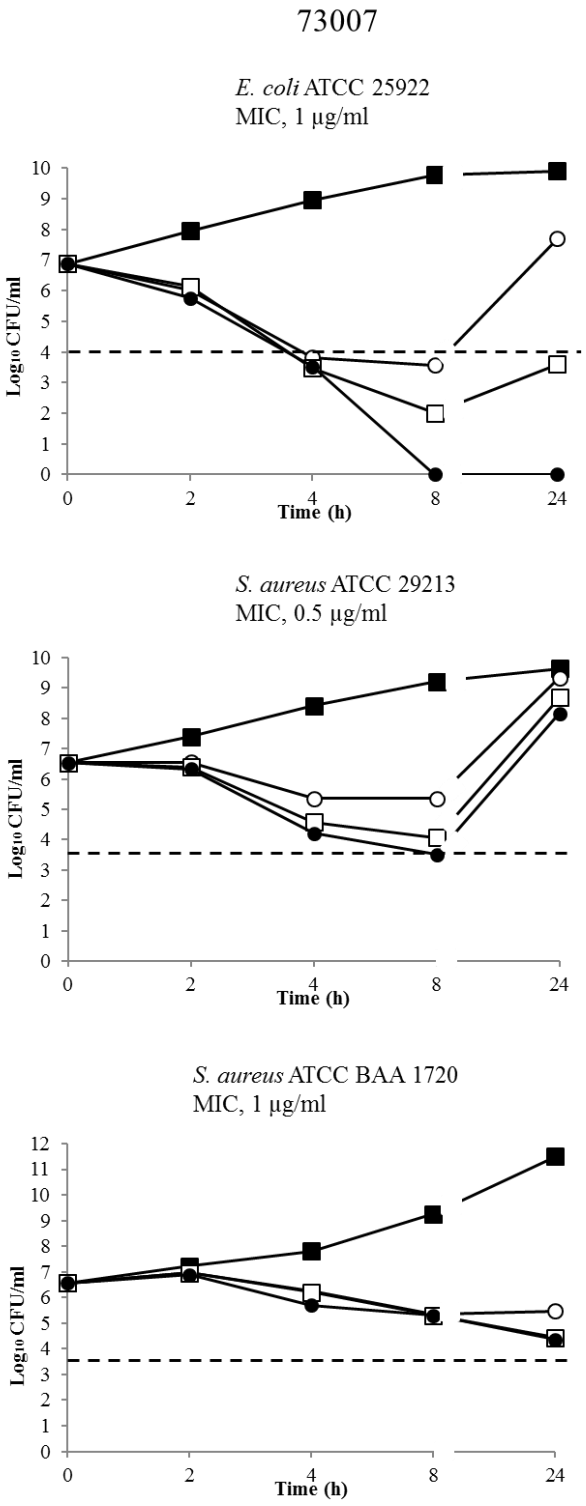

Figure 3.2.3 Killing kinetics of 73007 molecule against three control strains: *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA 1720. All experiments were performed in triplicate. The Threshold corresponding to the 3 Log_{10} CFU/ml reduction was identified by black dashed line. Standard deviations of the reported values were always below 0.5 Log_{10}. ■, growth control; ○, 1×MIC; □ 2×MIC; ●, 4×MIC.

3.2.3 Time-kill performance of 73009

The 73009 was the single NBTI compound tested also against *P. aeruginosa* ATCC 27853 in time-kill experiments (figure 3.2.4). A bactericidal effect was obtained against both Gram-negative reference strains. In particular, against *E. coli* ATCC 25922, this compound appeared to be effective at concentration equal to 1 µg/ml (2×MIC) after 8 hours incubation. A concentration equal to 2 µg/ml (4×MIC) had a bactericidal activity already after 4 hours reducing to zero viable counts after 8h. No regrowth was also observed over 24 h. The 73009 molecule was less active against *P. aeruginosa* ATCC 27853, with a bactericidal activity after 8 h, using 8 µg/ml (4×MIC) of this compound. No bactericidal effect was observed against both *S. aureus* strains at the tested concentrations during the 24 hours of incubation time.

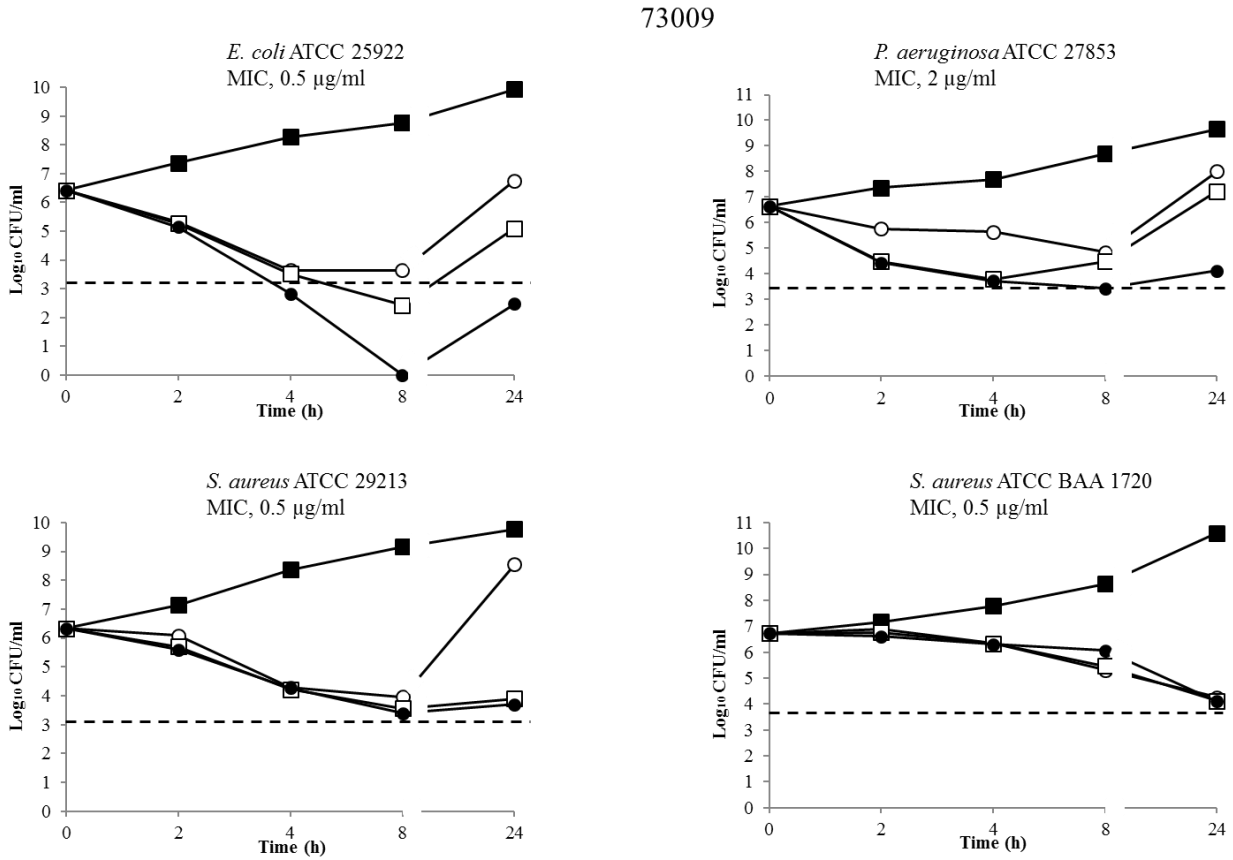

Figure 3.2.4 Killing kinetics of 73009 molecule against four control strains: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA 1720. All experiments were performed in triplicate. The Threshold corresponding to the 3 Log_{10} CFU/ml reduction was identified by black dashed line. Standard deviations of the reported values were always below 0.5 Log_{10}. ■, growth control; ○, 1×MIC; □ 2×MIC; ●, 4×MIC.

3.2.4 Time-kill performance of 73011

This compound showed the lowest activity (figure 3.2.5): a bactericidal activity was observed against *E. coli* ATCC 25922 only, using a high concentration of the molecule (8 µg/ml, 4×MIC). No bactericidal effect was observed against both *S. aureus* strains at all tested concentrations despite its good activity in bacterial growth inhibition (low MIC values).

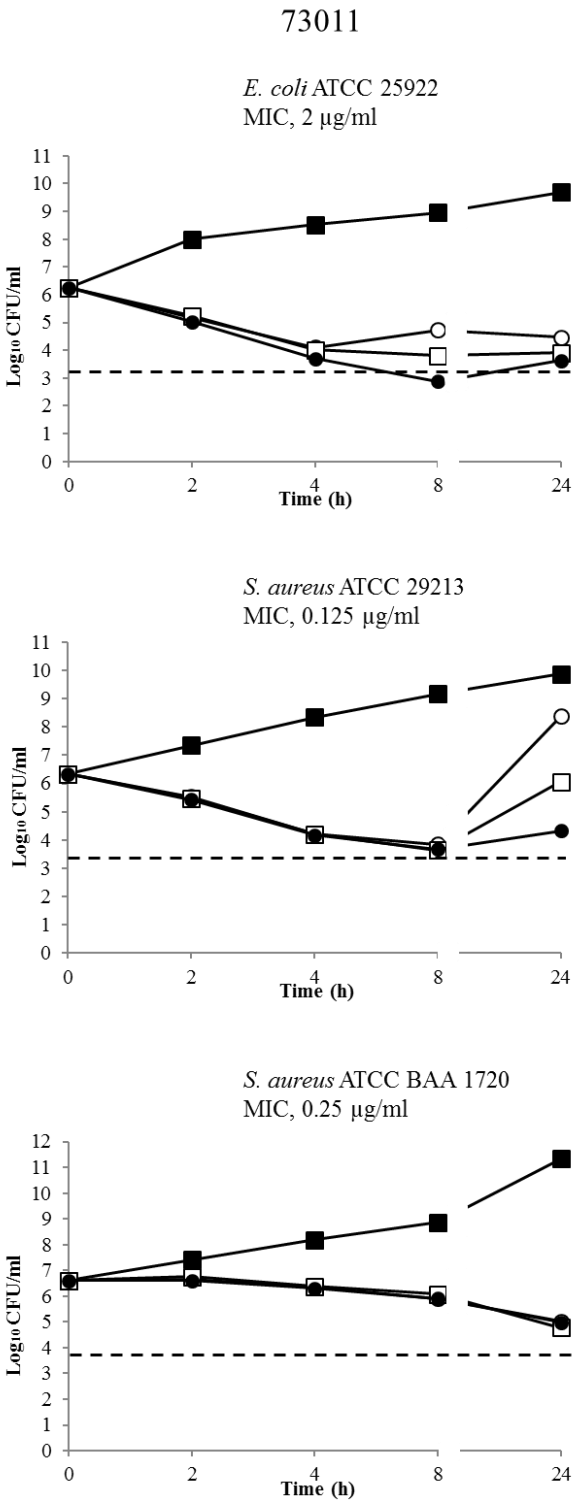

Figure 3.2.5 Killing kinetics of 73011 molecule against three control strains: *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA 1720. All experiments were performed in triplicate. The Threshold corresponding to the 3 Log_{10} CFU/ml reduction was identified by black dashed line. Standard deviations of the reported values were always below 0.5 Log_{10}. ■, growth control; ○, 1×MIC; □ 2×MIC; ●, 4×MIC.

3.2.5 Time kill performance of 73012

After MIC determination, the 73012 molecule resulted effective only against the two *S. aureus* strains (MIC ≤ 2 μg/ml). Therefore, its time kill performances were investigated only against staphylococci. However, all the different concentrations tested in the assays displayed no bactericidal activity against both staphylococcal strains (see figure 3.2.6).

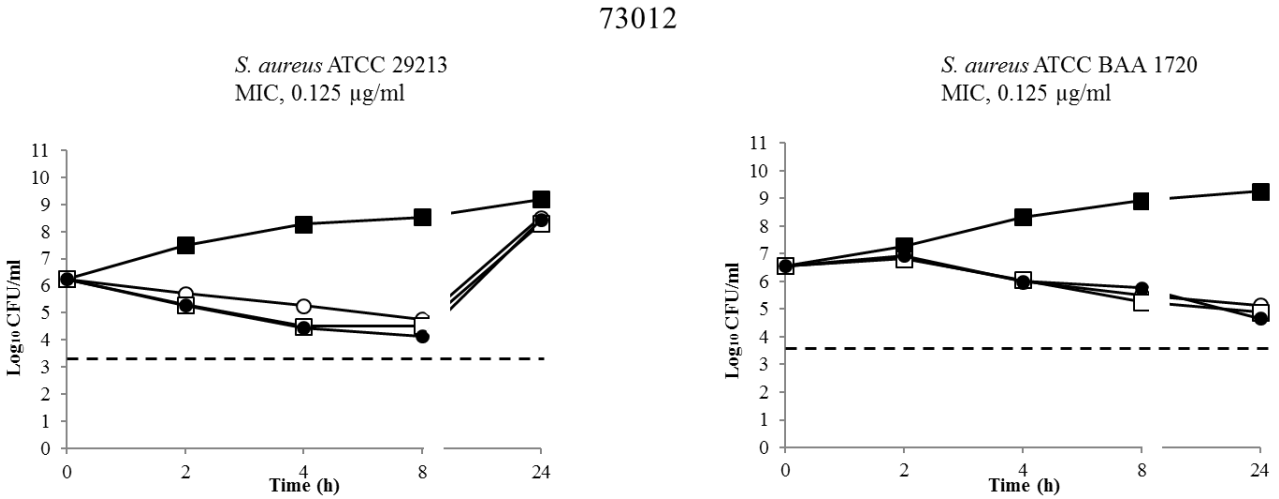

Figure 3.2.6 Killing kinetics of 73012 molecule against the two staphylococci: *S. aureus* ATCC 29213, *S. aureus* ATCC BAA 1720. All experiments were performed in triplicate. The Threshold corresponding to the 3 Log_{10} CFU/ml reduction was identified by black dashed line. Standard deviations of the reported values were always below 0.5 Log_{10}. ■, growth control; ○, 1×MIC; □ 2×MIC; ●, 4×MIC.

3.2.6 Time-kill performance of 73013

The 73013 molecule showed a bactericidal activity against all the three strains (figure 3.2.7). A 3 Log_{10} CFU/ml viable count reduction was observed using all tested concentration (0.5, 1 and 2 µg/ml) after 8 hours incubation against *E. coli* ATCC 25922. Concentrations equal to 1 and 2 µg/ml (2×MIC and 4×MIC) maintain the efficacy over the 24 hours. As regard staphylococci, this compound was effective against both *S. aureus* ATCC 29213 and *S. aureus* ATCC BAA 1720 at 24 hours using a concentration of 0.5 µg/ml (4×MIC and 2×MIC, respectively). Against *S. aureus* ATCC BAA 1720, 1 µg/ml (4×MIC) was also bactericidal at 24h.

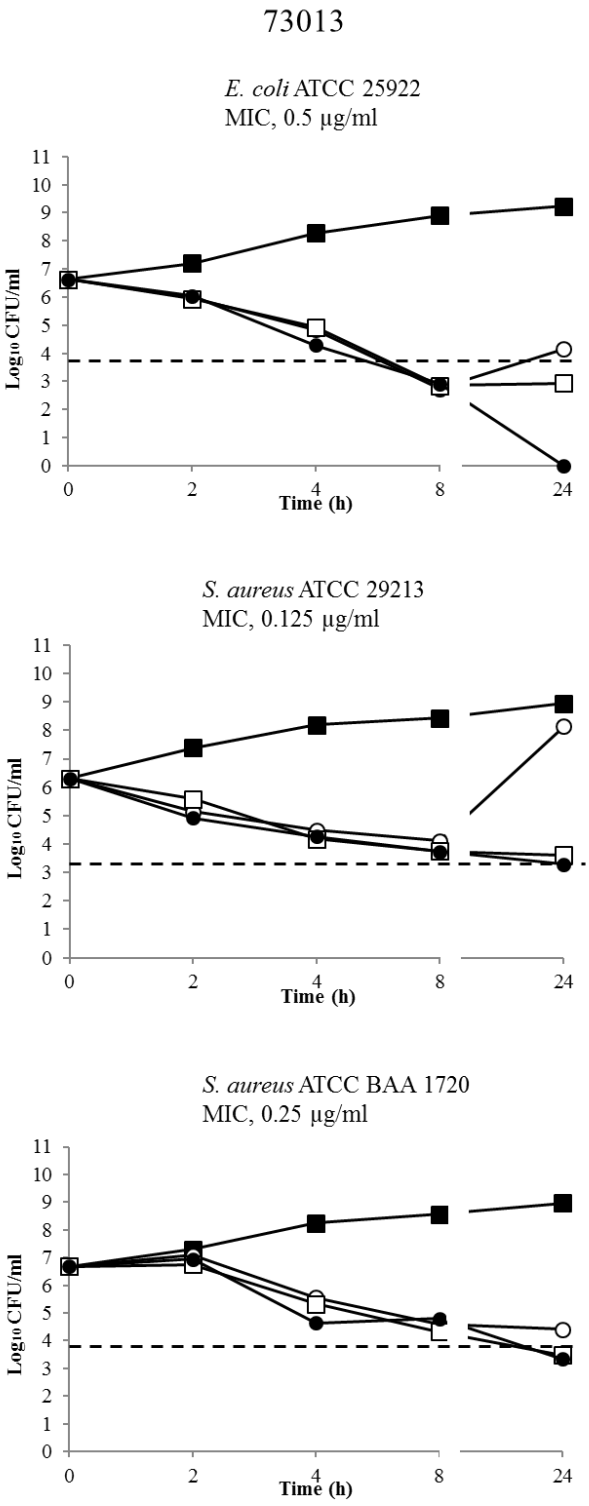

Figure 3.2.7 Killing kinetics of 73013 molecule against three control strains: *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA 1720. All experiments were performed in triplicate. The Threshold corresponding to the 3 Log_{10} CFU/ml reduction was identified by black dashed line. Standard deviations of the reported values were always below 0.5 Log_{10}. ■, growth control; ○, 1×MIC; □ 2×MIC; ●, 4×MIC.

3.2.7 Time-kill performance of 73015

The 73015 molecule showed a bactericidal activity against all the three strains (figure 3.2.8). The viable count reduction was observed using all tested concentration (0.5, 1 and 2 µg/ml) against *E. coli* ATCC 25922 after 8 hours. This compound showed a bactericidal activity also against both *S. aureus* reference strains but at higher concentrations: 2 µg/ml (4×MIC) resulted bactericidal against *S. aureus* ATCC BAA 1720 after 24 hours incubation time whereas concentrations equal to MIC, 2×MIC, and 4×MIC had no bactericidal activity against *S. aureus* ATCC 29213. Indeed, to record bactericidal activity against *S. aureus* ATCC 29213, in time kill assay, it was necessary to test two further concentrations (8×MIC and 16×MIC) compared to those routinely tested: both concentrations (4 µg/ml and 8 µg/ml respectively) were effective after 24 hours incubation, confirming MBC testing results.

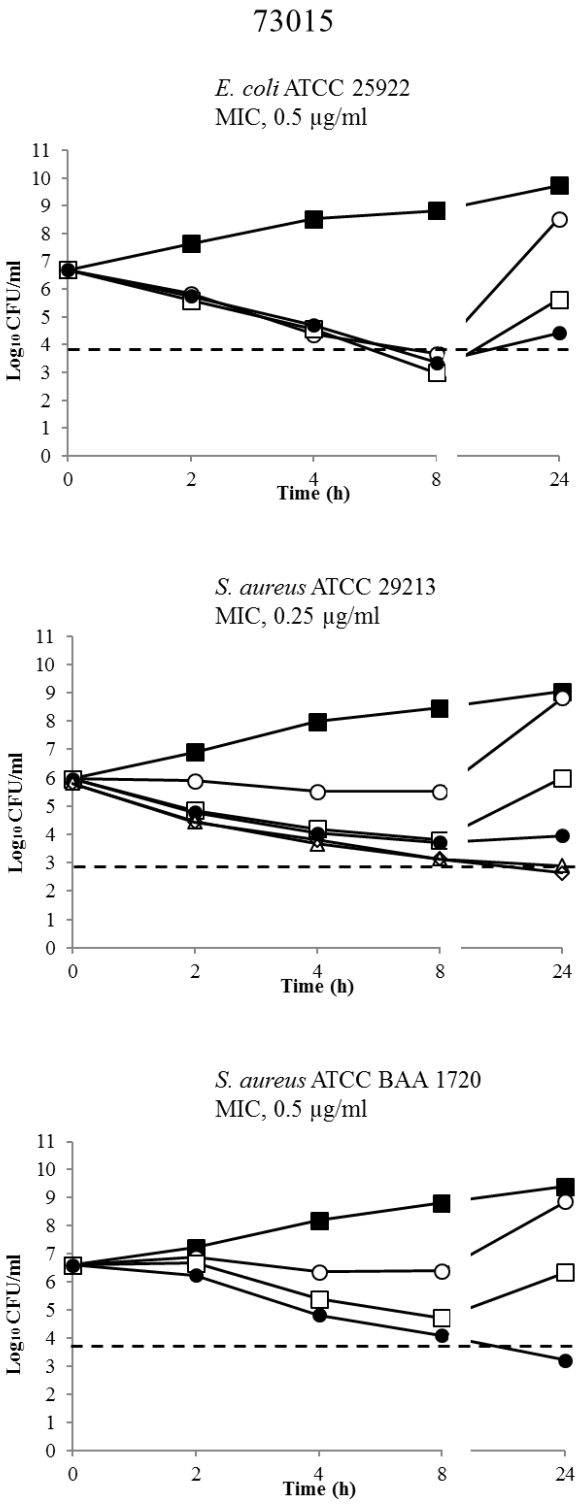

Figure 3.2.8 Killing kinetics of 73015 molecule against three control strains: *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA 1720. All experiments were performed in triplicate. The Threshold corresponding to the 3 Log_{10} CFU/ml reduction was identified by black dashed line. Standard deviations of the reported values were always below 0.5 Log_{10}. ■, growth control; ○, 1×MIC; □ 2×MIC; ●, 4×MIC; Δ 8×MIC; ◊ 16×MIC.

3.2.8 Time-kill performance of 73016

Although low MIC values of the 73016 molecule were recorded especially with *S. aureus* strains, the different concentrations tested in each time kill assay displayed no bactericidal activity against all control strains during 24 h incubation time (see figure 3.2.9). Moreover, a regrowth at 24 h occurred in all strains with all tested concentrations.

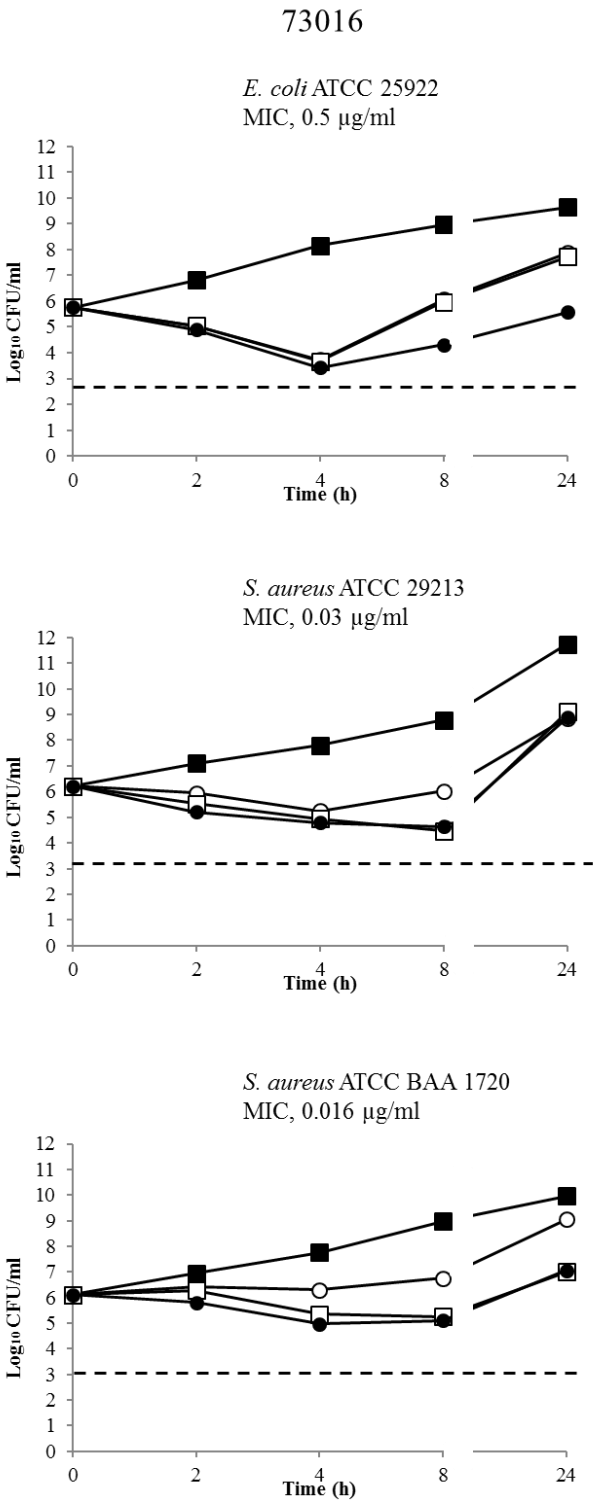

Figure 3.2.9 Killing kinetics of 73016 molecule against three control strains: *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA 1720. All experiments were performed in triplicate. The Threshold corresponding to the 3 Log_{10} CFU/ml reduction was identified by black dashed line. Standard deviations of the reported values were always below 0.5 Log_{10}. ■, growth control; ○, 1×MIC; □ 2×MIC; ●, 4×MIC.

3.3 PAE testing

PAE testing of 73015 and 73016, were performed against *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *S. aureus* ATCC BAA 1720 and PAE values were compared with those obtained for ciprofloxacin. The post antibiotic effect of ciprofloxacin against the methicillin resistant *S. aureus* ATCC BAA 1720 was not evaluated due to the high level of ciprofloxacin resistance of strain (MIC 128 µg/ml). Persistent suppression of bacterial growth after intermittent antimicrobial exposure appeared to be a feature of every antimicrobial agent: indeed, this phenomenon was observed with each tested drug against all organisms. The duration of PAEs differed for specific drug/microorganism combinations and increasing concentrations of all antibiotics were associated with a progressive increasing of the PAE. (see table 3.3.1).

PAEs on the three control strains after 1 h exposure to the 73015 molecule are graphically shown in figure 3.3.2.

Bacterial growth suppression with this molecule was higher on *E. coli* ATCC 25922: the bacterial growth resumption of *E. coli* was more time-consuming than those of the two staphylococci. Overall, the PAE values of 73015 ranged from 14 min to 98 min for *E. coli* ATCC 25922 from 10 to 58 min for the *S. aureus* strains (see table 3.3.1).

The PAE of 73016 molecule was also evaluated: data for all control strains following exposure for 1 hour at different concentrations of this compound are plotted in figure 3.3.3. This molecule proved to be more effective than the previous compound in the suppression of bacterial growth, even after its removal by dilution of broth cultures. The PAE values of 73016 ranged from 20 to 100 min for *E. coli* ATCC 25922 and from 20 to 78 min for the two staphylococci (table 3.3.1).

Table 3.3.1 *In vitro* PAEs of 3 antimicrobials on the three control strains after 1 h exposure to different concentrations.

Organism and drug	**MIC (µg/ml)**	**PAE (min)**		
		1×MIC	4×MIC	16×MIC
***E. coli* ATCC 25922**				
73015	0.5	14	66	98
73016	0.5	20	95	100
ciprofloxacin	0.008	10	55	120
***S. aureus* ATCC 29213**				
73015	0.25	33	54	58
73016	0.03	43	73	78
ciprofloxacin	0.5	25	25	35
***S. aureus* ATCC BAA 1720**				
73015	0.5	10	20	25
73016	0.016	20	50	70
ciprofloxacin	**128**	ND*	ND	ND

*ND means not determined

After 1 h exposure, ciprofloxacin proved to be more effective against *E. coli* ATCC 25922 than *S. aureus* ATCC 29213, however, PAEs were observed at each tested concentrations against *S. aureus* strain (table 3.3.1). PAEs on *E. coli* ATCC 25922 and *S. aureus* ATCC 29213, after ciprofloxacin exposure, are graphically shown in figure 3.3.4.

Ciprofloxacin displayed an effective killing activity during the hour exposure and a long-term effect after drug removal against *E. coli* ATCC 25922 (figure 3.3.4). The time required for normal growth resumption of *E. coli* ATCC 25922 increased from 10 to 120 min respect to the unexposed culture. Shorter PAEs of ciprofloxacin, compared with those obtained for the other NBTIs, were recorded for *S. aureus* ATCC 29213. PAEs duration ranged from 25 to 35 min.

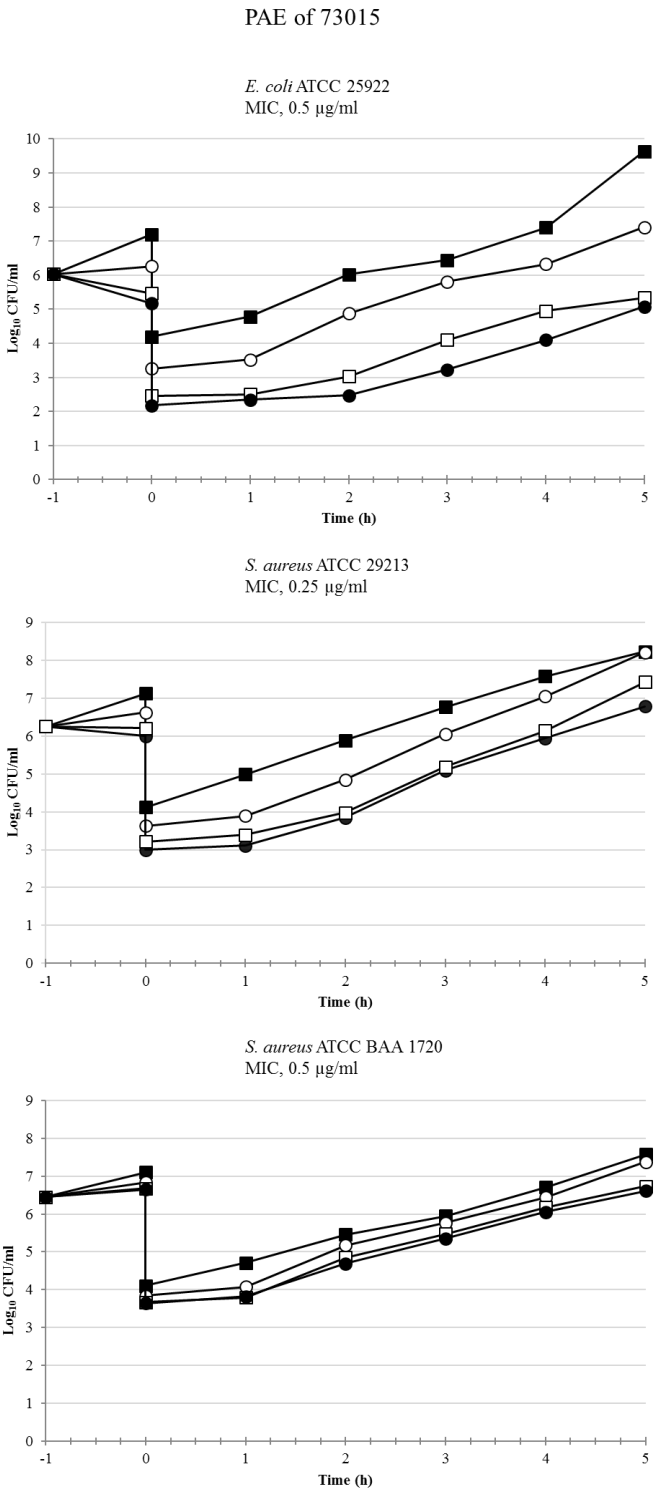

Figure 3.3.2 PAEs induced by 1-hour exposure to 73015 molecule of three control strains: *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA 1720. All experiments were performed in triplicate. Standard deviations of the reported values were always below 0.5 Log_{10}. ■, growth control; ○, 1×MIC; □ 4×MIC; ●, 16×MIC.

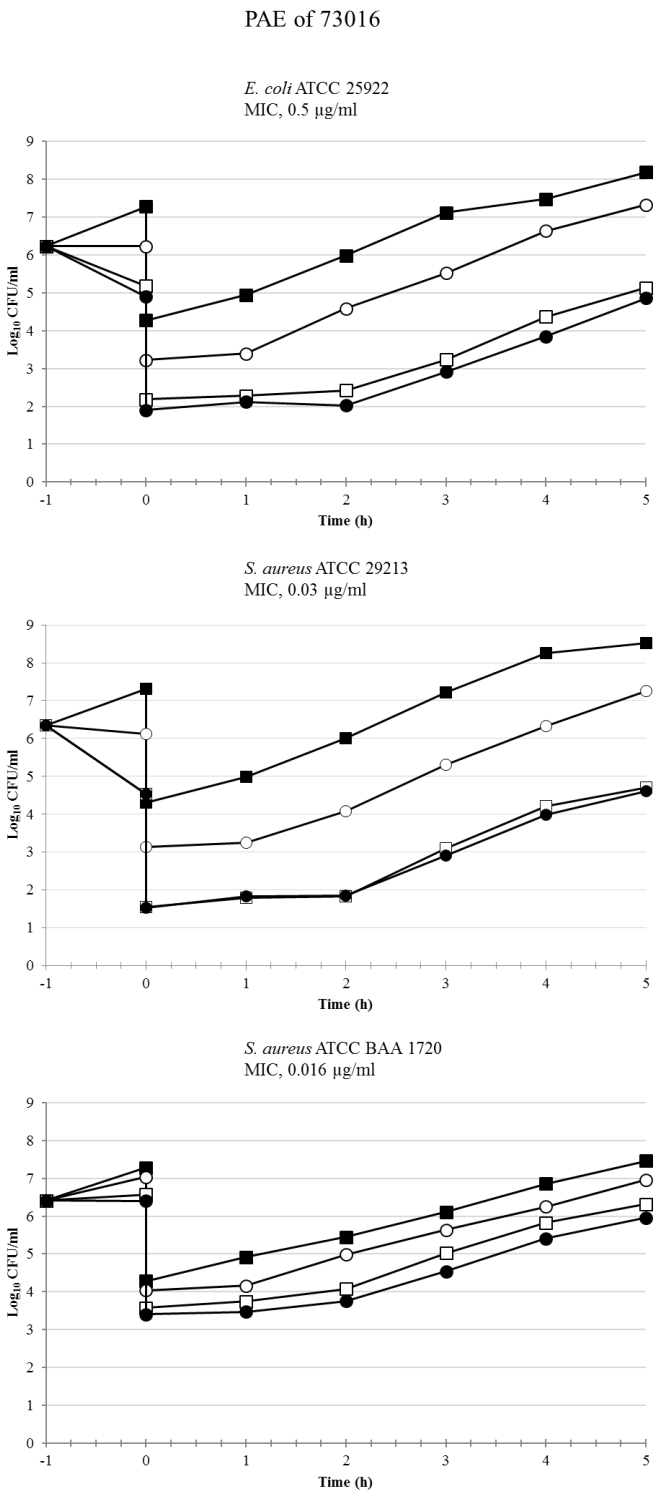

Figure 3.3.3 PAEs induced by 1-hour exposure to 73016 molecule of three control strains: *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA 1720. All experiments were performed in triplicate. Standard deviations of the reported values were always below 0.5 Log_{10}. ■, growth control; ○, 1×MIC; □ 4×MIC; ●, 16×MIC.

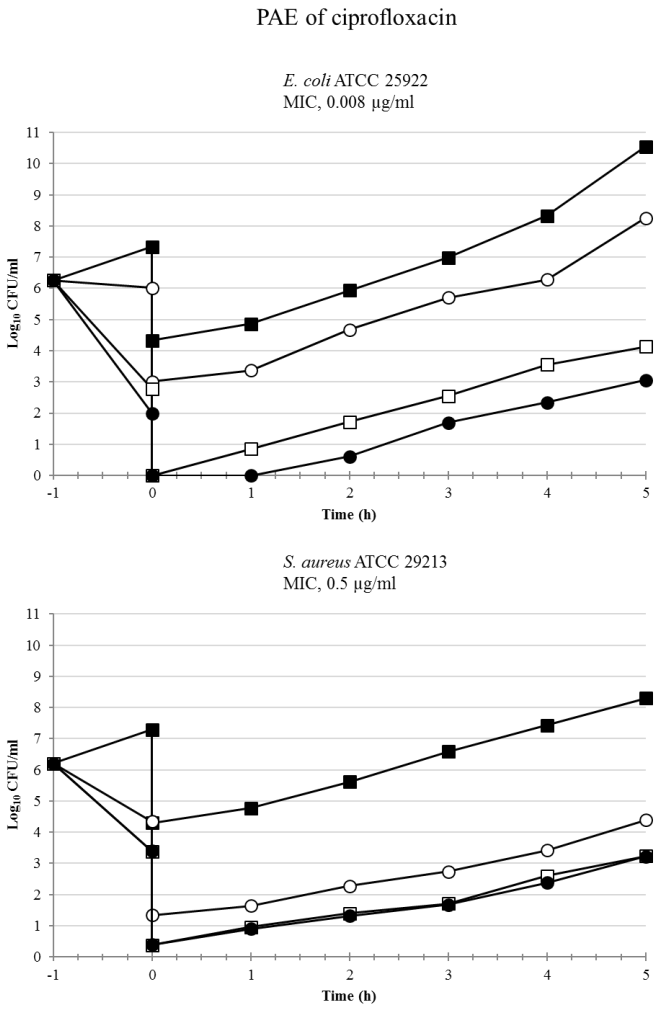

Figure 3.3.4 PAEs induced by 1-hour exposure to ciprofloxacin of *E. coli* ATCC 25922, *S. aureus* ATCC 29213. All experiments were performed in triplicate. Standard deviations of the reported values were always below 0.5 Log_{10}. ■, growth control; ○, 1×MIC; □ 4×MIC; ●, 16×MIC.

3.4 *In vitro* selection of staphylococcal resistance

Acquired antibiotic resistance to some antimicrobials (e.g. quinolones) can be achieved by spontaneous mutations in specific chromosomal loci. The mutation rate is defined as the *in vitro* frequency at which detectable mutants arise in a bacterial population in presence of a given antibiotic concentration (Martinez & Baquero, 2000). In this study the mutation frequency to fluoroquinolones and to some NBTIs in *S. aureus* has been determined. The acquired resistance in *S. aureus* mutants has also been characterized.

3.4.1 *In vitro* selection of *S. aureus* ATCC 29213 mutants

In table 3.4.1 are reported the results obtained from the *in vitro* selection of *S. aureus* ATCC 29213 mutant strains. The resistant mutants were obtained through single-step mutation method using ciprofloxacin and the molecules 73009, 73011, 73012, 73013, 73015, 73016. All resistant mutants were selected on plates supplemented with a maximum of 1 µg/ml of the single antimicrobials except for the molecules 73009 and 73015 (table 3.4.1). Resistant mutants to these compounds were obtained using 4 and 2 µg/ml respectively. Taking into account the novel NBTIs, the average mutation frequency of *S. aureus* ATCC 29213 was $2.55 \cdot 10^{-7}$. Selection of mutants was also performed on agar containing 1 µg/ml (2×MIC) of ciprofloxacin with mutation frequencies of $4 \cdot 10^{-8}$. Overall, all putative mutant strains showed increased MIC values (4 to 32-fold the MIC) if compared to those of the wild-type *S. aureus* ATCC 29213. Moreover, even after growth on antibiotic-free plates, the MICs of mutants were stable or, if decreased, still remained higher than the MIC of the wild-type strain. Mutants resistant to ciprofloxacin and to 73011

showed a further increase in MIC values after 5 more passages on antibiotic-free plates (see table 3.4.2).

Table 3.4.1 *In vitro* frequency of resistant mutants to ciprofloxacin and six novel NBTIs in *S. aureus* ATCC 29213

MIC-fold	ciprofloxacin	73009	73011	73012	73013	73015	73016
1×MIC	TNTC*	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
2×MIC	**$4 \cdot 10^{-8}$ (1 µg/ml)**	TNTC	TNTC	TNTC	TNTC	TNTC	**$6.7 \cdot 10^{-7}$ (0.06 µg/ml)**
4×MIC	$< 1 \cdot 10^{-8}$	TNTC	**$1.3 \cdot 10^{-7}$ (0.5 µg/ml)**	TNTC	**$2.6 \cdot 10^{-7}$ (0.5 µg/ml)**	**$1.4 \cdot 10^{-7}$ (2 µg/ml)**	$< 1 \cdot 10^{-8}$
8×MIC	$< 1 \cdot 10^{-8}$	**$1.1 \cdot 10^{-7}$ (4 µg/ml)**	$< 1 \cdot 10^{-8}$	**$2.2 \cdot 10^{-7}$ (1 µg/ml)**	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$
16×MIC	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$
32×MIC	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$	$< 1 \cdot 10^{-8}$

*TNTC means too numerous to count; in brackets the highest concentration of drug at which countable mutants developed

Table 3.4.2 MIC ranges of *S. aureus* ATCC 29213 mutants after single-step selection

Antimicrobial compound	**MIC wild-type (µg/ml)**	**MIC range mutants (µg/ml)***	**MIC range mutants (µg/ml)****	**MIC-fold increase**
ciprofloxacin	0.5	2 - 4	2 - 8	4× - 16×
73009	0.5	2 - >8	1 - >8	2× - >16×
73011	0.125	0.5 - 2	2 - 4	16× - 32×
73012	0.125	1 - 4	1 - 4	8× - 32×
73013	0.125	0.5 - 2	0.5 - 2	4× - 16×

*after 5 passages on plates containing the same antibiotic concentration used for selection
**after 5 more passages on drug-free plates

Resistant mutants selected on ciprofloxacin and on the molecules 73009, 73011, 73012, 73013, named SACIP3, SA009.9, SA011.3, SA012.5, SA013.11 respectively, have been further investigated for the occurrence and the position of point mutations in genes encoding DNA gyrase (*gyrA*, *gyrB*) and topoisomerase IV (*grlA*, *grlB*). Since these mutants displayed the highest increase in MIC value (16 or 32 MIC fold), they have been selected for sequencing analyses in order to explain the occurrence of resistance. The sequencing results are shown in table 3.4.3. The ciprofloxacin resistant mutant SACIP3 displayed a point mutation in *grlA* gene. Such mutation occurred in a hot-spot region, known as the Quinolone Resistance Determining Region (QRDR), resulting in the amino acid substitution of a serine (T**C**C) with a phenylalanine (T**T**C) at position 80 (table 3.4.3).

Table 3.4.3 Point mutations in DNA gyrase and topoisomerase IV genes of *S. aureus* ATCC 29213 mutants

		Amino acid substitution			
		DNA gyrase		*topoisomerase IV*	
Molecule	Mutant strain	*gyrA*	*gyrB*	*grlA*	*grlB*
ciprofloxacin	SACIP3	-	-	ser 80 phe T**C**C - T**T**C	-
73009	SA009.9	asp 83 asn **G**AC - **A**AC	-	-	-
73011	SA011.3	-	asp 437 ala G**A**C - G**C**C	-	-
73012	SA012.5	val 45 ileu **G**TA - **A**TA	-	-	pro 307 thr **C**CA - **A**CA
73013	SA013.11	met 121 lys AT**T**G - AA**A**G	-	-	-

As regard the mutants selected on novel NBTIs, no mutation was observed in the QRDR of *grlA* gene (corresponding to amino acid position 64 to 103, Ferrero *et al*., 1995) and all mutants showed point mutations on *gyrA* or *gyrB* genes. The mutations located on *gyrA* gene occurred in different positions among mutants (table 3.4.3). A single mutant, SA009.9, carried a point mutation within the QRDR of *gyrA* gene (corresponding to amino acid position 68 to 107 of the GyrA protein, Ferrero *et al*., 1995): this mutation determined the substitution of aspartate with an asparagine at amino acid position 83. In mutant strain SA011.3, a point mutation on the *gyrB* gene occurred in the amino acid position 437 (table 3.4.3). Only the mutant SA012.5, resistant to the molecule 73012, showed point mutations on both *gyrA* and *grlB* genes.

3.4.2 *In vitro* selection of *S. aureus* ATCC BAA 1720 mutants

In table 3.4.4 are reported the results obtained from the *in vitro* selection of *S. aureus* ATCC BAA 1720 mutant strains. The resistant mutants were selected by molecules 73015 and 73016 using different concentrations in agar plates (table 3.4.4). The 73015 and 73016 molecules led to the development of resistant mutants using 16 and 0.25 µg/ml, respectively (32×MIC and 16×MIC) and for both NBTIs an average mutation frequency of $1 \cdot 10^{-7}$ were determined.

Table 3.4.4 *In vitro* frequency of resistant mutants to two novel NBTIs in *S. aureus* ATCC BAA 1720

MIC-fold	73015	73016
MIC	TNTC	TNTC
2×MIC	TNTC	TNTC
4xMIC	TNTC	TNTC
8×MIC	TNTC	TNTC
16×MIC	TNTC	**$1.43 \cdot 10^{-7}$ (0,25 µg/ml)**
32×MIC	**$1 \cdot 10^{-7}$ (16 µg/ml)**	$< 1.4 \cdot 10^{-8}$

*TNTC means too numerous to count

Overall, all putative mutant strains showed increases in MICs that were up to 32-fold higher than the wild-type (*S. aureus* ATCC BAA 1720) MIC values. Moreover, the MICs of 73015 and 73016 further increased after 5 passages of mutant strains on antibiotic-free plates (table 3.4.5).

Table 3.4.5 MIC ranges of *S. aureus* ATCC BAA 1720 mutants after single-step selection

Antimicrobial compound	**MIC wild-type (µg/ml)**	**MIC range mutants (µg/ml)***	**MIC range mutants (µg/ml)****	**MIC-fold increase**
73015	0.5	2 - 4	2 - 8	4× - 16×
73016	0.016	0.06 - 2	0.25 – 0.5	16× - 32×

*after 5 passages on plates containing the same antibiotic concentration used for selection
**after 5 more passages on drug-free plates

Mutant strains displaying the highest increase in MIC values (16 or 32 MIC fold), have been selected for sequencing analyses. Indeed, *S. aureus* ATCC BAA1720 resistant mutants, selected on the molecules 73015 and 73016, named MRSA015.14, MRSA016.7, have been investigated for the occurrence and distribution of point mutations in *gyrA*, *gyrB* and *grlA*, *grlB* genes. Sequencing analyses on *grlA* gene of *S. aureus* ATCC BAA 1720 wild-type strain, revealed a single point mutation within the QRDR determining the substitution of a serine with a phenylalanine at amino acid position 80. This point mutation was obviously detectable in each *S. aureus* ATCC BAA 1720 mutant strain. Although induced by different molecules, all point mutations occurred in one of the two genes encoding the DNA gyrase. The sequencing results are shown in table 3.4.6.

Table 3.4.6 Point mutations in DNA gyrase and topoisomerase IV genes of *S. aureus* ATCC BAA 1720 mutants

Molecule	Mutant strain	Amino acid substitution			
		DNA gyrase		*topoisomerase IV*	
		gyrA	*gyrB*	*grlA*	*grlB*
73015	MRSA015.14	-	pro 456 leu C**C**A - C**T**A	-	-
73016	MRSA016.7	pro 44 leu C**C**A - C**T**A	-	-	-

The mutant strain, MRSA015.14, carried a point mutation on the *gyrB* gene and this mutation determined the shift from a proline to leucine at the amino acid position 456. The only mutation, carried by the mutant strain MRSA016.7, fell within the *gyrA* gene but outside of the QRDR (corresponding to amino acid position 68 to 107) causing the amino acid substitution at position 44 (table 3.4.6).

3.4.3 Conserved domains analysis

Functional domain of Topisomerase IV and DNA gyrase affected by point mutations, were identified using Conserved Domains search tool. The analysis revealed that all point mutations in *gyrA* gene resulting in amino acid substitution in the DNA gyrase CAP-like domain functioning as transcriptional activator. The point mutations in *gyrB* involved amino acid substitutions mapped to the enzyme topoisomerase primase (TOPRIM) nucleotidyl transferase/hydrolase domain.

Table 3.4.7 Effect of point mutation on functional domain of Topoisomerase IV and DNA gyrase

Molecule	**Gene target**	**Protein**	**Substitution**	**Domain**
Ciprofloxacin	*grlA*	Topoisomerase IV - A subunit	ser 80 phe	CAP*-like domain
73009	*gyrA*	DNA gyrase – A subunit	asp 83 asn	CAP-like domain
73011	*gyrB*	DNA gyrase – B subunit	asp 437 ala	TOPRIM** nucleotidyl transferase/hydrolase domain
73012	*gyrA*	DNA gyrase – A subunit	val 45 ileu	CAP-like domain
	grlB	Topoisomerase IV - B subunit	pro 307 thr	Transducer domain
73013	*gyrA*	DNA gyrase – A subunit	met 121 lys	CAP-like domain
73015	*gyrB*	DNA gyrase – B subunit	pro 456 leu	TOPRIM nucleotidyl transferase/hydrolase domain
73016	*gyrA*	DNA gyrase – A subunit	pro 44 leu	CAP (catabolite-activator protein)-like domain

*CAP means catabolite-activator protein
**TOPRIM means topoisomerase primase

3.5 Susceptibility of clinical strains to 73015 and 73016 NBTIs

In the present study, *A. baumannii* and MRSA strains of clinical origin were tested for their susceptibility to 73015 and 73016 molecules; MICs of ciprofloxacin against all clinical strains were first determined. A comprehensive comparison of the activities of 73015, 73016 and ciprofloxacin against *A. baumannii* and MRSA strains are presented in table 3.5.1. Resistance phenotype of clinical *A. baumannii* and MRSA strains has been determined by Clinical Microbiology Laboratory, Ospedali Riuniti of Ancona. *A. baumannii* strains were resistant to all drugs routinely screened by regional hospital (including levofloxacin). The colistin resulted the only therapeutic option for treatment of infections caused by these multidrug resistant strains. All *A. baumannii* isolates were resistant to ciprofloxacin (MIC from 8 to >128 µg/ml). MICs of 73015 and 73016 against clinical *A. baumannii* strains ranged both from 0.125 to 2 µg/ml. The 73015 and 73016 MIC values at which 50% and 90% of *A. baumannii* tested was inhibited remained equal or below the resistance breakpoint arbitrarily set at 2 µg/ml.

All MRSA strains were resistant to levofloxacin. Resistance to erythromycin, clindamycin and gentamicin occurred in the majority of strains (68.18%, 18.18%, 40.91%, respectively). All isolates were susceptible to linezolid, vancomycin and teicoplanin. Similarly to *A. baumannii*, all MRSA isolates were resistant to ciprofloxacin (MIC from 4 to 128 µg/ml).

Table 3.5.1 Comparative activities (MIC) of ciprofloxacin, 73015 and 73016 against clinical multidrug resistance *A. baumannii* and methicillin resistant *S. aureus*

Organism (no. of tested isolates)	Antimicrobial agent	MIC (µg/ml)*		
		RANGE	50%	90%
A. baumannii (38)	ciprofloxacin	8 - >128	32	>128
	73015	0.125 - 2	0.25	1
	73016	0.125 - 2	0.5	1
S. aureus, methicillin-resistant (24)	ciprofloxacin	4 - 128	8	128
	73015	0.03 - 1	0.25	0.5
	73016	0.016 - 0.5	0.03	0.125

* 50% and 90%, MICs at which 50 and 90% of isolates tested are inhibited, respectively

MICs of 73015 against these clinical strains ranged from 0.03 to 1 µg/ml. The 73015 MIC for 50% and 90% were below the resistance breakpoint arbitrarily set at 2 µg/ml. (see table 3.5.1). On the contrary, 73016 molecule was more active than 73015 molecule with MIC ranged from 0.016 to 0.5 µg/ml. Concentrations of 73016 able to inhibit 50% and 90% of tested MRSA strains were the lowest (0.03 and 0.125 µg/ml, respectively), confirming the good efficacy of this compound against MRSA.

Chapter 4 – DISCUSSION

The increasing antimicrobial resistance in health care settings and the subsequent lack of effective antimicrobials is of worldwide concern (Holmes *et al*., 2016). It is well established that antimicrobial resistance dramatically reduces the probability of successfully treating infections and leads to the increase of the morbidity and mortality associated with common bacterial diseases (Cassir *et al*., 2014). Since the discovery of penicillin in 1928, antimicrobial resistance has been linked to antibiotic use, although recent studies on bacteria from permafrost samples documented the existence of resistance genes even 30,000 years ago (D'Costa *et al*., 2011). Rising of antimicrobial resistance in microorganisms is a natural phenomenon and its selection has been driven by antimicrobial exposure in several settings, such as health care systems, agriculture, and natural environments (Rolain *et al*., 2013). Antimicrobial resistance represents an ongoing challenge that requires a multifaceted approach including (i) biomedical innovation, (ii) more conscious antibiotic consumption, (iii) prevention of health-care-associated infections by multidrug resistant bacteria and their environmental spread and (iv) avoidance of clinical and veterinary misuse (Cassir *et al*., 2014). Insight of the scientific basis on antimicrobial resistance is essential to counteract this public health threat. All this should include the understanding of resistance mechanisms, enabling novel approaches to diagnostics and therapeutics, and of the main drivers affecting antimicrobial resistance in society and environment in order to develop an appropriate intervention policy (Ardal *et al*., 2016; Dar *et al*., 2016; Mendelson *et al*., 2016).

Nowadays, diseases caused by multidrug resistant bacteria have increasingly resulted untreatable with conventional antimicrobials (Frieri *et al*., 2016). Hospital infections caused by multidrug resistant bacteria are constantly occurring (Boucher & Corey, 2008) and *ESKAPE* pathogens represent new paradigms in pathogenesis, transmission and resistance (Rice, 2008) being responsible of the most severe microbial diseases (Tang *et al*., 2014). Indeed, nosocomial infections are microbiologically heterogeneous and antibiotic usage constantly selects pathogens resistant also to last line antimicrobials (French, 2010).

Bacterial resistance continues to spread and strains also resistant to newly developed antibiotics are raising (Cassir *et al*., 2014) but the rate at which novel antimicrobial molecules are being developed is decreasing (ECDC/EMEA, 2009). Over the last decade, the significant failure of exploiting novel antibacterial targets (Silver, 2011) can be due to several developing barriers that impede the discovery of novel antibiotics (Akova, 2016b). Nonetheless, some efforts in the research and development of novel antibacterial agents have been made and resulted in compounds with potent antimicrobial activity against multidrug resistant pathogens (Lahiri *et al*., 2015). Ideally, novel compounds should have a well differentiated mode of action compared to currently used antibiotics in order to overcome the existing resistance mechanisms of bacteria (Perez *et al*., 2008; Karaiskos & Giamarellou, 2014). Among desirable targets for the development of new antibacterial agents, bacterial topoisomerases are an interesting field of research and the quinolone class of antibiotics provides a clinical validation of this target (Silver, 2011). This has promoted discovery and development of both new quinolones and other unrelated compounds that target bacterial DNA gyrase and/or topoisomerase IV with improved human safety and activity against clinical multidrug

resistant isolates (Bradbury & Pucci, 2008; Wiles *et al*., 2010). Due to the historical success of new topoisomerase inhibitors, their research and development continues to the present day. Bacterial topoisomerase inhibitors can be classified as quinolones, quinolone-like compounds, and non-quinolones (Pucci & Wiles, 2014). Actually, novel (non-fluoroquinolone) bacterial type II topoisomerase inhibitors, designated by some authors (Collin *et al*., 2011; Reck *et al*., 2011) as NBTIs, have been described as a promising class of compounds able to inhibit both bacterial DNA gyrase and topoisomerase IV (Bax *et al*., 2010; Miles *et al*., 2013; Dougherty *et al*., 2014). Drugs possessing dual target activity should reduce the development of resistance in bacteria, due to the unlikely occurrence of two simultaneous mutations in both essential targets (Azam *et al*., 2015). Therefore, these NBTIs could represent a new class of clinically relevant antimicrobial agents (Bradbury & Pucci, 2008).

In agreement with the medical need to find novel broad spectrum antimicrobial agents (Tomaras & Dunman, 2015), a series of NBTIs has been synthesized and selected by Angelini (ACRAF) laboratories. These molecules, should have a mechanism of action similar to that of fluoroquinolones, interacting to a similar extent with the two bacterial topoisomerase type II, DNA gyrase and topoisomerase IV. This work has been focused on the study of the effectiveness of these NBTIs. Molecular and microbiological methods have been applied in order to evaluate the susceptibility of bacterial reference strains, to assess the occurrence of resistance in *S. aureus* and to investigate the susceptibility of multidrug resistant isolates of clinical origin.

The susceptibility of the strains to these NBTIs was compared to that to ciprofloxacin and levofloxacin. Basing on MIC results, ciprofloxacin and levofloxacin resulted effective only against *E. coli* ATCC 25922 and *S.*

aureus ATCC 29213 whereas the methicillin resistant *S. aureus* ATCC BAA 1720 strain turned out to be highly resistant to both antimicrobials. Fluoroquinolone resistance of this reference strain was explained not only by the point mutation within QRDR of GrlA (ParC) protein on the hot spot localised at amino acid position 80, which is known to confer fluoroquinolone resistance (Takahata *et al*., 1996; Roychoudhury *et al*., 2001), but also by the carriage of a *norA* gene (Holden *et al*., 2004) that encodes a broad spectrum energy-dependent efflux system (Kaatz & Seo, 1995). These characteristics were obtained from the analysis of the complete sequence of *S. aureus* ATCC BAA 1720 (designated as MRSA 252 at https://www.atcc.org/products/all/BAA-1720.aspx) genome available on NCBI under accession number BX571856.1. About the novel compounds, 6 out of 12 molecules resulted effective (breakpoint arbitrarily set at MIC $\leq$ 2 μg/ml) simultaneously against all the three control strains, encompassing both Gram-positive and -negative. It is worth noting that even the *S. aureus* ATCC BAA 1720 control strain, highly resistant to second generation quinolones (ciprofloxacin and levofloxacin), was susceptible to several molecules. This susceptibility suggested that these molecules were not affected by the common quinolone resistance mechanisms able to increase MIC of ciprofloxacin up to 128-fold (Redgrave *et al*., 2014). The MIC results were in line with ability of the non-fluoroquinolone NBTIs to inhibit bacterial growth of both Gram-positive and -negative strains, reported in literature (Pucci & Wiles, 2014). On the whole, in terms of bacterial growth inhibition, intended as low MIC values, almost all compounds had a greater efficacy against the *S. aureus* control strains than the *E. coli*. Particularly, the 73016 seemed to be a very promising molecule showing the lowest MIC values against both *S. aureus* ATCC 29213 and *S. aureus* ATCC BAA

1720 (0,03 and 0,016 µg/ml, respectively). The better growth inhibition displayed by these novel compounds against *S. aureus* strains resulted in agreement with the antibacterial activity showed by other broad spectrum NBTIs (Mani *et al*., 2006; East *et al*., 2009; Trzoss *et al*., 2012;) that exhibited efficacy, especially against Gram-positive strains (Pucci & Wiles, 2014).

Results from minimal bactericidal concentration tests, have revealed that about all compounds, for which it has been feasible performing the assay, showed that bactericidal activity were achieved using concentrations of one- or two-fold MIC values. A considerable number of inhibitors tested in this work was highly active against Gram-positive microorganisms, and the Gram-negative *E. coli* strain. Antibacterial activities of the three compound tested (73009, 73015 and 73016) against *P. aeruginosa* ATCC 27853 were limited. MIC/MBC ranged from 2 to >8 µg/ml revealing *P. aeruginosa* reference strain as only slightly susceptible or even resistant to these molecules. Conversely, the bactericidal activity of different molecules was more evident against *E. coli* strain although at high concentrations. In time-kill studies, the molecules 73013 and 73015 possessed a comparable activity, proving to be the most effective compounds. Both molecules were bactericidal against all reference strains and caused the 3 Log_{10} reductions in viable cell counts within 24 h. These two molecules exhibited a bactericidal activity very similar to levofloxacin and higher than ciprofloxacin that was bactericidal only against 2 out of 3 reference strains. The simultaneously efficacy of 73013 and 73015 in time-kill performances against all control strains, although at different time and concentrations, encourages to deepen the study of these molecules since a new antimicrobial agent should preferentially have a broad spectrum activity (Brown & Wright, 2016). Surprisingly,

73016 molecule, whose MIC values were the lowest, was not bactericidal in time-kill experiments at each times and concentrations displaying no 3 Log_{10} reduction in bacterial viable counts.

Post antibiotic effect (PAE) was the term used by Mc Donald and colleagues (1977) to describe the suppression of bacterial growth that persist after short exposure of microorganisms to antimicrobials. This phenomenon emphasises that the effect is due to a prior drug exposure rather than to persisting sub-minimal inhibitory concentration of drug (Lorian 4th edition, 1996). In this work, persistent suppression of bacterial growth after antimicrobial exposure, appeared to be a feature of every antimicrobial agent tested: this phenomenon was observed with each tested molecules against all organisms. Indeed, drugs that do not demonstrate PAE should require more frequent administrations during therapy, than those that do exhibit such effect (Lorian 4th edition, 1996). However, the duration of PAEs differed among specific drug/microorganism combinations. All strains exhibited a lag in the resumption of normal growth: the persistent suppressive effect after 1h-exposure to 73015 and 73016 was documented and was comparable to that obtained with ciprofloxacin. These findings were in line with the literature, that reports PAE values of ciprofloxacin and other fluoroquinolones ranging from 120 to 150 min against *E. coli* and from 60 to 240 min against *S. aureus* (Lorian 4th edition, 1996). Also Spangler and colleagues (2000) have been reported measurable PAEs from 0h to 3h for grepafloxacin, and other fluoroquinolones (ciprofloxacin, levofloxacin and sparfloxacin). The occurrence of PAE is not only a “microbiologic curiosity” but it has also a clinical relevance (den Hollander *et al*., 1998). As a matter of fact, the longest periods of growth suppression were observed with the 73016 after 1-hour exposure of all strains. Actually, post

antibiotic effect is a pharmacodynamic phenomenon. Therefore, even if this compound showed no bactericidal activity in time-kill experiments, the low MIC and its long PAEs do not exclude it from being considered one of the most interesting compounds. The clinical implication of long PAEs consists of the possibility of increasing the intervals between drug administrations, thus influencing antimicrobial dosing regimens and allowing fewer daily doses, and potentially the reduction of treatment costs and selection of resistance (Lorian *et al*., 1989; Odenholt-Tornqvist, 1993; Lorian 4th edition, 1996; Spangler *et al*., 2000).

The frequency of mutation measures all the mutants present in a given population, irrespective of whether the mutation events occurred early or late during the process. In the case of antibiotic resistance, the mutation rate is defined as the *in vitro* frequency at which detectable mutants arise in a bacterial population in the presence of a given antibiotic concentration. Antibiotic resistance can be achieved, for instance, by horizontal acquisition of resistance genes (carried by plasmids or transposons), by recombination of foreign DNA into the chromosome, or by point mutations in different chromosomal loci (Martinez & Baquero, 2000). In this research work, *S. aureus* resistant mutants were obtained with all molecules. This aspect should not be of particular concern because all existing drugs are able to select resistant strains *via* different mechanisms since antimicrobials resistance is a natural process that has been observed for as long as the first antibiotics were discovered (French, 2005). Conversely, it would be more appropriate to focus not only on concentrations but also at which rate spontaneous resistance to new antibiotics develops (Martinez & Baquero, 2000). Overall, *S. aureus* resistant mutants were not obtained at concentrations higher than 4 µg/ml, except for the 73015 that resulted able to select mutants up to 16 µg/ml.

Frequencies of spontaneous development of resistance to new antibiotics were low (just one mutant per 10 million bacterial cells on average), and slightly higher than that of ciprofloxacin.

The average mutation rate (~10^{-7}) of these NBTIs were comparable to (Mani *et al*., 2006; Lahiri *et al*., 2015) or higher (East *et al*., 2009) than the frequency at which *S. aureus* mutants, resistant to other non-fluoroquinolone topoisomerase inhibitors, developed. The elevation in MIC values of all selected mutants was very consistent, achieving an increase of up to 32 fold the MIC of wild type strain. Mutation is a common mechanism of resistance induced by many synthetic antibacterial agents, such as the fluoroquinolones and oxazolidinones, although resistance in these cases, arises less easily (Woodford & Ellington, 2007). The topoisomerase type II genes sequence of wild type strains along with the genes sequence of mutants have been analysed in order to explain the resistance to these novel compounds. Development of resistance to fluoroquinolones and to compounds with comparable activity is due to the amino acid substitutions in DNA gyrase and/or topoisomerase IV genes and the accumulation of mutations generally increases MIC values (Hawkey, 2003; Ruiz, 2003). As expected, the ciprofloxacin resistant *S. aureus* ATCC 29213 mutant (SACIP3) showed only a point mutation in *grlA* gene. It occurred in the QRDR, on the hot-spot localized on amino acid position 80, determining the substitution of a serine with a phenylalanine. The ciprofloxacin MIC increase (16-fold) of this mutant strain was in line with literature (Piddock,1999; Hooper, 2000). The fluoroquinolones or similar compounds induce mutations at first on *grlA* or *grlB* genes in the Gram-positive bacteria (Jacoby, 2005), as also occurred in SACIP3 mutant. In contrast the new molecules induced point mutations in *gyrA* and *gyrB* genes in both *S. aureus* strains although in

positions that differed among drugs and mutants. Indeed, it is well known that non-fluoroquinolone topoisomerase inhibitors are able to induce point mutations at first in one or both DNA gyrase encoding genes (Mani *et al*., 2006; Black *et al*., 2008; East *et al*., 2009; Eakin *et al*., 2012; Lahiri *et al*., 2015). Moreover, these molecules, like other NBTIs do not induce the same target mutations caused by fluoroquinolones (Pucci & Wiles, 2014). Computational design and X-ray crystallography of a NBTI reported in literature (Eakin *et al*., 2012), further highlight the potency of these enzyme inhibitors with improved antibacterial activity likely due to cellular mode of action through GyrB. In addition, Bax and colleagues (2010) have been determined by crystallography that the binding sites for their NBTIs in the DNA-gyrase complex of *S. aureus* do not appear to overlap with the two fluoroquinolone binding sites. In this study the analysis of the conserved domains on the genes carried point mutations revealed that the functional domain targeted was always TOPRIM domain in GyrB and CAP-like domain on GyrA and GrlA but point mutations occurred in different positions (determining diverse amino acid shift and affecting different parts of the same domain) among mutants.

All these results could explain why *S. aureus* ATCC BAA 1720 was susceptible to several NBTI molecules despite the carriage of *norA* gene and a mutated *grlA* gene conferring resistance to ciprofloxacin, levofloxacin and other fluoroquinolones (Roychoudhury *et al*., 2001; Holden *et al*., 2004) but not to NBTIs.

Actually, it has been demonstrated that NBTIs possess a novel mode of action different from that of fluoroquinolones, without the formation of DNA-cleavage complex and the stabilization of a cleaved complex intermediate (Bax *et al*., 2010; Shapiro & Andrews, 2012). Recently, structural advances in the field of bacterial topoisomerases (Bax *et al*.,

2010; Vos *et al*., 2011; Laponogov *et al*., 2013) have significantly improved the understanding of this complex machinery. This novel binding mode explains why NBTIs are able to overcome target-mediated fluoroquinolone resistance (Bax *et al*., 2010). Indeed, also the novel compounds analysed in this work appears to possess this mode of action. This mechanism seems distinct from that of fluoroquinolones as suggested not only by the functional domain analyses previously discussed but also by the good *in vitro* antibacterial activity against relevant Gram-positive and Gram-negative species. The efficacy of 73015 and 73016 against two *ESKAPE* pathogens is of great relevance. Both molecules turned out to be very effective against multidrug resistance *A. baumannii* and MRSA strains of recent clinical isolation. Only few therapeutic options remain available against such pathogens: colistin, tigecycline and in few cases imipenem to treat Gram-negative; daptomycin, vancomycin and linezolid for Gram-positive infections. These results are of particular significance because it has been reported that the presence of multi drug resistant *Acinetobacter* spp. in the healthcare environment is of particular concern since they are notoriously difficult to eradicate once established (Maragakis & Perl, 2008). To compound the situation, the mortality rate of patients with *A. baumannii* infections in hospitals and in intensive care units has reached 23% and 43%, respectively (Lin & Lan, 2014).

The 73016 was highly able to inhibit the growth of MRSA isolates displaying the lowest MIC_{50} and MIC_{90} (0.03 and 0.125 µg/ml, respectively) and confirming once again itself as a suitable compound against this difficult to treat pathogen. Indeed, MRSA infections remains a public health priority in Europe and strategies targeting healthcare settings remain essential to continue reducing the spread of MRSA throughout the Europe (EARS-net, 2014). The efficacy of both 73015 and

73016 against such bacteria is of importance because pan-drug resistant bacteria of clinical origin, resistant not only to fluoroquinolones but especially to last-line antimicrobial agents, such as colistin and carbapenems, are now occurring (Boucher *et al*., 2009). Moreover, a lot of people die worldwide for infections due to multi-drug resistant Gram-negative and -positive pathogens (Falagas & Kasiakou, 2005; Falagas & Bliziotis, 2007; Boucher & Corey, 2008; Urban *et al*., 2008). These *ESKAPE* pathogens are responsible for the bulk of antimicrobial resistant infections (Tang *et al*., 2014) amongst critically ill and immunocompromised individuals (Rice, 2010). Nowadays, control of antimicrobial resistance in bacterial pathogens is a challenge to limit the high morbidity and mortality (Klevens *et al*., 2007, Akova, 2016a). Multidrug resistance bacteria are difficult to treat and may even be untreatable with conventional antibiotics. Currently, there are no effective therapies, no successful prevention measures and only a few antibiotics are available as treatment options and alternative antimicrobial therapies (Frieri *et al*., 2016). Just as new quinolones have been introduced over the years, there are expectations that quinolone-like derivatives and NBTIs will eventually reach the market. Such new compounds offer the advantage of inhibiting clinically validated targets while potentially overcoming existing antibiotic resistance (Pucci & Wiles, 2014).

These molecules could be a clinically useful alternative for the treatment of severe infections caused by these pathogens, particularly those resistant or not fully susceptible to both fluoroquinolones and newly developed antibiotics. The present study has demonstrated that these compounds could be of interest because they act on a clinically validated antibacterial target through a novel mechanism of inhibition and retain activity against quinolone resistant isolates. These results are preliminary, but very

promising and prompted further studies to better elucidate the potential of these compounds and to develop a new broad spectrum antimicrobial agent. Future development of these compounds could enhance and extend the clinical utility of bacterial topoisomerase inhibitors beyond the clinically valuable and commercially successful quinolone antibiotics. Bacterial topoisomerase inhibitors should continue to play an important role in the battle against bacterial pathogens for many years to come.

Chapter 5 - REFERENCES

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