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Monitoring antibiotics in the environment. Study of Quinoxaline derivatives bioactivity

Dissertação para obtenção do Grau de Doutor em
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"One sometimes finds what one is not looking for."

Sir Alexander Fleming

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RESUMO

Os agentes antimicrobianos revolucionaram a medicina e promoveram um aumento da esperança média de vida das populações humanas, em todo o mundo. Este tipo de fármacos é utilizado não só em medicina humana como em veterinária, no tratamento e na prevenção de infecções, e em algumas regiões no mundo, como promotores de crescimento, garantindo uma maior e melhor produção animal. A utilização de antimicrobianos na produção animal faz com que resíduos destes fármacos contaminem o produto final e sejam, eventualmente, distribuídos na cadeia alimentar humana. Resíduos de agentes antimicrobianos, provenientes do consumo animal e humano, estão também presentes em esgotos, águas superficiais ou em lençóis de água. Ainda não se conhecem todas as consequências desta contaminação, mas há indícios de alterações na microbiota autóctone. O uso destes fármacos foi rapidamente seguido do aparecimento de resistência, o que levou à diminuição de eficácia e de compostos disponíveis. Portanto, a disseminação de antimicrobianos no ambiente também está associada ao aumento da resistência a este tipo de drogas.

O trabalho desenvolvido pretendeu estabelecer métodos de monitorização da presença de antimicrobianos em alimentos de origem animal, verificar se a presença de antimicrobianos no ambiente, designadamente em águas, em concentrações sub-inibitórias pode contribuir para a selecção de bactérias resistentes e caracterizar a actividade biológica de um conjunto de compostos, da família das quinoxalinas, como potenciais novos agentes antimicrobianos.

A fim de alcançar estes objectivos, foram utilizadas técnicas cromatográficas, para detecção e quantificação de agentes antimicrobianos, técnicas de microbiologia e biologia molecular para avaliação do comportamento de bactérias sob pressão selectiva. Foram, também, utilizadas várias estirpes de microrganismos eucariontes e procariontes, de forma a avaliar a actividade antimicrobiana de derivados *N*-óxido da quinoxalina. Recorreu-se, ainda, a culturas celulares, para avaliar a toxicidade destes potenciais novos antimicrobianos. Um novo método cromatográfico foi desenvolvido para a quantificação das formas reduzida e oxidada da glutationa, de forma a inferir o stress oxidativo celular provocado pela exposição aos compostos derivados da quinoxalina com comprovada actividade antimicrobiana.

Os resultados obtidos confirmam que as técnicas cromatográficas de HPLC-DAD são ferramentas potentes no controlo de qualidade alimentar. Indicam, ainda, que a presença de quantidades sub-inibitórias de agentes antimicrobianos no ambiente tem influência na dinâmica da população bacteriana de *Escherichia coli* sensível e resistente à ciprofloxacina.

Uma avaliação da actividade biológica de derivados de quinoxalina indicou os compostos estudados como potenciais novos agentes antimicrobianos, que demonstraram baixos efeitos tóxicos em linhas celulares e dano celular oxidativo de pequena extensão.

Palavras-chave: Antimicrobianos, Ambiente, Alimentos, Resistência a antimicrobianos, Quinoxalinas.

ABSTRACT

Antimicrobial agents have revolutionized medicine and promoted an increase in average life expectancy of human populations worldwide. These drugs are used not only in human medicine but also in veterinary practice, in the treatment and prevention of infections, and in some regions in the world, as well as growth promoters, ensuring a greater and better animal production. The use of antimicrobial agents in animal production causes contamination of the final product with drug residues that are eventually distributed in human food chain. Residues of antimicrobial agents provenient from human and animal consumption are also present in sewage, surface water or ground water. It is still unknown all the consequences of this contamination, but there are indications of changes in indigenous microbiota. The use of these drugs was quickly followed by the emergence of resistance, which led to decreased efficacy and compounds available. Therefore, the spread of antimicrobial agents in the environment is also associated with increased resistance to such drugs.

The presented work intended to establish methods for monitoring the presence of antibiotics in animal foods, evaluate if the presence of antimicrobial agents in the environment at sub-inhibitory concentrations can contribute to the selection of resistant bacteria and characterize the biological activity of a number of compounds of the quinoxaline family as potential new antimicrobial agents.

In order to achieve these objectives, chromatographic techniques were used for detection and quantification of antimicrobial agents, methods of microbiology and molecular biology to evaluate the behavior of bacteria under selective pressure. Various strains of prokaryotes and eukaryotes microorganisms were also used to evaluate the antimicrobial activity of *N*-oxide derivatives of quinoxaline. We used, also, cell cultures to assess the potential toxicity of these new antibiotics. A new chromatographic method was developed to quantify the reduced and oxidized forms of glutathione, in order to infer the cellular oxidative stress induced by exposure to the quinoxaline derivative compounds with proven antimicrobial activity.

The results confirm that the chromatographic HPLC-DAD methods are powerful tools in monitoring food quality. They also indicate that the presence of sub-inhibitory amounts of ciprofloxacin in water may influence the dynamic of susceptible and resistant to ciprofloxacin *Escherichia coli* population.

An assessment of the biological activity of quinoxaline derivatives indicated the compounds studied as potential new antimicrobial agents who have shown low toxicity in cell lines and oxidative cell damage in small extent.

Keywords: Antimicrobials, Environment, Food, Antimicrobial Resistance, Quinoxalines.

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ABBREVIATION INDEX

2A3CQNX	2-Amino-3-cyanooylquinoxaline-1,4-dioxide
2HF	2-Hydroxyphenazine-*N*,*N*-dioxide
2M3BenzoylQNX	2-Methyl-3-benzoylquinoxaline-1,4-dioxide
2M3BQNX	2-Methyl-3-benzyloylquinoxaline-1,4-dioxide
2MQNX	2-Methylquinoxaline-1,4-dioxide
3M2QNXC	3-Methyl-2-quinoxalinecarboxamide-1,4-dioxide
5-HT	5-Hydroxytryptamine
6-APA	6-Aminopenicillanic acid
7-ACA	1-Aminocephalosporanic acid
AA	Arachidonic acid
AIDS	Acquired immunodeficiency syndrome
AML	Amoxicillin
AMOX	Amoxicillin
AMPA-R	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor
AMR	Antimicrobial resistance
AQ	Acenaphtho[1,2-*b*]quinoxaline
ATCC	American Type Culture Collection
ATP	Adenosine-5'- triphosphate
CDC	Center for Disease Control and Prevention
CFU	Colony-forming unit
CIP	Ciprofloxacin
CISA	Centro de Investigação em Saúde e Ambiente
CLOR	Chloramphenicol
CLOX	Cloxacillin

CLSI	Clinical and Laboratory Standards Institute
CV	Coefficient of variation
Cys	Cysteine
DAD	Diode Array Detector
DHFR	Dihydrofolate reductase
DHPP	Dihydropteridine pyrophosphate
DHPS	Dihydropteroate synthetase
D-MEM	Dubelco-Modified Eagle's Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Desoxirribolnucleic acid
DOI	Digital object identifier
DTNB	Ditionitrobenzoic acid
EARSS	European Antimicrobial Resistance Surveillance Sustem
ECD	Electron Capture Detector
EDTA	Ethylaminotetracetic acid
EEA	Excitatory amino acid
ESAC	European Sulveillance of Antimicrobial Consumption
EtOH	Ethanol
FA	Folic acid
FCT-MEC	Fundação para a Ciência e Tecnologia - Ministério da Educação e Ciência
FDA	Food and Drug Administration
FDNB	2,4-Dinitrofluorobenzene
FID	Flame Ionization Detector
FL	Fluorescent Detector
FOX	Cefoxitin
FPD	Flame Photometric Detector
G-	Gram-negative
G+	Gram-positive

GC	Gas Chromatography
GI50	50% Growth inhibition
GPCR	G-protein coupled receptor
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GS_T	Total glutathione
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
HSV	Herpes symplex vírus
IMA	Indomethacin
INQUI	Indeno-1-one[1,2-*b*]quinoxaline
IRD	Infrared Detector
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid Chromatography
LD	Limit of Detection
LEV	Levofloxacin
LOX	Lipoxygenase
LQ	Limit of quantification
MAO	Monoamine oxidase
MDR	Multi-drug resistant
MeOH	Methanol
MHPQ	3-methyl-2-(2-hydroxyphenyl)quinoxaline
MIC	Minimum inhibitory concentration
MMtHPQ	3-methyl-2-(3-methoxy-4-hydroxyphenil)quinoxaline
MPA	Mycophenolic acid
MPQ	3-methyl-2-phenylquinoxaline
MRL	Maximum residue limit
mRNA	Messenger ribonucleic acid

MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSE	Methicillin-resistant *Staphylococcus epidermis*
MS	Mass Spectrometer
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NGP	Natural growing products
NOR	Norfloxacin
NPD	Nitrogen-Phosphorus Detector
NSAID	Non-steroidal anti-inflammatory drug
OD	Optical density
OGTT	Oral glucose tolerance test
OLED	Organic light-emitting diode
PABA	*p*-Aminobenzoic acid
PBP	Penicillin-Binding Protein
PCA	Plate count agar
PHOLED	Phosphorescent organic light-emitting diodes
PHPQ	3-Phenyl-2-(2-hydroxyphenyl)quinoxaline
PPQ	2,3-Diphenylquinoxaline
PYCC	Portuguese Yeast Culture Collection
QD	Quinoxalinedione
QNX	Quinoxaline -1,4-dioxide
R	Resistant
RAoSMC	Rat aortic smooth muscle cell
RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic acid
ROS	Reactive oxigen species
RP	Reverse Phase
RT	Reverse transcriptase
S	Susceptible

SMC	Smooth cell
SOD	Superoxide dismutase
sp.	Specie
SPE	Solid phase extraction
spp.	Species
SSA	5-Sulfosalicylic acid
SULFO	Sulfomethoxazole
SXT	Sulfamethoxazole/Trimetropim
TB	Tuberculosis
tBOOH	Tertiary-butylhydroperoxide
TCA	Trichloroacetic acid
TCD	Thermal Conductivity Detector
TCY	Tetracyclin
TFA	Trifluoroacetic acid
TIC	Ticarcillin
TNB	2-Nitro-5-thiobenzoate
tRNA	Transfer ribonucleic acid
TSA	Tryptic soy agar
TSB	Tryptic soy broth
USA	United States of America
UTI	Urinary tract infections
UV	Ultra-violet
VRE	Vancomycin-resistant Enterococcus
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organization
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2*H*-tetrazolium-5-carboxanilide
YEPD	Yeast extract peptone dextrose

Chapter 1: General plan and main objectives

1 GENERAL PLAN AND MAIN OBJECTIVES

The discovery of antibiotics, in the 1930s, revolutionized the medicine world. It became possible to treat serious lethal bacterial infections. Enthusiasm took over the medical community and the consequence was overprescribing.

Bacteria communities and cells can easily evolve and become adapted to changing environmental conditions, often through modification in the genome [1]. When exposed to antibiotics, bacteria find ways to avoid their deletery effects. Abusive and inappropriate use of antibiotics can, thus, result in bacterial resistance to antibiotics. The eradication of these resistant bacteria requires higher doses of medicine or stronger antibiotics. Because of antibiotic overuse, certain bacteria have become resistant to some of the most powerful antibiotics available today [2, 3].

In the past 40-50 years bacteria have become increasingly unsusceptible to the antibiotics in our medical arsenal. At the same time, scientific community and pharmaceutical industry have developed only a small number of new drugs to take the place of those that have become useless (Figure 1.1). As antibiotic effectiveness decreases, patients require longer, more toxic and expensive treatments. As a consequence, hospitals have themselves become spots of highly resistant pathogens, like methicillin-resistant *Staphylococcus aureus* (MRSA), increasing a paradoxal risk: hospitalization may actually kills instead of heal [4].

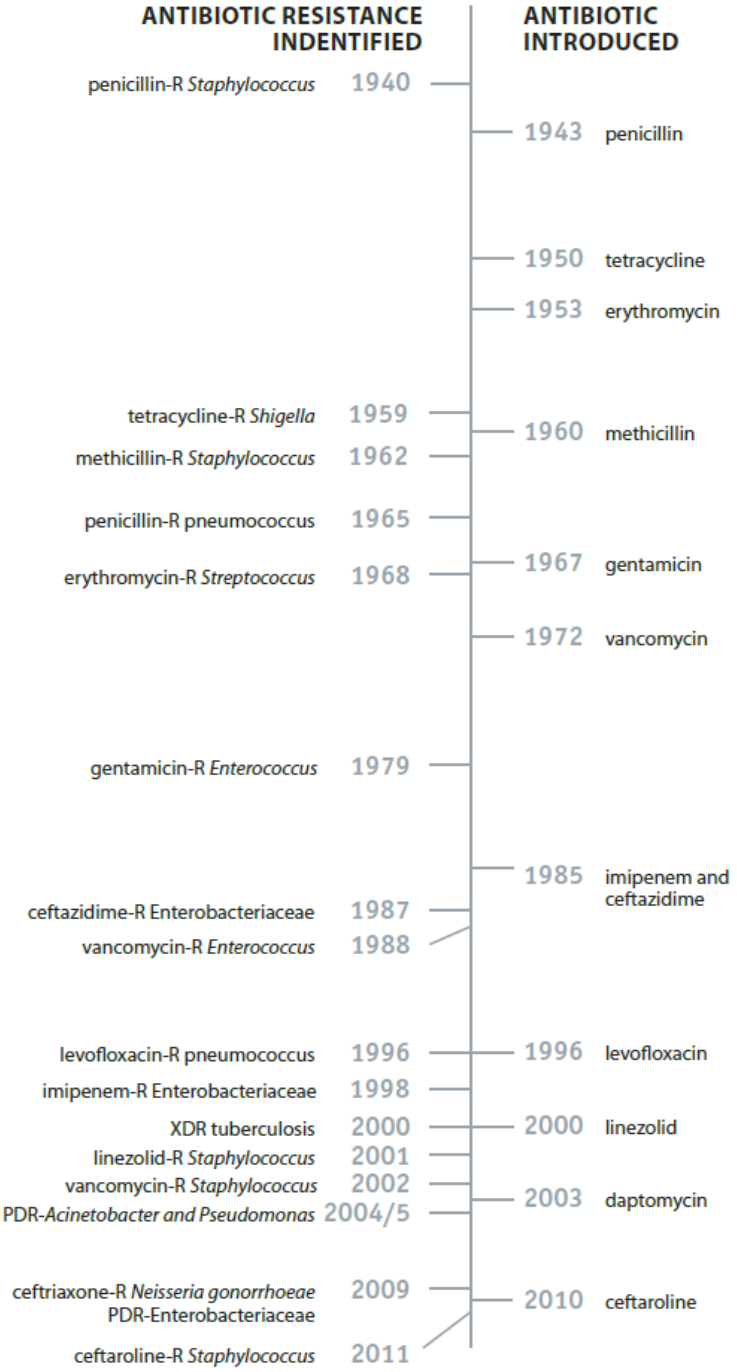


Figure 1.1: Antibiotic introduction and resistance occurrence (Data from CDC, "Antibiotic Resistance Threats in the United States, Threats Report 2013" [4]).

Antibiotics are not used only in human medicine. Animal production for consumption has taken gigantic proportions and animals are usually kept in large groups and the animal density is high. These are conditions for illness introduction and spread. Antibiotics then begun to be used for animal treatment, as well as for preventing infection appearance. Antibiotics are also used as growth promoters, since these properties have been identified and since the livestock industry deals with profit rates, and thereby achieves a faster and larger animal production. In the middle 1990s, multi-drug resistant strains of antibiotics were found in livestock and in food from animal origins. These resistant strains were mostly related to the antibiotics used as growth promoters. The use of these antibiotics in veterinary medicine and as growth promoters may result in trace residues of these substances in food from animal origin and have consequences in the human commensal flora [5, 6]. Nowadays, the use of antibiotics as growth promoters is forbidden in Europe [7], as "*part of the Commission's overall strategy to tackle the emergence of bacteria and other microbes resistant to antibiotics, due to their overexploitation or misuse".* In the USA, the use of antibiotics is still allowed, although there are appeals for the reduction and ban of these drugs [8].

Antimicrobialy active residues have been also detected in surface waters, soil and manure. The input of these microbial active substances is associated to municipal sewage, hospital effluents and agricultural and livestock activities [9-12].

The worldwide spread of resistance to antimicrobials has become "one of the world's most pressing public health problems." (Centers for Disease Control and Prevention - CDC). World Health Organization (WHO) established a set of recommendations, in 2001, in order to limit the emergence and spreading of antimicrobial resistance [13].

In this perspective, this research work intends to make a monitoring of the presence of antimicrobial residues in surface water and in food of animal origin (milk), by the development of a multiresidue antibiotic detection method, including the most

used and representative of different classes of antimicrobials. Because one of the most important consequences of the contamination with antimicrobial residues is the selection of antibiotics resistant bacteria, a model system with ciprofloxacin and *Escherichia coli* strains resistant and susceptible to that antimicrobial was used to assess such an effect. Knowledge of these effects is crucial for understanding the consequences of the use and disposal of antimicrobials.

One of the current problems related to resistance to antimicrobial agents is the lack of new substances that can replace those that have lost effectiveness. Thus, this study intends to evaluate the antimicrobial and antitumor activity, as well as toxicity of some quinoxaline derivatives. This family of compounds has been evaluated by several research groups in recent years. Additionally, there are data indicating them as a possible alternative to the fight against resistant bacterial strains, thus replacing other antimicrobials that are currently losing effectiveness.

1.1 MAIN OBJECTIVES

The main objectives proposed for this work were:

a) Develop chromatographic methods in order to evaluate antibiotic residues presence in several samples, as water and milk;

b) Compare the behavior of ciprofloxacin-resistant and susceptible strains of *Escherichia coli* in the presence of sub-inhibitory concentrations of that antimicrobial drug;

c) Evaluate antimicrobial activity and cellular toxicity of quinoxaline derivatives in human cells;

d) Determine the oxidative stress in liver extract caused by exposure to quinoxaline derivatives solutions.

Specific objectives were traced in order to achieve the main goals for this work and are described along the text, in each chapter.

1.2 GENERAL FRAMEWORK

The present work is divided into 9 chapters. The present chapter (**Chapter 1**) presents the framework of the developed work and the main objectives. In **Chapter 2**, is presented a general introduction to the main topic of antibiotics, resistance to antibiotics and the public health concerning. A state-of-the-art review on quinoxaline derivatives biological activity is also presented. The content of this chapter is under review at the *European Journal of Medical Chemistry* (Manuscript Number: EJMECH-D-13-01270). Another paper was published in the development of the present work, addressing the resistance topic: Bacterial Resistance, Ricardo Ferraz, Cristina Prudêncio, Mónica Vieira, Ruben Fernandes, João Paulo Noronha, Zeljko Petrovski, *Biochemistry & Pharmacology*: Open Access, 1-7, 2012 (doi:10.4172/2167-0501.1000e138). Part of this article is textually exposed in section 2.3 of this document.

In **Chapter 3** is presented the development of a new chromatographic method for antibiotic detection and its application on the analysis of surface water and food samples. The contents of this chapter was submitted to the international journal Talanta (Manuscript Number: TAL-D-13-02953) and is under review.

Chapter 4 reports the behavior of several bacterial strains under sub-inhibitory concentrations of ciprofloxacin in a time course assay. Susceptibility and phenotypic

profile was evaluated. The contents of this chapter are being submitted to an international peer review journal.

Chapter 5 reports the antimicrobial activity of quinoxaline derivatives tested. This chapter has been published at *Microbiological Research* (http://dx.doi.org/10.1016/j.micres.2013.06.015).

Chapter 6 presents the results obtained on the cellular toxicity in the human cells assays performed in order to evaluate quinoxaline derivatives as potential antimicrobial agents. The content of this chapter has been submitted to *European Journal of Medicinal Chemistry* (Manuscript Number: EJMECH-S-13-02430) and is under review.

Chapter 7 reports the oxidative damage results obtained for liver extract exposed to the quinoxaline derivatives. The content of this chapter has been submitted to *Journal of Biotechnology* (JBIOTEC-D-13-01223) and is under review.

Discussion of the results obtained and major conclusions are presented in **Chapter 8**. Future perspectives and current works are explained in **Chapter 9**.

Chapter 2: State of the Art

2 INTRODUCTION

"From the beginning of human existence, their struggle for survival was established with other living beings, of all sizes. Some of these beings are so small that they were not seen nor touched. However, these microorganisms, in the battle for survival, inflicted heavy losses to the human species. There were pests, such as in Egypt, pests such as the bubonic plague and, more recently, epidemics such as influenza and cholera."

The natural products were the earliest therapeutic substances used by man, since there were observations that these substances controlled the symptoms of some diseases. Mercury, for example, had been used since the sixteenth century in the treatment of syphilis. Quinine and ipecacuanha root were brought to Europe in the seventeenth century and are effective against parasites of malaria and amoebic dysentery.

Man has used antimicrobial drugs for centuries without truly realizing its potential. It took several years to be able to identify and distinguish, at the microscope, bacteria, fungi and viruses. Antimicrobial drugs are the oldest therapy used to combat specific microorganisms such as bacteria and yeast. Nowadays, we look at the infections as minor diseases, but before the 1950s, cases of death at very young ages were very frequent due to infections such as tuberculosis, pneumonia, meningitis, diphtheria, pertussis, measles and influenza. With the discovery of antibiotics there was a revolution in the practice of medicine and life expectancy has increased significantly [14, 15].

The Modern Era of Antibiotics begins in the decades of 1930-1940, when man managed to control infections caused by streptococci and pneumococci. Some authors consider the beginning of this age with the onset of the clinical use of sulfonamides in 1936. Other authors consider that, officially, antibiotics were discovered in the 1920s,

specifically in September 1928, by the renowned Scottish chemist Sir Alexander Fleming, who observed the behavior of certain substances, such as the presence of the fungus *Penicillium notatum*, which restricted the normal growth of *Staphylococcus aureus*. Since then, antibiotics are used on a large scale, in fighting infections by microorganisms.

Some authors argue that the history of antibiotics begins with the use of alkaloids, starting in 1619, date that recall the earliest records of the first effective treatments for malaria and dysentery with quinine and root extracts of ipecacuanha, respectively [16, 17]. Several scientists, such as Joseph Lister [18], Louis Pasteur, Jules François Joubert [19], Paul Ehrlich [20], among others, identified the antimicrobial potential of different products.

Currently, there are several known classes of antibiotics. The first to be used were of natural origin, produced by bacteria, but at the present days, the vast majority of antimicrobial active ingredients used are of synthetic or semi-synthetic origin. Molds and soil organisms are the most used today for the production of antibiotics, but technological evolution enabled a better production and purification of these substances [21].

2.1 ANTIBIOTICS HISTORY

The term antibiosis, used to designate the antibiotic function, was first proposed by Vuillemin, in 1889, and described the antagonism between living beings in general. The term antibiotic was used, for the first time, by Selman Waksman, in 1941, to describe molecules produced by microorganisms that were antagonists of growth or living of other organisms around them [21, 22]. The common use of the term made it

applicable also to antimicrobial agents of synthetic origin, such as the quinolones and sulfonamides.

The discovery of natural origin substances with antibacterial effects occurred with the discovery of penicillin. Fleming, after a period of absence of the laboratory, observed inhibition of growth of a culture of staphylococci around a mold, which accidentally appeared in his plate [23]. The mold *Penicillium notatum* produces the substance inhibiting the growth of bacteria, later identified as penicillin [24]. The substance that Fleming was referring to is benzylpenicillin or Penicillin G. Howard Florey and Ernst Chain followed up the discovery of Fleming, and shared the Nobel Prize in Physiology or Medicine with him, in 1945. The main achievement was the successful isolation of penicillin and its extraction it with good yields [25]. Florey and Chain proved, further, its non-toxicity, using models of mice previously infected with streptococci. In 1959, the 6-aminopenicillanic acid (6-APA), shown in Figure 2.1, was extracted enzymatically by Batchelor [26]. The 6-APA was achieved through a simple mechanism and in a good yield. This product was the starting point for achieving semi-synthetic penicillins, which present improved spectrum of activity compared to penicillin G. The first semi synthetic penicillin produced was ampicillin, which has demonstrated antibacterial activity against Gram-negative microorganisms [27-29]. Penicillin and its derivatives are included in the **β-lactam** group of antibiotics that share a β-lactam ring as common feature. Cephems, carbapenems and monobactams are the other sub-classes that complete this group of antibiotics.

β-Lactams are, since their application, the more prescribed antibiotics in human and veterinary medicine [30]. The structure of this class of antibiotics is based on a β-lactam cyclic system attached to a thiazolidine ring, forming the 6-aminopenicillanic acid (6-APA). This structure is obtained by the condensation of a valine molecule and a cysteine molecule, originating the characteristic double system. This structural element is essential to β-lactams biological activity and, if the β-lactam ring is destroyed, by metabolic transformation or chemical alteration, the antibacterial activity is lost [31].

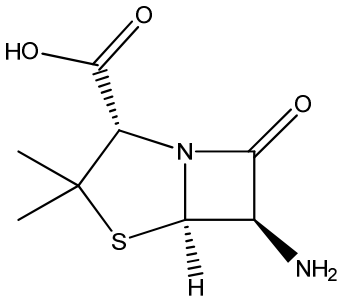
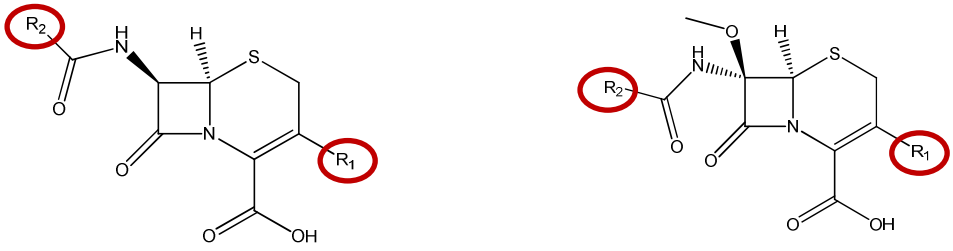


Figure 2.1: Chemical representation of 6-aminopenicillanic acid (6-APA).

β-lactams are divided in four major classes (penicilins, cephems, carbapenems and monobactams) and a β-lactamase inhibitors group (Table 2.1-Table 2.9).

The **cephem** group of β-lactams includes cephalosporins and cephamycins. **Cephalosporins** (Figure 2.2) are semi-synthetic antibiotics, derived from cephalosporin C, a natural compound, produced by *Cephalosporium acremonium*. Cephalosporin C was discovered in 1948, by Giuseppe Brotzu [32]. Brotzu noticed that *Salmonella typhi*, the bacterial strain that caused the typhoid fever, was inhibited by substances produced by *C. acremonium*. The cephalosporin active chemical nucleus is the 7-aminocephalosporanic acid (7-ACA), similar to 6-APA, as it possesses also a β-lactam ring. Cephamycins and cephalosporins are very similar structurally and are all classified as cephalosporins, by some authors [33, 34]. **Cephamycins** (Figure 2.2) are naturally produced by members of the *Streptomyces* genus and characterized by the 7-α-methoxyl chemical group. This sub-class of β-lactams were first reported in 1972, by Stapley and Miller, from the Merck Sharp and Dome Research Laboratories [35].



Cephalosporin chemical nucleus **Cephamycin chemical nucleus**

Figure 2.2: Structural core representation for cephalosporin (left) and cephamycin (right).

Carbapenems (Figure 2.3), another sub-class of β-lactam antibiotics, were developed from thienamycin, a natural product produced by *Streptomyces cattleya*, discovered in 1976 [36]. Thienamycin revealed chemical and biological instability. Therefore, scientists developed new compounds, more stable and profitable [37]. Structurally, carbapenems are also similar to penicillin, but they present a carbon atom, instead of a sulfur atom, in the position 1 of the β-lactamic structure

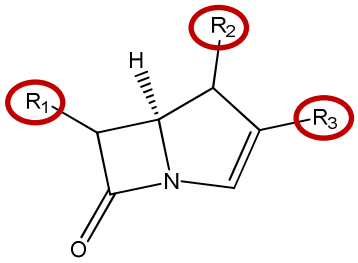


Figure 2.3**:** Representation of the chemical nucleus of carbapenems.

Monobactams are a sub-class of β-lactam antibiotics of synthetic origin. They may also be produced by actinomycetes, unlike cephalosporin and penicillin. These compounds present a chemical structure different from other β-lactams, since they are monocyclic (Figure 2.4) [38, 39]. The denomination of monobactams was employed by Sykes [40] and this class of compounds were discovered about 50 years after Fleming's finding. Aztreonam was the first synthetic monobactam reported and the first, from a series of compounds, that actually reached medicine practice [41].

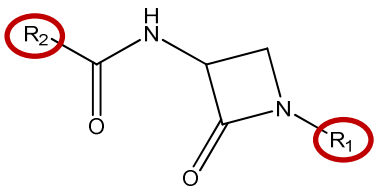


Figure 2.4: Representation of monobactam core structure.

There is another functional sub-class of compounds, included in the β-lactam antibiotics, the β-lactamase inhibitors. Bacteria, after exposure to β-lactam antibiotics, have developed mechanisms of resistance to these drugs, through the action of β-lactamase enzymes that hydrolyze the active β-lactam ring, thus deactivating the antibacterial action. The β-lactamase inhibitors present higher affinity to the β-lactamase enzymes than the β-lactams, and bind irreversibly to them [42]. The clavulanic acid, sulbactam and tazobactam are the compounds that belong to this sub-class of β-lactams.

The β-lactam antibiotics are bactericidal, since they interfere in the cell wall synthesis, specifically by inactivation of enzymes involved in the synthesis of peptidoglycan, a fundamental biomolecule in cell wall constitution, leading to cell death due to osmotic pressure. The β-lactams target enzymes are the Penicillin-Binding Proteins (PBPs) that are located at the cytoplasmatic membrane [43].

In addition to β-lactam antibiotics, there are other classes of antibiotics with very different chemical structures that present different antibacterial activity.

In the 1930s, a series of discoveries, carried out in Germany and France, were called "the miracle of miracles," for the man provided an effective weapon to overcome bacterial infections: the **sulfonamides** or sulfa drugs [14]. These drugs were active only on some bacterial strains and had no effect on strains that caused lethal infections. The assembly referred to as sulfa drugs or sulphonamides have a common set of atoms that form the sulfonamide functional group (Figure 2.5).

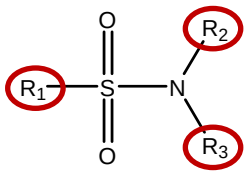


Figure 2.5: Representation of the chemical structure of sulfonamides.

Sulfonamides are antimicrobial agents with considerable use in human and veterinary medicine. These antimicrobial agents were the first to be used to fight bacterial infections in human medicine [44].

In the 1940s, the sulfonamides were considered drugs of choice for the treatment of bacterial infections in humans. Currently, and in human medicine, the sulfonamides are used only in specific infections, such as urinary tract infections(UTI), but have also a wide application in veterinary medicine.

In 1908, Gelmo and collaborators have synthesized, for the first time, a sulfonamide, while searching for new azo dyes [45]. Following this work, Höerlin and colleagues found that dyes with sulfanyl group present in its composition had a higher affinity for silk and wool proteins [46], but also promoted some protection against septicemia in mice caused by hemolytic streptococci [47]. This discovery led Eisenberg, in 1913, to observe that chrysoidine showed bactericidal properties [47, 48], and, in 1919, Jacobs and Heidelberger [47] detected that some azo dyes, synthesized from hydrocupreine and diazotized sulfonamides , presented bactericidal action. In 1932, Domagk was awarded the Nobel Prize in Physiology or Medicine for his discovery of the therapeutic *Prontosil*® (*p*-aminobenzenesulfonamide, synthesized by Gelmo), which showed protective action, in rats and rabbits, against staphylococci and hemolytic sterptococci. Although not convinced that *Prontosil*®, that was so effective in animal models, could be effective in humans, Domagk administered a dose of it to his daughter, when she came down with an infection caused by streptococci. The recovery was complete, but this fact Domagk concealed until 1935, when the results become available for clinical trials in patients [49]. Following this discovery, several sulfonamides were synthesized in the 1930s, and many of these compounds revealed antibacterial activity against various strains of pneumococci and other sterptococci. In 1941, several sulfapyrimidines were presented, with higher antibacterial activity and

lower toxicity than the sulfonamides synthesized previously [50]. Presently, there are more than 5000 known compounds belonging to the class of sulfonamides, but only about 30 are used in human and veterinary medicine [51, 52]. Of these, we highlight 5, with main antibacterial application: sulfanilamide, sulfamethoxazole, sulfacetamide, sulfadiazine and sulfamerazine. From the central structure of sulfonamides (aniline group + sulfonium group) several compounds were synthesized and have pharmaceutical application as antibacterial agents. Some of the derivatives obtained may also act as diuretics and antidiabetogenic agents [50-52].

Sulfonamides exhibit activity against Gram-positive and Gram-negative bacteria as well as against certain fungi and protozoans, and therefore are classified as broad spectrum antibiotics [53].

Glycopeptides are complex heterocyclic molecules, constituted of a multi-peptide backbone with several sugars attached to it. These molecules are bulky and cannot penetrate the outer membrane of Gram-negative bacteria, reducing, thereby, their spectrum of action to Gram-positive bacteria [54]. Vancomycin, the first glycopeptide, was isolated in 1953, by Edmund Komfeld, from *Amycolatopsis orientalis* (formerly *Nocardia orientalis*), present in soil samples. It is a rare example of a haloorganic natural compound, with two chlorine atoms [55]. Bleomycin was discovered in 1966, by Hamao Umezawa [56], and is produced by *Streptomyces verticillus*. This antibiotic was found when Umezawa was looking for compounds with antitumor activity. Bleomycin is used as an anticancer agent, in Hodgkin's lymphoma, squamous cell carcinoma and testicular cancer. It is also used in the treatment of plantar warts and in the chemical pleurodesis procedure [57]. Teicoplanin was discovered in the early 1990s and corresponds to a mixture of closely related compounds [58, 59]: five major components and four minor components. Telavancin, a semi-synthetic lipoglycopeptide, was approved in late 2000s [60].

Aminoglycoside is another class of antibiotics, and this name is related to its chemical structure. Aminoglycosides consist of two or more aminosugars linked to an aminocyclitol nucleus, an aminosubstituted cyclic polyalcohol. The different aminoglycosides are distinguished by the amino sugars in their constitution [61].

Aminoglycosides were introduced in 1944, with streptomycin, discovered by Selman Waksman [62]. Streptomycin is of natural origin, produced by *Streptomyces griseus*. Its discovery was an important goal, since it presented activity against *Mycobacterium tuberculosis*. Neomycin was the subsequently aminoglycoside, and was presented in 1949, extracted from *Streptomyces fradiae*, followed by kanamycin, that is produced by *Streptomyces kanamyceticus* [63, 64]. Amikacin, a derivative of kanamycin was introduced in 1972, and was the first semi-synthetic aminoglycoside [65]. In 1963, gentamicin was introduced, extracted from *Micromonospora purpurea*, an actinomycete, and netilmicin, a semi-synthetic derivative of sisomicin, produced by *Micromonospora* species, was presented in 1976 [66, 67]. The name of aminoglycosides follow the rule: substances originated from the *Streptomyces* genus present the suffix –mycin and those originated from the *Micromonospora* genus are named with the suffix –micin [68].

Another sugar related class of antibiotics is the **macrolides**. This class of antibiotics is characterized by the presence, in their structure, of a macrolide ring, a 14-, 15- or 16- macrocyclic lactone ring substituted by several sugars [69, 70]. Macrolide antibiotics were discovered in 1952, with the introduction of erythromycin, a natural product of *Streptomyces erythreus*. This compound is susceptible to acidic conditions, like those experienced in the stomach passage. With the purpose of adjust this behavior, scientists modified erythromycin structure. Semi-synthetic macrolides derivatives, like azithromycin and clarithromycin, were discovered by Croatian and Japanese drug companies, respectively, between late 1970s and early 1980s [54].

Tetracyclines were described in 1945 by Benjamin Duggar [71], on his research on *Streptomyces* species, although there are reports of tetracycline present in bones of

ancient Egyptians, from the fourth century [72]. Chlortetracycline was the first tetracycline described, extracted from *Streptomyces aureofaciens*. The following compounds were oxytetracycline and tetracycline. These antibiotics are used in human medicine for decades and, as they are broad spectrum and low cost antibiotics, are widely used in veterinary medicine, for treatment and production of animals, since they avoid infections in the livestock facilities, and as growth promoters [73]. The more common used tetracyclines are tetracycline itself, chlortetracycline, oxytetracycline and doxycycline [74]. The chemical structure of this class of antibiotics is derived from a hydronaphthacene nucleus containing four fused rings.

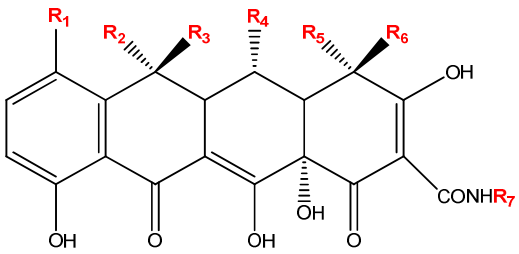


Figure 2.6: Tetracycline general structure.

Tetracycline antibiotics have similar chemical structures. They are strong chelating agents and biological membranes present a good permeability to these compounds. Due to their good lipossolubility, allied to low cost, residues of tetracyclines have been detected and reported in several food products and water [75].

Quinolones appeared in 1962 in the development of therapies for malaria. Quinolones are not natural substances of microbial origin, like penicillin, but have been synthesized, like the sulfonamides. The first substance of the class to which it was assigned antibacterial properties was nalidixic acid [76].

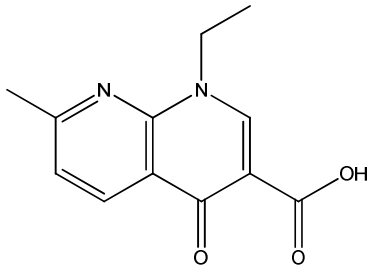


Figure 2.7: Structural representation of Nalidixic Acid.

The use of this substance was limited due to their narrow spectrum of activity, low maximum amounts of application and toxicity. In the 1980s, when resistance to antibacterial agents used, by strains of *Shigella* spp. and *Escherichia coli*, was detected, this class of antibacterial agents regained the attention of scientists, in the treatment of diarrhea and UT infections [76, 77]. It was the beginning of a period of synthesizing new substances, in order to replace the β-lactams and macrolides, looking for broader action spectra and less toxicity. The first quinolones developed from the nalidixic acid were pipemidic acid, cinoxacin and oxolinic acid. The skills acquired in the development of new drugs and the perception that simple changes in the side chains of the main structure caused significant changes in the action of these substances led to the development of more than 10,000 substances. However, only about 2% of these were subjected to the clinical trials and only some 20 substances have reached the market. Quinolones developed from the nalidixic acid exhibit activity against Gram-positive and Gram-negative bacteria, anaerobic bacteria and pathogenic mycobacteria [78]. Flumequine was the first fluoroquinolone substance, which includes a fluorine atom at the C-6 position, in its constitution, which demonstrated increasing applications of this class of antibacterials.

Quinolones (Figure 2.8) are structurally based on a 4-quinolone nucleus [79, 80], which is derived in four groups of compounds, called generations.

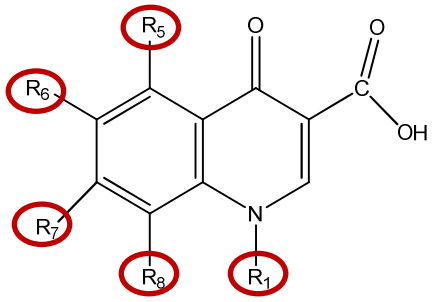


Figure 2.8: Structural representation of quinolone nucleus - adapted from [79].

Quinolones available for human and veterinary medicine use are divided, on the basis of their spectrum of activity, in four generations [79]. This classification is not unanimous among all authors. Flumequine is classified as a fluoroquinolone of 1st generation, together with nalidixic acid and its first derivatives, and is no longer used clinically. The 2nd generation quinolones, all fluoroquinolones with a fluorine atom at the C-6 position, have the advantage of greater effectiveness against Gram-negative bacteria. The most relevant quinolones of this generation are ciprofloxacin (CIP) and norfloxacin (NOR), as well as levofloxacin (LEV), which currently have clinical application. Are also part of this generation, sparfloxacin and grepafloxacin, which have improved activity against Gram-positive bacteria. The 3rd generation of quinolones, besides showing activity against Gram-positive organisms, yet has activity against anaerobes. This increase in the spectrum of action was obtained by introducing a methoxy group, at position C-8, and pyrrolidines or alkyl-substituted piperazines at position C-7. Trovafloxacin (or naphthyridone), although not presenting a methoxy substitution at C-8, belongs to this generation of quinolones and was the developed substance with wider spectrum recorded, but presented hepatotoxicity, so its clinical use was short lived [76]. Some authors consider that the quinolones with the methoxy group belong to the 4th generation [80, 81]. The 4th generation of quinolones, for some authors, is called des-fluoroquinolone generation [76], since encompasses substances free of fluorine at the C-6 position. The presence and location of fluorine atom in quinolones of 2nd and 3rd generations is associated with genotoxicity risks and side effects in the central nervous system. The introduction, in the central active molecule,

of substituents with antibacterial capacity increasingly improved, led to the removal of fluorine atom in position 6. Garenoxacine is one of the substances belonging to the 4th generation of quinolones [82], according to this classification, and contains no fluorine in its constitution.

Lincosamides, oxazolidinones and streptogramines are classes of antibiotics usually grouped together because they present similar mode of action and antibacterial spectra. They are active essentially against Gram-positive bacteria

Lincomycin, the first **lincosamide** antibiotic, was discovered in 1963, by Mason and co-workers [83], and is a natural product of *Streptomyces lincolnensis*. Lincomycin was supplanted by clindamycin, a semisynthetic derivative, due to its improved antibacterial activity [84]. Chemicaly, lincosamides are characterized by the presence of an alkyl 6-amino-6,8-dideoxy-1-thio-D-*erythro*-α-D-*galacto*-octopyranoside [83]. Lincosamides present antibacterial activity similar to macrolides [85] and resistance to erythromycin, a macrolide, affects lincosamides as well [54].

Oxazolidinones are a synthetic class of antibiotics. Linezolid was the first oxazolidinone approved for clinical use [86]. Some of the oxazolidinone antibiotics are the last generation of antibiotics used against Gram-positive pathogens and against multidrug-resistant bacteria, such as *Methicilin-resistant Staphylococcus aureus* (MRSA) [87]. The oxazolidinone essential chemical structure, or pharmacophoric template, is characterized by the structure in Figure 2.9.

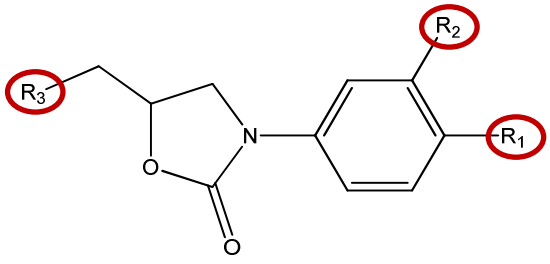


Figure 2.9: Oxazolidinones pharmacophoric template (Adapted from [87]).

Since oxazolidinones are a completely synthetic class of antibiotics, many investigation groups are developing new synthetic routes in order to obtain new effective antibiotics [86, 87], especially against multidrug-resistant bacteria.

Each member of the **streptogramines** family corresponds to two antibiotics, components A and B, produced as synergetic mixtures by various species of *Streptomyces* [54] and was first isolated from *Streptomyces graminofaciens* [88]. The two compounds in the streptogramin antibiotics are structurally different and present, alone, anti-bacterial activity, being bacteristatic. The combination of the two compounds has a bactericidal action improved relatively to the isolated components. The component A corresponds to a polyunsaturated peptide and component B to a hexadipeptide [89].

The imidazoles were introduced in the late 1950s and are a group of nitroheterocyclic compounds with antimicrobial activity that covers almost all microbial spectra, since they present activity against viruses, protozoa, helminthes and bacteria [54, 90].

The highly selective effect of these drugs is due to reduction of these drugs by nitroreductase enzymes resulting in the formation of highly reactive free radical species. Studies revealed mutagenic activity in bacteria and carcinogenic in rodents, although there is no proved carcinogenicity or mutagenicity in human beings [91].

Depending on the nature of substituents and the position of the nitro group, the nitroimidazole derivatives can show various pharmacological activities. The **5-nitroimidazoles** are the class of imidazoles that present antibacterial activity. The best known 5-nitroimidazol is metronidazole [54], a white to pale-yellow powder, insoluble in water. Metronidazole is active against anaerobic bacteria and resistant strains are so uncommon that it is the elected treatment for infections of this kind of bacteria. Related 5-nitroimidazoles are dimetridazole, ipronidazole, ronidazole.

Lipopeptide class of antibiotics is a structurally diverse group of metabolites produced from bacterial and fungal genera [92], specially *Pseudomonas* and *Bacillus*, that present antimicrobial, antitumour, immunosuppressant and surfactant activities. These compounds present differences in the length and composition of the lipid moiety as well as in the type, number and configuration of the amino acids in the peptide chain.

There are other classes of antibiotics, like the antibacterial polimyxins, chloramphenicol and bacitracin, the antituberculosis rifamycins, isoniazid, pyrazinamide and etambuthol, the antifungal amphotericin B, imidazolic and equinocandines; and antiparasitic benzimidazoles and avermectins [54].

In the 30 years following the use of sulfonamides and penicillin, there was the development of many substances with antimicrobial activity. Some of these substances have been discovered through the observation method, as happened with the first cephalosporin C, in wastewater [93, 94], and are classified as natural. Other substances were developed from modifications in natural substances and are classified as semi-synthetic [95]. This pace of discovery and introduction of new drugs has slowed in 1960 and only in 2000 emerged a new class of antibacterial agents, the oxazolidinones, which was followed, in 2003, by the lipopeptides [95]. Most drugs introduced between the late 1960s and early twenty-first century corresponds only to changes to the chemical structures already known. These changes have always aimed at broadening the spectrum of action of antimicrobial agents and fight the resistance that have emerged, each time with less time between the introduction of the drug and its expression [95-98].

The discovery and introduction of new classes of antimicrobial agents is a challenging and hard task, as well as extremely costly, as evidenced by the records of the Food and Drug Administration (FDA) in approving new drugs (Figure 2.10, Figure 2.11) for the last three decades [95, 98, 99].

Some of the substances developed demonstrated improvements in relation to the "generations" above, such as cephalosporins, others, which although disclosing similar activity to substances already in use, were shown to be safer and / or can be administered in dosages of greater benefit to patient [95].

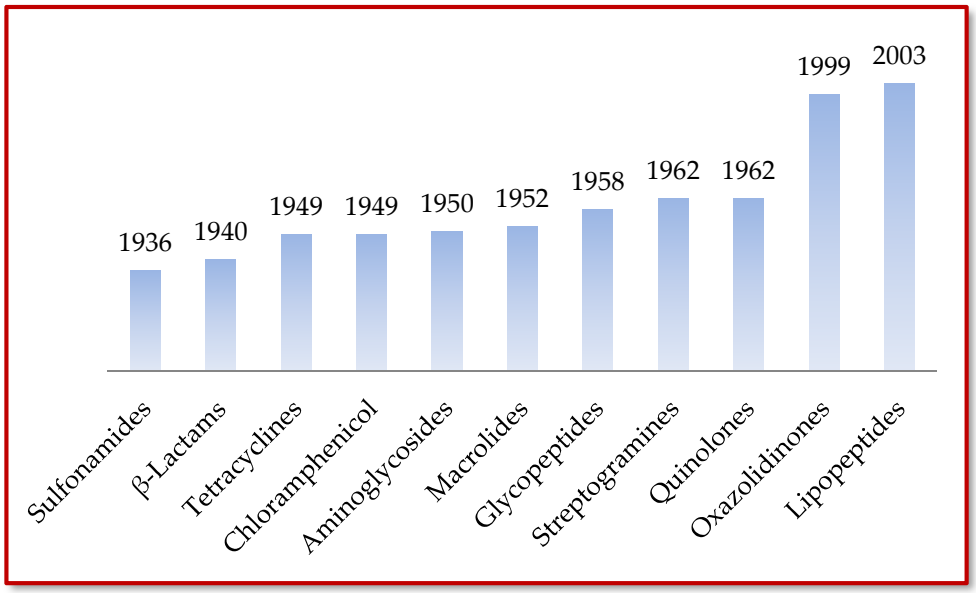


Figure 2.10: Representation of chronological approval of antibiotics classified by classes (adapted from *Lack of development of new antimicrobial drugs: a potential serious threat to public health* [100], with licence)

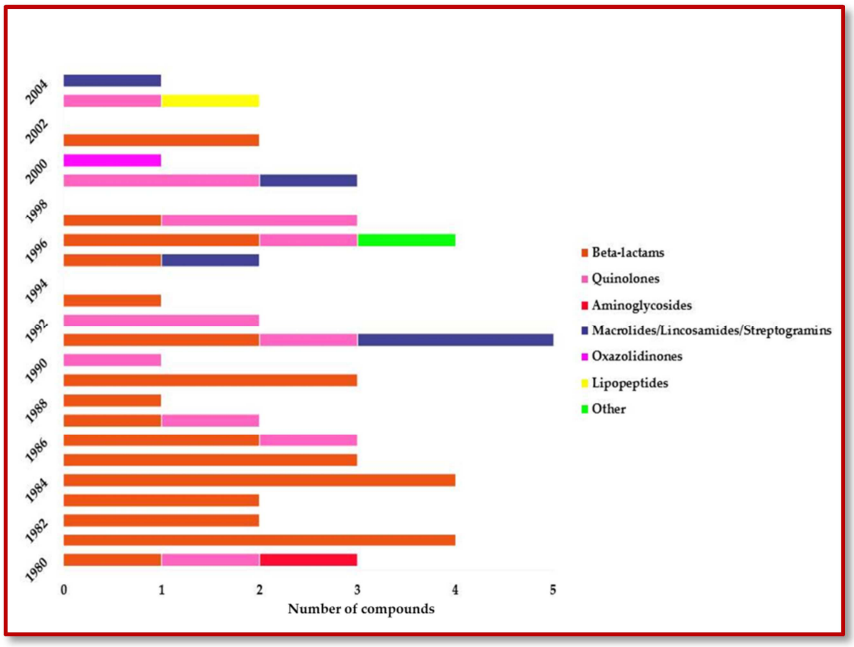


Figure 2.11: Number of new molecules, distributed by class of antibiotic, over the last decades (adapted from Powers, J. H. [98])

Nowadays, the need for new antibacterial agents is urgent due to the emergence of resistance to this type of drug, and the resistance to more than one class of antimicrobial compounds, reaching to the multidrug resistance (resistance to more than three antimicrobial classes [101]), is the most worrisome. Paradoxically, the pharmaceutical industry has increasingly invested less in research directed to the development of new antibacterial substances [99, 102].

2.2 CLASSIFICATION OF ANTIBIOTICS

Antibiotics can be classified in several ways, given the different characteristics they present.

The classification of these substances can be made according to its spectrum of action, which can be divided in broad or narrow.

Broad spectrum antibiotics act against a wide range of bacterial strains, both Gram-positive and Gram-negative. Some of these antibiotics are the most prescribed in clinical practice and are prescribed whenever the infectious bacteria is unknown. An example of a broad-spectrum class of antibiotics is the quinolones, which can act against Gram-negative and Gram negative bacteria, except first generation quinolones [103].

In contrast, **narrow spectrum antibiotics** have their action limited to a specific family of bacteria and are prescribed when the infectious bacterium is known. The glycopeptide class of antibiotics, that act only against Gram-positive bacteria, is an example for narrow spectrum antimicrobials [54].

Antibacterial agents can be classified as bacteriostatic or bactericidal. This classification divides them into those that lead to cell death (bactericidal) and those that inhibit cell growth (bacteriostatic). This classification is not very accurate, because this effect varies with the type of organism against which the antibacterial operates. The β-lactam antibiotics are classified as bactericidal [104] and the sulfonamides as bacteriostatic [50].

Antimicrobial agents may be classified, also, by their mechanism of action. The known mechanisms of action of antibiotics are divided in five groups (Figure 2.12). There are the *i*) bacterial cell wall synthesis inhibitors, *ii*) inhibitors of protein synthesis, *iii*) inhibitors of DNA/RNA precursors biosynthesis, *iv*) inhibitors of DNA/RNA synthesis and *v*) and disruptors of cell membrane function [3, 104].

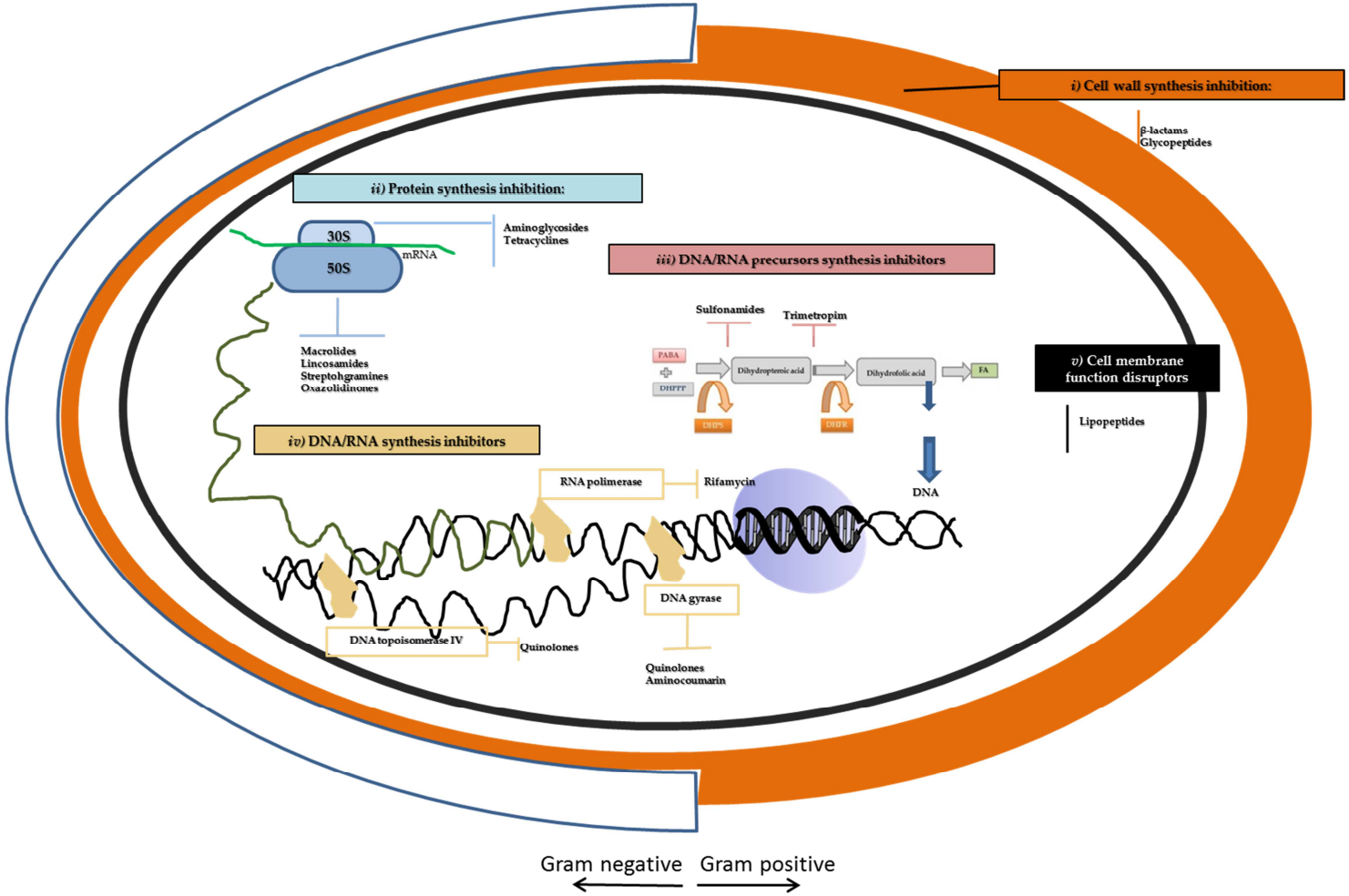


Figure 2.12: Schematic representation of the mechanism of action of antimicrobial agents (Adapted from [3]).

There are also new classifications proposed, such as chromatographic classification [105, 106], that are still not adopted in human and veterinary medicine.

The classification considered the more appropriate is the one which groups antibiotics according to their chemical structure [107, 108]. According to this classification, antibiotics may be divided into the following groups or classes: β-lactams, sulfonamides, glycopeptides, aminoglycosides, macrolides, tetracyclines, quinolones, lincosamides, oxazolidinones, streptogramins, 5-nitroimidazoles, lipopeptides, and other [109].

The antibiotics, divided in structurally related chemical formulas, present similar modes of action and activity spectra.

2.2.1 B-LACTAMS

β-Lactam antibiotics share a β-lactam ring system, a cyclic amide attached to a β-carbon, and are divided into penicillins (penams), cephems, carbapenems and monobactams [104, 110]. There are also the β-lactamase inhibitors [111].

When the β-lactam ring is fused with a five-membred ring, the compound can be: penicillin (Table 2.1), when fused with a thiazolidine; or carbapenem (Table 2.7), when fused with a pyrrolidine. When the β-lactam ring is fused with a six-membred ring, the compound is a cephem (3,6-dihydro-2*H*-1,3-thiazin). Cephems are divided into cephalosporins (

Table 2.2 to Table 2.5), cephamycin (Table 2.6), oxacephems (3,6-dihydro-2*H*-1,3-oxazine) and carbacephems (1,2,3,4-tetrahydropyridine) [104, 112]. A carbapenem is defined as the 4:5 fused ring lactam of penicillins with a double bond between C-2 and C-3 but with the substitution of carbon for sulfur at C-1 [113]. The monobactams are monocyclic β-lactam rings (not fused to any other ring system) [104, 114].

Clavulanic acid, sulbactam and tazobactam, the β-lactamase inhibitors (Table 2.9) are structurally similar to penicillins, differing from them by the presence of a leaving group at position C-1 of the five membered ring. Alone, they present little antimicrobial activity [111, 113].

β-lactam antibiotics inhibit the growth of bacteria by inactivating proteins involved in the synthesis of peptidoglycan, a macromolecule constituted by a cross-linked chain of sugars (*N*-Acetylmuramic acid and *N*-Acetylglucosamine) and amino acids, that is part of the cell wall structure. Both Gram-positive and Gram-negative bacteria cell wall has peptidoglycan in its constitution: Gram-positive species have multiple layers of peptidoglycan external to the cell membrane and have a thick cell wall; the Gram-negative bacteria have a thiner cell wall with the peptidoglycan positioned between an outer membrane, composed of phospholipids and lipopolyssacharides, and the cell membrane. Peptidoglycan is fundamental in cell wall structure, to provide support and strength, and helps maintain the osmotic pressure. Its synthesis is dependent on the action of penicillin-binding protein (PBPs) or transpeptidases [115, 116]. β-lactams inhibit a family of PBPs involved in several stapes of biosynthesis of peptidoglycan, binding to the serine active-site of the enzyme, through the β-lactam ring [54, 117]. This action leads to inhibition of bacterial growth and cell lysis happens, by activation of autolytic enzymes, and thus β-lactams have bactericidal action [118].

Table 2.1: β-Lactam class of antibiotics – Penicillin: Biological Properties

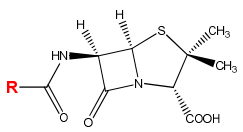
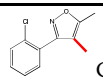
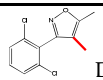
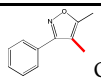
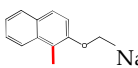
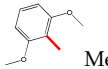
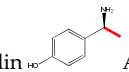
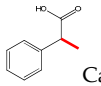
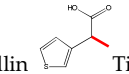
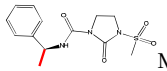
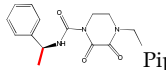
Sub class	Pharmacophore template	Derivative Structure (R)	Spectrum of action
Penicillin [104]		 Penicillin G or Benzylpenicillin  Penicillin V  Clometocillin	**Narrow spectrum; beta-lactamase sensitive (pH sensitive)** **Gram-positive cocci:** *Staphylococcus* spp. (non-penicillinase-producing); *Streptococcus* spp., *Pneumococcus* spp. **Other Benzylpenicillin sensitive microorganisms:** *Neisseria gonorrhoeae*, *Actinomyces bovis*, *Streptobacillus moniliformis*, *Listeria monocytogenes* e *Leptospira* spp.,*Treponema pallidum* **At high seric levels:** *Escherichia coli*, *Proteus mirabilis*, *Salmonella* spp., *Shigella* spp., *Enterobacter aerogenes*, *Alcaligenes faecalis*, *N. meningitidis*, *Clostridium tetane*
		 Cloxacillin  Dicloxacillin  Oxacillin  Nafcillin  Methicillin	**Narrow spectrum; beta-lactamase resistant** **Gram-positive cocci:** *Staphylococcus* spp., *Streptococcus* spp., *Pneumococcus* spp.
		 Ampicillin  Amoxicillin  Epicillin  Carbenicillin  Ticarcillin  Mezlocillin  Piperacillin	**Large spectrum; Aminopenicillins, Carboxypenicillins, Ureidopenicillins** **Gram-positive:** *Streptococcus* spp, *Staphylococcus* spp. (non-penicillinase-producing), *Bacillus anthracis*, *Clostridium spp.*, *Enterococus faecalis*, *Corynebacterium* spp. and others **Gram-negative:** *Haemophylus influenzae; Neisseria gonorrhoeae; Escherichia coli , Neisseria meningitidis; Proteus mirabilis*, *Salmonella* spp., *Shigella* spp., *Bordatella pertussis*, *Pasteurella septica*, *Vibrio cholerae*, *Helicobacter pylori*

Table 2.2: β-Lactam class of antibiotics – Cephems: Biological Properties (First Generation Cephalosporins)

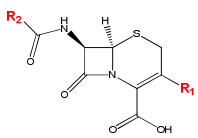
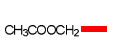
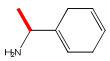
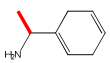
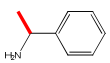
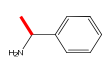
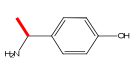
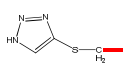
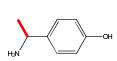
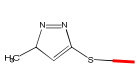
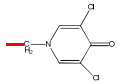
Sub class	Pharmacophore template	Derivative Structure		Name	Spectrum of action
		R1	R2		
Cephems Cephalosporin - First Generation [104]				Cefacetrile	**Broad Spectrum for Gram-positive; Moderate spectrum for aerobic Gram-negative[119]** **Gram-positive:** Penicillinase-producing, methicillin-susceptible *Staphylococcus* and *Streptococcus* beta-hemolytic, *Streptococcus pneumonia*. (No MRSA or MRSE activity). **Gram-negative:** *Proteus mirabilis*, some *Escherichia coli*, *Klebsiella pneumoniae, Enterobacter aerogenes, Haemophylus influenza.*
				Cefradine	
				Cefroxadine	
				Cefaloglycin	
				**Cephalexin**	
				**Cefadroxil**	
				Cefatrizine	
				Cefazedone	

Table 2.2: Continued

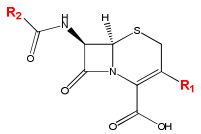
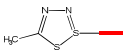
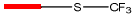
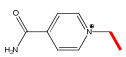
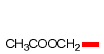
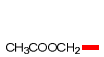
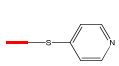
Sub class	Pharmacophore template	Derivative Structure		Name	Spectrum of action
		R1	R2		
Cephems – Cephalosporins – First Generation [104]				Ceftezole	
				**Cefazolin**	
				Cefazaflur	**Broad Spectrum for Gram-positive; Moderate spectrum for aerobic Gram-negative [119]**
				Cephalotin	**Gram-positive:** Penicillinase-producing, methicillin-susceptible *Staphylococcus* and *Streptococcus* beta-hemolytic, *Streptococcus pneumonia*.(No MRSA or MRSE activity)
				Cefaloridine	**Gram-negative:** *Proteus mirabilis*, some *Escherichia coli*, *Klebsiella pneumoniae, Enterobacter aerogenes, Haemophylus influenzae*
				Cephalonium	
				Cefapirin	

Table 2.3: β-Lactam class of antibiotics – Cephems Biological Properties (Second and Third Generation Cephalosporins)

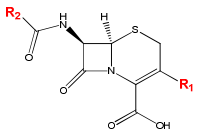
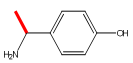
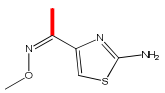
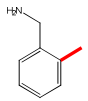
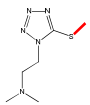
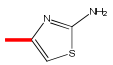
Sub class		Pharmacophore template	Derivative Structure		Name	Spectrum of action
			R1	R2		
Cephems	Cephalosporins – Second Generation [104]				**Cefaclor**	**Moderate spectrum** **Gram-positive:** Less than first-generation. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus* Group B, *Streptococcus mitis*, *Bordetella pertussis* **Gram-negative:** Greater than first-generation: *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Enterobacter aerogenes Neisseria gonorrhoeae*, *N. meningitides*, *Escherichia coli*; *Klebsiella* spp., *Proteus mirabilis*, *Providencia* spp., *Proteus rettgeri*, *Moraxella catarrhalis*, *Salmonella* spp **Anaerobic:** Gram-negative and Gram-positive (*Peptostreptococcus*).
					Cefonicid	
					Cefprozil	
					**Cefuroxime**	
					Cefuzonam	
					**Cefamandole**	
					Ceforanide	
					Cefotiam	

Table 2.3: Continued

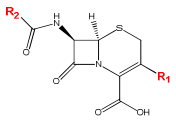
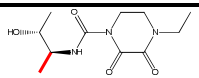
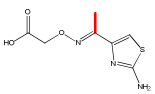
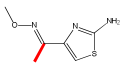
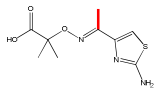
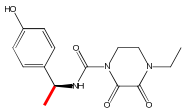
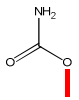
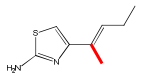
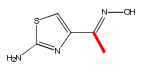
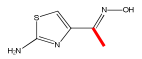
Sub class	Pharmacophore template	Derivative Structure		Name	Spectrum of action
		R1	R2		
Cephems Cephalosporins – Third Generation [104]				Cefbuperazone	**Broad spectrum** **Gram-negative:** *Aeromonas hydrophila*; *Bordetella pertussis*; *Moraxella catarrhalis*; *Citrobacter freundii*; *Citrobacter koseri, Escherichia coli*, *Enterobacter* spp., *Haemophilus* penicillinase-producing strains, *Klebsiella pneumonia, Klebsiella oxytoca, Staphylococcus* Methicillin sensitive strains, *Morganella morganii*; *Neisseria gonorrhoeae, Neisseria meningitides, Proteus mirabilis, Salmonella* spp., *Serratia* spp., *Shigella* spp., *Veillonella* spp., *Yersinia* spp. **Gram-positive:** Less than first generation. *Bacillus subtilis, Clostridium perfringens, Corynebacterium diptheriae, Erysipelothrix insidiosa, Propionibacterium, Streptococcus pneumoniae, Streptococcus* spp. **Gram-variable:** *Eubacterium* group, *Borrelia burgdorferi*
				**Cefixime**	
				**Ceftriaxone**	
				**Ceftazidime**	
				Cefoperazone	
				Cefcapene	
				Cefdaloxime	
				Cefdinir	

Table 2.3: Continued

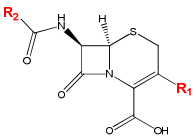
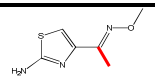
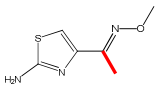
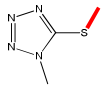
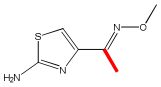
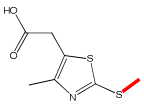
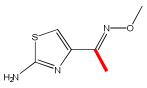
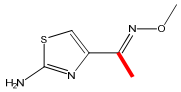
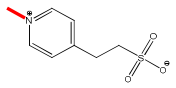
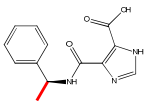
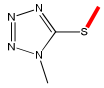
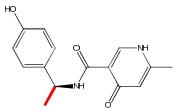
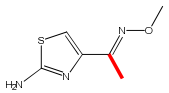
Sub class	Pharmacophore template	Derivative Structure		Name	Spectrum of Action
		R1	R2		
Cephems Cephalosporins – Third Generation [104]				Cefditoren	**Broad spectrum** **Gram-negative:** *Aeromonas hydrophila, Bordetella pertussis, Moraxella catarrhalis, Citrobacter freundii, Citrobacter koseri, Escherichia coli, Enterobacter* spp., *Haemophilus* penicillinase-producing strains, *Klebsiella pneumonia, Klebsiella oxytoca, Staphylococcus* Methicillin sensitive strains, *Morganella morganii, Neisseria gonorrhoeae, Neisseria meningitides, Proteus mirabilis, Salmonella* spp., *Serratia* spp., *Shigella* spp., *Veillonella* spp., *Yersinia* spp. **Gram-positive:** *Bacillus subtilis, Clostridium perfringens, Corynebacterium diptheriae, Erysipelothrix insidiosa, Propionibacterium, Streptococcus pneumoniae, Streptococcus* spp. **Gram-variable:** *Eubacterium* group, *Borrelia burgdorferi*
				Cefetamet	
				Cefmenoxime	
				Cefodizime	
				**Cefotaxime**	
				Cefpimizole	
				Cefpiramide	
				Cefpodoxime	

Table 2.3: Continued

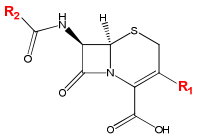
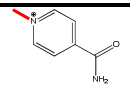
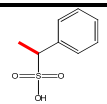
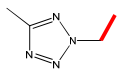
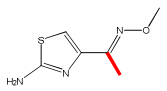
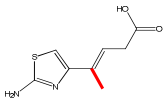
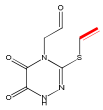
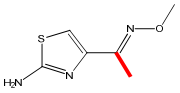
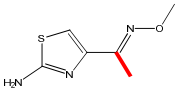
Sub class		Pharmacophore template	Derivative Structure		Name	Spectrum of action
			R1	R2		
Cephems	Cephalosporins – Third Generation [104]				Cefsulodin	**Broad spectrum** **Gram-negative:** *Aeromonas hydrophila, Bordetella pertussis, Moraxella catarrhalis, Citrobacter freundii, Citrobacter koseri, Escherichia coli, Enterobacter* spp., *Haemophilus* penicillinase-producing strains, *Klebsiella pneumonia, Klebsiella oxytoca, Staphylococcus* Methicillin sensitive strains, *Morganella morganii; Neisseria gonorrhoeae, Neisseria meningitides, Proteus mirabilis, Salmonella* spp., *Serratia* spp., *Shigella* spp., *Veillonella* spp., *Yersinia* spp. **Gram-positive:** *Bacillus subtilis, Clostridium perfringens, Corynebacterium diptheriae, Erysipelothrix insidiosa, Propionibacterium, Streptococcus pneumoniae, Streptococcus* spp. **Gram-variable:** *Eubacterium* group, *Borrelia burgdorferi*
					Cefteram	
					Ceftibuten	
					Ceftiolene	
					Ceftizoxime	

Table 2.4: β-Lactam class of antibiotics – Cephems Biological Properties (Fourth Generation Cephalosporins)

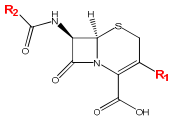
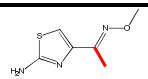
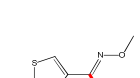
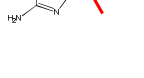
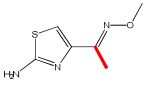
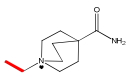
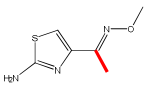
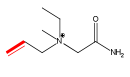
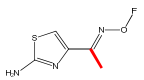
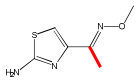
Sub class	Pharmacophore template	Derivative Structure		Name	Spectrum of action
		R1	R2		
Cephems Cephalosporins – Fourth Generation [104]				Cefepime	**Broad Spectrum** **Aerobic Gram-positive:** *Staphylococcus aureus*, *Staphylococcus epidermidis*, *S. hominis* e *S. saprophyticus*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, other *Streptococcuos* beta-hemolitic, *S. bovis S. Viridans*. **Aerobic Gram-negative:** *Acinetobacter calcoaceticus*, *Aeromonas hydrophila*, *Capnocytophaga* spp., *Citrobacter* spp. (*C. diversus* and *C. freundii*), *Campylobacter jejuni*, *Enterobacter* spp. (*E. cloacae*, *E. aerogenes* and *E. sakazakii*), *Escherichia coli*, *Gardnerella vaginalis*, *Haemophilus ducreyi*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Hafnia alvei*, *Klebsiella* spp. (*K. pneumoniae*, *K. oxytoca* and *K. ozaenae*), *Legionella* spp, *Morganella organii*, *Moraxella catarrhalis*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pantoea agglomerans*, *Proteus* spp., *Providencia* spp., *Pseudomonas* spp., *Salmonella* spp., *Serratia* spp., *Shigella* spp., *Yersinia enterocolitica*. **Anaerobic:** *Bacteroides* spp., *Clostridium perfringens*, *Fusobacterium* spp., *Mobiluncus* spp., *Peptostreptococcus* spp., *Prevotella melaninogenica*, *Veillonella* spp.
				Cefozopran	
				Cefpirome	
				Cefquinome Used in veterinary medicine	
				Cefeclidine	
				Cefluprenam	
				Cefoselis	

Table 2.5: β-Lactam class of antibiotics – Cephems Biological (Fifth Generation Cephalosporins)

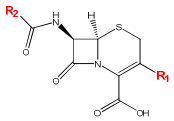
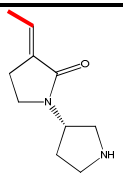
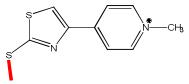
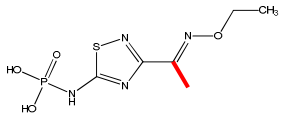
Sub class	Pharmacophore template	Derivative Structure		Name	Spectrum of action
		R1	R2		
Cephalosporins – Fifth Generation [120-122]				Ceftobiprole	**Extended Gram-positive spectrum** **MDR Gram-positive:** Methicillin-resistant *Staphylococcus aureus*, vancomycin-intermediate *S. aureus*, penicillin-resistant *Streptococcus pneumonia,* vancomycin-resistant *Enterococcus faecalis* **Gram-negative**: wild-type Enterobacteriacea, *Pseudomonas aeruginosa*
				Ceftaroline fosamil	**Extended Gram-positive spectrum** **MDR Gram-positive:** Methicillin-resistant *Staphylococcus aureus*, vancomycin-intermediate *S. aureus*, vancomycin-resistant *S, aureus*, penicillin-resistant *Streptococcus pneumonia* **Gram-negative:** *Haemophilus influenza, Moraxella catarrhalis,* wild-type Enterobacteriacea

Table 2.6: β-Lactam class of antibiotics - Cephems Biological Properties (Cephamycins)

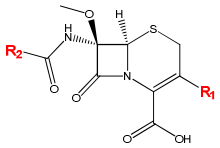
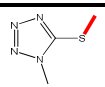
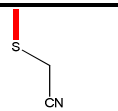
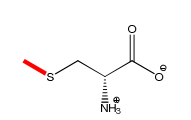
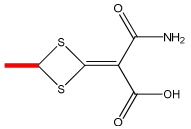
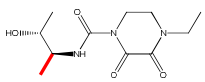
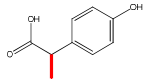
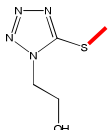
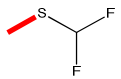
Sub class		Pharmacophore template	Derivative Structure		Name		Spectrum of action
			R1	R2			
Cephems	Cephamycins [104]				Cefmetazole		**Broad spectrum** **Aerobic Gram-positive**: *Staphylococcus*; *Streptococcus* beta-hemolitic from group A and B, *Streptococcus pneumoniae*, Streptococcus spp. (excluding group D), *Enterococcus* **Aerobic Gram-negative:** *Neisseria gonorhoeae*, *Neisseria meningitidis*. *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella* spp., *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus vulgaris*, *Proteus mirabilis*, *Providencia* spp., *Providencia rettgeri*, *Salmonella* spp., Shigella spp. *Serratia marcescens*. *Bacteroides* spp., *Bacteroides fragilis*, *Bacteroides melaninogenicus*, *Fusobacterium* spp., *Acinetobacter calcoaceticus* var. anitratum, *Acinetobacter calcoaceticus* var. Iwoffi, *Alcaligenes faecalis*, *Citrobacter* spp., *Enterobacter* spp., *Flavobacterium* spp., *Pseudomonas* spp., most of *Enterococcus* strains, *Enterobacter* spp., methicillin-resistant *Staphylococcus*, *Listeria monocytogenes*. **Anaerobic Gram-positive**: *Clostridium* spp., *Clostridium perfringens*, *Eubacterium* spp., *Propionibacterium acne* **Anaerobic Gram negative:** *Veillonella* spp.
					Cefminox		
					Cefotetan	These cephems are sometimes grouped with 2nd-generation cephalosporins	
					**Cefoxitin**		
					Cefbuperazone		
					Latamoxef	This structure is an oxacephem and is sometimes grouped with 3rd-generation cephalosporins:	
					Flomoxef	This structure is an oxacephem and is sometimes grouped with 4th-generation cephalosporins:	

Table 2.7: β-Lactam class of antibiotics - Carbapenems Biological Properties

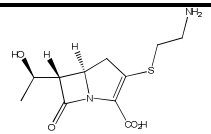
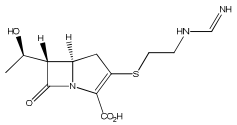
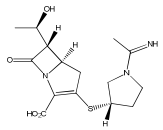
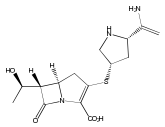
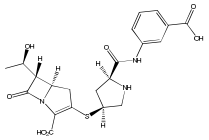
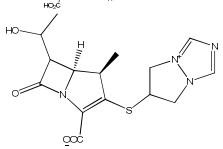
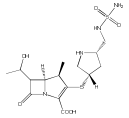
Sub class	Pharmacophore template	Name	Spectrum of action
Carbapenems [104]		Tienamicin	**The broadest spectrum of activity and greatest potency against Gram-positive** (excluding Staphylococcus oxacillin-resistant) **and Gram-negative** (excluding *Acinetobacter*, *H. influenzae*, *Stenotrophomonas* and *Pseudomonas*) **bacteria.**
		Imipenem	
		Panipenem	
		Meropenem	
		Ertapenem	Often used as "last-line agents" or "antibiotics of last resort" when patients with infections become gravely ill or are suspected of harboring resistant bacteria [113].
		Biapenem	
		Doripenem	

Table 2.8: β-Lactam class of antibiotics - Monobactams Biological Properties

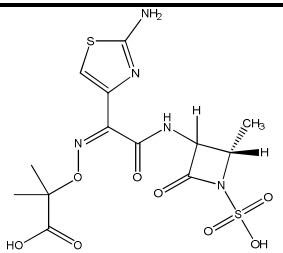
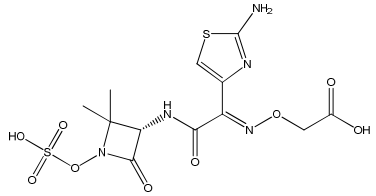
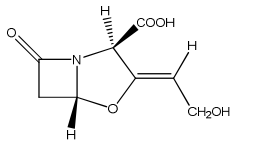
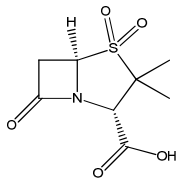
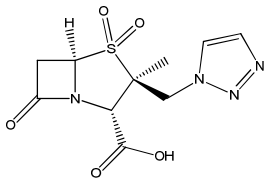
Sub class	Pharmacophore template	Name	Spectrum of action
Monobactams [104]		Azetronam	Active only against aerobic and facultative **Gram-negative bacteria** [123]**:** *Acinetobacter anitratus, Escherichia coli* and *Proteus mirabilis.*
		Tigemonam	**Gram-negative:** *Escherichia coli, Klebsiella* spp., *Enterobacter aerogenes, Citrobacter diversus, Proteus* spp., *Providencia* spp., *Aeromonas hydrophila, Salmonella* spp., *Shigella* spp., *Serratia marcescens, Yersinia enterocolitica. Haemophilus* spp., *Neisseria* spp., *Branhamella catarrhalis* [124].

Table 2.9: β-Lactam class of antibiotics - β-lactamase inhibitors Microbiological Properties

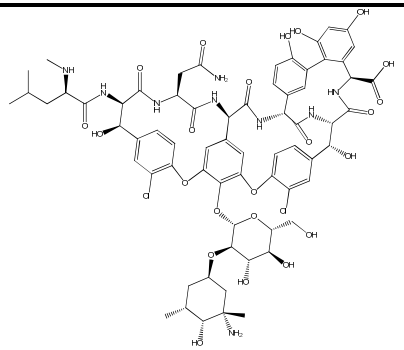
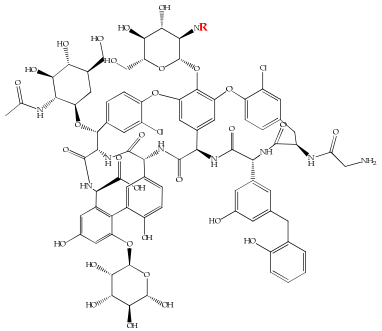
Class	Chemical Structure	Name	Combined activity
β-lactamase inhibitors			
Broad-spectrum [104]		Clavulanic acid (carbapenem)	**Amoxicillin + Clavulanic acid:** **Aerobic Gram-positive:** *Staphylococcus aureus*, *Staphylococcus* coagulase negative (including *Staphylococcus epidermidis*), *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus* sp., *Corynebacterium* spp, *Bacillus anthracis*, *Listeria monocytogenes*. **Anaerobic Gram-positive:** *Clostridium* spp.,*Peptococcus* spp., *Peptostreptococcus* spp. **Aerobic Gram-negative:** *Haemophilus influenzae*, *Moraxella catarrhalis*, *Escherichia coli*, *Klebsiella* sppp., *Proteus mirabilis*, *Proteus vulgaris*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Salmonella* spp., *Shigella* spp., *Bordetella pertussis*, *Brucella* spp., *Vibrio cholerae*, *Pasteurella multocida*, *Gardnerella vaginalis*, *Heliobacter pylori*, *Legionella* spp., *Yersinia enterocolitica*. **Anaerobic Gram-negative:** *Bacteroides* spp. (including *B. fragilis*), *Fusobacterium* spp.
		Sulbactam	**Ampicillin + Sulbactam:** **Aerobic Gram-positive:** *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pmeumoniae, Streptococcus faecalis, Streptococcus* spp **Aerobic Gram-negative:** *Haemophilus influenza* and *H. parainfluenzae* (both β-lactamase positive and negative), *Branhamella catarrhalis*, *Escherichia coli*, *Klebsiella* spp, *Proteus* spp, *Morganella morganii*, *Citrobacter* spp, *Enterobacter* spp, *Neisseria gonorrhoeae*, *Neisseria meningitides* **Anaerobic Gram-negative:** *Bacteroides fragilis*
		Tazobactam	**Piperacilina + Tazobactam:** **Aerobic Gram-positive:** *Streptococcus* (*S. pneumoniae*, *S. pyogenes*, *S. bovis*, *S. agalactiae*, *S. viridans*), *Enterococcus* (*E. faecalis*, *E. faecium*), *Staphylococcus aureus* (*S.aureus* methicillin non-resistant), *S. saprophyticus*, *S. epidermidis* (Staphylococcus coagulase-negative), *Corynebacteria*, *Listeria monocytogenes*, *Nocardia* spp. **Anaerobic Gram-positive:** *Peptostreptococcus* spp., *Clostridia* spp, *Actinomyces* spp **Aerobic Gram-negative:** *Escherichia coli*, *Citrobacter* spp., *Klebsiella* spp., *Enterobacter* spp., *Proteus vulgaris*, *Proteus mirabilis*, *Providencia rettgeri*, *Providencia stuartii*, *Plesiomonas shigelloides*, *Morganella morganii*, *Serratia* spp, *Shigella* spp, *Salmonella* spp., *Pseudomonas aeruginosa* and other *Pseudomonas* spp., *Xanthomonas maltophilia*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Moraxella* spp., *Acinetobacter* spp., *Haemophilus influenzae*, *H. parainfluenzae*, *Pasteurella multocida*, *Yersinia* spp., *Campylobacter* spp., **Anaerobic Gram-negative**: *Bacteroides* spp, *Fusobacterium*spp., *Veillonella* spp **Gram-variable:** Eubacterium group, *Gardnerella vaginalis*

2.2.2 GLYCOPEPTIDES

Glycopeptides are formed by two sugars and a heptapeptide domain (a string of amino acids), and five of these amino acids are common to all glycopeptides [125], differing only on position 1 and 3 amino acids and substituents on these residues (Table 2.10). The structure bearing the seven amino acids is called aglycone and is the one responsible for the biological activity of this class of antibiotics [118, 125]. This aglycone structure is the one responsible for the biological activity of this class of antibiotics and the substituents, located on the outside of the bracelet-like configuration adopted by the aglycone structure, do not seem to have a significant effect on antibiotic activity, but may increase hydrophobicity [125], allowing these molecules to cross the cytoplasmatic membrane, and reach the aminoacids, binding to them and inhibiting their addition to the peptidoglycan chain. In particular, they bind to acyl-D-alanyl-D-alanine.

Glycopeptides, as β-lactam antibiotics, act on the biosynthesis of peptidoglycan, binding to aminoacids through the aglycone moiety, by means of hydrogen bonding [118]. Glycopeptides are, for the same reasons that β-lactams, bactericidal.

Table 2.10: Chemical structure and Biological Properties for some Glycopeptide Antibiotics

Class	Chemical Structure	Name	Spectrum of action
Glycopeptides [118, 125]		Vancomycin	**Narrow spectrum.** **Gram-positive:** *Staphylococcus* spp, *Streptococcus* spp, *Listeria monocytogenes*, *Entherococcus faecalis* and *E. faecium*, *Clostridium difficile* and other Clostridia, *Bacillus cereus* and *B. subtilis*, *Corynebacterium* spp, *Actinomyces* spp
		Teicoplanin Teicoplanin $A_2$1 Teicoplanin $A_2$2 Teicoplanin $A_2$3 Teicoplanin $A_2$4 Teicoplanin A_2 5	

2.2.3 SULFONAMIDES

Sulfonamides are white crystals, usually poorly soluble in water. The corresponding sodium salts make them water soluble. These compounds exhibit amphoteric behavior (Figure 2.13), but usually behave as weak organic acids and are more soluble in alkaline than acidic environments. Sulfonamides with therapeutic interest have pKa between 4.8 and 8.6. In some ranges of pH, sulfonamides may be in their zwitterionic form. This feature has a great importance for the antibacterial activity. According to Struller [52], the anionic form of the drug has greater activity than the neutral form, and the acidic form activity is substantially reduced.

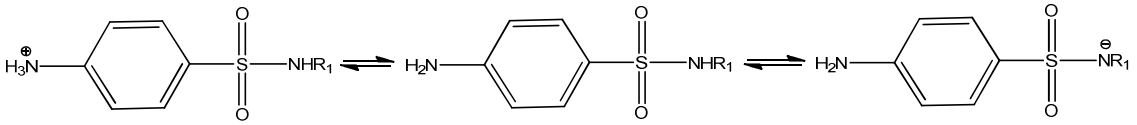


Figure 2.13: Anfoteric forms of sulfonamides.

Sulfonamides greatly alter their solubility with variation in pH, which makes it possible that sulfonamides crystals may precipitate in renal tissue, causing crystalluria in patients under treatment. To overcome this effect, the sulfonamides can be administered in the form of sodium or disodium salts, in order to increase their solubility [126].

Some compounds are used as pro-drugs, such as ftalilsulfathiazol, which is hydrolyzed in the intestine and releases the sulfathiazole, for regeneration of the *p*-NH_2 group, essential for the antibacterial activity [127].

The sulfonamides are characterized by the active functional group [R_1-SO_2-*N*(R_2;R_3)] and are similar in structure to *p*-aminobenzoic acid (PABA) (Table 2.11). This class of antibiotics acts like their antimetabolite, competing with PABA in the synthesis of folic acid (FA). FA is essential for the growth of all the cells, participating in the synthesis and repair of DNA, and functions as a cofactor in certain biological reactions [128]. The FA is present, as vitamin B9, in diet, reaching the cytoplasm across the membrane of eukaryotic cells [129]. However, this diffusion does not occur across the membranes of bacterial cells and, therefore, it has to be synthesized by those from PABA

[130]. The sulfonamides, by interfering in the synthesis of FA, inhibiting dihydropteroate synthetase (DHPS), (Figure 2.14), prevent bacterial growth [51, 131].

By intervening in the normal production of FA , sulfonamides prevent bacterial growth, and therefore have bacteriostatic action [132]. The action of sulfonamides is reduced in the presence of excess of PABA and similar molecules, as local anesthetics procaine and tetracaine [133].

Trimetropim (a diaminopyrimidine), is an inhibitor for dihydrofolate reducyase (DHFR), an enzyme that converts dihydrofolic acid into folic acid. Alone, it is bactericidal. This compound is usually administrated in combination with sulfonamides, tradicionally sulfamethoxazol, improving their action [134]. The combination of sulfonamides and trimetropim is bactericidal.

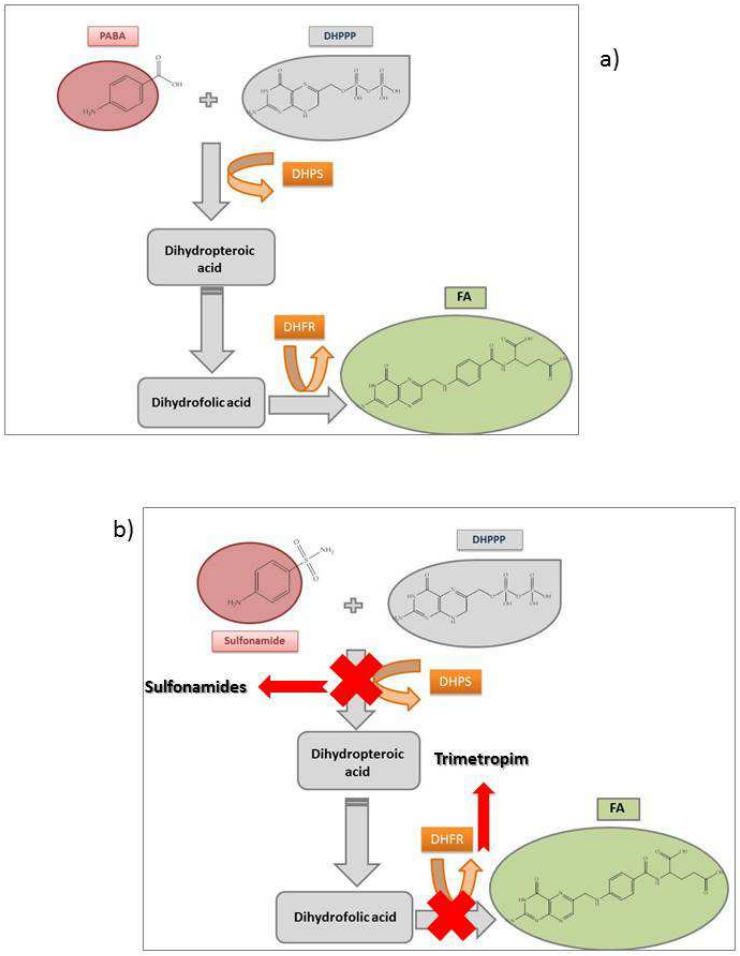
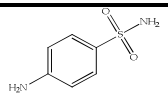
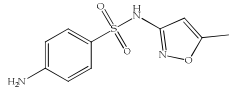
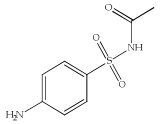
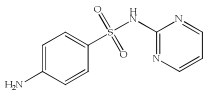
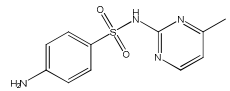
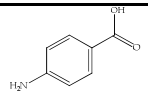


Figure 2.14: Synthesis of FA: a) normal pathway for FA synthesis from PABA; b) sulfonamides action on FA synthesis by dihydropteroate synthetase (DHPS) inhibition and trimetropim action on dihydrofolate reductase (DHFR) inhibition. This combination of compounds (sulfonamide + trimetropim) is normally used in order to improve efficacy.

Table 2.11: Chemical Structure and biological data for some Sulfonamides and p-aminobenzoic acid (structures built from software Cambridge Chem3D Pro 12.0)

Chemical Structure	Trivial Name		Spectrum of action
	Sulfonilamide		**Sulfonamide (Sulfametoxazol, sulfadiazine)+trimetropim:** **Cocos:** Branhamella catarrhalis **Gram-negative:** *Haemophilus influenzae* (β-lactamase positive, β-lactamase negative), *Haemophilus parainfluenzae*, *E. coli*, *Citrobacter freundi*, *Citrobacter* spp., *Klebsiella* spp., *Enterobacter cloacae*, *Enterobacter aerogenes*, *Hafnia alvei*, *Serratia* spp., *Proteus mirabilis*, *Proteus vulgaris*, *Morganella morganii*, *Shigella* spp., *Yersinia enterocolitica*, *Yersinia* spp., *Vibrio cholerae*. *Edwardsiella tarda*, *Alcaligenes faecalis*, *Pseudomonas cepacia*, *Burkholderia pseudomallei*. *Brucella*, *Listeria monocytogenes*, *Nocardia asteroides*, *Pneumocystis carinii*, *Cyclospora cayetanensis*. **Partially sensitive microorganisms:** *Staphylococcus* spp. (coagulase- negativo), *Streptococcus pneumoniae* (penicillin sensitive and penicillin resistant), *Haemophilus ducreyi*, *Providencia rettgeri*, other *Providencia* spp., *Salmonella typhi*, *Salmonella enteritidis*, *Stenotrophomonas maltophilia* (formerly *Xanthomonas maltophilia*), *Acinetobacter lwoffi*, *Acinetobacter anitratus*, *Aeromonas hydrophila*
	Sulfamethoxazole		
	Sulfacetamide		
	Sulfadiazine	*Haemophilus ducreyi*, *Nocardia* sp., *Actinomyces* sp., *Calymmatobacterium granulomatis* and *Toxoplasma gondii*	
	Sulfamerazine		
	PABA - *p*-aminobenzoic acid		

2.2.4 AMINOGLYCOSIDES

Aminoglycosides are a class of antibiotics used, mostly to combat infections due to aerobic Gram-positive bacterial strains. Aminoglycosides are hydrophilic molecules formed by an aminocyclitol ring linked to one or more amino sugars, through a glycosidic bond (Table 2.12). The most widely used classification refers to ring 1 as the amino sugar bonded in position 4 to the aminocyclitol ring (2-desoxistreptomycin) that corresponds to ring 2. The third ring is the amino sugar bonded in position C-5 or C-6 of the aminocyclitol ring. There may be a fourth ring, bonded to ring 3. Most of the compounds with clinical applicability have a 2-desoxistreptomycin as aminocyclitol nucleus, which can be substituted in positions C-4 and C-5 or C-4 and C-6 (Figure 2.15).

Aminoglycosides act on the 30S ribosomal subunit. Bacterial ribosomes (70S) are made up of two subunits: 30S and 50S [135]. The 30S ribosomal subunit binds to mRNA and initiates protein synthesis, so aminoglycosides act as inhibitors of protein synthesis. The 30S subunit does not exist in eukaryotic cells, so the action of this class of compounds is bacterial selective, but there may be damage on eukaryotic cells due to nonspecific binding to ribosomes and/or nucleic acids [136].

Aminoglycosides are highly polar at physiological pH, so they cannot diffuse through bacterial membrane. There is a specific mechanism of transport, and it is not energy dependent, on the outer membrane of Gram-negative bacteria, but energy-dependent on the passage through cytoplasmic membrane. It has been demonstrated that the higher the transmembrane potential, the greater the antibacterial activity of the aminoglycoside [137]. Once in the cytosol, aminoglycosides bind to the 30S ribosomal subunit. The specific mechanism of action is complex and energy-dependent. Shortly, this class of antibiotics interferes in the impairment of the proofreading process that controls translational accuracy, causes misreading and/or premature protein synthesis. The output of this action are aberrant proteins, that may bond to cell wall, and the damage extend varies from one compound to another [136].

Aminoglycosides are bactericidal antibiotics and are synergistic with antibiotics that act on the cell wall synthesis, because these facilitate aminoglycoside penetration into bacterial cytosol.

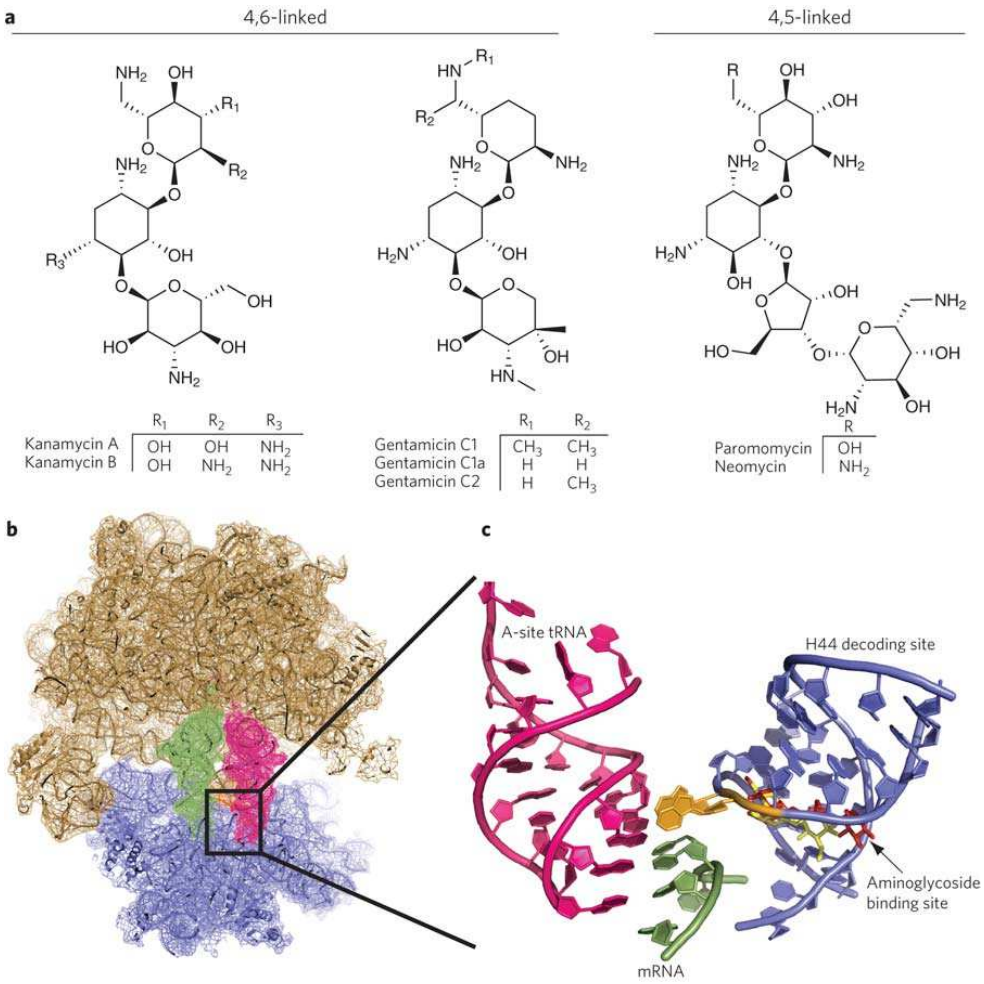


Figure 2.15: Aminoglycoside antibacterial mechanism of action: a) general chemical structural of some aminoglycoside antibiotics; b) 70S prokaryotic ribosome structure (30S subunit in blue and 50S subunit in brown); c) amplification of the binding site of aminoglycosides (16S, in blue), that disturbs protein synthesis. Gentamicin (yellow) and neomycin (red) are bonded to the decoding site. (Adapted from Feldman et al [135], with license).

Table 2.12: Chemical Structure and Biological Properties for Aminoglycoside Antibiotics

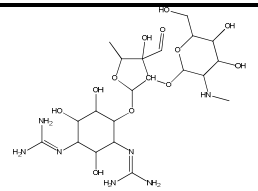
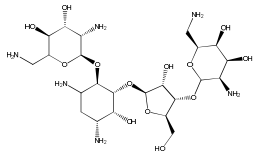
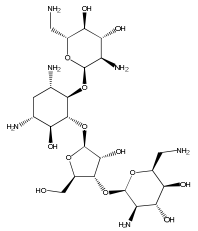
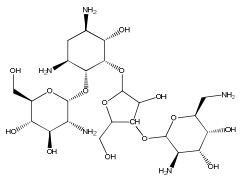
Chemical Structure	Trivial Name		Spectrum of action
	Streptomycin		**Broad-spectrum** **Aerobic Gram-negative bacilli.** Very active, including Enterobacteriaceae (*Escherichia coli*, *Klebsiella*, *Proteus*), *Pseudomonas aeruginosa*, *Acinetobacter*, *Providencia*. Minimally active against *Hemophilus*. **Aerobic Gram-positive:** Staphylococci, Streptococci (eg: Enterococci, Group B streptococci, viridans streptococci) aminoglycosides (are not the preferred agents). **Other.** *Mycobacterium tuberculosis* (amikacin, streptomycin) and *Mycobacterium-avium* intracellulare (amikacin). **Anaerobes:** are not susceptible aminoglycosides to their target site. Aminoglycosides may be used **in combination with cell-wall active drugs** such as beta-lactams and vancomycin for **synergy against Staphylococci and Streptococci.**
	Neomycin	Charged positively at physiologic pH	
	Framycetin	Nephrotoxicity and Ototoxicity associated	
	Paromomycin		

Table 2.12: Continued

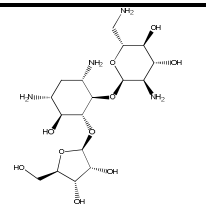
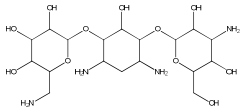
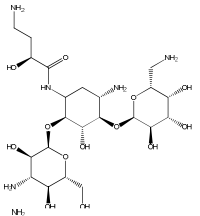
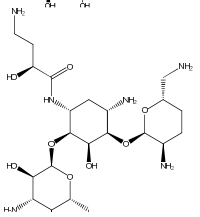
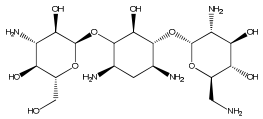
Chemical Structure	Trivial Name		Spectrum of action
	Ribostamycin		
	Kanamycin		**Broad-spectrum**
	Amikacin	Charged positively at physiologic pH Nephrotoxicity and Ototoxicity associated	**Aerobic Gram-negative bacilli.** Very active, including Enterobacteriaceae (*Escherichia coli*, *Klebsiella*, *Proteus*), *Pseudomonas aeruginosa*, *Acinetobacter*, *Providencia*. Minimally active against *Hemophilus*. **Aerobic Gram-positive:** Staphylococci, Streptococci (eg: Enterococci, Group B streptococci, viridans streptococci) aminoglycosides (are not the preferred agents). **Other.** *Mycobacterium tuberculosis* (amikacin, streptomycin) and *Mycobacterium-avium* intracellulare (amikacin). **Anaerobes:** are not susceptible aminoglycosides to their target site. Aminoglycosides may be used **in combination with cell-wall active drugs** such as beta-lactams and vancomycin for **synergy against Staphylococci and Streptococci.**
	Arbekacin		
	Bekanamycin		

Table 2.12: Continued

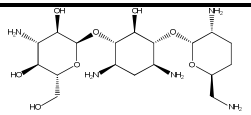
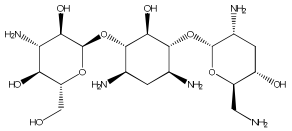
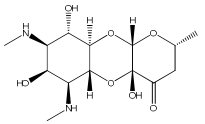
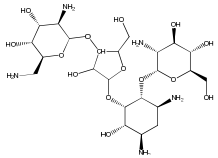
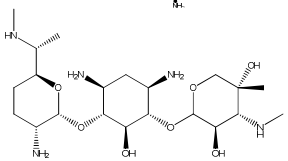
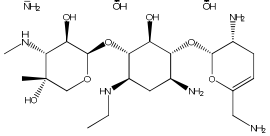
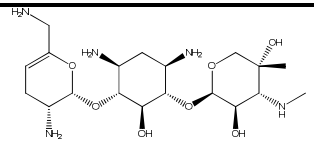
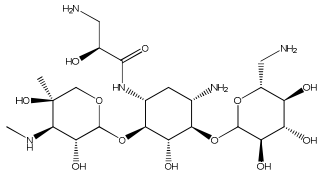
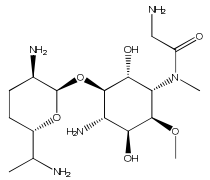
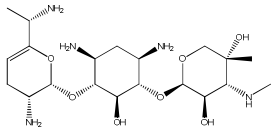
Chemical Structure	Trivial Name		Spectrum of action
	Dibekacin		
	Tobramycin		**Broad-spectrum**
	Spectinomycin	Charged positively at physiologic pH	**Aerobic Gram-negative bacilli.** Very active, including Enterobacteriaceae (*Escherichia coli*, *Klebsiella*, *Proteus*), *Pseudomonas aeruginosa*, *Acinetobacter*, *Providencia*. Minimally active against *Hemophilus*.
	Paromomycin	Nephrotoxicity and Ototoxicity associated	**Aerobic Gram-positive:** Staphylococci, Streptococci (eg: Enterococci, Group B streptococci, viridans streptococci) aminoglycosides (are not the preferred agents).
	Gentamicin		**Other.** *Mycobacterium tuberculosis* (amikacin, streptomycin) and *Mycobacterium-avium* intracellulare (amikacin).
	Netilmicin		**Anaerobes:** are not susceptible aminoglycosides to their target site.
			Aminoglycosides may be used **in combination with cell-wall active drugs** such as beta-lactams and vancomycin for **synergy against Staphylococci and Streptococci.**

Table 2.12: Continued

Chemical Structure	Trivial Name		Spectrum of action
	Sisomicin		**Broad-spectrum**
	Isepamicin	Charged positively at physiologic pH	**Aerobic Gram-negative bacilli.** Very active, including Enterobacteriaceae (*Escherichia coli*, *Klebsiella*, *Proteus*), *Pseudomonas aeruginosa*, *Acinetobacter*, *Providencia*. Minimally active against *Hemophilus*.
	Astromicin	Nephrotoxicity and Ototoxicity associated	**Aerobic Gram-positive:** Staphylococci, Streptococci (eg: Enterococci, Group B streptococci, viridans streptococci) aminoglycosides (are not the preferred agents). **Other.** *Mycobacterium tuberculosis* (amikacin, streptomycin) and *Mycobacterium-avium* intracellulare (amikacin). **Anaerobes:** are not susceptible aminoglycosides to their target site.
	Verdamicin		Aminoglycosides may be used **in combination with cell-wall active drugs** such as beta-lactams and vancomycin for **synergy against Staphylococci and Streptococci.**

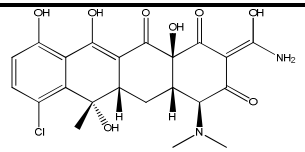
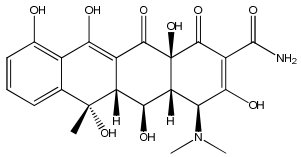
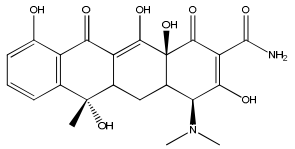
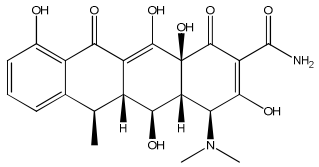
2.2.5 TETRACYCLINES

Tetracyclines (TCY) are formed by four hydrophobic fused rings, diversely substituted, mostly by oxygenated hydrophobic groups (Table 2.13). TCY penetrate Gram-negative outer membrane crossing porins, protein channels through which molecules can diffuse [138]. Concentration in cytosol is dependent on the pH gradient between the outer medium and cytosol and there is no clear explanation, yet, whether this transport is performed by diffusion or energy-dependent mechanisms.

TCY act on the 30S ribosomal subunit (as aminoglycosides), so they are included in the protein synthesis inhibition class of compounds. The acute mechanism is not known, but there is evidence of interference on tRNA binding site [3, 139].

Some TCY (chlortetracycline, tetracycline, doxycycline) exhibit bacteriostatic action, and others exhibit bactericidal action (chelocardin, 6-thiatetracycline). The latest reveal low protein synthesis inhibition [139].

Table 2.13: Chemical structure and Biological Properties for Tetracycline Antibiotics

Chemical Structure	Trivial Name		Spectrum of action
	Chlortetracycline		
	Oxytetracycline	Used as growth promoter, in animal production [139].	**Broad-spectrum** **Gram-negative**: Escherichia coli, Haemophilus influenza, Chlamydia spp., *Escherichia coli*, *Klebsiella pneumoniae*, *Borrelia burgdorferi*, *Borrelia afzelii*, *Borrelia garinii*, *Vibrio cholerae*, *Treponema pallidum*, spp., *Yersinia pestis*
	Tetracycline		**Gram-positive:** staphylococci, propionibacteria, *Bacillus anthracis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Bordetella pertussis*, *Moraxella catarrhalis*
	Doxycycline		

2.2.6 MACROLIDES

Macrolides are 14-, 15- or 16- membered lactone rings substituted by several rings [69, 70]. In the 15- membered rings, an amino acid additional function is present in the lactone ring, and this sub-class is named "azalides" (Table 2.14). The 16- membered macrolides are stable at acidic pH, while 14- and 15- membered analogous reveal some instability.

Macrolides act at bacterial cells binding to the 50S ribosomal sub-unit, reversibly, thus interfering in the protein synthesis. Macrolides block the peptide bond formation or the translocation of peptidyl tRNA. Some studies propose that macrolides also favor the synthesis of incomplete peptides [140]. This class of antibiotics has a mechanism of action similar to that of streptogramins, lincosamines and chloramphenicol [141].

The mode of action of macrolides is essentially bacteriostatic, but at high concentrations, they are bactericidal.

Table 2.14: Chemical Structure and Biological Properties for Macrolide Antibiotics

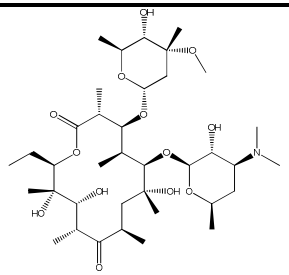
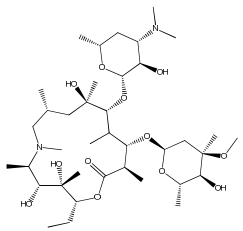
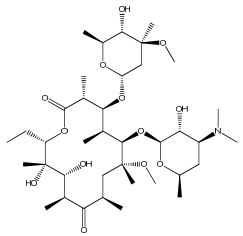
Chemical Structure	Trivial Name		Spectrum of action
	Erythromycin		**Broad-spectrum**
	Azithromycin	Improved action against Gram-negative bacteria, compared with erythromycin [142]	**Gram-positive (aerobic and anaerobic):** *Streptococcus* sp., *Listeria monocytogenes, Mycoplasma pneumoniae, Mycoplasma hominis, Staphylococcus aureus, Propionibacterium acnes, Clostridium perfringens, Peptococcus niger*
	Clarithomycin	Significant activity against Mycobacteria	**Gram-negative (aerobic and anaerobic):** *Haemophilus influenzae, Haemophilus parainfluenzae, Neisseria gonorrheae, Legionella pneumophila, Pasteurella multocida, Helicobacter pylori, Campylobacter jejuni, Chlamydia trachomatis, Chlamydia pneumoniae, Branhamella catarrhalis, Ureaplasma urealyticum*, *Bordetella pertussis, Borrelia burgdorferi, Bacteroides melaninogenicus*

Table 2.14: Continued

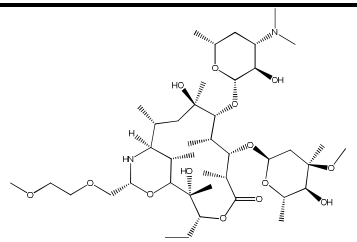
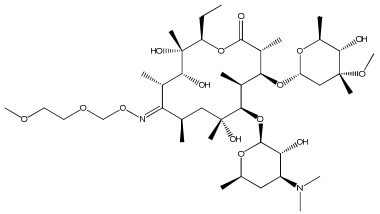
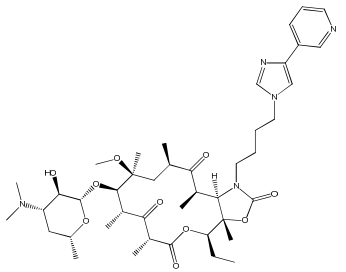
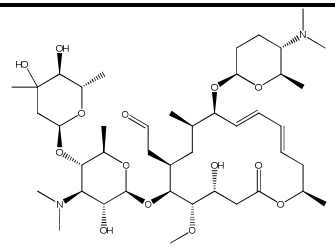
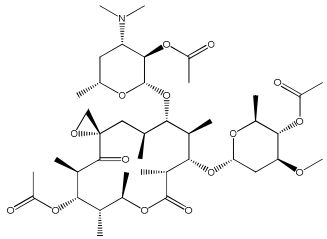
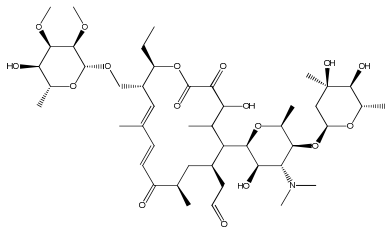
Chemical Structure	Trivial Name		Spectrum of action
	Dirithromycin		**Broad-spectrum**
	Roxithromycin		**Gram-positive (aerobic and anaerobic):** *Streptococcus* sp., *Listeria monocytogenes, Mycoplasma pneumoniae, Mycoplasma hominis, Staphylococcus aureus, Propionibacterium acnes, Clostridium perfringens, Peptococcus niger* **Gram-negative (aerobic and anaerobic):** *Haemophilus influenzae, Haemophilus parainfluenzae, Neisseria gonorrheae, Legionella pneumophila, Pasteurella multocida, Helicobacter pylori, Campylobacter jejuni, Chlamydia trachomatis, Chlamydia pneumoniae, Branhamella catarrhalis, Ureaplasma urealyticum, Bordetella pertussis, Borrelia burgdorferi, Bacteroides melaninogenicus*
	Telithromycin	Approved only for Italy and Turkey	

Table 2.14: Continued

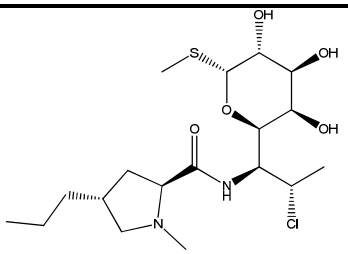
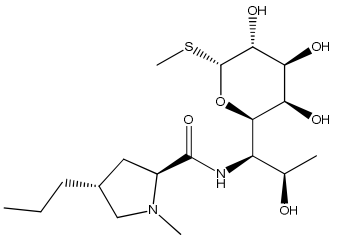
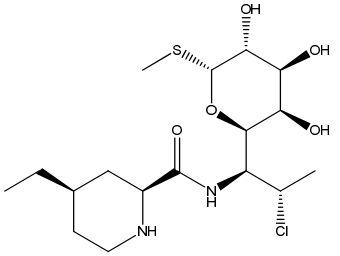
Chemical Structure	Trivial Name		Spectrum of action
	Spiramycin	Approved in Europe, but not in USA FDA	**Broad-spectrum**
	Troleandomycin		**Gram-positive (aerobic and anaerobic):** *Streptococcus* sp., *Listeria monocytogenes, Mycoplasma pneumoniae, Mycoplasma hominis, Staphylococcus aureus, Propionibacterium acnes, Clostridium perfringens, Peptococcus niger* **Gram-negative (aerobic and anaerobic):** *Haemophilus influenzae, Haemophilus parainfluenzae, Neisseria gonorrheae, Legionella pneumophila, Pasteurella multocida, Helicobacter pylori, Campylobacter jejuni, Chlamydia trachomatis, Chlamydia pneumoniae, Branhamella catarrhalis, Ureaplasma urealyticum, Bordetella pertussis, Borrelia burgdorferi, Bacteroides melaninogenicus*
	Tylosin	Used in animals	

2.2.7 LINCOSAMIDES

Lincosamides comprise a propylhygrinic acid linked to an amino sugar (Table 2.15).

Like the macrolide class of antibiotics, lincosamides act at the 50S ribosomal sub-unit of prokaryotic cells, inhibiting protein synthesis. They interfere in the elongation process, binding close to the peptidyl tranferase center, avoiding peptide bonds formation [140].

Table 2.15: Chemical Structure and Biological Properties for Lincosamide Antibiotics

Chemical Structure	Trivial Name	Spectrum of action
	Clindamycin	**Aerobic Gram-positive:** *Staphylococcus* sp. (penicilinase and non-penicilinase producing), *Streptococcus* (exception to *Streptococcus faecalis*), *Pneumococcus* **Anaerobic Gram-negative**: *Bacteroides* spp.; *Fusobacterium* spp. **Anaerobic Gram-positive:** *Propionibacterium*, *Eubacterium*, *Actinomyces* spp. **Gram-positive**: *Peptococcus* spp., *Peptostreptococcus* spp., *Microaerophilic* sp.
	Lincomycin	
	Pirlimycin	

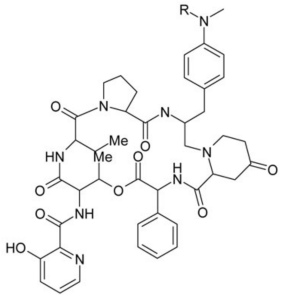
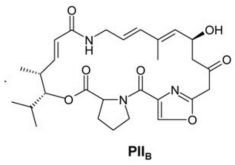
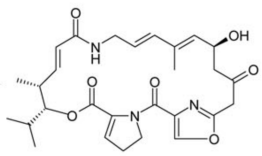
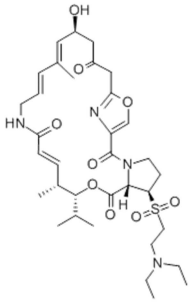
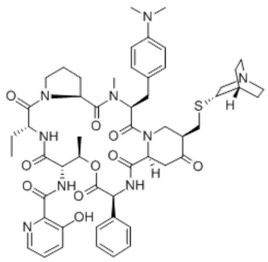
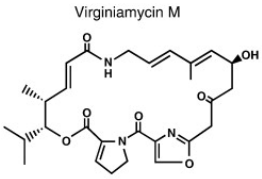
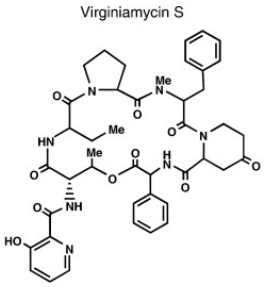
2.2.8 STREPTOGRAMINES

Streptogramine is a class of antibiotics that comprise a pair of synergistic chemical structures: a depsipeptide and a lactone macrocycle (A and B, Table 2.16) [54, 88].

Streptogramines bind to the 30S ribosomal sub-unit, inhibiting protein synthesis (like macrolide and lincosamide classes of antibotics) [140]. However, this compounds exhibit a double mechanism, inhibiting the incorporation of tRNA and the translocation of mRNA.

The two components of the streptogramine antibiotics are, alone, bacteriostatic, but, bactericidal when combined [141].

Table 2.16: Chemical Structure and Biological Properties for Streptogramine Antibiotics

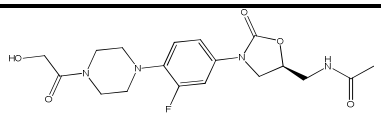
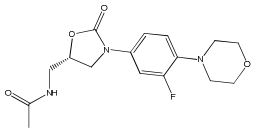
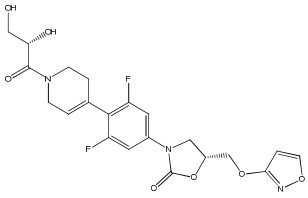
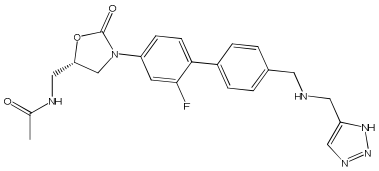
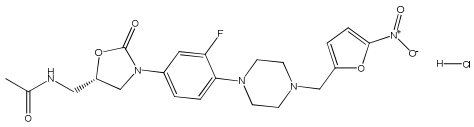
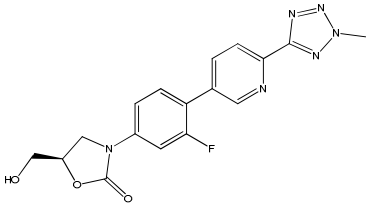
Chemical Structure		Trivial Name	Spectrum of action
A	**B**		
	 	Pritinamycin [143] MDR Gram-positive	**Extended-spectrum MDR Gram-positive:** vancomycin-resistant *Staphylococcus aureus* (VRSA), vancomycin-resistant *Enterococcus* (VRE), vancomycin-resistant *Enterococcus faecium [144]*
		Quinupristin/Dalfopristin [145, 146] MDR Gram-positive	
		Virginiamycin [147]	

2.2.9 OXAZOLIDINONES

Oxazolidinones are heterocyclic organic compounds. The heterocyclic structure is a five membered ring, with oxygen and nitrogen as heteroatoms [148]. Several derivatives have been synthetized and rely on substitutent groups to the five membered ring (Table 2.17) [149].

Oxazolidinones bind to the 50S ribosomal sub-unit and it is thought that they inhibit formation of the 70S ribosome. Binding of oxazolidinones inhibits translocation of the peptide chain, during formation of the peptide bond. This mechanism of action, although similar in location, is different from the protein synthesis inhibitors, since its major action is to binding P site, inhibit initiation complex and translocation of peptidyl-tRNA [150]. This group of compounds exhibit bactericidal mode of action.

Table 2.17: Chemical Structure and Biological Properties for Oxazolidinone Antibiotics

Chemical Structure	Trivial Name	Spectrum of action
	Epetrezolid	
	Linezolid	MDR Gram-positive
	Posizolid	
	Radezolid	**Extended-spectrum MDR Gram-positive** **Limited activity against Gram-negative bacteria**
	Ranbezolid [151]	
	Torezolid	

2.2.10 QUINOLONES

Due to the presence of a carboxylic acid functional group and one or more amine functional groups, quinolones act as amphoteric substances. In the pH range from 6 to 8 (between pKa of –COOH and $–NH_2$), these compounds are lipid-soluble and able to cross membranes [152]. Quinolones are soluble in acid and basic solutions, as well as in polar organic solvents, but are insoluble in non-polar solvents.

Quinolones are classified into generations, but this classification is some kind arbitrary and based, essentially, in the spectrum of application of the drugs [81].

The activity of the quinolone derivatives differs with the substitutions made to the core structure. Ciprofloxacin is preferentially used against Gram-negative infections, whereas Moxifloxacin is more used against Gram-positive bacteria. Norfloxacin is a weak antibacterial drug against Gram-positive organisms and Levofloxacin has intermediate activity [76].

Quinolones cross the bacterial membrane through porines and reach the cytoplasm. The mechanism of action of quinolones is based on the inhibition of DNA replication. During DNA replication, the double-stranded DNA (that is present in every bacteria DNA [153]) needs to unwind into single stranded DNA. This unwinding of DNA in the bacteria is performed by an enzyme named DNA-gyrase or DNA-topoisomerase. DNA-gyrase is a topoisomerase type II enzyme that unwinds the DNA by introducing negative supercoils and can also help relax positive supercoils [154-156]. Four types of topoisomerases were identified in bacterial DNA. Topoisomerases type I and III are not very susceptible to quinolone drugs, whereas topoisomerases type II and IV are extremely affected by these drugs. Both quinolone target topoisomerases are tetrameric structures, divided in four subunits. Topoisomerase II (or DNA-gyrase) has two A monomers, named GyrA, and two B monomers, GyrB. This enzyme is responsible for the negative supercoiling of DNA. The topoisomerase IV also has A and B monomers, encoded by the genes *parC* and *parE* and is involved in the relaxation of supercoiled DNA. In Gram-negative bacteria, quinolones inhibit, preferentially, DNA-gyrase. In Gram-positive

organisms, topoisomerase IV is the preferential target [157]. Quinolones inhibit these enzymes by binding to the A-subunit, preventing DNA replication and protein synthesis.

Quinolones have excellent activity against Gram-negative bacteria and good activity against Gram-positive bacteria and mycobacteria. They have no activity against anaerobic bacteria and streptococci [152]. By inhibiting DNA replication, this class of drugs promotes cell death, thus acting as bactericidal agents.

Table 2.18: Chemical structure and Biological Properties of quinolones classified according to generation – adapted from [76]

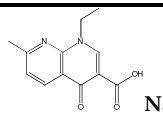
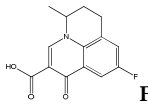
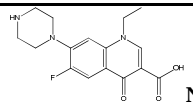
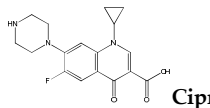
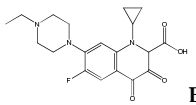
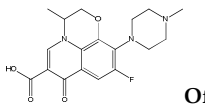
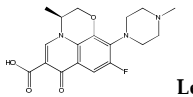
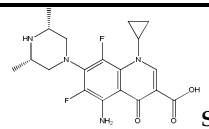
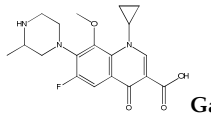
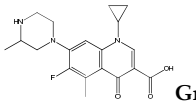
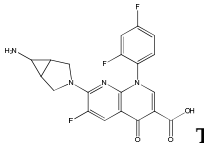
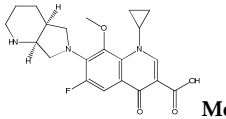
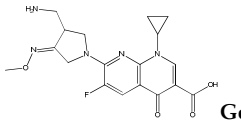
Chemical Structure/Trivial Name	IUPAC name / Molecular Formula/ Molecular Weight (g·mol^{-1})	Gen	Spectrum of Action
 **Nalidixic Acid**	1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid $C_{12}H_{12}N_2O_3$ MW: 232,24	**1st**	**Almost narrow spectrum against Gram-negative strains** (but not Pseudomonas species) **[158, 159]**
 **Flumequine**	9-fluoro-5-methyl-1-oxo-1,5,6,7-tetrahydropyrido[3,2,1-ij]quinoline-2-carboxylic acid $C_{14}H_{12}FNO_3$ MW: 261,25		
 **Norfloxacin**	1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid $C_{16}H_{18}FN_3O_3$ MW: 319,33	**2nd**	**Broad spectra and potent activities against aerobic and anaerobic gram-positive** organisms (including *Staphylococcus aureus* but not *Streptococcus pneumoniae*) **and gram-negative bactéria** (including Pseudomonas species) and some atypical pathogens **[159, 160]**
 **Ciprofloxacin**	1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid $C_{17}H_{18}FN_3O_3$ MW: 331,34		
 **Enrofloxacin**	1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-3,4-dioxo-1,2,3,4-tetrahydroquinoline-2-carboxylic acid $C_{19}H_{22}FN_3O_4$ M W: 375,39		
 **Ofloxacin**	9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-3,7-dihydro-2*H*-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylic acid $C_{18}H_{20}FN_3O_4$ MW: 361,37		
 **Levofloxacin**	(S)-9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-3,7-dihydro-2*H*-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylic acid $C_{18}H_{20}FN_3O_4$ MW: 361,37		

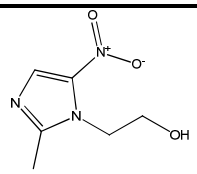
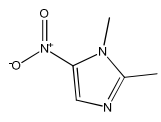
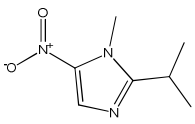
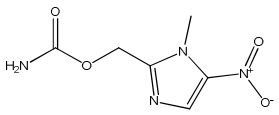
Table 2.17: Continued

Chemical Structure/Trivial Name	IUPAC name / Molecular Formula/ Molecular Weight (g·mol^{-1})	Gen	Spectrum of Action
 **Sparfloxacin**	5-amino-1-cyclopropyl-7-((3R,5S)-3,5-dimethylpiperazin-1-yl)-6,8-difluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid $C_{19}H_{22}F_2N_4O_3$ MW: 392,40		
 **Gatifloxacin**	1-cyclopropyl-6-fluoro-8-methoxy-7-(3-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid $C_{19}H_{22}FN_3O_4$ MW: 375,39	**3rd**	**Expanded activity against gram-positive organisms** (including penicillin-sensitive and penicillin-resistant *S. pneumoniae*) **and atypical pathogens [159]**
 **Grepafloxacin**	1-cyclopropyl-6-fluoro-5-methyl-7-(3-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid $C_{19}H_{22}FN_3O_3$ MW: 359,39		
 **Trovafloxacin**	7-(6-amino-3-azabicyclo[3.1.0]hexan-3-yl)-1-(2,4-difluorophenyl)-6-fluoro-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid $C_{20}H_{15}F_3N_4O_3$ MW: 416,35		
 **Moxifloxacin**	1-cyclopropyl-6-fluoro-7-((4aR,7aR)-hexahydro-1*H*-pyrrolo[3,4-b]pyridin-6(2*H*)-yl)-8-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid $C_{21}H_{24}FN_3O_4$ MW: 401,43	**4th**	**Enhanced spectrum of antimicrobial activity, which includes obligate anaerobes, penicillin-resistant gram-positives, and atypical respiratory pathogens [161]**
 **Gemifloxacin**	(*Z*)-7-(3-(aminomethyl)-4-(methoxyimino)pyrrolidin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid $C_{18}H_{20}FN_5O_4$ MW: 389,38		

2.2.11 5-NITROIMIDAZOLES

5-Nitroimidazoles are heterocyclic organic compounds, insoluble in water. The antimicrobial toxicity of 5-nitroimidazoles is dependent on reduction of its nitro moiety to the nitro anion radical and other compounds, including nitroso and hydroxylamine derivatives. They are administrated as pro-drugs and must be activated by 1-electron reduction of the nitro group [162]. The reduction products cause damage to macromolecules, and have been shown to cause DNA degradation and strand breakage, acting as bacteriostatic [163]. The selective toxicity of this class of compounds for anaerobic bacteria and protozoa is due to the redox potential of the components of their electron transport chain [164]. Mutagenic and carcinogenic activity is associated to 5-nitroimidazoles, so treatment must be supervised [165].

Table 2.19: Chemical structures and Biological Properties of 5-Nitroimidazoles

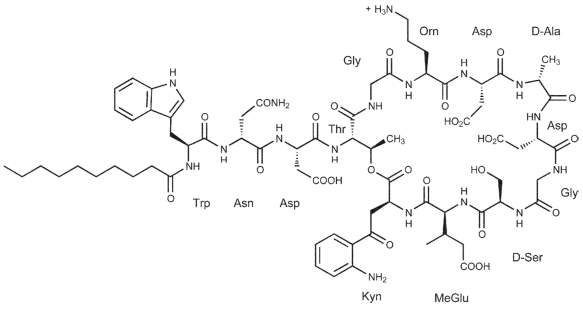
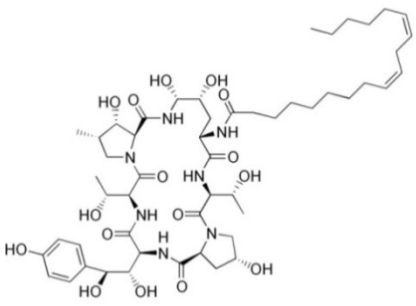
Chemical Structure	Trivial Name	IUPAC name / Molecular Formula/ Molecular Weight ($g \cdot mol^{-1}$)	Spectrum of Action
	Metronidazole	2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol $C_6H_9N_3O_3$ MW: 171,15	
	Dimetridazole	1,2-dimethyl-5-nitro-1*H*-imidazole $C_5H_7N_3O_2$ MW: 141,13	Active against **anaerobic bacteria:** *Helicobacter pylori*, *Gardnerella vaginalis* Exhibit activity against **other microbial organism:** *Entamoeba hystolitica*, *Giardia lamblia*, *Trichomonas vaginalis*.
	Ipronidazole	2-isopropyl-1-methyl-5-nitro-1*H*-imidazole $C_7H_{11}N_3O_2$ MW: 169,18	Used essentially in veterinary medicine, as anti-fungal and anti-protozoa [166]l
	Ronidazole	(1-methyl-5-nitro-1*H*-imidazol-2-yl)methyl carbamate $C_6H_8N_4O_4$ MW: 200,15	

2.2.12 LIPOPEPTIDES

Lipopeptides consist of a loop of amino acids and a hydrophilic fatty acid chain. They present surfactant properties and so are capable of penetrating and disrupting the cell membranes of all types of bacteria [54].

Equinocandine is a lipopeptide synthetically modified and presents anti-fungal activity. It inhibits the synthesis of glucan in the cell wall [167].

Table 2.20: Chemical structures and Biological Properties of some Lipopeptides

Chemical Structure	Trivial Name	Spectrum of Action
	Surfactin	**Gram-positive and Gram-negative**
	Daptomycin [168]	**MDR Gram-positive**, among them glycopeptide-resistant enterococci, methicillin-resistant staphylococci, streptococci, corynebacteria [169].
	Echinocandin	**Anti-fungal** activity, especially against *Candida* spp. [170, 171]

2.3 ANTIBIOTIC RESISTANCE: A PUBLIC HEALTH CONCERNING

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Bacterial Resistance

Ricardo Ferraz, Cristina Prudêncio, Mónica Vieira, Ruben Fernandes, João Paulo Noronha, Zeljko Petrovski,:

Antimicrobial resistance (AMR) is the resistance of a microorganism to a substance [13]. This resistance may be natural or acquired [3]. Resistant organisms can be bacteria, fungi, viruses and even some parasites. These microorganisms become able to avoid the attack of antimicrobials, which makes these drugs ineffective and infections become persistent and the risk of spread is increased. The development of resistant strains may occur when a given strain is exposed to an antibiotic and may be accelerated by inappropriate use of this kind of drugs and the poor infection control. These defense mechanisms may be transferred between certain types of bacteria, which lead to an increase in AMR. Although the development of AMR is a natural phenomenon, certain human actions actually accelerate the emergence and spread of them.

Shortly after its discovery, in the late 1920s, the antibiotics have been used successfully in the fight against various bacterial infections, but soon the first resistant bacterial strains to the drugs used appeared [172]. Whenever an antibiotic was discovered and available to the population, resistance to that drug emerged. The first resistance to penicillin was found only a few months later its usage, early in 1945 [173, 174], but, to vancomycin, it took about 30 years to appear, in 1987 [3]. This fact may be related to the limited use of vancomycin, but widespread use of penicillin.

Some bacterial species are intrinsically resistant to some antibiotics. For example, Mycobacteria are resistant to β-lactams and glycopeptides, because these classes of antibiotics act on the peptidoglycan synthesis and Mycobacteria present very low permeability to antibiotics, mostly due to an unusual cell wall structure, thicker than in many other bacteria, which is hydrophobic, and rich in mycolic acids/mycolates [175]. A microorganism is intrinsically resistant to an antibiotic if it does not possess the antibiotic target in its structure or if it is impermeable to the drug. This behavior is usually termed as not susceptible instead of resistant.

Resistance may, however, be acquired. The introduction of antibiotics, through human and veterinary medicine, and in animal production on a large scale, has been followed by the emergence of resistant strains of bacteria, primarily susceptible to them [176]. The rate of development and spread of resistance is unpredictable and the increase of acquisition of resistance has decreased enormously the appliance of important antibiotics but, on the other hand, is a stimulus to the development and discovery of new effective drugs. There is no clear explanation for the large differences observed in the rate and extent of acquisition of resistance among different species of bacteria. Even when exposure to a particular selective pressure is similar, results can be very different, as is the case of *Staphylococcus aureus*, with about 90% of ampicillin resistant strains, whereas only 40-50% of the strains of *Escherichia coli* are resistant to this antibiotic [3]. However, some studies indicate that the removal of certain antibiotics in clinical practice may lead to lowering of resistant strains [177]. On the other hand, the introduction of new antibiotics changes the selection of prevalent resistant strains, as happened in the 1960s, with the introduction of cephalosporins and penicillins β-lactamase stable to solve the problem of staphylococcal infections. This practice led to the development of resistance among Gram-negative bacteria, which have become the major pathogens in hospitals [3].

There are two main types of acquired resistance: the mutational and the transmissible resistance [178]. The mutational resistance is present in any bacterial

population, where there are always cells that become resistant spontaneously. These cells appear with a frequency of 10^{-9} in each bacterial generation and therefore do not pose a great danger on the spreading of resistance during antibiotic therapy, but many antimicrobial agents select this type of resistance and care as to be taken in long-term therapy. The transmissible resistance occurs when genes conferring resistance are transferred from a resistant bacterial cell to a susceptible one or hereditarily. The simultaneous transfer of resistance to several unrelated antimicrobial agents has already been demonstrated [3, 178]. This mechanism is much more effective in the dispersion of resistance through bacteria formerly susceptible, then the mechanism of mutation of individual cells. The resistance spread is enhanced in the presence of selective pressure caused by a given antibiotic.

Concerns about antibiotic resistance have appeared in regular time intervals, since the introduction of antimicrobial chemotherapy, but nowadays, this question is more relevant than ever [179]. There are even suggestions that the resistance to antibiotics is now so common that there is a risk of turning the situation prevailing in the pre-antibiotic era. Indeed, the situation in which we live is disturbing, but it is not hopeless, since it is still possible to treat the infections that appear most, however, this may indicate that drugs that are effective yet may be more toxic and less effective for those bacteria with acquired resistance [3]. Currently, it is known that the intensive and extensive use of antimicrobials in human medicine, veterinary medicine and even in animal production leads to increased bacterial resistance worldwide [180, 181].

In human medicine, the vast amount of prescribed antibiotics is a major factor of increasing resistant bacteria. The fact that they are not always prescribed properly [182] or that patients do not finish the course of treatment are also important points [183]. There is also the extremely important problem of resistant and multidrug resistant bacterial prevalence in hospital environment [184]. Antibiotics are used in animals, as well. Sometimes, the drug used is exactly the same, and there are cases where the antibiotic used in veterinary or animal production is a substance not used in humans.

The main concern about antibiotic use in animals as been the residues of these compounds in animal origin products, rather than the development of bacterial resistance, but there are studies pointing to transference of resistant bacteria from animals to humans [185, 186].

There is increasing data demonstrating the selective ability of low concentrations of antibiotics to strains with low and high level of resistance [2, 187]. The existence of resistant bacteria in animals from environments relatively intact is an indicator and may be explained by the presence of sub-inhibitory levels of antibiotic [188, 189]. Exposure to sub-inhibitory levels can cause, in bacteria, changes in pathogenicity, resistance [190, 191]. All these data suggest that the presence of sub-inhibitory concentrations of antibiotics may have an important role in the maintenance and proliferation of resistant strains [180].

The concentration of antibiotics in natural environments is obviously associated with the use of such drugs. These concentrations vary significantly in the environment: close to the pharmaceutical and hospital effluent may reach levels of the order of mg/mL [192-194], in aquatic environments and soil, these concentrations are significantly lower [195]. Residues of antibiotics were also found in foods as well as bacteria resistant to several antibiotics, including those used in animal growth promotion [196-199].

AMR is a multifactorial problem, with respect to human and veterinary medicine, environment and society. To solve this problem, we must, first, accept the natural evolution of bacteria that can adapt to all kinds of environments [180], and try to avoid the development of resistance, or fight them with a more rational use of existing antimicrobial agents and develop new effective compounds.

Recent outbreaks, like the one found in Germany for *E. coli* O104 as well as the emergence of multi-drug resistant organisms, such as gram-negative *Enterobacteriaceae* associated to the New Delhi metallo β-lactamase evidence this problem, that not only

has public health implications, but also at an economic and social level, threatening global safety. The latest studies reported also the significant financial burden on health care-associated infections (HAIs) in the USA. In the UK, approximately 9% of hospitalized patients acquire an infection after post-admission to hospital which increases the budget in the health care system [179]. This could lead to the use of non-antibiotics compounds that have antimicrobial properties. These molecules could act through a new mechanism, or may interfere with the developed resistance mechanism, and could revert the resistance phenotype, previously presented. In the last case, for example, the mechanism of action may be: an alteration of membrane permeability to antibiotics, inhibition of efflux pumps, or the inhibition of β-lactamases, when the resistance mechanism is present. In this context, it is important to know and study the resistance mechanisms, and also study non-antibiotics compounds as antimicrobial agents [179].

2.4 QUINOXALINE: THE LIGHT AT THE TUNNEL?

In search of alternatives to antimicrobial agents currently used, in order to respond to the landscape of loss of efficacy due to the emergence of resistance, several research groups have evaluated several families of compounds, among them the quinoxaline.

Mostly in the last two decades, several quinoxaline derivatives have been tested and presented antimicrobial activity, as antifungal [200-202] and antibacterial agents [200-210]. The antibacterial activity observed covers Gram-negative and Gram-positive bacteria [204, 206, 209, 210], including *Mycobacterium* [202, 205, 207, 208, 211, 212]. There are also data pointing to activity against multidrug resistant *Mycobacterium tuberculosis* [212]. There are quinoxaline derivatives that show antiprotozoan activity

[213, 214], especially antiamoebic [215-217] and broadening the range of the antimicrobial activity of this heterocyclic family of compounds.

Quinoxaline derivatives present other biological properties, as anticancer [218-220], antioxidant and anti-inflammatory [221, 222].

Quinoxaline is a heterocyclic organic compound that can be also named benzopyrazine or 1,4-diazanaphthalene. It is formed by a benzene ring condensed with a pyrazine. This structure is present in some antibiotics used nowadays, like Echinomycin, Levomycin and Actinoleutin [223]. When an oxygen atom is attached to each nitrogen atom in the heterocyclic structure, the bioreducing potency of these compounds is enhanced, leading to more powerful antimicrobial and antitumor activity [207, 224].

Quinoxalines, beyond its biological properties, are used in other types of applications, such as inhibitors of metal corrosion [225-227] and in colorimetric methods for metal detection [228], acting as selective and sensitive agents.

To frame the evaluation of biological activity of quinoxaline derivatives, it was necessary to make a survey of existing studies involving this family of compounds. The aim of this State of the Art review was to survey the main biological characteristics of quinoxaline.

2.5 QUINOXALINE: A STATE OF THE ART REVIEW

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Quinoxaline, its derivatives and biological applications: A state of the art review

Joana A. Pereira, Rúben Fernandes, M. Natália D.S. Cordeiro, Ana M. Pessoa, Cristina Prudêncio, João Paulo Noronha, Mónica Vieira

2.5.1 ABSTRACT

Quinoxaline derivatives are an important class of heterocycles compounds, where N replaces some carbon atoms in the ring. Quinoxaline molecular formula is $C_8H_6N_2$, formed by two aromatic rings, benzene and pyrazine. It is rare in natural state, but their synthesis is easy to perform. Modifying its structure is possible to obtain a wide variety of antimicrobial activities such as antibacterial, anti-tubercular, antiviral, antifungal, anti protozoan activity (amoebic, malarial and leishmanial), anti-diabetic, anti-atherogenic, anti-inflammatory, anticancer, anti-glaucoma, anti-depressant presenting AMPA receptor antagonism. The large scope of biomedical application results from modifications of its structure, whose analysis provides data for future new biomedical applications. They are also important in industry due to, for example, Cu^{2+} detection in colorimetric sensors.

In this review the State of the Art will be presented, which includes a summary of the progress made over the past years in the knowledge of the structure and mechanism of the quinoxaline and quinoxaline derivatives, associated medical and biomedical value, and in the efforts made towards the development of new quinoxaline derivatives with medical applications, as well as their industrial value in fields that can be of biomedical interest, such as Cu^{2+} detection in colorimetric sensors.

Keywords: Quinoxaline, Quinoxaline derivatives, biological activity, therapeutic application, biomedical applications.

2.5.2 INTRODUCTION

Quinoxaline derivatives are an important class of heterocycles compounds, in which N replaces one or more carbons atoms of the ring [229], and the approved number for the quinoxaline ring system is shown in Figure 2.16, where 2 and 3 are designated α-positions [230]. They are important in industry due to their power to inhibit the metal corrosion [225, 231, 232], in the preparation of the porphyrins, since their structure is similar to the chromophores in the natural system, and are also usefull in the electroluminescent materials [233-235]. In pharmacological industry they are considered promising molecules since they show wide biological properties [202, 214, 229, 236, 237]. For exemple, they can be usded against bacteria, fungi, virus, leishmania, tuberculosis, malaria, cancer, depression, and neurological activities, among others. All these activities are possible due to the quinoxaline structure since its nucleus, in numerous cases, acts as a precursor to assembly a large number of quinoxaline derivatives, which consequently, provide a large number of new compounds for diverse applications [229].

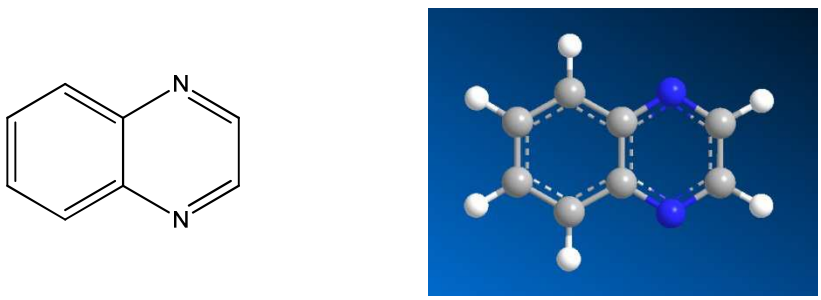


Figure 2.16: Quinoxaline compound

Quinoxaline is formed by two aromatic rings, benzene and pyrazine. For this reason is also called benzopyrazine, and is described as a bioisoster of quinoline, naphatelene and benzothiophene [238]. The atoms S and N play an important role in the ring since they stabilize ion radical species and extended π-conjugation facilitate in decreasing coulombic repulsion [239]. Molecular weight of the quinoxaline is 130.1466400, with a molecular formula of $C_8H_6N_2$, and it is a white crystalline powder [229].

Chemically, quinoxaline has a low melting point (27-34 ºC), is soluble in water, and produces monoquaternary salts when treated with quaternizing agents, like methyl sulfate and methyl *p*-toluenesulfate [230, 238]. The quaternary salts of 2-alkylquinoxalines are unstable and can be converted into complex colored products when under oxidation [230]. It is weakly basic with a pKa of 0.60 in water at 20 ºC, and nitration occurs only under forcing conditions (Conc. HNO_3, Oleum, 90 ºC), resulting in the formation of two compounds: 5-nitroquinoxaline (1.5%) and 5,7-dinitroquinoxaline (24%) [238]. Its second pKa is - 5.52, meaning that quinoxaline is significantly diprotonated only in a strongly acidic medium [230].

Quinoxaline has a dipole moment of 0.51 Debey in benzene ring, and their first and second ionization potentials, measured by photonelectron spectroscopy, are 8.99 and 10.72 eV, respectively [230]. However it is not known certainly from which orbital the first electron is lost, since highest-occupied π-orbital and non-bonding orbitals are very close in energy. The heat of atomization was calculated to be 79.739 eV by a self-consistent field molecular orbital treatment [230]. Molecular orbital calculations of the π-electron density were made, and shown that the highest electron density at the carbon ring is at positions C-5 and C-8, followed by positions C-6 and C-7, and the lowest at C-2 and C-3 positions [230].

Table 2.21: Quinoxaline Physical and Chemical Properties

Quinoxaline Properties

Structural Formula	$C_8H_6N_2$
Molecular Weight/g·mol^{-1}	130.15
Acidity (*pKa*)	0.56
Second *pKa*	-5.52
Melting Temperature	29-30 ºC
Natural State	White crystaline powder
Dipole Moment	0.51 Debey
Ionization (1st/2nd)	8.99/10.72 eV
Heat of atomization	70.74eV

Most of quinoxaline compounds are rare in natural state, being of synthetic origin. The method used to synthesized quinoxalines is to condense *o*-disubstituted benzene with a two carbon synthon. Therefore, the condensation of *o*-phenylenediamine with α-dicarbonyl compounds result in quinoxaline formation (Figure 2.17**)** [230].

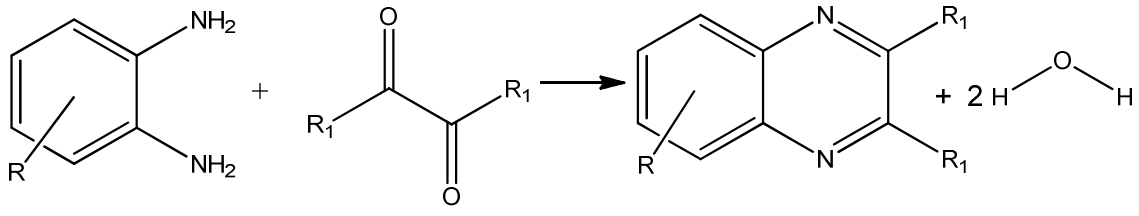


Figure 2.17: Quinoxaline synthesis representation.

Quinoxaline and their derivatives could be converted in both mono and di-*N*-oxides by oxidation with peracids [230].

2.5.3 BIOLOGICAL ACTIVITY

The study of quinoxaline, and its derivatives, has become a subject of interest in recent years, due to their wide variety in biological activity and also their therapeutic applications. Although rare in nature, synthesized quinoxaline and derivatives are included in various antibiotics such as echinomycin, levomycin and actinomycin, which are known to inhibit the growth of Gram-positive bacteria and also active against transplant tumors [204, 238]. Echinomycin, for example have quinoxaline compound in their structure, and also some recognized drugs, like Brimonidins, alleviates glaucoma symptoms [229].

The vast scope of synthesized quinoxaline and derivatives potentials is well referenced and published in a wide range of scientific journals. We analyze in detail, transversally and in context the relevant scientific data pertaining great quinoxaline and potentials derivatives in literature.

2.5.3.1 ANTIMICROBIAL ACTIVITY

2.5.3.1.1 ANTIBACTERIAL ACTIVITY

The antimicrobial-resistance is a serious threat to global public health, result of

the widely disseminated and careless use of antimicrobials, and demands a continuous effort in order to seek for better antimicrobial agents effective against resistant pathogenic microorganisms [238, 240-242]. There are a wide range of quinoxaline derivatives with antimicrobial activity documented.

A new series of 8-chloro-1,4-substituted-[1,2,4]-triazole[4,3-*a*] quinoxaline derivatives (Figure 2.18) (Table 2.22) was synthesized and screened for antimicrobial and antioxidant activities [243]. The antibacterial activity was screened against Gram-positive *Staphylococcus aureus* and *Bacillus subtilis*, and Gram-negative *Proteus vulgaris* and *Klebsiella pneumoniae*, using chloramphenicol as reference drugs [241]

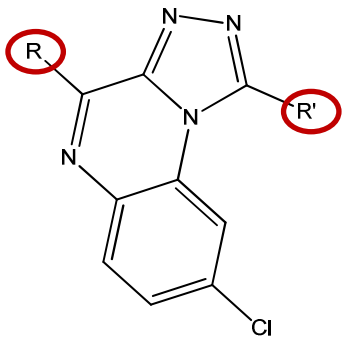


Figure 2.18: 8-chloro-1,4-substituted[1,2,4]triazolo[4,3-*a*] quinoxaline derivatives core structure.

Ammar *et al.* [244], have synthesized thieno[2,3-*d*]pyrimidines and pyrrolo[3,4-*b*]quinoxalines which antibacterial activity were tested against *S. aureus* and *E. coli*.

Also, an in vitro fluorescence polarization assay demonstrated that a library of quinoxaline derivatives, prepared to target non-structural protein 1 of influenza A (NS1A), disrupted the dsRNA-NS1A interaction to varying extents, which lead to the development of anti-influenza drugs [245].

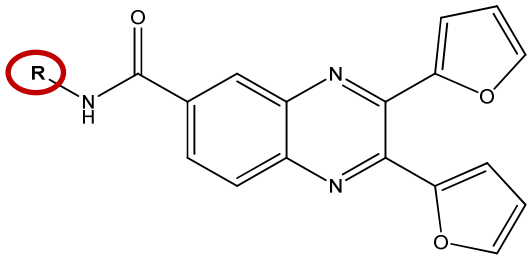


Figure 2.19: 2,3-difuryl-4-quinoxaline-R-metilcarboxamide derivatives

In such study, researchers have prepared a library based on 2,3-difuryl-4-quinoxaline-**R**-metilcarboxamide derivatives (Figure 2.19**)**, with 2-furyl groups at position 2 and 3, and phenyl group in position 6 through an amide linker. Among all the compounds in the library, those listed in Table 2.22 have shown the highest effectiveness. These compounds do not inhibit NS1A-dsRNA interactions by interfering with dsRNA but by the binding to NS1A dsRNA-binding domain itself. Also the compound 2 was able to inhibit influenza A virus growth [245].

Table 2.22: Published experimental data (percentage of Binding, Intercalation, IC_{50} and IG_{50}) of quinoxaline derivatives and their substituents

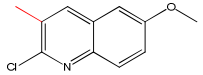
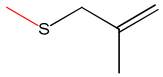
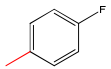
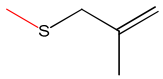
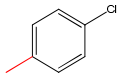
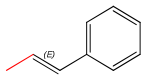
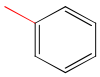
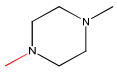
Main Compound	Compound	R	R′	R′′	% Binding at 50 μM	% Intercalation	IC_{50} μM	IG_{50} μM	Reference
8-chloro-1,4-substituted-[1,2,4]-triazolo[4,3-*a*]-quinoxaline derivatives	1			-	-	-	-	-	[241]
	2			-	-	-	-	-	
	3			-	-	-	-	-	
	4			-	-	-	-	-	
	5			-	-	-	-	-	
	6			-	-	-	-	-	
	7			-	-	-	-	-	

Table 2.2: Continued

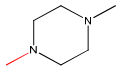
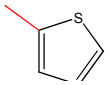
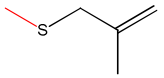
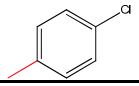
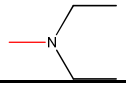
Main Compound	Compound	R	R′	R″	% Binding at 50 μM	% Intercalation	IC_{50} μM	IG_{50} μM	Reference
8-chloro-1,4-substituted[1,2,4]-triazolo-[4,3-*a*]-quinoxaline derivatives	8	—CH_3		-	-	-	-	-	[241]
	9			-	-	-	-	-	
	10			-	-	-	-	-	
2,3-difuryl-4-quinoxaline-R-metilcarboxamide derivatives	1	3-OMe-Ph-	-	_	74.0	4.5	6.2	_	[245]
	2	2-Furyl-	-	_	79.5	5.9	3.5	_	
1-(thiazolo-[4,5-*b*]cquinoxaline-2-yl)-3-phenyl-2-pyrazolines derivatives	1	—H	—H	-	-	-	6.76	-	[246]
	2	—Br	—H	-	-	-	4.98	-	

Table 2.2: Continued

	3	—Cl	—H	-	-	-	1.09	-
1-(thiazolo-[4,5-*b*]cquinoxaline-2-yl)-3-phenyl-2-pyrazolines derivatives	4	—H	—CH_3	-	-	-	2.34	-
	5	—Br	—CH_3	-	-	-	1.45	-
	6	—Cl	—CH_3	-	-	-	0.72	-
	Metronidazole	-	-	-	-	-	1.69	-

Table 2.2: Continued

Main Compound	Compound	R	R′	R″	% Binding at 50 μM	% Intercalation	IC_{50} μM	IG_{50} μM	Reference
2-alkylcarbonyl and 2-benzoyl-3-trifluromethylquinoxaline-1,4-di-*N*-oxide derivatives	1	—H	—H	-	-	-	-	1.02	[236]
	2	—Cl	—Cl	-	-	-	-	0.42	
	3	—F	—F	-	-	-	-	0.52	
	4	—F	—F	-	-	-	-	0.15	
	5	—H	—H	-	-	-	-	0.49	
6-arylamino-2,3-bis(pyridin-2-yl)-7-chloro-quinoxaline~-5,8-diones	1	—H	—Cl	H	-	-	1.5	-	[247]
	2	—H	—OH	H	-	-	5.5	-	
	3	—H	—F	H	-	-	1.0	-	
	4	—H	—CF_3	H	-	-	1.1	-	
	5	—H	—OCF_3	H	-	-	1.0	-	
	6	—H	—OCH_3	H	-	-	3.5	-	
	7	—H	—H	H	-	-	3.1	-	
	8	—Cl	—Cl	H	-	-	1.0	-	
	9	—F	—F	F	-	-	1.2	-	
	10	-	-	-	-	-	>100	-	
	MPA	-	-	-	-	-	1,0	-	

2.5.3.1.2 ANTITUBERCULAR ACTIVITY

Tuberculosis (TB) is a contagious disease, caused by the infection of *Mycobacterium tuberculosis*, which have a high rate of mortality in the world. About 3 million people die every year from TB, and 8 million new cases estimated each year, which 95% of them occur in developing countries [248-250].

The therapy used in these days to fight TB consists in the administration of one of three drugs (isoniazid, rifampin or pyrazinamide) for 2 months, followed by 4 months of follow-up therapy with isoniazid and rifampin. However, the arising of multidrug resistant (MDR) TB it is required the development of new therapeutic agents, with a unique mechanism of action, able to treat MDR forms of the disease.

Several studies has been described, concerning synthesis and biological activity of a large amount of quinoxalines and 1,4-di-*N*-oxide quinoxaline derivatives, where compounds such as 7-chloro-3-(*p*-substituted)-phenylaminoquinoxaline-2-carbonitrile-1,4-di-*N*-oxide, 6,7-dichloro-2-ethoxycarbonyl-3-methylquinoxaline-1,4-di-*N*-oxide and 3-acetamide-6,7-dichloroquinoxaline-2-carbonitrile-1,4-di-*N*-oxide derivatives have been shown to inhibit *M. tuberculosis* to a rate of 99 to 100% [207, 241]. However, it is observed that the lack of the two *N*-oxide groups lead to the loss of the antimycobacterial activity [207, 251, 252]. Some novel condensed bridgehead nitrogen heterocycles of quinoxalines have been synthesized and showed activity against *M. tuberculosis* H_{37}Rv species [222, 238, 241]. The compounds 3-methyl-2-phenylthioquinoxaline-1,4-dioxides generally showed a good activity against *M. tuberculosis* in the preliminary in vitro evaluation and exhibited Minimum Inhibitory Concentration (MIC) between 0.39 and 0.78 μg/mL (rifampicin MIC = 0.25 μg/mL) [202].

It was also reported the activity of 3-methyl-9-substituted-6-oxo-6,9-dihydro-

3*H*-[1,2,3]-triazolo-[4,5-*h*]-quinolonecarboxylic acids and their esters of as a new class of anti-infective agents against multidrug resistance (MDR) *M. tuberculosis*, with no cytotoxicity reported [212].

2.5.3.1.3 ANTIVIRAL ACTIVITY

Viruses such as Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) belong to the *Herpesviridae* family, are double-stranded DNA [253], and share high homology in genome structure and DNA sequence. These viruses can cause various illness states from asymptomatic infection to fulminant disseminated diseases, including labial herpes, keratitis, genital herpes, and encephalitis [254, 255].

There are a wide number of drugs for treatment of HSV infections like acyclovir, ganciclovir, penciclovir, valaciclovir (converted to acyclovir) and famciclovir (converted to penciclovir) [253, 256], being acyclovir the most common drug used. However, there are drug-resistant strains of HSV emerging and increasing [253, 257], leading to the search of new antiviral drugs.

Quinoxalines have a variable antiviral activity, suggesting that their activity depends on specific substitution patterns. Novel series of al 6*H*-indolo[2,3-*b*]-quinoxalines were synthesized and evaluated for antiherpes virus activity and the compound 2,3-dimethyl-(dimethylaminoethyl)-5*H*-indolo[2,3-*b*]-quinoxaline had the major antiviral activity. This specific compound was tested for its antiviral effect and action mechanism, showing the capacity to inhibit replication of herpes simplex virus type 1, cytomegalovirus, and varicella-zoster virus in tissue culture, in concentrations of 1 to 5 μM, depending on the virus amount and cell type used in the assay. Also the compound 2,3-dimethyl-6-(dimethylaminoethyl)-6*H*-indolo[2,3-*b*]-quinoxaline (Figure 2.20**)** have showed high activity against herpes virus, and derivatives with 6-(2-

dimethylaminoethyl) side chain, due to their DNA binding properties, showed an improved biological activity [229].

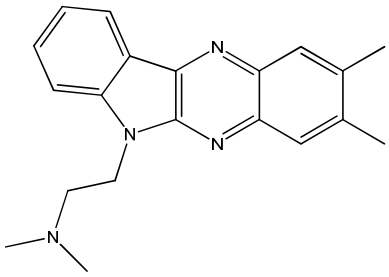


Figure 2.20: 2,3-dimethyl-6-(dimethylaminoethyl)-6*H*-indolo[2,3-*b*]-quinoxaline

There is also reference to IndQloquinoxalines (2,3-dimethyl-6-(dimethylaminoethyl)-6*H*-indolo[2,3-*b*]-quinoxaline) with capacity to inactivate virions in high concentrations (around 300 μM), and decrease the synthesis of viral DNA and protein at lower concentrations (around 3 μM) [241].

Concerning human immunodeficiency virus type 1 (HIV-1), which is the agent causative of acquired immunodeficiency syndrome (AIDS) [258-260], there are a wide number of clinical drugs used to fight the disease, such as non-nucleoside reverse transcriptase (RT) inhibitors, which interact with a specific allosteric non-substrate binding site on HIV-1 RT [238]. Compound 6-chloro-3,3-dimethyl-4-(isopropenyloxycarbonyl)-3,4-dihydroquinoxalin-2(1*H*)-thione (Figure 2.21) was synthesized and evaluated for enzyme activity, and was found to be a very potent inhibitor for both HIV-1 RT activity and HIV-1 replication in tissue cultures. Although, like some other non-nucleoside RT inhibitors, this compound was not effective against HIV-2 RT [241].

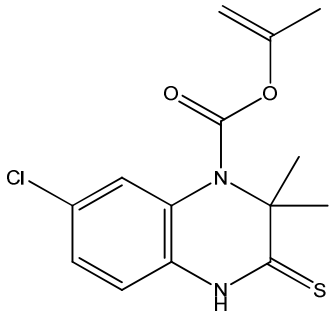


Figure 2.21: 6-chloro-3,3-dimethyl-4-(isopropenyloxycarbonyl)-3,4-dihydroquinoxalin-2(1*H*)-thione.

2.5.3.1.4 ANTIFUNGAL ACTIVITY

Prevalence of fungal diseases has increased significantly in the past 50 years. Fungal diseases manifest themselves differently, including mycoses in the skin, hair, nails, but also as systemic mycoses, being the last one an issue of great medical concern due to the increase in the immunocompromised patient population [261].

One of the most common fungal infections is candidiasis, caused by *Candida albicans*, a diploid fungus that grows both as yeast and filamentous cells [262, 263]. This fungus can also develop resistance to antimycotic drugs that already exist in the market [264], being important a constant search for new drugs and treatments.

Thieno[2,3-*d*]-pyrimidines and pyrrolo[3,4-*b*]-quinoxalines were synthesized, have antifungal activity, and were tested against *C. albicans* [238, 244].

Researchers also reported some 2-sulphonylquinoxalines and 3-[(alkylthio)methyl]quinoxaline-1-oxide derivatives as compounds with high antifungal activity [202], and also pyrazoloquinoxalines which were observed to be active against fungal infections [229].

2.5.3.1.5 ANTIPROTOZOAN ACTIVITY

2.5.3.1.5.1 ANTIAMOEBIC ACTIVITY

Entamoeba histolytica is a protozoan responsible for the amoebiasis infection [265, 266], causing amoebic colitis, brain and liver abscess, being the second leading cause of death worldwide. The traditional treatment used is based in antiamoebic compounds such as nitroimidazoles, but not always effective, raising the possibility of drug resistance, leading to the search of new compounds able to fight the infection successfully [246].

Some 1-(thiazole[4,5-*b*]-quinoxaline-2-yl)-3-phenyl-2-pyrazolines derivatives produced (Figure 2.22), were found to be a potent inhibitor of HM1:IMSS strain of *E. histolytica*, where the presence of 3-bromo or 3-chloro substituents on the phenyl ring and 4-methyl group on the pyrazoline ring affected antiamoebic activity to a great extent [246].

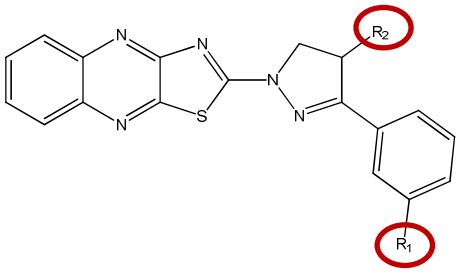


Figure 2.22: 1-(thiazole[4,5-*b*]quinoxaline-2-yl)-3-phenyl-2-pyrazolines core.

In such study metronidazole was used as the reference drug and had a 50% inhibitory concentration of IC_{50} 1.69–1.82 μM, and compound 6 (Table 2.22) showed great effectiveness, being the most active [246].

2.5.3.1.5.2 ANTIPARASITIC ACTIVITY

Leishmaniasis is a parasitic disease cause by protozoan of the genus *Leishmania* in tropical and subtropical areas of the World, and despite all efforts to fight this disease about 1-2 million new cases are registered every year [241, 267]. Most of the drugs available against leishmaniasis are expensive and require a long treatment and are becoming more and more ineffective [214].

Malaria is also a tropical parasitic disease, cause by *Plasmodium falciparum*, leading to over a million deaths annually, and rising, probably due to a resistance increasing, requiring the development of cheaper and more effective drugs [214, 268-270].

Carlos Barea, *et al* [214], synthesized 14 new 3-amino-1,4-di-*N*-oxide quinoxaline-2-carbonitrile derivatives. These compounds were evaluated for their in vitro antimalarial and antileishmanial activity against *P. falciparum* (Colombian FCR-3 strain) and *Leishmania amazonensis* (strain MHOM/BR/76/LTB-012A). The study showed that compounds with one halogenous group in position 6 and 7 provide an efficient approach for further development of antimalarial and antileishmanial agent.

2.5.3.2 ANTIDIABETIC ACTIVITY

Diabetes Mellitus is a disease caused by the dysfunction of glucose homeostasis, in which glucose levels appear abnormal with tendency to hyperglycemia. Diabetes type 1 is insulin-dependent and requires a daily subcutaneous injection of insulin, while diabetes type 2 is non-insulin-dependent and can be treated with several drugs such as sulfonylureas, nateglinide, biguanides, among others. However these treatments have limited efficacy and tolerability, and could cause severe side effects

[271]. In this regard, new transition metal complexes of quinoxaline-thiosemicarbazone ligands L^1H_2 and L^2H_2 (Figure 2.23) were prepared. The ligands were explored with copper and zinc complexes in diabetes induced Wister rats. The compounds $[ZnL^1(H_2O)]$ and L^2H_2 have showed prominent reduction in blood glucose level and the complexes $[CuL^1(H_2O)]$, $[ZnL^1(H_2O)]$ and $[CuL^2(H_2O)]$ have exhibited good activity in oral glucose tolerance test (OGTT) and showed low toxicity [237].

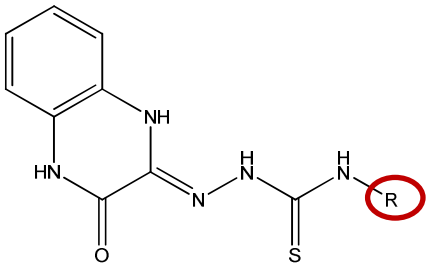


Figure 2.23: Ligands L^1H_2 and L^2H_2. For L^1H_2, R=CH_3 and for L^2H_2, R=C_6H_5.

Also (*N*-arylcarbamoyl and *N*-aryl thiocarbamoyl)hydrazinequinoxalin-2-(1*H*) (Figure 2.24**)** have been reported as mild hypoglycaemic agents [229].

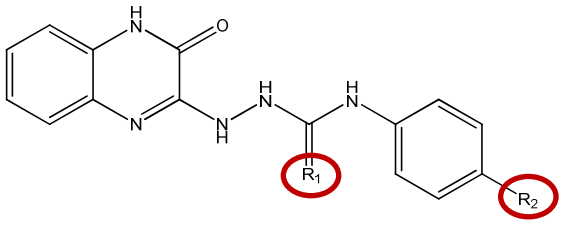


Figure 2.24: (*N*-arylcarbamoyl and *N*-arylthiocarbamoyl) hydrazinequinoxaline-2-(1*H*) compounds core.

2.5.3.3 ANTI-INFLAMMATORY ACTIVITY

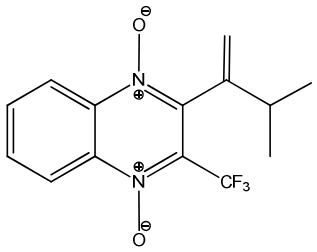
Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in therapeutics, generally for the treatment of the pain and inflammation. Nevertheless its long-term usage can lead to significant side effects like gastrointestinal lesions, bleeding, and nephrotoxicity. Due to the reasons mentioned it is important the discovery of new safer anti-inflammatory drugs [272-274].

Quinoxaline 1,4-di-*N*-oxide derivatives such as 4-(7-fluoro-3-methyl-quinoxalin-2-yl)-6-(3,4,5-trimethoxy-phenyl)-pyrimidin-2-ylamine and 2,6,7-trimethyl-3-[5-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-1*H*-pyrazol-3-yl]quinoxaline, showed an *in vivo* anti-inflammatory effect, higher than one reference drug, IMA (indomethacin), and *in vitro* decreasing values of LOX (lipoxygenase). LOX is an enzyme essential to arachidonic acid (AA) metabolism, which leads to the formation of leukotrienes, a type of pro-inflammatory mediator involved in processes like fever, asthma and cardiovascular disease [221, 275]. It was demonstrated that the incorporation of pyrimidine, thiazolopyrimidine, pyrazolopyridine, pyridopyridine, *p*-chlorophenyl, *p*-methoxyphenyl or pyridine nucleus to quinoxaline moiety cause significant anti-inflammatory activity and also analgesic [272].

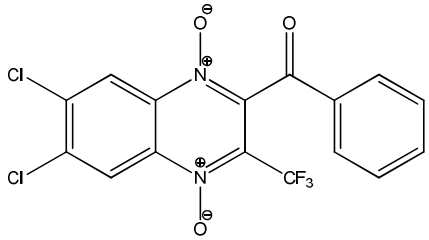
2.5.3.4 ANTICANCER ACTIVITY

Quinoxaline nucleuses exhibit potential anticancer activity, which makes them an important basis for the anticancer drugs [241]. A new series of 2-alkylcarbonyl and 2-benzoyl-3-trifluromethylquinoxaline-1,4-di-*N*-oxide derivatives was synthesized and evaluated for *in vitro* antitumor activity against a 3-cell line panel (MCF7 (breast), NCIH 460 (lung) and SF-268 (CNS)), and then evaluated in full panel of 60 human

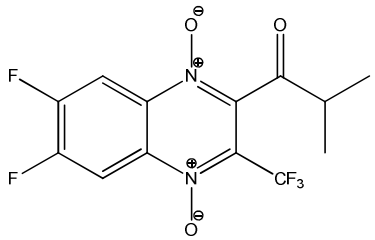
tumor cell lines, derived from nine cancer cell types. It was showed that, in general, anticancer activity depends on the substituents in the carbonyl group, increasing the activity in the order: ethyl<isopropyl<*tert*-butyl<phenyl-ones. Among these the compounds (Figure 2.25) 2-(3-methylbut-1-en-2-yl)-3-(trifluoromethyl)quinoxaline-1,4-di-*N*-oxide (Compound 1), 2-benzoyl-6,7-dichloro-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide (Compound 2), their difluorinatedanalogs (6,7-difluoro-2-isobutyryl-3-(trifluoromethyl)quinoxaline 1,4-di-*N*-oxide and 2-benzoyl-6,7-difluoro-3-(trifluoromethylquinoxaline-1,4-di-*N*-oxide) (Compound 3 and 4), and 2-(2,2-dimethylpropanoyl)-3-(trifluoromethyl)quinoxaline-1,4-di-*N*-oxide (Compound 5) were the most active, with higher anticancer activity with mean GI_{50} (Growth Inhibition) values of 1.02, 0.42, 0.52, 0.15, and 0.49 μM, respectively (Table 2.22) [236, 241].



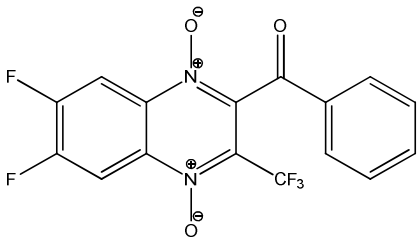
Compound 1: 2-(3-methylbut-1-en-2-yl)-3-(trifluoromethyl)quinoxaline-1,4-dioxide



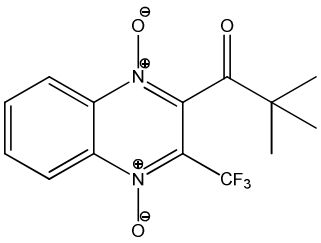
Compound 2: 2-benzoyl-6,7-dichloro-3-(trifluoromethyl)quinoxaline-1,4-di-*N*-oxide



Compound 3: 6,7-difluoro-2-isobutyryl-3-(trifluoromethyl)quinoxaline-1,4-dioxide.



Compound 4: 2-benzoyl-6,7-difluoro-3-(trifluoromethyl)quinoxaline-1,4-di-*N*-oxide



Compound 5: 2-(2,2-dimethylpropanoyl)-3-(trifluoromethyl)quinoxaline-1,4-di-*N*-oxide

Figure 2.25: Quinoxaline derivatives with anticancer activity.

2.5.3.5 ANTIGLAUCOMA ACTIVITY

Glaucoma is the designation to refer the diseases that affect the optic nerve, involving the loss of retinal ganglion cell sin, a characteristic pattern of optic neuropathy, and excavations of the nerve head [276-278]. Almost 67 million people worldwide are affected by glaucoma, remaining the leading cause of irreversible blindness, responsible for 14% of blindness after cataract and trachoma [279, 280].

Alphagan® (Brimonidin) is a relatively selective alpha-2 adrenergic receptor agonist, and its composition consists in 5-bromo-*N*-(2*H*-imidazol-2-yl)quinoxalin-6-amine (Figure 2.26**)**. This drug works as an antiglaucoma agent, due to its power to reduce the intraocular pressure, alleviating the symptoms of glaucoma [229, 241].

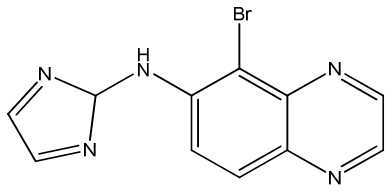


Figure 2.26: *Alphagan*® chemical structure (5-bromo-*N*-(2*H*-imidazol-2-yl)quinoxalin-6-amine)

2.5.3.6 ANTIATHEROSCLEROTIC ACTIVITY

After artery injury, abnormal proliferation and migration of vascular smooth cells (SMCs) into the intimal layer of the arterial wall occurs, proliferating and synthesizing extracellular matrix components, playing an important role in coronary artery atherosclerosis and restenosis after an angioplasty [281, 282].

A series of 6-arylamino-2,3-bis(pyridin-2-yl)-7-choloroquinoxaline-5,8-diones (Figure 2.27) were synthesized and screened for their inhibitory activity on rat aortic smooth muscle cell (RAoSMC) proliferation. IC_{50} (Inhibition Concentration) values were determined and compared to the positive control mycophenolic acid (MPA) (Table 2.22), and most of the compounds showed good activity, and the quinoxaline-5,8-diones were found as potent antiatherosclerotic agents [229, 241, 247].

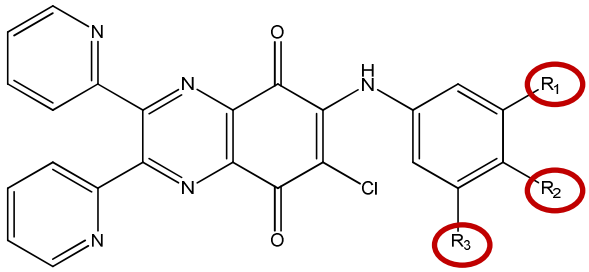


Figure 2.27: 6-arylamino-2,3-bis(pyridin-2-yl)-7-choloroquinoxaline-5,8-diones core.

2.5.3.7 ANTIDEPRESSANT ACTIVITY

5-Hydroxytryptamine (5HT), commonly known as serotonin, is a neurotransmitter involved in a great number of physiological and patho-physiological processes, acting through the receptor subtypes, from 5-HT_1 to 5-HT_7. Almost all of the receptors subtypes belong to the family of G-protein coupled receptor (GPCR), but the specific receptor subtype $5HT_3$ is a ligand gated ion channel [283, 284]. The antagonists to this receptor lead to various responses, such as anti-emetic action in cancer chemo-/radio-therapy induced nausea and vomiting, but also anti-depressant, anxiolytic, anti-phsycoticand anti-inflammatory activities. However, the drugs available to depression conditions have a delayed onset of action, which emphasizes the demand of new antidepressant drugs, with a safer and faster action [283, 284].

New series of structurally novel 3-substituted-2-carboxamides quinoxalinewere designed as 5-HT_3 receptor antagonists using ligand-based approach. All the compounds synthesized exhibited 5-HT_3 receptor antagonism, and some of them showed antagonism greater than the standard drug, ondansetron, like (3-ethoxyquinoxalin-2-yl)(4-methylpiperazin-1-yl)methanone and *N*-(2-(1*H*-indol-3-yl)ethyl)-3-ethoxyquinoxaline-2-carboxamide [284]. The compound (3-ethoxyquinoxaline-2-yl)(4-methylpiperazine-1-yl)-methanone showed the most

favorable 5-HT_3 receptor antagonism [241].

Also 3-benzyl-2-substituted quinoxalines were synthesized as novel monoamine oxidase A (MAO-A) inhibitors. The MAO inhibitors are very useful for the treatment of several neurological diseases, like Parkinson and depression. MAO-A inhibitors are used as antidepressant and antianxiety drugs. In this study, the final compounds were evaluated for their MAO-A inhibitory activity *in vitro*, using serotonin as substrate [238].

2.5.3.8 ANTI-GLUTAMATERGIC ACTIVITY

A major excitatory neurotransmitter in the central nervous system in mammalian species is the glutamic acid, an excitatory amino acid (EAA). Although, if an overstimulation of the postsynaptic glutamate receptors occurs, due to a high release of EAA, could result in neuronal death, and consequently induce neurodegenerative disorders such as Alzheimer and Huntington's disease [285-289]. AMPA-R (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor) antagonists have showed to have no side effects such as schizophrenia and protective activity in neural death, and many quinoxalinedione derivatives with competitive AMPA-R antagonistic activity have been synthesized and tested against the EAA receptor [290].

The compound 7-[4-[*N*-(4-carboxyphenyl)carbamoyloxy]methylimidazolyl]-3,4-dihydro-6-nitro-3-oxo-quinoxaline-2-carboxylic acid (GRA-293) was identified as a novel AMPA-R antagonist due to its high potency and good selectivity *in vitro*, and its potent neuroprotective effects in an animal model *in vivo*, than the known quinoxalinedione compounds used. These effects are due to a novel substituent, namely substituted benzene ring with urethane linkage to imidazole, in C-7 position, which leads to a potent AMPA-R affinity and contributes to therapeutic efficacy in animal models. This compound, with such characteristics, meets the criteria, in an injectable formulation, for use in the treatment of acute cerebral ischemia [290].

2.5.4 INDUSTRIAL AND ENVIRONMENTAL APPLICATIONS OF QUINOXALINES

In industry, the quinoxalines and derivatives have also various applications. They can be used to prevent metal corrosion, leading to a decrease in maintenance of some materials and equipment, and can be used to detect Cu^{2+}, for example. The Cu^{2+} detections are also important regarding medical applications, once it is a crucial transition-metal ion in the life processes, and are an example of transversal application from pure industrial value of quinoxalines to the biomedical field.

2.5.4.1 CORROSION INHIBITION

The use of acids is very common in industry, in fields such as pickling, industrial acid cleaning, acid descaling and oil well acidizing. However, the continuous use of acid solutions can lead to the metal corrosion, leading to enormous economic losses. Thence the importance of having corrosion inhibitors to minimize the metal dissolution and acid consumption, instruments malfunction and contamination [232, 291].

The most effective corrosion inhibitors used in industry to minimize these losses are organic compounds. Their inhibitory effect is reinforced by the presence of heteroatoms such as sulphur, nitrogen and oxygen, which will facilitate its adsorption on the mild steel surface following the sequence S>N>O [231, 291].

Other studies reveal that the adsorption in mild steel surface also depends on

the physicochemical properties of the inhibitor group, planarity of the system, presence of multiple adsorption active centers with lone pair and/or π orbitals, molecular size and electronic density at the donor atom. On this basis, the choice of effective corrosion inhibitors is done taking in consideration their structure, their mechanism of action and their electron donating ability [231]. Many *N*-heterocyclic organic compounds are good corrosion inhibitors, but some of them are highly toxic to both human beings and environment, leading to a continue search for eco-friendly and harmless *N*-heterocyclic compounds as corrosion inhibitors [227].

Quinoxaline derivatives are *N*-heterocyclic aromatic compounds that have been proved to be excellent corrosion inhibitors for mild steel in acidic media, easy to synthesize and ready available [226].

2,3-Quinoxalinedione (QD) (Figure 2.28) was used to study their corrosion inhibition properties for mild steel in 1M HCl, due to the presence of heteroatoms N and O, and π-electrons. It was shown that QD can act as a good corrosion inhibitor for mild steel in 1M HCl, with an inhibition efficiency of 88% at 10^{-3}M (measured through weight loss) [291].

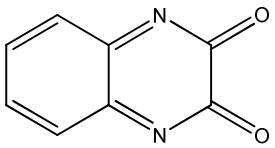


Figure 2.28: 2,3-quinoxalinedione (QD).

Indeno-1-one [2,3-*b*] quinoxaline (INQUI) (Figure 2.29), was synthesized and tested for inhibition corrosion of mild steel in 0.5 M H_2SO_4, and showed about 81% of inhibition efficiency at 10^{-6} M. This efficiency increases with INQUI concentration but decreases with immersion time [227].

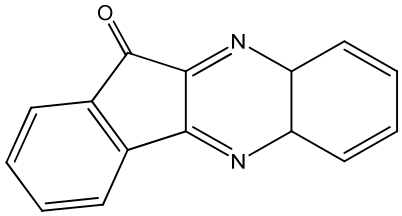


Figure 2.29: Indeno-1-one[2,3-*b*]-quinoxaline (INQUI).

Also acenaphtho[1,2-*b*]-quinoxaline (AQ) (Figure 2.30) was tested as a corrosion inhibitor for mild steel in 0.5 M H_2SO_4. AQ acts as an effective inhibitor for mild steel in acidic medium, with 80% of inhibition efficiency at 10^{-6} M (measured through weight loss) [226].

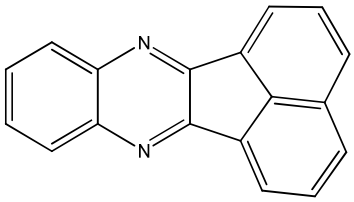


Figure 2.30: Acenaphtho[1,2-*b*] quinoxaline (AQ).

Quinoxaline derivatives, namely 3-methyl-2-phenylquinoxaline (MPQ), 2,3-diphenylquinoxaline (PPQ), 3-methyl-2-(2-hydroxyphenyl)quinoxaline (MHPQ), 3-phenyl-2-(2-hydroxyphenyl)quinoxaline (PHPQ) and 3-methyl-2-(3-methoxy-4-hydroxyphenyl)quinoxaline (MMtHPQ), have been shown through experimental studies to have high corrosion inhibition efficiencies for copper in nitric acid, with the order of inhibition efficiency being MMtHPQ> PPQ > MPQ > PHPQ > MHPQ [232].

2.5.4.2 CU^{2+} DETECTION - COLORIMETRIC SENSOR

Cu^{2+} is a transition-metal ion, crucial in the life processes, since it has an

important role as a catalytic cofactor for a variety of metallo-enzymes such as superoxide dismutase, cytochrome c oxidase, lysyl oxidase and tyrosinase, among many others. However when overloading, exhibit toxicity and could cause a variety of neurological diseases. Besides, it is also important in pollution matters, since the formulation of copper-containing pesticides uses various forms of copper, which in the end dissociates into Cu^{2+} [228, 292].

There are well developed technologies to detect Cu^{2+} such as inductively coupled plasma detectors, surface-plasmon resonance detectors, fluorescence anisotropy assays, quantum-dot-based assays, electrochemical sensors and fluorescence sensors. These are technologies with high sensitive and specificity in the Cu^{2+} detection, but also very expensive, due to the need of sophisticated instruments and highly trained operators. On the other hand, it is possible to use a naked-eye detection method, which gives a more fast response without involving any costly instrument. Although this method allows a good qualitative approach it presents a low sensitivity [228].

A colorimetric receptor ninhydrin–quinoxaline based was designed, synthesized and characterized, and exhibited high sensitivity and selectivity for Cu^{2+} in aqueous medium over a wide variation of cations such as Na^{+}, Mg^{2+}, Al^{3+}, Co^{2+}, Fe^{3+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+} and Pb^{2+}. In this study Cu^{2+} was added to the receptor solution giving a clear color change from olive green to pink. The detection limit was found to be 3.43×10^{-7} M which is one of the lowest for the Cu^{2+} in an aqueous solution by any naked-eye receptor [228].

2.5.4.3 OLED - ORGANIC LIGHT-EMITTING DIODE

Phosphorescent Organic Light-Emitting Diodes (PHOLEDs) are well known due to its high efficiency (possibility of 100% internal quantum efficiency) [293]. This property is driving the investment on research, leading to the synthesis of new compounds and materials as hosts, charge transporting materials and emitters. The high efficiency is due to PHOLED capacity to harvest both singlet and triplet excitons for light emission [294, 295]. However, triplet emitters tend to decrease the efficiency because of the concentration triplet-triplet annihilation during device operation, despite of owing long emissive lifetimes. The solution is to develop new bipolar host materials, which will contribute to the balanced transport of carriers and help to increase the probability of carrier recombination, and will grant better device stability due to its amorphous nature [295].

New series of carbazole/quinoxaline hybrids with 1,3,5-benzene core have been synthesized, and showed excellent thermal and morphological stabilities, due to their twisted geometry. These compounds, bipolar along with high thermal stability and favorable electrochemical properties, are promising for their use as host materials in red and green phosphorescent based OLEDs [295].

2.5.5 CONCLUSIONS

The literature survey reveals that quinoxaline and their derivatives have a wide application, with a great potential, being an important class of biological active compounds. This review tries to embrace quinoxalines features such as its biological activities and industrial value. Quinoxaline and its derivatives showed a wide field of application in medicine due to its biological activities that include antimicrobial and antiparasitic anti-diabetic, anti-atherosclerotic, anti-inflamatory, anticancer, antiglaucoma, antidepressant activities and also against the excitatory AMPA receptor

antagonist activity. The biological activities referred are very encouraging for the investigators and pharmacists, leading to new treatments and therapeutic agents that will benefit humanity. Regarding quinoxaline derivatives potential in industry, they have shown great outcomes in metal corrosion inhibition, showed to be excellent as host materials for use in phosphorescent OLEDs and led to an increase in both sensitivity and selectivity in Cu^{2+} detection in colorimetric sensors. These findings are very important for the industry, decreasing costs (inhibition metal corrosion) and increasing efficiency (OLED), and also for medical field, regarding Cu^{2+} detection, providing more accurate results. We hope, with this review, along with the easy process of quinoxaline derivatives synthesis, to lead to further development of the quinoxaline nucleus, allowing the research for new compounds, its features and potentials.

Chapter 3: Monitoring of antibiotic residues in milk

3.1 Objectives

3.2 Abstract

3.3 Introduction

3.4 Material and methods

3.5 Results

3.6 Discussion/Conclusion

3 MONITORING OF ANTIBIOTIC RESIDUES IN MILK

3.1 OBJECTIVES

The objectives in the present chapter were to determine the presence of antimicrobial residues in water and food samples, mainly at concentrations bellow the standard methods usualy used. A chromatographic method able to determine several antibiotics in the same analysis was needed, as well as a preliminary solid phase extraction (SPE) method for sample cleaning and concentration.

This chapter is a reproduction of the text submitted to the *Food Chemistry* (Manuscript Number: FOODCHEM-S-13-05971).

Antimicrobial multi-residue detection in milk samples

Mónica Vieira, Larissa Batista, Patrick Pais, Cristina Prudêncio, João Paulo Noronha

3.2 ABSTRACT

The presence of antimicrobial residues in foodstuffs constitutes a potential risk to the human health and undesirable effects on consumers, and nowadays these antimicrobial residues are also recognized as an emerging environmental problem.

This work describes the development of a sensitive and reliable method using HPLC with UV-diode array detection (HPLC-DAD) for simultaneous determination of five antibiotics, amoxicillin (AMOX), ciprofloxacin (CIP), sulphamethoxazole (SULFO), chloranfenicol (CLOR) and cloxaicillin (CLOX), in milk samples. The column, mobile

phase, temperature and flow rate were optimized to provide the best resolution of these analytes. The extraction method of the antibiotic residues involves the deproteinisation of the milk samples using trichloroacetic acid and centrifugation followed by a solid-phase extraction (SPE) clean-up. The recovery for ciprofloxacin was 94.54 ± 6.57%, ranging from 87 to 108%. The limits of quantification (LQ) for all these compounds were in the range of 2.43 ng/mL (CLOR) and 162 μg/mL (AMOX), which are lower than the maximum residue limits (MRLs) established by the European Union for the studied antibiotics in milk (Commission Regulation (EU) No 37/2010, OJ L15, 20.1.2010, pl1), making the method suitable for performing quality control analyses.

The proposed multi-residue HPLC-DAD method is a powerful and popular alternative for the determination and confirmation of antibiotic residues in milk industries and authorities for quality control.

3.3 INTRODUCTION

3.3.1 ANTIMICROBIAL AGENTS AND ANTIBIOTICS

Antimicrobial agents are natural or synthetic products produced in laboratory, capable of destroying microorganisms or inhibiting their growth. The antimicrobial main goals is preventing and/or treating a microbial infection and, if possible, preserving the usual flora of the organism [296]. An antimicrobial, on the other hand, is a more restricted term, that refers to the natural source of antimicrobial agent [297] Since the discovery and introduction of antimicrobial agents in therapeutics, these have been considered as a “miraculous” drug in the actions against bacteria induced diseases [298].

Most part of antimicrobial agents clinically used in human beings belongs to five main groups: β-lactams (penicillin and cephalosporin), macrolides, aminoglycosides, tetracycline and quinolones. In animals, most used antibiotics belong to classes of β-lactams, aminoglycosides, tetracycline, macrolides, and sulfonamides [299].

Antibiotics are widely used in dairy cattle management for the treatment of disease and as dietary supplements, administrated orally as feed additives or directly by injection and this use, especially when they are not used properly, may result in drug residues present in animal origin food, for example in milk [300].

3.3.1.1 AMINOGLYCOSIDES

Aminoglycoside antibiotics have an aminoglycoside moiety linked via a glycosidic bond to an aminocyclitol ring moiety. The aminoglycoside antibiotics most commonly used in veterinary medicine are gentamicin, neomycin, dihydrostreptomycin and streptomycin. Residues of aminoglycoside are believed to damage kidneys and cranial nerves causing hearing loss. The aminoglycosides are extremely polar, hydrophilic compounds that have little tendency to be retained on nonpolar reversed-phase liquid chromatography (LC) columns [300].

3.3.1.2 B-LACTAMS

The β-lactam antibiotics include penicillins and cephalosporins are widely used in cattle raising. There are concerns about this kind of antibiotics mostly because of

allergic reactions in some individuals. The most used technique for β-lactam detection and quantification is LC, but also GC and gel electrophoresis are widely used [300].

3.3.1.3 CHLORAMPHENICOL, FLORFENICOL AND THIAMPHENICOL

As broad spectrum antibiotics chloramphenicol, florfenicol and thiamphenicol are suitable for the treatment of a variety of infectious organisms. A small percentage of humans exposed to chloramphenicol got aplastic anemia so this drug is not approved for use in food producing animals in EU and in the USA, but florfenicol has been approved for the treatment of bovine respiratory disease. These lower polarity antibiotics are easily extracted from biological matrices and have strong UV absorption so they have a lot of reported LC and gas chromatography (GC) methods available for detection and quantification [300].

3.3.1.4 TETRACYCLINES

Tetracyclines are amphoteric and form crystalline salts with acids and bases. Widely used at subtherapeutic levels in cattle feeds for prophylaxis these antibiotics are generally used for the treatment of bovine mastitis. The chromatographic approach most used for determination of tetracycline in milk is LC [300].

3.3.1.5 MACROLIDES

The macrolide antibiotics are most effective against gram-positive organisms and are used to treat a wide range of infections. Being relatively recent there are only a

few studies about chromatographic methods for determination of macrolides in milk [300].

3.3.1.6 AMINOCYCLITOLS

There are two commonly used aminocyclitol antibiotics used in food producing animals, apramycin and spectinomycin both hydrophilic and do not contain UV chromophores. Both of them are forbidden in lactating dairy cattle. Determination of these antibiotics is based in LC methods [300].

3.3.1.7 FLUOROQUINOLONES

Fluoroquinolones are a developed class of antibacterial drugs used against infections in humans and animals. They are synthetized drugs and is relevant to consider residues of these drugs in food as they may increase the level of drug resistant pathogens that are infective to humans. Enrofloxacin and sarafloxacin have been approved for use in chicken and turkey in USA although prohibition of extra label use of fluoroquinolones in food producing animals. Despite being relatively recent the use of this compounds there is at least one reported LC method for determination of ciprofloxacin, difloxacin, enrofloxacin and sarafloxacin residues in milk [300].

3.3.2 VETERINARY USE OF ANTIBIOTICS

Antibiotics are widely used in veterinary as therapeutic for bacterial infection, diseases prophylaxis and as growth promoters (food additives) [301]. This drugs are used to avoid epidemic propagation of infection diseases, prevention of animals' zoonosis transference to humans and possible food born diseases, as well as improving the efficiency of animal production and food stuff, like meat, milk or eggs, as well as for security for human consume [302].

Based on World Health Organization (WHO) some antibiotics or substances that are given to animals used for alimentation are the same that are used in human medicine. In livestock those drugs are extensively used to prevent diseases and promote animal growing, what leads to a simultaneous and mass administration of those, making this the main difference between human medicine use of these drugs and veterinary use of them [303].

Not all countries have embracing statistics about quantity of veterinary drugs used in cattle raising, but it is estimated that more than 70% of those compounds are antibiotics [304]. In USA, in 1999, there was a use around 9300 tons of antibiotics in cattle raising, being 87% used for therapeutic issues, both preventive or therapeutic, and the remaining was used to promote a better efficiency of production and animal growth [305]. In Europe, during the same period, there was a use around 3900 tons and in more recent studies around 4600 tons of antibiotics used in European Union countries, in 2005, mainly tetracycline, β-lactam and cephalosporin [306].

3.3.2.1 ANTIBIOTICS USE IN THERAPEUTIC AND PROPHYLAXIS

Despite being mostly used to promote growing, antibiotics are equally important tools for treatment, maintain a healthy condition and animals' well-being in cattle raising. Nowadays, this drugs are being used as therapy according to objectives of use, dose, and duration of treatment [307].

Therapeutic treatment with antimicrobials' main goal is control bacterial infection and can be promoted in an individual or group treatment, preferable in an injectable way as sick animals have an impaired food and water intake [308]. The most frequent kind of diseases treated with antimicrobials in cattle raising are gastro enteric, respiratory, cutaneous, and reproductive ones [309]. In dairy cows, mastitis is the most common infectious disease that leads to bigger economic losses related to high frequency, treatment expenses, production reduction and reduction of productive life of the animal [310, 311]. Mastitis is an inflammation of mammary gland promoted by gastro enteric pathogens like *Escherichia coli* and *Salmonella* spp. and respiratory ones like, *Pasteurella* spp. and *Haemophilus* spp. [312] and it can be classified as sub clinic or clinic mastitis. In subclinical mastitis there is no visible signals of inflammation of mammary gland and milk has normal macroscopically appearance, although its composition is modified and microorganisms may be isolated from it. On the other hand, clinical mastitis can be diagnosed more easily has long has the milk clearly presents an abnormal aspect and mammary glands present inflammation [313]. Main antimicrobials given to dairy cows are β-lactams, as penicillin and cephalosporin, aminoglycosides, chloramphenicol, tetracycline and macrolides [300, 314].

For prophylactic use, are used dosages under the recommended therapeutic dose, many times based on empiric and not scientific knowledge [308]. This kind of treatment has been widely used in cattle raising has a way of preventing diseases occurrence and usually is administrated mixed with food and/or water. This is a questionable strategy for already sick or infected animals, because those will not consume enough water or food and they will spread the agent to other population members. This way, there is not an efficient prevention of future occurrence of the disease [307, 308]. The efficiency of this kind of drug requires the use of the correct antimicrobial for the target bacteria. The clinical trial (sensitivity tests and bacterial culture) and veterinary experience are fundamental tools that must identify the agent. After identification, the drug must be chosen according to current pharmacological

information and therapeutic principles, being indispensable the respect for label instructions referring to correct dose, treatment duration and administration pathways [307].

3.3.2.2 ANTIBIOTICS USE AS GROWING PROMOTERS

Some studies point that antibiotics used as growing promoters started in the forties [302] and during the following decade were published in scientific articles some results pointing out that antibiotics efficiency when associated to feeding or water of cattle in doses above the recommended dose for therapeutic use was benefic [315, 316].

The use of antibiotics in cattle raising as growing promoters has the objective of promoting a better performance of the animal, decrease mortality due to clinical and subclinical problems and improve the food conversion, resuming, improving a better animal development [317]. The action mechanism of a growing factor is not totally described, but some studies point that these drugs might act by eliminating infection diseases by purging of microbial flora implying destruction of pathogenic bacteria from gastrointestinal system of the animal allowing a better digestion and metabolism [318, 319]. In this situation antibiotics are used in concentrations below usual, making it insufficient to eliminate all pathogenic bacteria from the organism, allowing adaptation and afterwards appearing of bacteria resistant strains [320].

No matter the proved contribution in animal performance, antibiotics with grow promoting effect are now being seen as risk factors to human health due to high consume of animal products like milk and meat. Part of the resistant bacteria end spreading and creating resistances to human organism defenses [321]. In 2006, European Union forbidden the use of antibiotics as grow promoters in production of meat at the same time that dramatically changed the exigencies for aviary meat importation [322].

New alternatives are being tested trying to substitute antibiotics as grow promoters, the natural growing promoters (NGPs), namely probiotics and prebiotics. Probiotics are defined as food supplements based in living microorganisms that affect in a positive way the host organism promoting a better intestinal microbial balance [323] and prebiotics are supplements indigestible for the host animal that are selectively fermented by beneficial gut bacteria and, therefore, support a healthy gut microflora [324]. According to Gibson and Roberfroid [325] prebiotics are "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" [325]. Some compounds might be classified as prebiotics, namely carbohydrates, lipids, fibers, although short chained oligosaccharides, like other mananoligosaccharides, frutoligosaccharides and glucoligosaccharides are the most studied for presenting better results [324, 326].

3.3.3 ANTIBIOTICS RESIDUES

The incorrect use of antibiotics in cattle treatment, prevention of diseases or with grow promoting effect may left residues in animal origin products and may become toxics in the consumers organism, namely allergic reactions in hypersensitive individuals, or can cause indirect problems by inducing resistant bacteria strains [327].

To protect consumers from exposing to potentially prejudicial compounds veterinary drugs can only be officially registered after extensive security studies and residues evaluation. On those evaluations are defined the Maximum Residues Limits (MRL) and the shortage period of it [328]. According to regulation Nº 470/2009 from European Parliament, MRL is the maximum concentration of residues, expressed in

mg/kg of fresh tissue that is acceptable in food stuff as a result of one veterinary drug use in animals raised to human alimentation.

Some European studies point that various antibiotics residues of veterinary use have been detected in relatively low concentrations in soil samples [329], superficial waters and phreatic waters [330] being considered insufficient to cause toxic effects in exposed organisms.

A study was made in United Kingdom and some antibiotics as amoxicillin, benzylpenicillin, trimetoprim and sulfadiazine were chosen to environmental monitoring due to excessive use by local population, potential transportation in environment as well as toxicity of those compounds [331]. Another group of researchers made a study in Germany that reveals that in the analyzed water samples it was detected few quantities or even none of antibiotics' residues from groups tetracycline, β-lactam e fluoroquinolone, despite that on the same samples it was found a big frequency of molecular residues from sulfonamides, macrolides and lincosamides. The hypothesis to the absence of tetracycline is its big fotodegradation ratio, and for the prevalence of sulfonamides, macrolides and lincosamides the main justification may be sewers' deposition in water courses [332].

3.3.4 ANTIBIOTICS: WATER AND ENVIRONMENT PROBLEMATIC

Nowadays studies about antibiotics' waste in environment come to bigger interest as many of those compounds are frequently found in effluents of wastewater treatment plants and natural water with concentrations in the order of μg/L and ng/L [333].

Antibiotics' waste in environment may present adverse effects in aquatic and terrestrial organisms. Physical, chemical and biological processes regulate the behavior and destiny of antibiotics in environment after interaction between those compounds and the soil, processes like absorption/adsorption, transformation (degradation), and transport (leaching and superficial flow) [305]. Those processes depend on antibiotic's physical-chemical properties (molecular structure, hydrophobia, solubility, etc.) as well as the soil structure and climatic conditions [304].

Utilization of antibiotics in cattle raising represents one of the environmental antibiotics' ways that leads to environmental contamination directly by animals' excretions and diet or indirectly by fertilizers utilization on soil [334, 335]. Some factors may influence the quantity of excreted antibiotic in environment, namely the kind of substance, dosage, animal's specie or age. Studies reveal that up to 95% of the active compounds given to animals may be integrally eliminated without suffering any metabolizing in animal's digestive tract and even when the molecule is metabolized some of the metabolism products excreted might continue having bioactivity [304].

Excessive antibiotics use may lead to environmental problems as hydric resources contamination or antibiotics resistance [333]. Studies put to evidence that a bacteria is drug resistant when it can *in vitro*, exposed to the antimicrobials concentrations usual achieved in blood under clinical use recommendations, and these studies also put to evidence that antimicrobials are not inducing resistances but acts as a selector of the most resistant strains from a specific population [336]. This phenomenon is due to arbitrary prescription of antimicrobials, excessive use of those drugs and propagation of resistant pathogens from one country to another through infected travelers carrying those strains [337].

Epidemiological surveillance network from all around the World collect data referring to microbial susceptibility patterns, namely its regional and temporal variation in a way that aims to achieve new orientations to therapeutic

recommendations and clinical exercise decisions. In Europe, the system to monitoring the evolution of bacterial resistance, *The European Antimicrobial Resistance Surveillance System* (EARSS), exists since 1998 and integrates nowadays a group of 32 countries, including Portugal [338]. EARSS has available in real time collected data from public hospital laboratories, in various countries, about the susceptibility of seven relevant bacteria in European countries, allowing, this way, the analysis of different patterns of resistance across time in different regions or countries [338]. Besides EARSS exists also on European continent, since 2001 an international network denominated, *European Surveillance of Antimicrobial Consumption,* (ESAC) that promotes surveillance to the consume of antibiotics and that links national level systems, providing data that pretends to help understanding the emergency of bacteria resistance and recommend the indications for antibiotics prescription in ambulatory and hospital service [339].

The importance of studies about antimicrobial resistance in cattle raising nowadays is on the existence of antibiotic resistant microorganisms and is directly connected with the indiscriminate use of those drugs, both in prophylaxis and disease treatment, but mostly as growth promoters, for improving the alimentary efficiency and growing rate in different species [340].

The use of the same drugs in human and veterinary medicine raises another problem: spread of resistant strains may accelerate the inefficacy of some antimicrobial agents. Both humans and animals may act as resistance reservoirs and they may spread from one to another. The use of sub-inhibitory concentrations of antimicrobial agents as growth promoters enhances the survival of resistant strains, thus favoring their prevalence and leading to antimicrobial agents' failure [186, 341].

3.3.5 ANTIBIOTICS DETECTION AND IDENTIFICATION

There are some used tests for detection of antibiotics in food samples. Microbial inhibition of immunological assay are two examples of screening tests used to detect antibiotic residues present in food samples, like milk, although there are some drawbacks about this kind of tests as they cannot identify which antibiotic is present on the sample, the presence of high somatic cell counts may result in false positives and also it may detect quantities of antibiotic below the legal limits making that sample unavailable to consume. Liquid chromatography and gas chromatography are the most commonly used techniques to have a sensitive and specific analytical identification and quantification of antibiotics residues [342]. With chromatographic techniques it is possible to have multianalysis, specificity, accuracy, precision, reproducibility and high sensibility. The sensibility limit is dependent, essentialy, on the detection method used.

Gas chromatography (GC) is a powerful tool for the detection and quantification of substances liable to be vaporized without undergoing decomposition. For this reason, and because many antibiotics are thermolabile, it is not the most used chromatographic technique. GC can use multiple detectors such as flame ionization detector (FID), thermal conductivity detector (TCD), flame photometric detector (FPD), electron capture detector (ECD), the nitrogen-phosphorus detector (NPD), infrared detector (IRD) and mass spectrometer (MS), a highly sensitive method [343]. Several studies reporting antibiotic detection by GC have been presented [344-349].

High Precision Liquid Chromatography (HPLC) is the most utilized technique for antibiotic detection. The most common detectors coupled to HPLC are the ultra-violet detector (UV) [350, 351], the diode array detector (DAD) [352, 353], the fluorescent detector (FL) [354] and mass spectrometer MS [314, 355].

The DAD makes the method more selective, since it allows measurements at various wavelengths, reducing the matrix effect. Using this method of detection

reliable determinations are possible to different classes of antibiotics in many biological and environmental matrices [356, 357].

Although extensively used, chromatographic methods have some drawbacks. Any process that involves a chromatographic analysis is extremely complex, demands trained personnel and specific equipment. Usually it is a time consuming process and expensive.

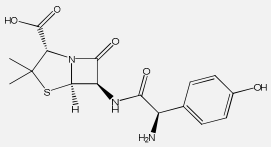
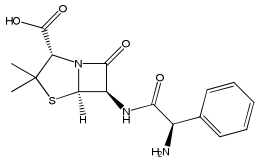
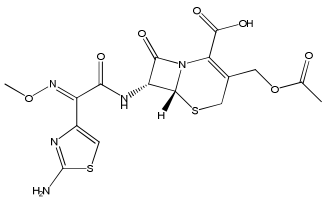
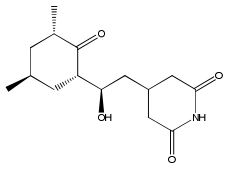
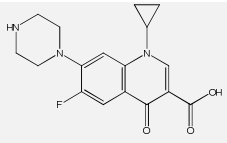
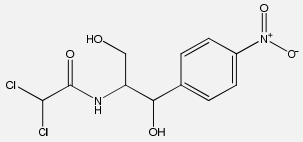
In the present work, a multidrug detection chromatographic method (HPLC-DAD) was developed to measure some antibiotic residues in food and water samples. Time limitation allowed the method to be applied only to milk samples.

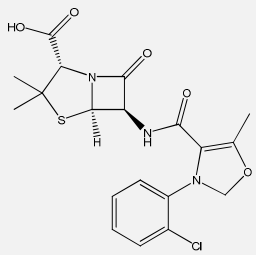
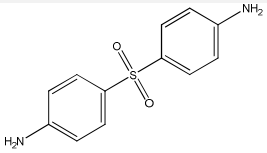
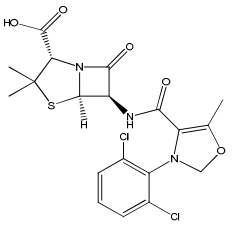
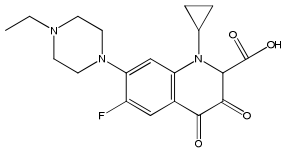
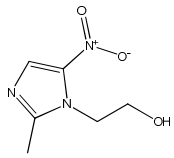
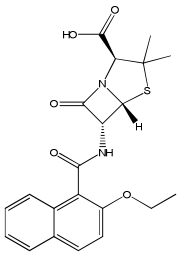
3.4 MATERIAL AND METHODS

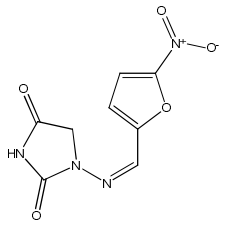
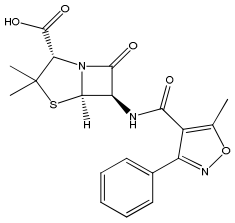
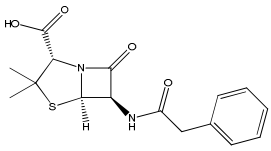
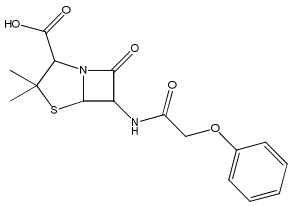
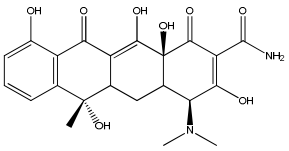
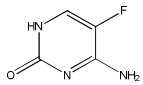
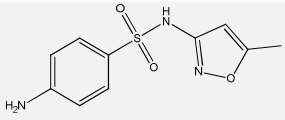
3.4.1 STANDARD ANTIMICROBIAL SOLUTIONS

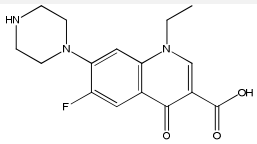
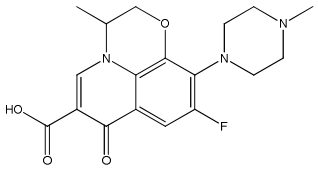
Twenty one antimicrobial agents (Table 3.1) were tested in the present study. In order to develop a multiresidue analysis, several hypotheses were tested and some of the 21 compounds had to be discharged. The developed method performs the simultaneous analysis of five antimicrobial agents, in acidic medium. The selection relayed on the antibiotic class and peak resolution observed.

Table 3.1: Antimicrobial agents tested for multiple detection method development. Grey marked compounds are the utilized in the chromatographic method (Grey cells indicate the antimicrobial agents selected for the developed chromatographic method).

Name	Structural Formula	Molecular weight/ g.mol^{-1}	Solubility
Amoxicillin (AMOX)	 $C_{16}H_{19}N_3O_5S$	365.4	pK_{a1} = **2.8** pK_{a2} = **7.2** Soluble in: Water, MeOH, EtOH Stability: pH 3.5-6
Ampicillin (AMP)	 $C_{16}H_{18}N_3NaO_4S$	371.4	pK_{a1} = **2.7** pK_{a2} = **7.3** Soluble in: Water, MeOH, EtOH, Acetone, DMF, DMSO Stability: pH 3.5-5.5
Cefotaxim (CEFO)	 $C_{16}H_{16}N_5NaO_7S_2$	455.47	
Cycloheximide (CIHEX)	 $C_{15}H_{23}NO_4$	281.35	
Ciprofloxacin (CIP)	 $C_{17}H_{18}FN_3O_3$	331	pKa_1. 8.62 pKa_2: 6.16.
Chloranphenicol (CLOR)		323	

Cloxacillin (CLOX)	$C_{11}H_{12}Cl_2N_2O_5$  $C_{19}H_{18}ClN_3O_5S$	435.88	pK_a = **2.7** Soluble in: Water, MeOH, EtOH, DMF, DMSO Stability: pH 6.0-7.5
Dapsone (DDS)	 $C_{12}H_{12}N_2O_2S$	248.3	
Dicloxacillin (DICLOX)	 $C_{19}H_{17}Cl_2N_3O_5S$	470.3	pK_a = **2.7** Soluble in: Water, MeOH, EtOH, DMF, DMSO Stability 24 ºC/7d
Enrofloxacin (ENRO)	 $C_{19}H_{22}FN_3O_3$	359.4	pK_a = **2.7** Soluble in: Water, MeOH, EtOH, DMF, DMSO Stability: 2-8 ºC
Metronidazole (METRO)	 $C_6H_9N_3O_3$	171.15	
Nafcillin (NAFCI)	 $C_{21}H_{22}N_2O_5S$	414.47	

Nitrofurantoin (NITRO)	 $C_8H_6N_4O_5$	238.16	
Oxacillin (OXA)	 $C_{19}H_{18}N_3O_5S$	401.4	pK_a = **2.7** Soluble in: Water, MeOH, EtOH, DMF, DMSO Stability: 24 ºC/d; 4 ºC/7d
Penicillin G (PENG)	 $C_{16}H_{17}KN_2O_4S$	372.5	pKa = 2.8 Soluble in: Water, MeOH, EtOH, DMF, DMSO Stability: pH 6.5 + 4 ºC Storage 2-8 ºC
Penicillin V (PENV)	 $C_{21}H_{22}N_2O_5S$	350.4	pKa = 2.7 Soluble in: Water, MeOH, DMSO, DMF Stability: pH < 5; rap. inactivation: pH 9-11 < 37 ºC
Tetracyclin (TCY)	 $C_{22}H_{24}N_2O_8$	444.4	
5-Fluorocytosin (FLUC)	 $C_4H_4FN_3O$	129.1	
Sulfamethoxazole (SULFO)		253.28	

	$C_{10}H_{11}N_3O_3S$	
Norfloxacin (NOR)		319.33
	$C_{16}H_{18}FN_3O_3$	
Ofloxacin (OFLO)		361.37
	$C_{18}H_{20}FN_3O_4$	

The antimicrobials chosen represent the β-lactam (penicillin and cephalosporin); sulfonamides, quinolones and chloramphenicol.

A stock solution for each antimicrobial studied (AMOX, CIP, SULFO, CLOR, CLOX, Sigma-Aldrich, Co., USA) was prepared with a concentration of 0,8 mg/mL, dissolving the weighted powder in mobile phase (80% TFA 0.02% pH = 2.2; 20% CH_3CN). TFA (trifluoroacetic acid), CH_3CN (acetonitrile) and standard antimicrobial powders were from Sigma-Aldrich, Co., USA Four standard solution were prepared by dilution of the stock solution for each antimicrobial agent. Final concentrations ranged from 10 to 200 μg/mL. The five antimicrobial agents selected by the developed method were prepared in a mixture containing all the five. Standard dilutions were prepared as described above, ranging the same concentrations.

3.4.2 SAMPLES COLLECTION

In the present study eighteen samples of different commercial brands and kinds (fat, medium fat, low fat, dehydrated, without lactose) of milk were used and analyzed. Samples were randomly chosen in different commercial stores in Porto. After the gathering the samples were identified and kept refrigerated at -80 ºC until laboratory analysis.

3.4.3 PROCEDURE FOR SAMPLE TREATMENT

These dairy products samples were previously unfrozen at environment temperature and homogenized in vortex for 1 minute. Afterwards, 2 mL were taken into a *falcon* tube, 3 mL of tricloroacetic (TCA, Sigma-Aldrich, Co., USA) in water at 20% were added and then samples were centrifuged at 8000 rpm during 10 minutes. Supernatant was recovered and stored at -80 ºC, until analysis.

3.4.4 PROCEDURE FOR SAMPLE ANALYSIS AND IDENTIFICATION

Chromatogram obtained on calibration of the mixture of antimicrobial agents is presented in Figure 3.1.

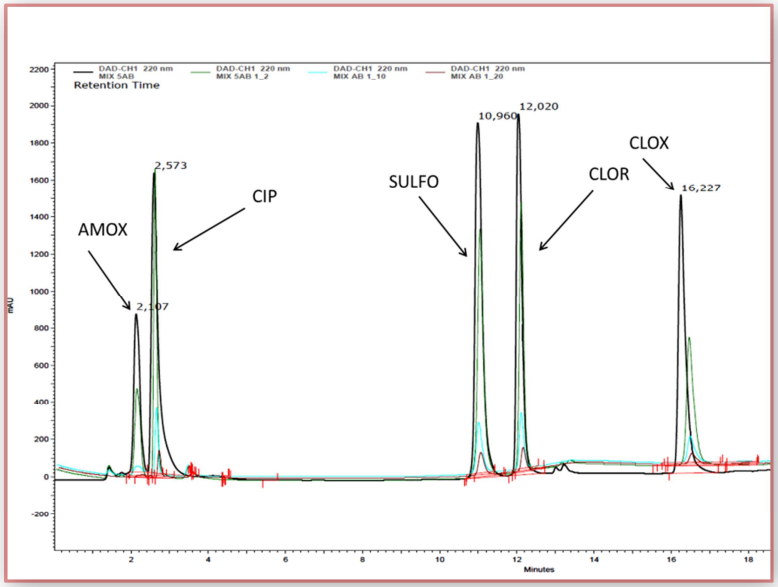


Figure 3.1: Calibration: Chromatograms for the four concentration standard tested. (AMOX: amoxicillin; CIP: ciprofloxacin; SULFO: sulfamethoxazole; CLOR: chloramphenicol; CLOX: cloxacillin)

The 18 samples were split into four different samples as referred in Table 3.2, totalizing 72 samples. In A group samples were directly analyzed in HPLC; In B group it was added 200 µL of the antibiotic ciprofloxacin and afterwards analyzed in HPLC; In group C the sample was cleaned-up using SPE technique and then analyzed in HPCL; finally to the D group was added 200 µL of enrofloxacin before use of SPE technique and HPLC analysis.

Table 3.2: Sample distribution over 4 groups of analysis

Number of samples	Group	Kind of sample
18	A	Sample
18	B	Sample + AB

18	C	Sample + SPE
18	D	Sample + AB + SPE

Legend: AB: Antimicrobial agent; SPE: Sample clean-up by solid phase extraction

The A group corresponds to the sample only previously deproteinized, as suggested by van Bruijnsvoort *et al.* [358].The B group corresponds to the sample spiked with 200 μL of CIP, to a final concentration of 160 μg/mL. The C group is the same as group A, but samples were subjected to a clean-up procedure, through SPE. The D group is the same as B group, but also after clean-up with SPE.

3.4.5 SOLID PHASE EXTRACTION (SPE)

SPE extraction used a system with capacity for 12 samples simultaneous analysis and a vacuum bomb Laboport Knf Neureberger. SPE-C18 with 500 mg and 3 mL volume cartridges were used (Lichrolut RP-18 Standard PP tubes).

A sample method of *clean-up* and pre-concentration was developed, for SPE in acid environment: 2 mL methanol (Sigma-Aldrich, Co., USA), 2 mL distilled water, 2 mL of previously deproteinized sample, 4 mL de distilled water, 4 mL distilled water and methanol (1:1) are passed consecutively through the cartridge to clean and retain the desired antibiotic analite. 4 mL de methanol and 3 mL ammonium in methanol

solution 16% (w/v) was passed through the cartridge and collected in a clean propylene 15 mL falcon tube. This extract was kept at 4 ºC until HPLC analysis. 1 mL was filtered (Pall Life Sciences® GHP Acrodisc 13 mm Ø Syringe filter 0.45 μm GHP membrane; GHPolypro 47 mm Ø 0.45 μm hydrophilic Polypropylene Membrane filters) and transferred to 1 mL vials before injection.

3.4.6 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY – DIODE ARRAY DETECTOR (HPLC-DAD)

The equipment used was a Hitachi® HPLC LaChrom Elite system, constituted for a quaternary bomb HTA L-2130, a vacuum degassing of solvent, in line, incorporated, auto sampler L-2200. The used detector was DAD L-2455, LaChrom Elite serie and column hoven L-2300, LaChrom Elite serie. The collect and treatment of chromatographic data was made using Software EZChrom Elite, Lachrom Elite serie.

Identification and quantification of antimicrobial residues in dairy products were made with HPLC-DAD equipped with a Purospher® STAR C18 chromatographic collumn, with 5 μm particle size and 4.6 x 250 mm internal dimensions, with the hoven at 40 ºC. Samples were kept at 4 ºC in the auto sampler tray and were used separation conditions in gradient, with a two solvent mobile phase application: acetonitrile and water with trifluoroacetic acid (TFA) 0.02% (pH = 2.2), with a flow rate of 1 mL min^{-1}, during 25 minutes. Samples injections and patterns were made in triplicated with 20 μL volume. Detection was made with a fotodiode system, ranging from 190 to 400 nm.

3.5 RESULTS

3.5.1 CALIBRATION OF A MIXTURE OF FIVE ANTIBIOTICS

Four standard mixture solutions were injected ($n = 3$) and calibration curves for each antimicrobial agent were drawn. In Figure 3.2 and Table 3.3 are presented the results obtained.

Selectivity was determined by analysis of a matrix antimicrobial agent free and no interfering substances were detected. Linearity was assessed by the determination of the calibration curve for each antimicrobial agent, in the mixture of five antimicrobial agents, at four different concentrations.

It was verified that alterations on the mobile phase pH and concentration of the organic eluent influenced the time of retention for all the antimicrobial agents tested. For milk samples, the matrix pH was adjusted to 2.2 and maintained at all stages of treatment, to ensure the stability of the antimicrobial.

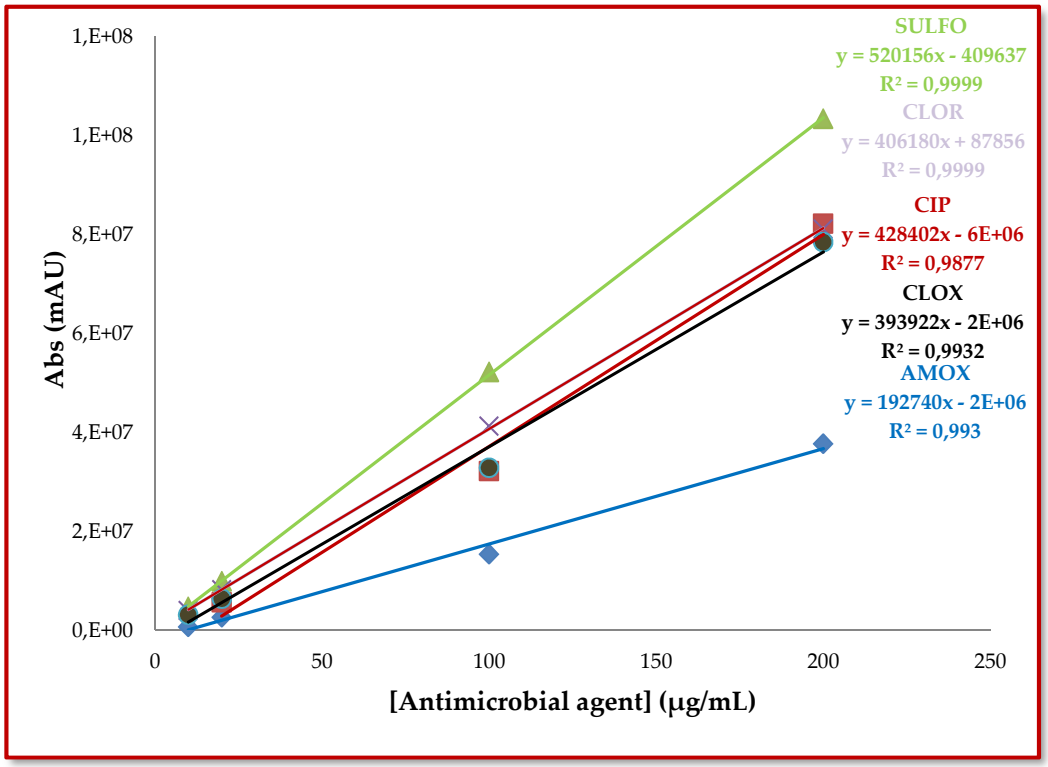


Figure 3.2: Calibration curves for a five antimicrobial agents mixture. (SULFO: sulfamethoxazole; CLOR: chloramphenicol; CIP: ciprofloxacin; CLOX: cloxacillin; AMOX: amoxacillin)

Table 3.3: Limits of Detection and Quantification for the five Antimicrobial Agents studied

	AMOX	CIP	SULFO	CLOR	CLOX
LD (µg/mL)	0.162569	0.044277	0.020993	0.00243	0.135142
LQ (µg/mL)	0.492633	0.134173	0.063616	0.007363	0.409523

3.5.2 QUANTIFICATION OF ANTIBIOTICS IN MILK SAMPLES

Milk samples analysed without any previous treatment, except deproteinization, reveald chromatographic profiles difficult to analyse, due to the presence of

interferences. No antimicrobial residues were, however, detected, for the antimicrobial agents tested. Clean-up of milk samples, with SPE improved the chromatographic analysis, allowing the evaluation of antibiotic residues.

Spiked samples, with CIP, at a final concentration of 117 μg/mL, were cleaned-up and analysed. Results are presented in Table 3.4.

Table 3.4: CIP quantification of spiked milk samples

Sample	[CIP] / μg/mL	CIP Recovery (%)
1D	79,8	68,21[a]
2D	127,5	108,94
3D	117,5	100,46
4D	107,9	92,20
5D	105,2	89,93
6D	73,9	63,18[a]
7D	118,2	101,00
8D	111,1	95,00
9D	108,5	88,10
10D	104,5	89,28
11D	101,7	86,89
12D	104,1	88,96

[a]: Samples not included in mean CIP recovery %

The mean value for [CIP] obtained by sample analysis was 110.61 ± 7.69 μg/mL. The percentage of CIP recovery was 94.54 ± 6.90%, ranging from 86.69 to 108.94%.

3.6 DISCUSSION/CONCLUSION

Antimicrobial residues in animal origin food are a public health issue. The presence of these compounds may have consequences in human health, as allergies and alteration of the commensal flora.

In the present study, the chromatographic method developed proved to be appropriate to simultaneous quantification of five antimicrobial agents selected from a pool of twenty. The clean-up step was proved to be an indispensable step to obtain matrices more clean and improved the detection and quantification of antimicrobial agents. The recovery of the antimicrobial agent ciprofloxacin was determined and evaluated as 94.54 ± 6.57%.

The antimicrobial agents proved to be sensible to pH variations and to organic eluent concentrations, so all the samples had to be treated at the optimized pH = 2.2.

LD and LQ determined for the presented chromatographic method is below the European MRL's for the antimicrobial agents studied (Commission Regulation (EU) No 37/2010, OJ L15, 20.1.2010, pl1), thus proving to be an efficient method for quality control on food stuff. The developed method presents LD from 0.00243 μg/mL (CLOR) to 0.162569 μg/mL (AMOX). The recovery percentage determined for ciprofloxacin (94.54 ± 6.57%).indicates that the SPE sample clean-up is efficient and allows reliable measurments on milk samples.

In conclusion, the chromatographic methods are potent tools in residues quantification, in animal origin food matrices. As reported by previous studies, HPLC associated to a DAD detector technique proved to be appropriate and reliable in the detection of antimicrobial and may be applied in food quality control.

The sample preparation and analysis demonstrated to be a delicate operation and required several adjustments and total mastery of the techniques used. The equipments specifications and data interpretation require specifis skills and demands operators preparation on analytical chemistry.

Chapter 4: Survival of Resistant and susceptible bacterial strains in the presence of sub-inhibitory concentration of ciprofloxacin

4 SURVIVAL OF RESISTANT AND SUSCEPTIBLE BACTERIAL STRAINS IN THE PRESENCE OF SUB-INHIBITORY CONCENTRATION OF CIPROFLOXACIN

4.1 OBJECTIVES

In this chapter the main goal was to compare the survival and phenotypic stability of bacterial strains of *Escherichia coli* in water contaminated with sub-inhibitory concentrations of ciprofloxacin.

Bacterial survival was monitored by cultivation, phenotype stability was assessed based on antibiograms, cultures' purity and authenticity by genotyping and ciprofloxacin concentration was measured over time using a chromatographic method developed in this study.

4.2 INTRODUCTION

The environmental contamination by antimicrobial residues is a public health concern. Intensification of large-scale animal production is on the top of the causes for contamination of water and soil [359], but human antibiotic consumption is also a source of environmental contamination [360, 361].

Antimicrobial agents are administered to livestock at therapeutic doses, to treat infections that were diagnosed, or to prevent illness, prophylactically. Antimicrobial

agents are also used as feed additives to increase the rate of growth and to improve feed efficiency. In Europe, antibiotic use as growth promoters was completely baned since January 2006, as a strategy for improvement of public health conditions and tackle antimicrobial resistance. In world regions where this procedure is allowed, the doses used in feed aditives are much lower than those used for treatment and may promote bacterial resistance to antimicrobial agents used in human and animal medicine[7, 359]. The environmental contamination by antimicrobial residues occurs normally at sub-inhibitory concentrations, as a result of partial degradation in the human and other animal bodies and also due to a dilution effect. Percentages of excretion of the non-metabolized antimicrobial compounds up to 75%-90% of the administrated drug have been reported [359, 362]. Another source is animal waste which is discharged to the soil and its drug content reach, eventually, surface and groundwater. Sludge storage and manure produced by these animals are pointed as environmental entries for antimicrobial agents [363]. Manure is also used in agricultural production, as fertilizer of crop fields, increasing crop yield. The antibiotics present in manure may adsorb to soil or be transported through runoff, leaching and infiltration and may also reach surface and groundwater [9, 359]. Human consumption and excretion of antibiotics also contaminates aquatic environments as wastewater effluents drive the drugs to water resources [364].

There are major concerns about the influence of the occurrence of antimicrobial agents, even at sub-inhibitory concentrations, in the environmental biota, especially in aquatic environments [362]. Little is known about the mechanisms involved, but there is association between subtherapeutic antimicrobial concentrations and increase of resistance among bacterial population [362, 365]. Previous studies demonstrated the presence of resistant bacteria in the final effluent resultant from wastewater treatment in municipal wastewater treatment plants and even in drinking water [364-366]. Some of these bacteria can share the habitat with human and animal commensal bacteria

and, in gene transfer events, involving antibiotic-resistance genes may create the possibility for the emergence of new resistance mechanism [360].

A better understanding of the impacts of antimicrobial residues on the microbial communities will contribute to improve environmental and human health risk assessment studies and to develop control measures. The present study was designed based on the hypothesis that in the presence of sub-inhibitory concentrations of ciprofloxacin, a bacterial strain of *Escherichia coli* resistant to this antibiotic would present a higher fitness than a susceptible isolate of the same species. To test this hypothesis, the survival and phenotypic stability of bacterial strains in water contaminated with sub-inhibitory concentrations of ciprofloxacin were compared.

4.3 MATERIALS AND METHODS

4.3.1 BACTERIAL STRAINS

Five *Escherichia coli* strains isolated from water environments were used - two of them from surface water (S3R9 and S3R22), one from an hospital effluent (H1Fc22) and two from urban wastewater (M3Ec1 and A1Fc22). Antibiotic resistance profiles were determined in previous works [367]. Except strain S3R9, the susceptible control, all strains were resistant to ciprofloxacin.

Bacteria were sub-cultured on Plate Count agar (PCA, Merck) and incubated for 24 hours at 37 ºC. Freshly prepared bacterial cells were transferred into a saline solution (0.09% NaCl) and optical density (O.D.) was adjusted to 0.2, at 620 nm, to standardize.the inoculum concentration.

4.3.2 EXPOSURE OF BACTERIA TO SUB-INHIBITORY CONCENTRATIONS OF CIPROFLOXACIN

In a first approach only two bacterial strains were used: a CIP resistant strain (S3R22) and a susceptible one (S3R9). Triplicate microcosm assays were prepared in 250 mL Erlenmeyer flasks containing 50 mL of sterile water spiked with different concentrations of ciprofloxacin. Pure cultures of strains S3R22 (ciprofloxacin resistant) or S3R9 (ciprofloxacin susceptible) were used as inocula to reach an initial O.D. of 0.2 at 620 nm. The bacteria were incubated, separately, in a 250 mL Erlenmeyer flask, in a 50 mL total volume in the presence of CIP. Volume of CIP (1 g/L) stock solution were added in order to reach final concentrations of 2.00, 0.50, 0.10 and 0.05 mg/L. Initial O.D. of microcosms was adjusted at 0,02 at 620 nm. Microcosms were incubated at 37 ºC, in a water bath, with agitation up to 6 weeks.

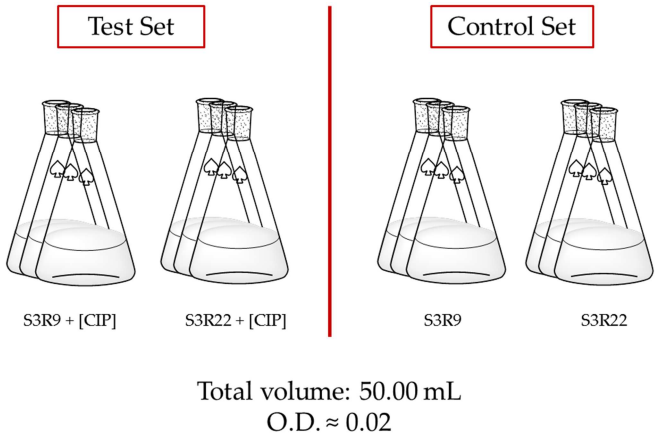


Figure 4.1: Procedure 1 – Monitoring of CIP Susceptible and resistant strains behavior along time.

At initial time (t=0) and every 7 days, an aliquot of 100 µL was taken from 2 flasks of each series and serially diluted. Colony-forming units (CFU) were enumerated over time, on PCA after 24 hours of incubation at 30 ºC.

In a subsequent experiment, microcosm were prepared under the same conditions described above but with a total volume of 20 mL and a mixture of the two strains. For CFU countings, the procedure adopted was the same explained above, but a PCA with CIP (2 mg/L) medium was used to recover the resistant strain alone.

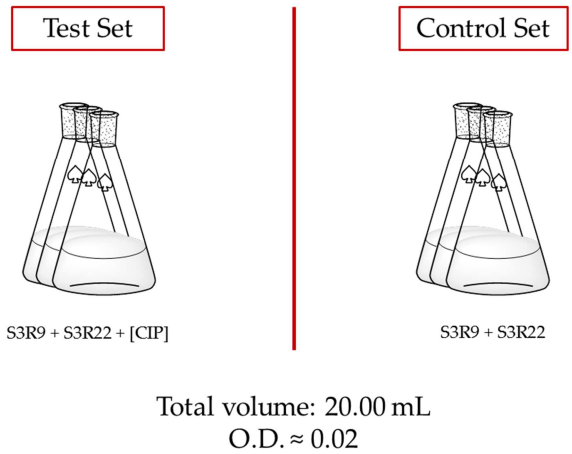


Figure 4.2: Procedure 2 – Monitoring of a mixture of two strains (susceptible and resistant to CIP) behavior along time.

Mixtures of susceptible strain S3R9 and each of the four resistant strains (S3R22, H1Fc22, A1Fc22 and M3Ec1) were prepared, in a multiwells plate, following the procedure described above, in a total volume of 150 µL. Plates were kept at 37 ºC in a microbiological incubator and countings of CFU were made at 0, 21 and 56 days.

4.3.3 CONFIRMATION OF STRAIN AUTHENTICITY

In order to confirm the authenticity of each of the isolates enumerated in the microcosms, randomly selected colonies, collected at the beginning and at the end of

each experiment, were genotyped using Random Amplified Polymorphic DNA (RAPD). The method used was described by Ferreira da Silva *et al* (2011) [368]. DNA was extracted from cells after 16 hours incubation at 30 °C. A single colony was suspended in a volume of 70 μL of ultra-pure water, boiled at 95 °C for 10 min, and cooled in an ice bath for 5 min. The suspension was centrifuged at 13,200 rpm for 2:30 min and the supernatant, containing the DNA, was stored at -20 ºC. Amplification reactions (25 μL) were prepared with: 14 μL of ultra-pure water; 2.5 μL of KCl buffer; 1.5 μL of MgCl (25 mM); 5 μL dNTP's (1 mM); 0.75 μL primer M13 (5'GAGGGTGGCGGTTCT3') (33 mM); 0.75 μL taq polymerase (10 μM) and 0.5 μL of cell lysates. Amplification was performed in a TPersonal Biometra thermociclator.

PCR products were analyzed in gel electrophoresis 1,5% (w/v) agarose (Bioscience). Genotypic profiles were compared visually, and only identical profiles were considered to represent the same strain.

4.3.4 ANTIBIOTIC RESISTANCE PHENOTYPES

To confirm the stability of the resistance profiles during the microcosms assays, the antibiograms of the bacterial isolates collected over time were determined based on the agar diffusion method, as described by CSLI [369]. Bacterial suspensions with O.D. = 0.2, at 620 nm, were spread on Mueller-Hinton agar plates and antibiotic disks were placed on the medium surface. Plates were incubated for 24 hours, at 37 ºC and antibiotic inhibition halos were measured. Six antibiotics from Oxoid were used: ciprofloxacin (CIP 5 μg), ticarcillin (TIC, 75 μg), tetracycline (TE, 30 μg), streptomycin (S, 10 μg), sulphamethoxazole/trimetropim (SXT, 25 μg) and amoxicillin (AML, 25 μg). These antibiotics were chosen according to the bacteria resistance phenotype previously determined [367].

4.3.5 CHROMATOGRAPHIC METHOD FOR CIPROFLOXACIN QUANTIFICATION

An Hitachi® High-Performance Liquid Chromatograph LaChrom Elite equipped with a HTA L-2130 quaternary pump; L-2300 column oven; L-2200 auto sampler; DAD L-2455 detector, LaChrom Elite series was used in the present work. Chromatographic collection and data treatment was performed by EZChrom Elite Software, Lachrom Elite series. A Purospher® STAR C18 chromatographic collumn was used, with 5 μm particle size and 4.6 x 250 mm internal dimensions.

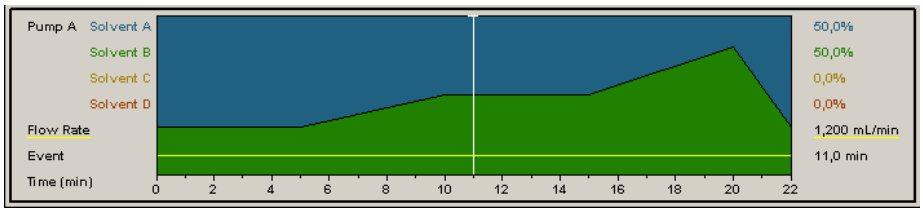
The procedure adopted for the quantification of CIP was adapted from that described by Kassab et al and Mostafavi *et al* [370, 371].

Analyses were performed with a column temperature of 40 ± 5 ºC under gradient conditions, according to the scheme shown below (

Table 4.1). A gradient mixture of KH_2PO_4 0.01% / methanol (Merck, Germany) was used as eluent. The pH of the aquose eluent was adjusted to pH = 3.0 with *o*-phosphoric acid (Merck, Germany). The flow rate was 1.2 mL/min and injection volume of 50.0 μL. Autosampler tray was maintained at 4 ºC. Mobile phase solutions were filtered and degasified; sterile syringe filter with cellulose acetate 0.45 μm (VWR, Portugal) were used for standards. The wavelength selected for the detection based on the absorption spectrum of the sample drawn was 276 nm. The column used was a Purospher® STAR C18 chromatographic collumn, with 5 μm particle size and 4.6 x 250 mm internal dimensions.

Table 4.1: Chromatographic method for CIP determination

Time (min)	% KH_2PO_4 0.01 M	% MeOH	Flow rate ($mL.min^{-1}$)
0.0	70	30	1.2
5.0	70	30	1.2
10.0	50	50	1.2
15.0	50	50	1.2
20.0	20	80	1.2
22.0	70	30	1.2



A stock solution of CIP (Sigma-Aldrich, Co., USA) was prepared by weighing 1.6 mg of antibiotic, diluted in mobile phase to a volume of 3.0 mL ([CIP] = 0.53 mg/mL). Five standards with concentrations ranging from 533.00 to 5.33 µg/mL were prepared from aliquots from stock solution. To obtain a calibration curve each standard was injected three times and the corresponding average area corresponding standard solution was plotted against to the respective concentration.

Samples were filtered with Pall Life Sciences® syringe filters (GHP Acrodisc 13 Ø Syringe filter 0,45 µm GHP membrane; GHPolypro 47 Ø 0,45 µm hydrophilic Polypropylene Membrane filters), before injection and then transferred to 2 mL vials. 50.00 µL of each sample was injected in triplicate.

4.3.6 STATISTICAL METHODS

To assess the distribution of the population, in order to decide whether a parametric or a nonparametric test should be used, the Shapiro-Wilk test was used. For different strains, in the same conditions, t-Student test was used when possible (normality situations) and Mann-Whitney test when normality was not observed. To evaluate the same strain in different conditions (with and without CIP), Wilcoxon test was applied (SPSS 19.0 for Windows) at a significance level (p) of 0.05.

4.4 RESULTS

4.4.1 AGITATED MICROCOSM ASSAYS

[CIP] of 2.00 mg/L, 0.50 mg/L, 0.10 mg/L and 0.05 mg/L was tested for only one strain/flask assays. The survival intervals observed are as presented in Table 4.2.

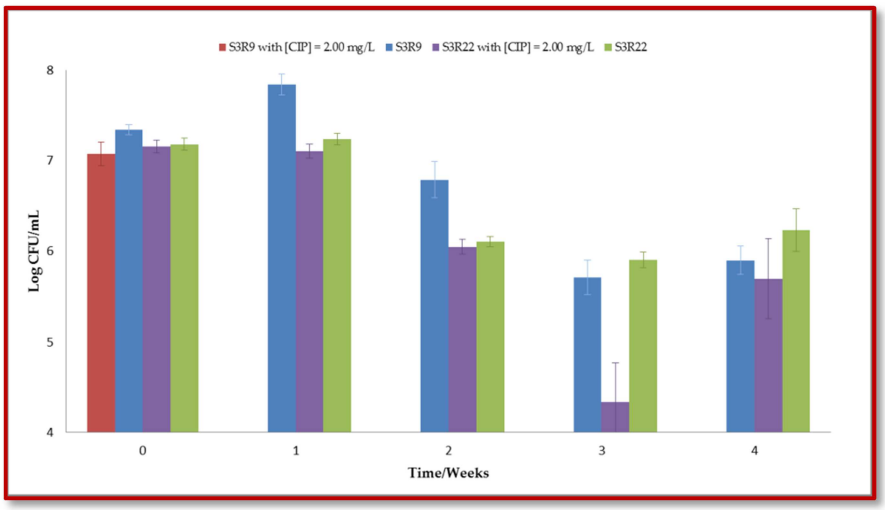
Table 4.2: Survival of *E. coli* strains S3R9 and S3R22 in water spiked with different concentrations of ciprofloxacin, monitored weekly

	Survival (weeks)*	
[CIP] / mg/L	*E. coli* S3R9	*E. coli* S3R22
2.00	< 1	4 < 6
0.50	< 1	6 < 10
0.10	< 1	6 < 10
0.05	5 < 6	9 < 11

*The detection limit was of 50 CFU/mL

The initial CIP concentration was 2.00 mg/L, but the susceptible S3R9 strain died before 7 days (Figure 4.3). [CIP] = 0.50 mg/L, 0.10 mg/L and 0.05 mg/L were tested in order to allow the survival of susceptible strain enough time to evaluate bacterial dynamics.

E. coli S3R9 was susceptible to CIP concentrations ranging from 2.00 to 0.10 mg/L and CFU countings were not possible at t = 7 days. *E. coli* S3R22 presented longer survival with lower CIP concentrations (Table 4.2).



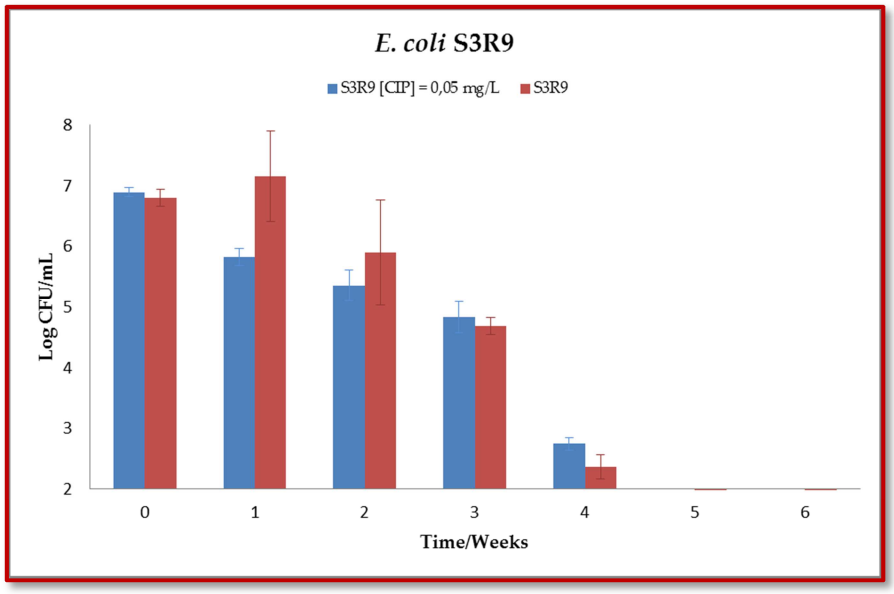


Figure 4.3: *E. coli* S3R9 and S3R22 behavior along time: top graph – [CIP] = 2.00 mg/L. The susceptible strain dies before 7 days of exposure; lower graph – *E. coli* S3R9 CFU countings for 4 weeks, under a [CIP] = 0.05 mg/L.

When conditions to allow *E. coli* S3R9 strain survival over 14 days were established ([CIP] = 0.05 mg/L), mixtures of two strains were monitored. Statistical differences were found between initial and final time counting per strain, but there was no statistical differences between strains' behavior at each time.

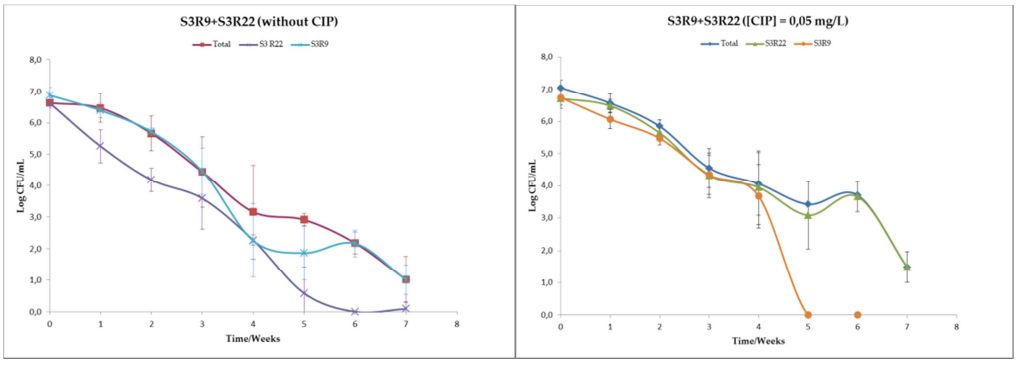


Figure 4.4: Log CFU/mL for mixtures of *E. coli* S3R9 and S3R22, with (right) and without (left) CIP.

It was observed that in a mixture without CIP, the susceptible strain lived longer than the resistant one, but this behavior was not observed in the mixture with CIP (Figure 4.4). Statistical analysis evidenced differences between the CIP resistant *E. coli* S3R22 behavior with and without the presence of the antibiotic. It was also observed that the CIP susceptible *E.coli* S3R9, when in the presence of CIP, lived less time than the resistant *E.coli* S3R22 (Figure 4.4). The results reflect two independent experiments under the same conditions.

Statisticaly significant differences where observed between *E.coli* S3R22 in the presence of CIP and both *E.coli* S3R9 and *E.coli* S3R22 behavior without CIP.

4.4.2 MULTIWELLS ASSAYS

Mixtures of two *E. coli* strains (a susceptible and a resistant to CIP) were monitored for 56 days. This experiment was repeated and the results are presented in Table 4.3. Isolation of the CIP susceptible strain for CFU countings was not possible for all mixtures due to their similarity. In Table 4.3 are represented the ratios observed for log CFU/mL between $t = 0$ days and $t = 21$ days.

Table 4.3: Log CFU/mL (t = 21)/ Log CFU/mL (t = 0) days for mixtures of *E. coli* S3R9 and CIP resistant *E. coli* strains in the presence and absence of CIP

Strain S3R9 with	**With [CIP] = 0.05 mg/L**		**Without CIP**	
	log CFU (tf) / log CFU (ti)		**log CFU (tf) / log CFU (ti)**	
	Mixture	**Resistant strain**	**Mixture**	**Resistant strain**
M3Ec1	0,8225	1,013	0,9549	0,7709
H1Fc2	1,021	1,013	0,8538	0,9971
A1Fc22	1,144	0,9243	1,148	1,167
S3R22	0,9517	0,9007	0,9278	0

As observed in the previous assays, the CIP resistant *E. coli* S3R22 is not measurable at t = 21 days, when in a mixture with *E. coli* S3R9 and without CIP, but is present in the similar mixture with CIP until t = 56 days. This performance may suggest some kind of interaction among the two strains, or the activation of some mechanism in *E. coli* S3R22, when CIP is available.

Statistical analysis of the data revealed that:

a) the CFU at final time are different from CFU at initial time for all mixtures and single resistant strains, which is an expected result since there are no available nutrients and a decrease in log CFU/mL is visible;

b) at t = 56 days, there are differences in CFU values between mixtures with and without CIP, for all pairs of strains, suggesting different behaviors in the presence and absence of selective pressure;

c) CIP resistant strains present different behavior in the presence of CIP, but similar in the absence of CIP, indicating a possible effect of CIP presence in the dynamics of the bacterial population.

4.4.3 BACTERIAL STABILITY

The RAPD profiles were analyzed at time zero and every 7 days, for flask assays, until both strains were not visible on PCA plate drops. For multiwells assays RAPD profiles were analyzed at t = 0; t = 21 days; t = 56 days. The authenticity of the atrains studied along time was confirmed for all the assays executed. As an example, RADP for CIP resistant *E. coli* H1Fc22 is presented in Figure 4.5.

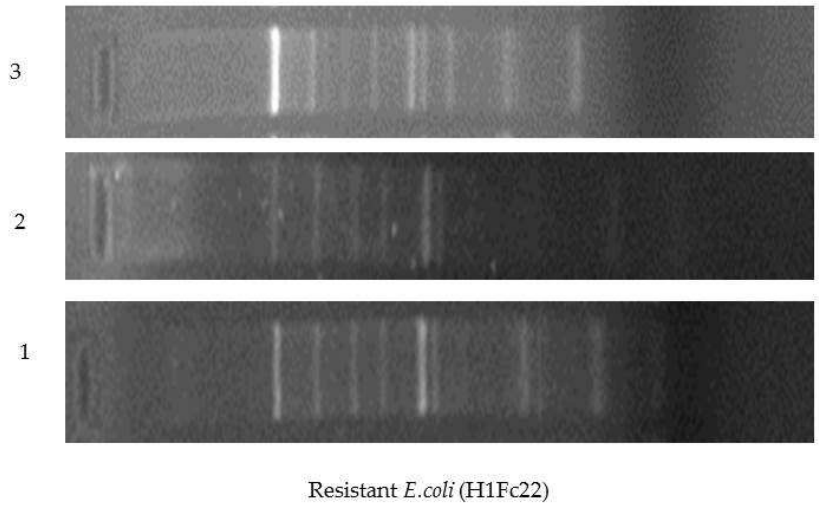


Figure 4.5: RAPD profile for *E.coli* CIP resitant strain (H1Fc22) at [CIP] = 0.05 mg/L, at: 1) 0 days, 2) 56 days, 3) freshly unfrozen cells.

4.4.4 RESISTANCE PHENOTYPES

No differences were found in the resistance phenotype for the bacterial strains used in the present work, under the conditions tested (time of exposure, CIP

concentration, mixture of bacteria, experimental vessel: Erlenmeyer and microplate wells, volume, temperature).

4.4.5 CIP CONCENTRATION ALONG TIME

The chromatographic method developed showed appropriate for the CIP quantifications. Several improvements were experimented and the outcome is summarized in 4.3.5.

The calibration curve was assessed by a series of four standard CIP solutions ranging from 533 μg/mL to 0.533 μg/mL (Figure 4.6).

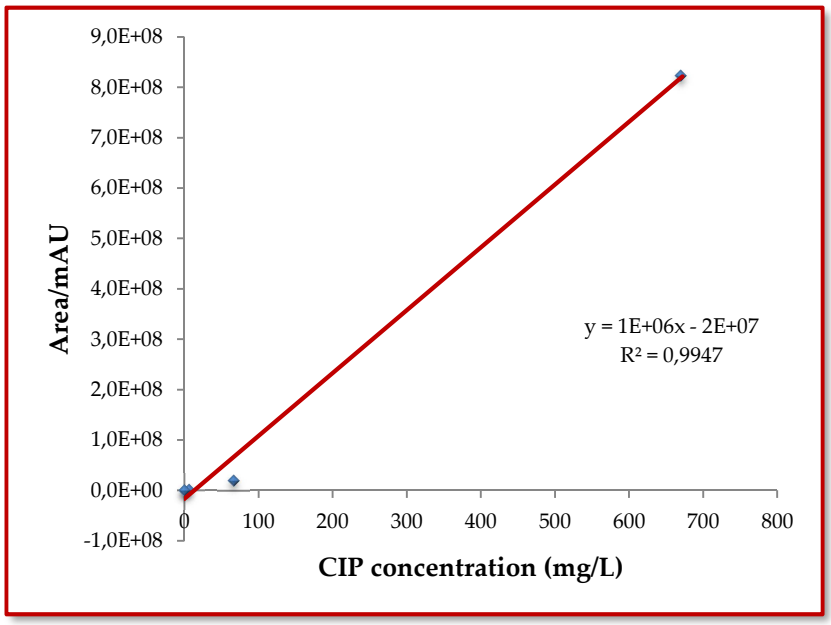


Figure 4.6: Calibration curve for CIP quantification by HPLC-DAD.

The limit of detection (LD) and the limit of quantification (LQ) were determined by the mathematical equations:

$$LD = 3.3 \times (s/S) \text{ and } LQ = 10 \times (s/S)$$

where **s** is the standard deviation of the response (blanks; n = 10) and **S** is the analytical curve slope (calibration curve). For the developed method, LD = 0.13 ng/L and LQ = 0.39 ng/L.

Only the flask assays were tested for CIP concentration along time. The volume used in the multiwells assays did not allow this verification.

For the assay with [CIP] = 0.05 mg/L, which was the concentration tested in both methodologies, there were no significant variations on the CIP concentration, for the period tested (6 weeks). These results may indicate that bacteria aren't using CIP as a carbon source, in nutrients absence.

Table 4.4: CIP quantification at t = 0 and t = 6 weeks

t = 0	**Area/mAU**	**[CIP]/mg/L**	**t = 6**	**Area/mAU**	**[CIP]/mg/L**
REP1	71900	0,0201	REP1	518585	0,0205
REP2	446823	0,0204	REP2	520506	0,0206
REP3	554881	0,0206	REP3	474334	0,0205
REP4	230124	0,0202	REP4	238031	0,0202
REP5	396922	0,0204	REP5	272845	0,0203
Mean	**0,02034 ± 0,000174**		**Mean**	**0,02042 ± 0,000147**	
	CV/%	**0,86**		**CV/%**	**0,72**

The differences observed between the CIP concentration in the flask (0.05 mg/L) and the mean value obtained by chromatographic quantification (0.02 mg/L) may be

related to the sample treatment. Nontheless, the observed [CIP] for initial and final times is not significantly different, indicating that [CIP] maintains along time, pointing to selective pressure preservation along time.

4.5 DISCUSSION/CONCLUSION

The studies on the survival of *E. coli* resistant and susceptible to ciprofloxacin strains performed suggest influence of sub-inhibitory concentrations of this antimicrobial agent in bacterial populations.

The results obtained for the agitated microcosm assays reveal the elimination of *E. coli* susceptible strain, in a short period of time (< 7 days) in the sub-inhibitory concentrations of CIP tested (2.00, 0.50 and 0.10 mg/L). The survival of this strain, for a period longer than 6 weeks, was verified only at [CIP] = 0.05 mg/L. At a CIP concentration of 0.05 mg/L, there are no significant differences between *E. coli* S3R22 resistant and *E. coli* S3R9 susceptible strain when tested in separate. On the other hand, in a mixture of these two strains, results indicate a longer survival of *E. coli* susceptible strain, when compared with the resistant strain *E. coli* S3R22, in the abcense of CIP and the oposite behavior in the presence of CIP.

On the multiwells assays, it was observed, again, a longer survival performance for *E. coli* S3R9 then *E. coli* S3R22, in the absence of CIP. This behavior was not verified for the other *E. coli* S3R9/resistant strain mixtures studied, indicating a possible particular interaction between these two strains. The results obtained also point to an influence on the bacterial populations studied, along time, by the presence of CIP once it was observed a significative difference in all the studied mixtures of *E. coli* strains between assays with and without CIP. Also, resistant strains demonstrated a similar behavior without the presence of CIP, but significant differences were observed when the antimicrobial agent was present.

The resistance phenotype of all the tested strains, under all the variables studied, was maintained, which indicates no genetic alterations or genetic information exchanging between the bacteria analyzed.

In conclusion, the present study suggests that the presence of CIP, even in sub-inhibitory concentrations, has influence on bacterial populations' performance. The bacterial populations' equilibrium is unequally perturbed by the presence of this antimicrobial agent.

The experimental design of this study only considers some of the vast variables present in natural environments. Further studies are needed in order to introduce other important factors, as antimicrobial agent class, nutrients availability and bacterial strains.

Chapter 5: Antimicrobial activity of quinoxaline derivatives

5 ANTIMICROBIAL ACTIVITY OF QUINOXALINE DERIVATIVES

5.1 OBJECTIVES

The main objectives in this chapter were to determine the antibacterial and antifungal activity for eight quinoxaline derivatives, using microbiological techniques.

Microbiological studies were performed and the corresponding results were submitted to an international journal. Publication of the full article was accepted on July, 17th 2013 and on line publication was finished on August, 6th 2013. This chapter corresponds to a textual transcription of that work.

Online publication complete: 6-AUG-2013

DOI information: 10.1016/j.micres.2013.06.015

5.2 ABSTRACT

Quinoxaline is a chemical compound that presents a structure that is similar to quinolone antibiotics. The present work reports the study of the antimicrobial activity of quinoxaline *N,N*-dioxide and some derivatives against bacterial and yeast strains. The compounds studied were quinoxaline-1,4-dioxide (QNX), 2-methylquinoxaline-1,4-dioxide (2MQNX), 2-methyl-3-Benzoylquinoxaline-1,4-dioxide (2M3BenzoylQNX), 2-methyl-3-benzylquinoxaline-1,4-dioxide (2M3BQNX), 2-amino-3-cyanoquinoxaline-1,4-dioxide (2A3CQNX), 3-methyl-2-quinoxalinecarboxamide-1,4-dioxide (3M2QNXC), 2-hydroxyphenazine-*N,N*-dioxide (2HF) and 3-methyl-*N*-(2-methylphenyl)quinoxalinecarboxamide-1,4-dioxide (3MN(2MF)QNXC). The prokaryotic strains used were *Staphylococcus aureus* ATCC 6538, *S. aureus* ATCC 6538P, *S. aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *E. coli* S3R9, *E. coli* S3R22, *E. coli* TEM-1 CTX-M9, *E. coli* TEM-1, *E. coli* AmpC Mox-2, *E. coli* CTX-M2 e *E. coli* CTX-M9. The *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* PYCC 4072 were used as eukaryotic strains. For the compounds that presented activity using the disk diffusion method, the minimum inhibitory concentration (MIC) was determined. The alterations of cellular viability were evaluated in a time-course assay. Death curves for bacteria and growth curves for *S. cerevisiae* PYCC 4072 were also accessed. The results obtained suggest potential new drugs for antimicrobial activity chemotherapy since the MIC's determined present low values and cellular viability tests show the complete elimination of the bacterial strain. Also, the cellular viability tests for the eukaryotic model, *S. cerevisiae*, indicate low toxicity for the compounds tested.

Keywords: Antimicrobial activity, quinoxaline *N,N*-dioxide derivatives, minimum inhibitory concentration, cellular viability

5.3 INTRODUCTION

Antimicrobial agents are largely used in treatment and prevention of microorganism infections. Among others, the misuse and, especially, the abusive use of this kind of drugs, in human health, veterinary and animal production, led to the development of drug-resistant and multidrug-resistant (MDR) microorganisms [372, 373]. In addition, the permanent contact with some antimicrobial drugs, besides the resistance development, allows the increase of allergies and respiratory complications which are affecting the human population worldwide [197, 374-376]. Resistant bacteria are increasing and the interval between the appearances of new and multi-drug resistant species is happening in short periods of time [377]. These conditions are becoming emergent public health issues in the sense that they compromise pharmacological activity and the efficacy of these antimicrobial agents [378, 379] and thus the heath of the population.

Because MDR bacteria are increasing worldwide human kind deals with the urgent need of development of new drugs with enhanced antimicrobial activity able to fight pathogens with no adverse effects [104]. It is also expected to develop drugs that can reverse the resistance observed overturning the actual bacterial profile. Some approaches have been developed in order to evaluate the bioactivity of numerous compound families against several strains of microorganisms [380, 381].

Quinoxaline is an organic heterocyclic compound that has been used as base of synthesis of bioactive derivatives and several investigation groups have demonstrated their potential in medical and pharmacological applications [203, 211, 382]. These studies point to chemotherapeutical interests regarding the anti-tumor, anti-bacterial, anti-fungal, and anti-viral including anti-HIV [383-386] applications of these

compounds. Relevant bioactivity has been reported in *Mycobacterium* spp. strains [211]. No studies were found reporting biological activity for the quinoxaline derivatives in the present study with the microbial strains used.

The quinoxaline derivatives with *N*-oxide and *N,N*-dioxide have particular interest since they present relevant anti-oxidant activity. Many compounds with nitrogen-oxygen bonds play important biological roles by releasing NO groups or by cellular deoxygenating [387, 388].

The present study pretends to be a contribution to the characterization of antibacterial and antifungal activity some *N,N*-quinoxaline derivatives.

The activity of these compounds was tested against bacteria and yeast in order to understand the biological activity in both eukaryotic and prokaryotic microbial models. In the present work Saccharomyces *cerevisae* and *Candida albicans* were used as representative models of eukaryotic microorganisms. Likewise, several strains of *Staphylococcus aureus* and *Escherichia coli* were used as prokaryotic representative models of Gram-positive and Gram-negative respectively.

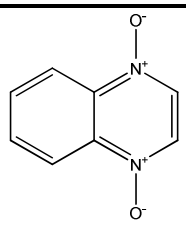
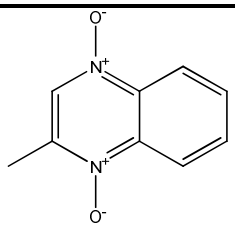
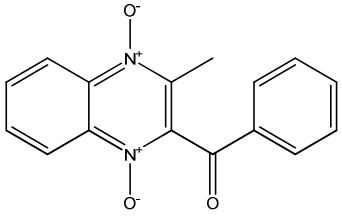
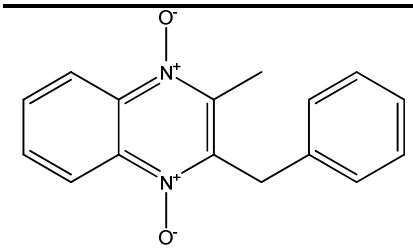
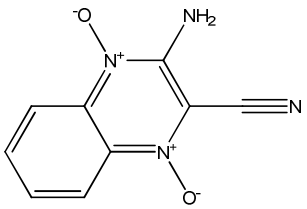
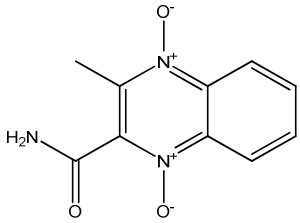
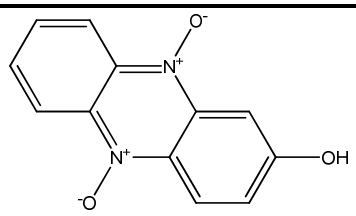
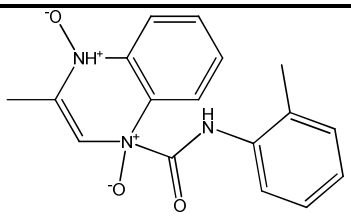
5.4 MATERIAL AND METHODS

5.4.1 QUINOXALINE *N,N*-DIOXIDE AND QUINOXALINE DERIVATIVES

The compounds used in the present study were previously used by some of our collaborators and were gently provided by the Center of Investigation in Chemistry of the University of Porto. Synthesis, spectra and thermochemical properties were already studied for the quinoxaline derivatives used in the present study (Table 5.1) [389-392]

Stock solutions of the compounds were prepared in a 500 mL volume at a 500 μg/L final concentration. Since the compounds are thermally stable, the solutions were sterilized in an autoclave (AJC Uniclave 88) for 20 minutes at 121ºC. From these solutions, standards were prepared at the final concentrations 500, 100, 50, 20, and 5 μg/L.

Table 5.1: Quinoxaline *N,N*-dioxide and quinoxaline derivatives.

Quinoxaline-1,4-dioxide (QNX)	2-methylquinoxaline-1,4-dioxide (2MQNX)	2-methyl-3-benzoylquinoxaline-1,4-dioxide (2M3BenzoilQNX)
		
2-methyl-3-benzylquinoxaline-1,4-dioxide (2M3BQNX)	2-amino-3-cyanoquinoxaline-1,4-dioxide (2A3CQNX)	3-methyl-2-quinoxalinecarboxamide-1,4-dioxide (3M2QNXC)
		
2-hydroxiphenazine-*N*-dioxide (2HF)		3-methyl-N-(2-methylphenyl)quinoxalinecarboxamide-1,4-dioxide (3MN(2MF)QNXC)
		

5.4.2 BACTERIAL STRAINS

The strains used in this study were stored deep frozen at -80 ºC. The selected strains, in order to evaluate the susceptibility of a bacterial cell model to the proposed compounds, included *S. aureus* ATCC 6538, *S. aureus* ATCC 6538P, *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *E. coli* S3R9 and *E. coli* S3R22 (a penicillin resistant strain and a multidrug resistant strain, respectively). It was included some *E. coli* strains harbouring broad spectrum β-lactamase TEM-1 and extended spectrum β-lactamases (ESBL) such as TEM-1 + CTX-M9, CTX-M2, CTX-M9 and the AmpC β-lactamase MOX-2.

5.4.3 YEAST STRAINS

The strains used in this study were *S. cerevisiae* PYCC 4072 (UNL, Portugal) and *C. albicans* ATCC 10231 and were also stored deep-frozen at -80 ºC.

5.4.4 MICROORGANISMS CULTURE AND ZONE INHIBITION

In order to assess the potential microbial activity of the compounds presented, disk diffusion method was used with two purposes. The first one was to determine the sensibility of the strains to known antibiotics, cefoxitin (FOX) and ciprofloxacin (CIP). The second was to assess the inhibition zone for new compounds. Bacteria and yeast cells were sub-cultured in broth agar (Tryptic Soy Broth - TSB) and incubated for 24

hours at 37 ºC. Freshly prepared bacterial cells were transferred into a saline solution (NaCl 0.9%, Carlo Erba Reactifs, France) and density was settled in the interval 0.09 and 0.10, corresponding to 0.5 McFarland (1 to 2 x 10^8 CFU/mL). The density of the solutions was measured at 625 nm using a spectrophotometer (Thermo Scientific Genesys 20). Solutions were spread onto a Mueller Hinton (MH; Cultimed, Spain) nutrient plate in a laminar flow cabinet. Yeast strains were spread onto Yeast Extract Peptone Dextrose (YEPD; Oxoid, Basingstoke, UK) nutrient plate, in laminar flow cabinet. Blank sterile disks were immersed in the standard solutions, with the final concentration of 500, 100, 50, 20, and 5 μg/L for each compound. Plates were incubated for 24 hours at 37 ºC and zone inhibition diameters were measured in millimetres. Each one of these bacteria was tested with cefoxitin disks (FOX) 30 μg and ciprofloxacin (CIP) 5 μg. Cefoxitin is a β-lactam and ciprofloxacin is a quinolone that has a similar structure of quinoxaline. The compounds studied have no established reference values regarding the sensitive/resistant behavior for the quinoxaline derivatives, so microdilution method was employed in order to determine de minimum inhibitory concentration. Strains studied were classified (Table 5.2) as Susceptible (S) or Resistant (R) according to Clinical Laboratory Standard Institute (CLSI) guidelines, by disk diffusion, considering the values of CLSI to the β-lactam and quinolone used in the present study. All results were confirmed by replica.

5.4.5 MINIMUM INHIBITORY ASSAYS

The minimum inhibitory concentration (MIC) for each quinoxaline derivative was estimated using the microdilution method, according to the CLSI [393-395]. The MIC's were determined for each strain/compound pairs that presented antimicrobial activity.

The microplates used consisted of 96 wells. The TSB was dispensed into several wells, 8 for each chemical compound. One of these 8 wells corresponded to the positive control (containing the culture medium and bacterial suspension) and another to the negative control (containing only the culture medium). The remaining wells were used to prepare volumetrically diluted in series from stock solution (1:1, 1:2, 1:4, 1:8, 1:16 and 1:32) for each compound and considering the concentrations that presented inhibition halo. Fresh prepared cultures of the microorganisms were suspended in a saline solution to a density of 0.100 at 625 nm. Each well was prepared to a final volume of 200 μL. The microplates were closed and incubated at 37 ºC, for 16 h to 20 h. The presence or absence of turbidity was verified and rechecked by inoculating a fraction of the wells in solid culture medium (Trypticase Soy Agar, TSA, Cultimed, Spain). The plates were incubated for 24 h at 37 ºC. The results obtained in the wells and plates were compared. All results were confirmed by replica.

5.4.6 CELLULAR VIABILITY OF BACTERIA

In order to evaluate the growing or death performance cellular viability was analyzed for all strain/quinoxaline derivative pairs that presented growth inhibition and for which MIC's were determined. Microbial suspensions were prepared at 0.5 McFarland density with TSB medium for bacterial strains. The solutions of the studied compounds were colored and the optical density (OD) measurements lack accuracy. In alternative to the construction of cellular viability and death curves by platting was observed in a time-course assay. For 24 hours, the presence of viable cells was tested and CFU/mL was determined. The time intervals were divided as presented in Table 5.3. A standard tube containing the studied strain and growing media was used as control and a test tube containing also the quinoxaline derivative in the MIC concentration determined previously was used as working solution. For CFU/mL

counting, optimum dilution was determined by serial dilutions with 10^{-1} factor, in saline solution, adding 1/10 of strain media and 9/10 of saline solution (Table 5.3). All results were confirmed by replica.

5.4.7 CELLULAR VIABILITY FOR EUKARYOTIC MODEL

The aim of this method was to evaluate the cellular viability effects of compounds that presented antibacterial activity on eukaryotic cells. The procedure was the same as described for bacteria, but the final concentration of compounds was 500 μg/L, corresponding to the highest MIC determined previously. Along 24 hours, CFU/mL was determined if viable cells were present. The time intervals were divided as presented in Table 5.3. All results were confirmed by replica.

5.5 RESULTS AND DISCUSSION

5.5.1 DISK DIFFUSION METHOD

The quinoxaline derivative compounds studied have no reference values for the disk diffusion method, according to CSLI. Nevertheless, CLSI guidelines were used for known antibiotics. For the results obtained by this method, the absence of inhibition zone for the compounds tested were considered an indicative of no antimicrobial activity against that strain. Table 5.4 shows antimicrobial activity results for each bacterial or yeast strain and each compound tested. All the compounds studied

showed no antimicrobial activity in the Gram-positive prokaryotic strains and eukaryotic strains since no inhibition zone was observed. On the other hand, the compounds only presented antimicrobial activity in Gram-negative prokaryotic strains, except for *E. coli* CTX-M2 and *E. coli* CTX-M9. The compound 2A3CQNX presented activity in four Gram-negative strains (*E. coli* ATCC 25922, *E. coli* S3R9, *E. coli* S3R22 and *E.coli* TEM-1); whereas 3M2QNXC was the compound that presented activity in a greater number of strains studied, namely, *E. coli* ATCC 25922, *E. coli* S3R9, *E. coli* S3R22, *E. coli* TEM-1 and *E. coli* AmpC MOX-2. The other compounds, QNX, 2MQNX and 2HF only presented activity in *E. coli* TEM-1. Several studies refer selective antibacterial activity. Meanwhile, others reveal activity in both Gram-positive and Gram-negative cells [396-398]. The results presented in Table 5.5 seem to indicate that *E. coli* S3R22 and *E.coli* TEM CTX-M9 are resistant to ciprofloxacin (5 μg) and *E. coli* AmpC MOX-2 is resistant to cefoxitin (30 μg). The other strains, *S. aureus* and *E. coli*, seem to be sensitive to both antibiotics.

5.5.2 MINIMUM INHIBITORY CONCENTRATION

For the compounds that presented activity using the disk diffusion method, minimum inhibitory concentration (MIC) was determined and presented in Table 5.6. These results can be compared with MIC values previously determined for cefoxitin and ciprofloxacin and presented in Table 5.2 [393]. The results are presented for each well, bacteria, growth medium and different concentrations of each compound. The results were validated with both negative (compound and growth medium that presented no turbidity) and positive controls (bacteria and growth medium that presented turbidity). The MIC values presented are smaller than those indicated by the CSLI to CIP and FOX, suggesting a more effective activity of quinoxaline derivatives with respect to those antibiotics.

Table 5.2: Zone diameter interpretative Standards (*Enterobacteriacceae*) and equivalent Minimal Inhibitory Concentration (MIC) according to CLSI document [394].

	Zone diameter Interpretative Standards (mm)			MIC (μg/mL)	
	R	**I**	**S**	**R**	**S**
Ciprofloxacin (CIP) (5 μg)	15	16-20	≥21	≥ 4	≤ 1
Cefoxitin (FOX) (30 μg)	14	15-17	≥18	≥ 32	≤ 8

R – Resistant

I – Intermediate

S - Susceptible

Table 5.3: Dilution factor and time intervals for UFC/mL determinations.

	Optimum dilution factor	Time for countings/min							
Bacteria	10^{-5}	0	30	60	90	120	180	1440	
Yeast	10^{-3}	0	30	60	90	120	180	210	440

Table 5.4:Disc diffusion diameters for the quinoxaline derivatives.

Compound / 500 ug/L / Strain	QNX	2MQNX	2M3BenzoilQNX	2M3BQNX	2A3CQNX	3M2QNXC	2HF	3MN(2MF)QNXC
	Inhibition Halo /mm							
S.aureus ATCC 6538								
S.aureus ATCC 6538P					0			
S.aureus ATCC 29213								
E.coli ATCC 25922	0	0	0	0	11	24	0	0
E.coli S3R9	0	0	0	0	16	26	0	0
E.coli S3R22	0	0	0	0	24	26	0	0
E.coli TEM-1 CTX M9	0	0	0	0	0	14	0	0
E.coli TEM-1	24	16	0	0	16	26	14	0
E.coli AmpC MOX-2	0	0	0	0	0	15	0	0
E.coli CTX M2								
E.coli CTX M9					0			
S.cerevisiae PYCC 4072								
C.albicans ATCC 10231					0			

Table 5.5: Classification of each strain as Susceptible (S) or Resistant (R) according to CLSI in presence of Cefoxitin disk (FOX) and Ciprofloxacin disk (CIP) by Disk Diffusion Method [394].

Antibiotic / **Strain**	**Cefoxitin (30 μg)**	**Ciprofloxacin (5 μg)**
S.aureus ATCC 6538	S	S
S.aureus ATCC 6538P	R	S
S.aureus ATCC 29213	S	S
***E.coli* ATCC 25922**	S	S
***E.coli* S3R9**	S	S
***E.coli* S3R22**	S	R
***E.coli* TEM CTX-M9**	S	R
***E.coli* TEM-1**	S	S
***E.coli* AmpC MOX-2**	R	S

S. aureus

S - Sensitive(CIP: zone inhibition ≥21 mm); (FOX: zone inhibition ≥22 mm)

R - Resistant (CIP: zone inhibition ≤15 mm); (FOX: zone inhibition <21 mm)

E. coli

S - Sensitive (CIP: zone inhibition ≥21 mm); (FOX: zone inhibition ≥18 mm)

R - Resistant (CIP: zone inhibition <15 mm); (FOX: zone inhibition <14 mm)

Table 5.6: Minimum inhibitory concentration (μg/L) results for each group Bacterial Strain/Compound by microdilution method, CLSI [394].

Strain	Compound	Minimum inhibitory concentration (μg/L)
***E.coli* ATCC 25922**	2A3CQNX	500
	3M2QNXC	350
***E.coli* S3R9**	2A3CQNX	320
	3M2QNXC	125
***E.coli* S3R22**	2A3CQNX	320
	3M2QNXC	200
***E.coli* TEM CTX-M9**	3M2QNXC	125
***E.coli* TEM-1**	QNX	256
	2MQNX	400
	2A3CQNX	100
	3M2QNXC	80
	2HF	329
***E.coli* AmpC MOX-2**	3M2QNXC	100

5.5.3 CELLULAR VIABILITY AND CFU VARIATION FOR PROKARYOTIC CELLS

Cellular viability was analyzed for all bacterial strain/quinoxaline derivative pairs that presented growth inhibition by disk diffusion method and for which MIC's were previously determined. Figure 5.1 shows an example of CFU/mL variation along time for Gram-negative prokaryotic strain (*E. coli* TEM-1). The results obtained show that every strain, in absence of any compound, grows up in the first minutes (viable cells), as expected [399-401] and then decreases the number of CFU/mL until 24 h. For

each bacterial strain/quinoxaline derivative pairs, the number of viable cells decreases with time, as expected, and at 24 h there are no viable cells. For *E. coli* TEM-1/ 2MQNX group the Figure 5.1 shows that there are no viable cells at 180 minutes. CFU/mL variation for other bacterial strains/quinoxaline derivative pairs presented a similar behavior to *E. coli* in presence and absence of compounds.

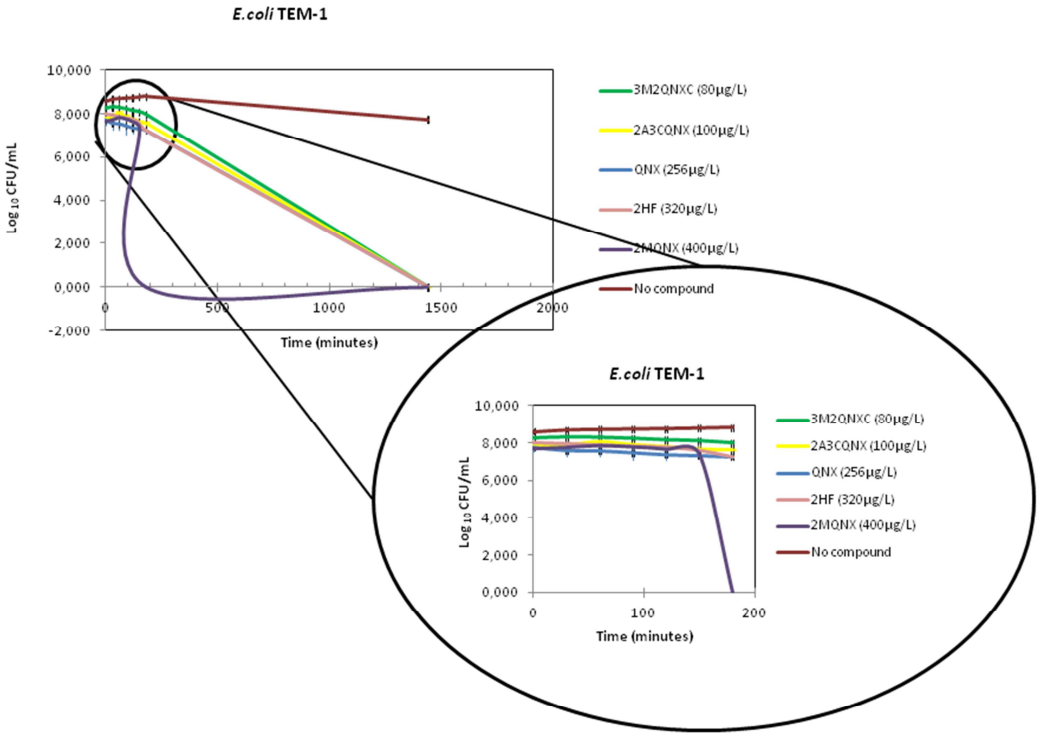


Figure 5.1: Example of variation for Gram negative prokaryotic strains (*E. coli* TEM-1) in the presence of quinoxaline derivative at MIC determined.

5.5.4 CELLULAR VIABILITY AND CFU VARIATION FOR EUKARYOTIC CELLS

Both eukaryotic strains, *C. albicans* and *S. cerevisiae*, grow up in the first minutes. This is followed by a decrease of cellular viability [402, 403]. By exposing *S. cerevisiae* to the compounds that presented antibacterial activity (QNX, 2MQNX, 3M2QNXC, 2A3CQNX, 2HF), the number of viable cells calculated by CFU increases in the time-course, as observed in Figure 5.2. These results seem to indicate that, in the presence of these quinoxaline derivatives, *S. cerevisiae* has a growthrate that increases. Surprisingly, all the quinoxaline derivatives studied showed no effect on growth or cell viability, when compared with the control. Actually, growth enhanced rather than declined. Naturally, we do not have at this point of the state of the art any explanation for these results that we intend to clarify in further studies.

Nevertheless, it seems promising that compounds that may be potential antimicrobial agents used in humans or veterinary, may not be toxic in eukaryotic cells.

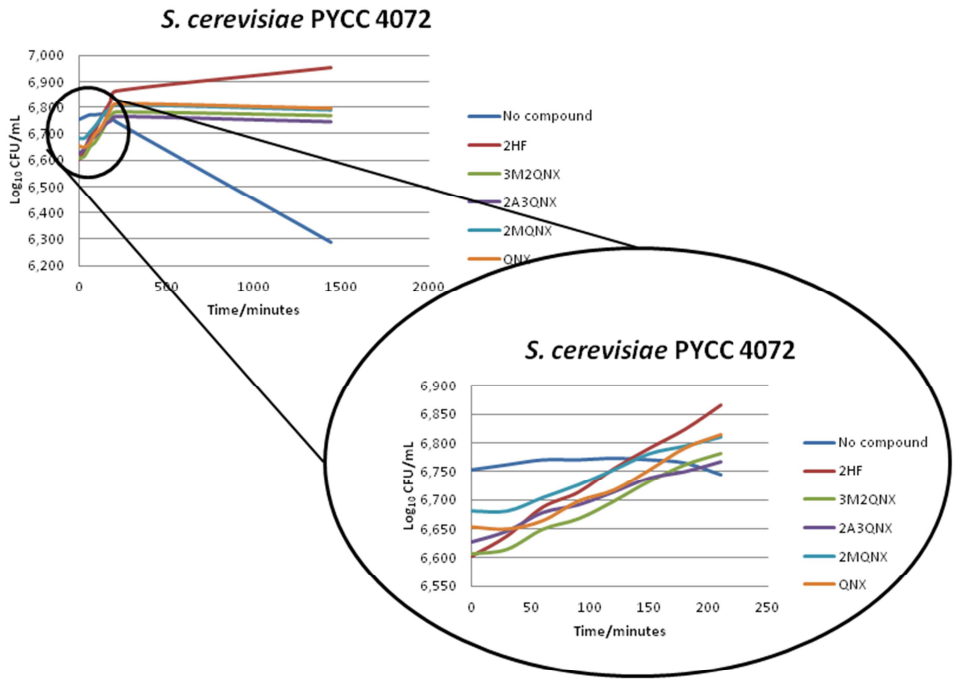


Figure 5.2: Variation along time for *S. cerevisiae* in absence/presence of the compounds tested.

5.6 CONCLUSIONS

Quinoxaline derivative compounds studied in the present work presented selective antimicrobial activity between both eukaryotic and prokaryotic strains. Antimicrobial activity was observed only in the Gram-negative strains studied, except in *E. coli* CTX-M2 and *E. coli* CTX-M9. We believe that these two strains may encode some kind of mechanism that confers resistance to this group of compound. No antimicrobial activity was observed for Gram-positive prokaryotic strains, possibly due to the presence of peptidoglycan in Gram-positive cell wall, permeability

properties of the membranes, or even due to metabolic dissimilarities between these two phylogenetic groups, that enables the entrance/activity of the compounds. This fact may also point to the need of these compounds to cross cellular walls to act. At the present time, there are no clues that might explain why some quinoxaline derivatives have a distinctive behavior between G+ and G- inhibition.

Moreover, we also have no data that supports why those quinoxaline derivatives which present antibacterial activity promote *S. cerevisae* growth. The mode of action of these quinoxaline derivatives is not clear yet. We intend to develop further studies in order to contribute to this elucidation. The results showed in the present study, should guide these work. We now know that they seem not to be effective in yeast and in gram positive strains. So, further studies on gram negative response will be conducted. Since the mechanism of action of these compounds is not known it is difficult at this point of the state of the art to discuss structure activity relationship based on cell viability studies.

With exception to 2M3BenzoylQNX, 3M3BQNX and 2MN(2MF)QNXC, that presented no activity against the strains studied, all the compounds presented activity against both quinolone and/or β-lactams Gram-negative resistant strains. The MIC values determined were between 80 and 500 μg/L, and are smaller than the admitted values for approved drugs with antibiotic activity (CIP and FOX) and with similar chemical constitution (CIP) (Table 5.2).

The cellular viability for the eukaryotic strain and the death rate for the prokaryotic strains studied, at MIC concentrations, suggest these compounds as potential new drugs with selective antibacterial activity for Gram-negative bacteria.

5.7 ACKNOWLEDGEMENTS

Thanks are due to Center of Health and Environmental Research (CISA) to assignment of Integration into Research Fellowship for performance this work, and Centre of Chemistry Investigation (CIQ) of the Chemistry Department, University of Porto, Portugal, for the supply of quinoxaline derivatives.

Funding: The authors thank Institute Polytechnic of Porto and Santander-Totta bank for a scholarship to CV and FCT-MES for a PhD grant SFRH/BD/48116/2008 to MV.

Chapter 6: Cell toxicity of quinoxaline derivatives

6 CELL TOXICITY OF QUINOXALINE DERIVATIVES

6.1 OBJECTIVES

Antimicrobial activity of quinoxaline derivatives 2HF, MQNX, QNX, 3M2QNXC and 2A3CQNX was observed previously (Chapter 6).

The main goal for the present chapter was to exploit biological/toxic effects of the quinoxaline derivatives with antimicrobial activity in eukaryotic cells to evaluate biological selectivity of these compounds.

The contents of this chapter has been submitted to *European Journal of Medicinal Chemistry* (Manuscript Number: EJMECH-S-13-02430) and is under review.

Bioactivity of quinoxaline 1,4-dioxide derivatives

Mónica Vieira, Joana Almeida, Pedro Coelho, Joana Costa, Marisa Coelho, João Costa-Rodrigues, Maria Helena Fernandes, João Paulo Noronha, Cristina Prudêncio, Rúben Fernandes

6.2 INTRODUCTION

Antibiotics were the great discovery of the 1920s and since then, the average life expectancy has increased. However, the use of antibiotics, not always in the most

accurate way, has led to the emergence of resistances. Currently, there is an urgent need to reinvent drugs for antimicrobial control.

6.2.1 ANTIBIOTIC CELLULAR EFFECTS OF ANTIBIOTICS

There are *in vitro* and *in vivo* studies revealing the proliferative and toxic effect of antibiotics [404, 405], namely β-lactams and quinolones, in both human and animal cell lines. The selectivity of bacterial cells to antibiotics is not evident and the effects on eukaryotic cells must be elucidated. There may be implications on tissue regeneration, as in orthopedic patients, or in tumor cells, that have altered metabolism [405] by exposure to xenobiotics. Eukariotic cell metabolic rate alteration is an important factor to consider when exploiting new therapeutics in order to prevent possible critical side effects.

6.2.2 QUINOLONES

Quinolone was discovered in the early 1960s, and is derived from quinine. Quinolone and its derivatives present an enormous clinical interest due to their antibiotic activity, as well as high potency, good bioavailability and broad-spectrum activity [406]. The first quinolone to be developed was the nalidixic acid, followed by flumequin, norfloxacin and enoxacin. There are two major groups of quinolone antibiotics: the quinolones and the naphthyridones. The two structures differ from each other in the position of nitrogen atoms in the two rings. The quinolones were used

essentially in the treatment of urinary tract infections and were neglected until the 1970s and 1980s, when fluoroquinolones were developed [407, 408].

Fluoroquinolones are broad-spectrum antibiotics, with particular activity against gram-negative organisms. The use of this type of antibiotics is not recommended in patients below the age of 18 years or in pregnant or breast-feeding. This recommendation relates to the fact that some animal studies indicate that fluoroquinolones interfere in the development of the bone, tendons, cartilage and even muscle [409, 410].

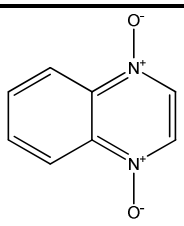
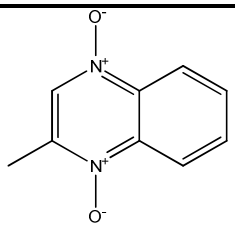
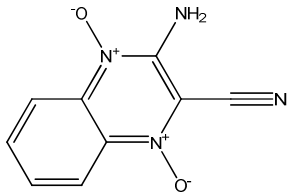
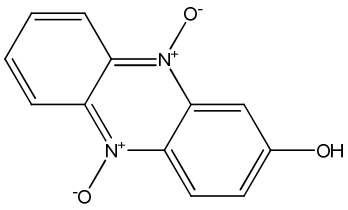
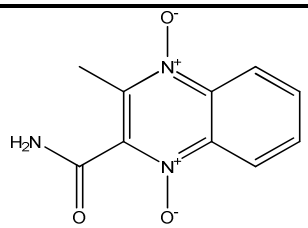
6.2.3 QUINOXALINE DERIVATIVES

Several research groups have demonstrated the potential of quinoxaline bioactive derivatives in medical and pharmacological applications [203, 211, 382].

The quinoxaline derivatives with *N*-oxide and *N*,*N*-dioxide have particular interest since they present relevant anti-oxidant activity. Many compounds with nitrogen-oxygen bonds play important biological roles by releasing NO groups or by cellular deoxygenating [208].

In the present study, five quinoxaline derivatives that demonstrated antimicrobial activity against gram negative strains [411], were tested in seven different cell lines, in order to determine their toxicity/proliferative effect. The structural similarity of quinoxaline and quinolone derivatives led us to investigate the toxicity of these new compounds in cell lines, in order to evaluate their potential applicability.

Table 6.1: Quinoxaline *N,N*-dioxide and quinoxaline derivatives that presented Gram negative antibacterial activity.

Quinoxaline-1,4-dioxide (QNX)	2-Methylquinoxaline-1,4-dioxide (MQNX)	2-Amino-3-cyanoquinoxaline-1,4-dioxide (2A3CQNX)
		
2-Hydroxiphenazine-*N*-dioxide (2HF)		**3-Methyl-2-quinoxalinecarboxamide-1,4-dioxide (3M2QNXC)**
		

6.3 MATERIAL AND METHODS

6.3.1 QUINOXALINE DERIVATIVES

Stock solutions of the five quinoxaline derivatives were prepared at a concentration of 10000 μg/L, by dissolving the solid compound in ultrapure water. Diluted solutions were prepared, in order to achieve the final concentrations in the wells were, for each compound, of 10.0; 1.00; and 0.01 μg/L, C_1, C_2 and C_3 respectively.

6.3.2 CELL LINES

In the present study, seven cell types were used, in order to determine the toxicity and antitumoral activity of the quinoxaline derivatives. The cells used were human gingival fibroblasts (GF), obtained from explants from healthy donors with 25-35 years old, after informed consent; human mesenchymal stem cells (purchased from Innoprot), human MG-63 osteoblast-like cells (osteosarcoma cell line, ATCC® CRL-1427™), murine 3T3-L1 fibroblasts (ATCC® CL-173™), murine skin melanoma B16-F10 (ATCC® CRL-6475™), murine brain BC3H1 cells (ATCC® CRL-1443™) and human colorectal adenocarcinoma HT29 (ATCC® HTB-38™).

6.3.3 CELL CULTURE AND *IN-VITRO* TREATMENT

B16-F10, 3T3-L1, colon, GF, MSC, and MG-63 cells were cultured in Dulbeco-Modified Eagle's Medium (D-MEM) containing 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, Co., USA), 1% Pen/Strep/AmphB (Gibco, Portugal). BC3H1 were maintained in D-MEM but supplemented with 20% fetal bovine serum (FBS). Cells were grown in a 5% CO_2 humidified atmosphere at 37 ºC. Treatments were carried out in serum-free mediumfor 24 hours. Controls were maintained in serum-free conditions.

6.3.4 TOXICOLOGICAL ASSAY

For the determination of toxicological effect of quinoxaline derivatives it was used the XTT (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide), from (Sigma-Aldrich, Co., USA) method that measures the mithocondrial activity.

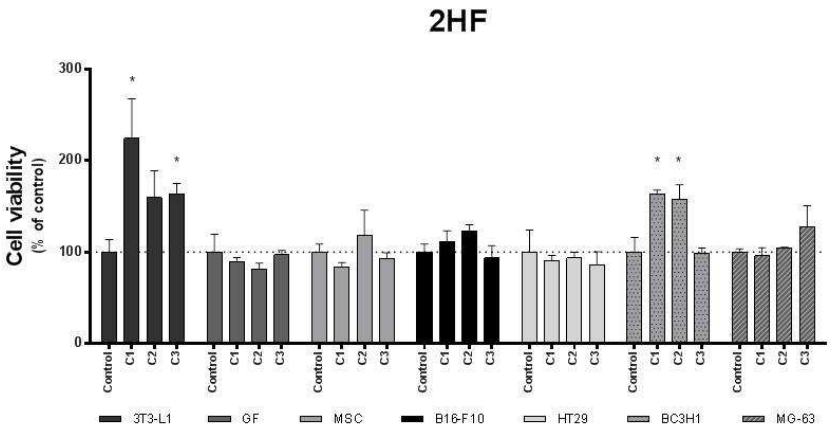
Cells were seeded in 96-well microplates at $1x10^4$ cells/mL and cultivated for 24 hours. To measure cell viability rate, after 24 hours incubation with the different bioactive compounds/concentrations, 20 μL of XTT reagent was added into each well followed by a three hours incubation period. Plates were then read at an absorbance of 450 nm. Results are expressed as the absorbance ratio: treated sample/control.

6.3.5 STATISTICAL ANALYSIS

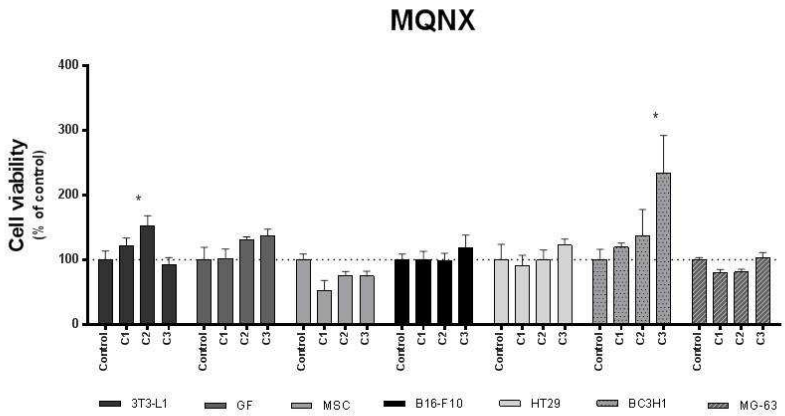
Statisticaly analysis was performed in SPSS (SPSS 19.0 for Windows), using t-Student test with a significance level of 0.05. The figures were designed in GraphPad (GraphPad Prism 6.02 for Windows).

6.4 RESULTS AND DISCUSSION

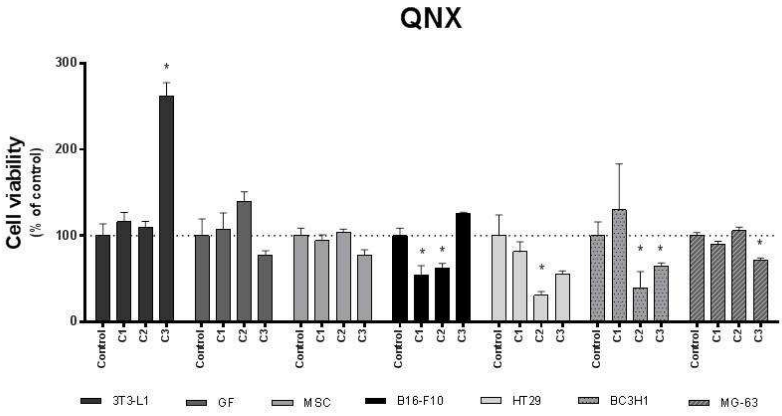
The influence in viability rate observed in cell lines studies is presented in Figure 6.1. Results obtained and statistical analysis are presented in Table 6.2.


2HF
Cell viability
(% of control)
300
200
100
0
Control
C1
C2
C3
3T3-L1
GF
MSC
B16-F10
HT29
BC3H1
MG-63

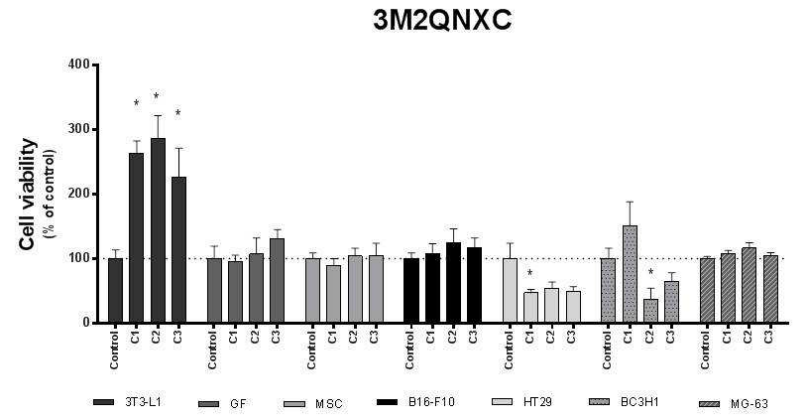
a)


MQNX
Cell viability
(% of control)
400
300
200
100
0
Control
C1
C2
C3
3T3-L1
GF
MSC
B16-F10
HT29
BC3H1
MG-63

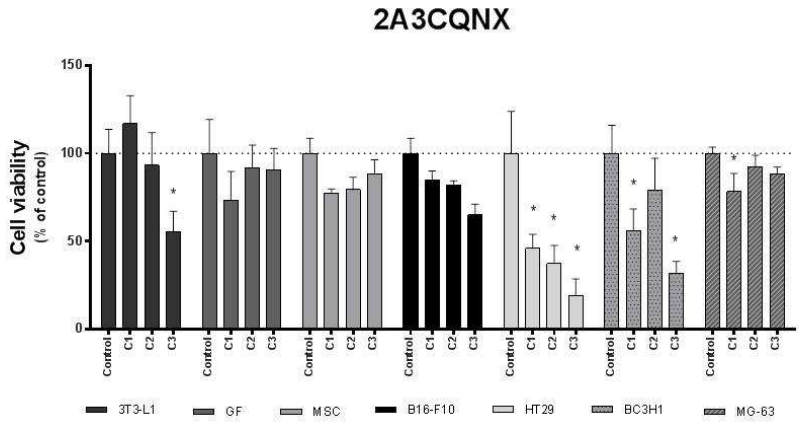
b)


QNX
Cell viability
(% of control)
300
200
100
0
Control
C1
C2
C3
3T3-L1
GF
MSC
B16-F10
HT29
BC3H1
MG-63

c)



d)



e)

Figure 6.1: Viability rate results for seven cell lines exposed to five quinoxaline derivatives with biological activity, measured after. a) exposure to 2HF; b) exposure to MQNX; c) exposure to QNX; d) exposure to 3M2MQNXC; e) exposure to 2A3CQNX. (*: $p < 0.05$; $n = 3$; results are expressed as *mean* ± *sd*; C1: 10^{-3}; C2 10^{-4}; C3: 10^{-6} µg/L). 3T3-L1: murine fibroblasts, GF: gingival fibroblasts, MSC: human mesenchymal stem cells, B16-F10: murine skin melanoma, HT29: human colorectal adenocarcinoma, BC3H1: murine brain cells, MG-63: osteosarcoma cell line.

Table 6.2: Sumary statistical analysis for metabolic rate of 7 cell lines exposed to quinoxaline derivatives

24 h	3T3L1	GF	MSC	B16-F10	HT29	BC3H1	MG-63
2HF	$p < 0.05$ C_1, C_3	NSD	NSD	NSD	NSD	$p < 0.05$ C_1, C_2	NSD
MQNX	$p < 0.05$ C_2	NSD	NSD	NSD	NSD	$p < 0.05$ C_3	$p < 0.05$ C_1
QNX	$p < 0.05$ C_3	NSD	NSD	$p < 0.05$ C_1, C_2	$p < 0.05$ C_2	$p < 0.05$ C_2, C_3	$p < 0.05$ C_1, C_2
3M2QNXC	$p < 0.05$ C_1, C_2, C_3	NSD	NSD	NSD	$p < 0.05$ C_1	$p < 0.05$ C_2	NSD
2A3CQNX	$p < 0.05$ C_3	NSD	NSD	NSD	$p < 0.05$ C_1, C_2, C_3	$p < 0.05$ C_1, C_3	$p < 0.05$ C_1

Legend: $p < 0.05$: statisticaly significant difference; C_1: 10^{-3}; C_2 10^{-4}; C_3: 10^{-6} µg/L; NSD: No significative differences. Green cells indicate metabolicrate acceleration; pink cells indicate metabolic rate diminuition. 3T3-L1: murine fibroblasts, GF: gingival fibroblasts, MSC: human mesenchymal stem cells, B16-F10: murine skin melanoma, HT29: human colorectal adenocarcinoma, BC3H1: murine brain cells, MG-63: osteosarcoma cell line.

Results reveal 3T3-L1 metabolic rate is promoted when exposed to 3M2QNX, 2HF, MQNX and QNX, with significative differences comparatively to control sample. These differences were observed in all concentrations tested for 3M2QNXC. On the other hand, when exposed to. 2A3CQNX, this cell line reveals significative metabolic rate decrease, at C_3.

The studied compounds and concentrations showed no signicative differences in metabolic rate for GF and MSC, suggesting no interference in cell activity.

B16-F10 metabolic rate presents no significative differences compared to the control, except for C_1 and C_2 concentrations of QNX. These results indicate that the compounds tested have no proliferative influence in this skin melanoma cell line. One of the compounds tested indicates, on contrary, decrease in metabolic rate.

2HF and MQNX have no significative influence in metabolic rate on human colorectal adenocarcinoma cell line HT29. QNX, 3M2QNXC and especialy 2A3CQNX reveal statisticaly significant decrease in metabolic rate.

BC3H1 murine brain cell line metabolic rate profile observed is similar to HT29. In 2HF and MQNX exposure results show a statisticaly significant difference in increase of metabolic rate, suggesting proliferation of this cell line.

MG-63 metabolic rate results indicate no statisticaly significant differences except for one of the tested concentrations of QNX, MQNX and 2A3CQNX.

Although in some compound/cell line tests there was a metabolic rate decrease for higher concentrations, it was observed an increase for the lowest tested concentration. This effect is shown, for example, in 3T3-L1 exposure to QNX.

6.5 CONCLUSIONS

Quinoxaline derivatives studied in the present work seem to have no significant toxic effects on primary cell lines (GF) and on osteoblast promoting cells, but proliferative effect on 3T3-L1 fibroblast-like cell line. Moreover, these findings suggest that, regarding to other quinolones, such as fluoroquinolone [412, 413], this group of quinoxaline derivatives may present an advantage, in terms of the adverse effects on the bone. It may be considered that lower concentrations of some quinoxaline derivatives can, not only not promote cell death, as well as present some protective effect.

There is no significant proliferative influence in tumor cell lines tested. On contrary, results show a negative influence on cell growth, suggesting some anti-tumoral effect. These results are in accordance with literature, where anti-tumoral

activity of the described quinoxalines has been observed in melanoma [414, 415], colon [415], glioma [416] and other tumoral human and animal cell lines [107, 218, 417].

Some studies revealed no significative proliferative influence on these cells when exposed to quinoxaline derivatives [244, 418]. However, some authors described morphological and biochemical modifications in BC3H1 cells when exposed to quinoxaline derivatives, suggesting that they might be toxic. These modifications led to pre-apoptotic modifications and programmed cell death pathway activation, which may explain some of the results above [419].

Further studies are needed to understand the influence of the studied quinoxaline derivatives in eukaryotic cells metabolic rates. However, our results are in line with some other studies involving other quinoxaline derivatives and cell lines.

Chapter 7: Oxidative cellular damage of quinoxaline derivatives

7.1 Objectives

7.2 Abstract

7.3 Introduction

7.4 Materials and methods

7.5 Results

7.6 Discussion/Conclusion

7 OXIDATIVE CELLULAR DAMAGE OF QUINOXALINE DERIVATIVES

7.1 OBJECTIVES

In order to evaluate the oxidative stress, it was necessary to develop a method for markers' quantification. There are many methods for the evaluation of the oxidative stress. One of the most widely accepted is the determination of the ratio of both reduced (GSH) and oxidized (GSSG) forms of glutathione. However, many of those methods are expensive, time-consuming and do not determinate individualy [GSH] and [GSSG]. They quantify the total glutathione and the reduced form. The first objective was to develop a chromatographic method in order to measure the ratio GSH/GSSG, an equilibrium involved in cellular oxidative stress.

The second objective was to assess the influence of quinoxaline derivatives in liver extract. Samples of liver extract were subjected to quinoxaline derivative solutions and oxidative stress was evaluated through the chromatographic method previously established.

The work described in this chapter has been submitted to the international journal *Journal of Biotechnology*, with the title **"Oxidative cellular damage of Quinoxaline derivatives"** (JBIOTEC-D-13-01223) and is under review.

Oxidative cellular damage of quinoxaline derivatives

Mónica Vieira, Vanda Peixoto, Ana Cláudia Pereira, Joana Costa, João Paulo Noronha, Cristina Prudêncio, Rúben Fernandes

7.2 ABSTRACT

The determination of both reduced (GSH) and oxidized (GSSG) forms of glutathione is a major method for the evaluation of oxidative status of an individual. These levels allow the calculation of the ratio of [GSH]/[GSSG] that is among the most reliable parameters of such state. In the present study, we have developed a new HPLC method that allows the simultaneous determination of both glutathione forms.

Chromatographic methods allow identification and quantification of multiple substances. The present work presents a new chromatographic method, based on an acidic aqueous mobile phase and a Diode Array Detector, avoiding the organic solvent usage. The developed method allowed the simultaneous quantification GSH and GSSG forms, thus the [GSH]/[GSSG] and was applied in C57BL6J mice liver extract exposed to the quinoxaline derivative compounds, in order to evaluate the oxidative damage of these compounds. The quinoxaline derivatives studied in the present work present potencial antimicrobial activity [411] and the effect of these potential new drugs on liver cells is an important fact to consider.

This new method presents several major advantages in contrast with other methods. We emphasize that this is a fast method, low cost and environmental friendly due to the absence of organic solvents such as methanol, hexane and acetonitrile.

Keywords: Glutathione, Oxidative Stress, Glutathione ratio, HPLC-DAD

7.3 INTRODUCTION

7.3.1 THE OXIDATIVE STRESS

Oxidative stress is an imbalance between reactive oxygen species (ROS) and antioxidant levels in a cell, and the inability to detoxify the resulting reactive metabolites or to repair the damage caused by reactive species in a biological system [420-423]. ROS include free radical intermediates such as superoxide anion radical ($O_2^{\bullet -}$; the most abundant under physiological conditions [421]), hydroxyl radical ($HO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$) and hydroperoxyl radical ($HO_2^{\bullet}$), and also non-radical intermediates such as hydrogen peroxide (H_2O_2), ozone (O_3), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$) and singlet oxygen ($^1O_2$). These compounds present high instability due to the existence of one, or more, unpaired electrons [424-426].

ROS can be generated from multiple mechanisms, such as: a) normal metabolic reactions as redox reactions during cell respiration. O_2 reduction to water implies a 1-2% electron leakage, generating $O_2^{\bullet -}$ at the ubiquinone and NADH dehydrogenase (complex I), as well as in complex III. This mechanism occurs at the outer membrane of the mitochondria, and is associated to large H_2O_2 production; b) radiation, exciting UV rays and ionizing X rays; c) xenobiotics and drug metabolism; d) activity of monoamine oxidase (MAO), which deaminates biogenic amines; e) in purine catabolism and formation of uric acid, in the reactions catalyzed by xanthine oxidase, a $O_2^{\bullet -}$ producing enzyme; f) in the reactions catalyzed by xanthine oxyreductase, NADPH oxidase, nitric oxide synthase, heme oxygenase and in the formation of peroxynitrite; g) during an inflammatory response.

The production of H_2O_2 and $O_2^{\bullet -}$ is a physiologic event in cells such as polymorphonuclear cells (PMN), eosinophils, monocytes, Kupffer cells and

macrophages, due to a highly specialized NADPH-dependent oxidase system located in the outer surface of the cell membrane, coupled to the action of superoxide dismutase (SOD) [421, 427, 428]. Also, during protein folding that takes place in the endoplasmic reticulum, a significant amount of $O_2^{\bullet -}$ is formed. In this process, the formation of disulphide bonds is an oxidative mechanism, since it involves the oxidation of sulphydryl groups of cysteine (Cys) residues [421, 427, 429].

At homeostatic levels, ROS are implicated in diverse activities on cell function, including activation of redox-sensitive transcription factors and activation of protein kinases [421], regulation of vascular tone and functions controlled by O_2 concentrations, enhancement of signal transduction from many membrane receptors (eg. the antigen receptor of lymphocytes) [422], fighting pathogens [428], among others[421, 422, 428]. However, when in excess, ROS can induce cell injury and a chronic inflammatory state that can trigger a cascade of free radical reactions, promoting secondary ROS generation and resulting in cellular modification and damage in DNA, carbohydrates, proteins and polyunsaturated fatty acids. This oxidative injury follows a general pattern that involves free thiol oxidation and formation of disulphide proteins, depletion of the ATP pool, free cytosolic Ca^{2+} increment, disintegration of cytoskeleton, increase in membrane lipid peroxidation, release of cytosolic compounds and DNA damage [423, 424, 430]. Examples of human disorders associated with increased oxidative state include cellular aging, brain dysfunction and neurodegenerative diseases, cancer, diabetes, rheumatoid arthritis and cardiovascular and renal diseases [422, 425, 431, 432].

Glutathione (GSH) is a thiol-containing tripeptide (γ-glutamyl-cysteinyl-glycine) that plays a major role in cellular protection against the toxicity induced by oxidant agents. GSH is also named as reduced glutathione in contrast with GSSG that is known as oxidized gluthatione. Regarding its antioxidant properties, GSH is involved in the detoxification of H_2O_2 or organic hydroperoxides into H_2O and respective alcohols by glutathione peroxidase (GPx), resulting in GSSG, a dimer of

GSH. Simultaneously, the Cys residues of GSH are oxidized forming disulfides (S-glutathionylation) (Figure 7.1).

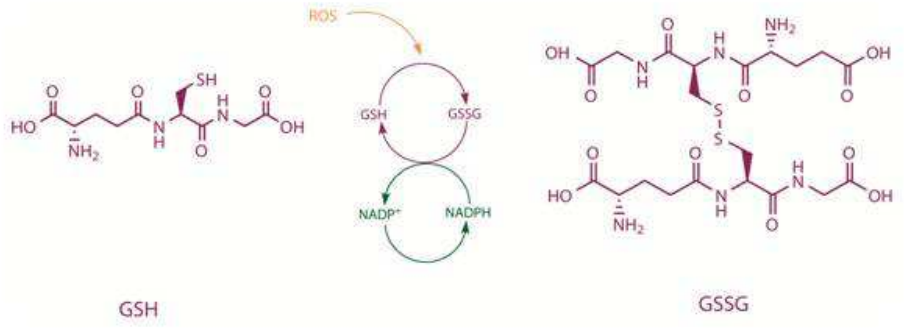


Figure 7.1: Schematic representation of the recycling process of glutathione. In the presence of ROS (reactive oxygen species), GSH (reduced form of glutathione) is oxidized to the GSSG form. In the presence of glutathione reductase, and using NADPH as an electron donor, GSSG can be reduced back to GSH.

GSH possesses a number of other important functions implicated in cell physiology and pathophysiology, such as amino acid transport through biological membranes, DNA synthesis and cell proliferation, regulation of nuclear matrix organization and maintenance of Cys residues on zinc-finger DNA-binding motifs in a reduced and functional state [433]. The millimolar levels of cellular GSH (5-10 mM) are distributed mainly between the cytosolic (about 80-85%) and mitochondrial (10-15%) compartments and the ratio [GSH]/[GSSG] determines the cellular redox state, with GSH representing the reduced form and GSSG [433, 434]. So it is expected that increased levels of oxidative stress implicate a lower [GSH]/[GSSG] ratio [423, 435].

Because abnormal blood levels of [GSH]/[GSSG] have been associated with several pathologies, like Alzheimer, Parkinson, diabetes, HIV infection, kidney failure, malignant disorders and alcoholism, among others [436-441], there is a crucial need of

analytical methods to determine reduced and oxidized glutathione forms present in biological samples, in order to elucidate about the redox state of such patients.

Traditionally, GSH and GSSG concentrations are determined by enzymatic methods, where the disulfide bond from Ellman reagent DTNB (dithionitrobenzoic acid), is cleaved by the thiol group from GSH. This reagent is largely used for thiol determination in biological samples [442].

This type of methods have the disadvantage of not allowing the direct determination of both GSH and GSSG forms, so this can be only achieved by calculating their levels through the reaction GS_T = GSH + 2 GSSG, with GS_T representing total glutathione [443].

Electroanalytical techniques are also applied to determine glutathiones levels and have shown to be highly sensitive and selective in the detection of a big number of compounds, and in particular to the detection of GSH. [444-448]

Another type of analytical methods use liquid chromatography combined with different detection techniques for the analysis of GSH and GSSG. These methods are based, generally, on derivatization through probes containing the reduced group –SH from GSH and containing GSSG after chemical reduction. Glutathiones can now be detected by many methods as liquid chromatography mass spectrometry (LC-MS), spectrophotometry, electrochemistry and capillary electrophoresis combined with electrochemical detection.

High Performance Liquid Chromatography (HPLC) technique enables simultaneous GSH and GSSG direct determination. However, to increase this method's sensitivity, it's reported the need of adding a derivative agent [442, 449]. Many HPLC procedures have been developed for the analysis of amino acid fluorescent derivatives. One of the most common approaches is the dansyl chloride derivatization, producing S-containing amino acids, like cysteine. There are also HPLC methods that react with amino group. The derivatization with 2,4-dinitrofluorobenzene (FDNB) has the

advantage of evaluating simultaneously GSH and GSSG in one single run. However, this method has the disadvantage of being time consuming due to the necessary derivatization and the inherent need to proper sample preparation [442, 450].

7.3.2 QUINOXALINE OXIDATIVE DAMADGE

Liver is the principal organ for drug metabolism, especially because it's where highest concentrations of most drug metabolizing enzymes are found. Drugs' metabolism is a set of metabolic pathways that transforms the chemical structure of the drug. The reactions of biotransformation often act to detoxify foreign compounds from cells, such as drugs and their metabolites.

Liver is a major metabolic organ and is involved in many biological processes as drug metabolism. In this sense, one of the most important is the detoxification of drugs such as antibiotics and its metabolites. Some antibiotics can cause direct damage to the liver, which can be quite severe in patients with chronic liver disease. For patients with a pre-existing liver disorder, the detoxification function of the liver is already compromised and substances that would normally be metabolized could actually accumulate in the liver or in the bloodstream [451]. Antibiotics that accumulate in this manner could become toxic to the body and its functions can change drastically from its original purpose [452]. The chemical reactions responsible for drug transformation are enzymatically catalyzed and may be influenced by pH, temperature and drug concentration [453].

Metabolic reactions for drug biotransformation are classified in phase I and phase II reactions, both with the final objective of transforming the drug in a more hydrophilic compound, in order to facilitate its excretion. Phase I reactions are characterized by the incorporation in the drug of polar chemical groups (hydroxide

and carbonyl groups, mostly) or by unmasking polar groups, through heterolytic cleavage [454]. These processes are dependent of a variety of enzymes of the cytochrome P450 superfamily (CYP450). In humans, CYP450 are located in mitochondrial inner membrane or in the endoplasmic reticulum of hepatocyte cells and are the major enzymes involved in drug metabolism (about 75% of total metabolism) [454]. Phase II reactions are also enzymatic and conjugate the transformed metabolites from phase I with charged species, as glutathione [455]. These metabolites have higher molecular weight and are less active than the original drug. The addition of large charged species makes them more polar and unable to cross cellular membranes by diffusion. The charged species act as affinity identifiers allowing cellular detoxification through specific membrane transporters [456].

Glutathione exists in reduced (GSH) and oxidized (GSSG) forms. In healthy cells, about 90% of total glutathione is in the reduced form. A decrease in this proportion, and thus alteration of the GSH/GSSG ratio, is indicative of oxidative stress. As previously presented glutathione is the major cellular antioxidant, neutralizing free radicals and oxygen reactive species (ROS). It also maintains exogenous antioxidants in the reduced active form [457]. The nitric oxide cycle, critical for life, is also regulated by glutathione [458] and glutathione participates in metabolic and biochemical reactions [459]. It is also important as a conjugate molecule added to xenobiotics in the liver during biotransformation.

In the present work, a practical HPLC method was developed, where glutathione molecules (reduced (GSH) and oxidized (GSSG)), were separated directly, without any further derivatization. The new HPLC method was optimised, which implies a shorter sample preparation and reduced reagents costs. That led to the determination, in short time, of the relationship between the two forms of glutathione. The sensibility and application range of the method was adequate to the analysis of different matrices.

By application of the chromatographic method, glutathione ratios were determined in order to assess oxidative damage inflicted by exposing hepatic cells to quinoxaline derivatives with biological activity.

In order to stablish this new method, it was used a number of samples of human patients with diabetes due to its reconized increase of ROS and glutathione concentration of both forms was determined. A commercial kit for determination of glutathione levels was used and values were compared with those obtained by the HPLC-DAD method developed.

7.4 MATERIALS AND METHODS

Reduced glutathione (GSH), glutathione oxidized (GSSG) supplied by Santa Cruz Biotechnology Inc.®; trifluoroacetic acid and ortho-phosporic acid were supplied by Merck. Ultrapure water was obtained by a GenPure TKA water purification system.

The glutathione kit assay was supplied by Sigma-Aldrich, Co., USA with catalog number CS0260. The plates were scanned in a Multiskan FC, from Thermo Scientific.

C57BL6J mice liver extract was used in oxidative damage analysis.

7.4.1 CHROMATOGRAPHIC SYSTEM

An Hitachi® High-Performance Liquid Chromatograph LaChrom Elite equipped with a HTA L-2130 quaternary pump; L-2300 column oven; L-2200 auto sampler; DAD L-2455 detector, LaChrom Elite series was used in the present work. Chromatographic collection and data treatment was performed by EZChrom Elite Software, Lachrom Elite series.

Glutathione separation was developed on a Purospher® STAR C18 chromatographic collumn, with 5 µm particle size and 4.6 x 250 mm internal dimensions. The column temperature was maintained constant at 40 ºC and autosampler tray was maintained at 4 ºC. The volume of injection was of 30 µL.

The wavelength range used was from 190 to 400 nm and chromatograms at 200 nm, 215 nm and 225 nm were collected, due to the maximum radiation absorption interval of the GSH and GSSG (Figure 7.2).

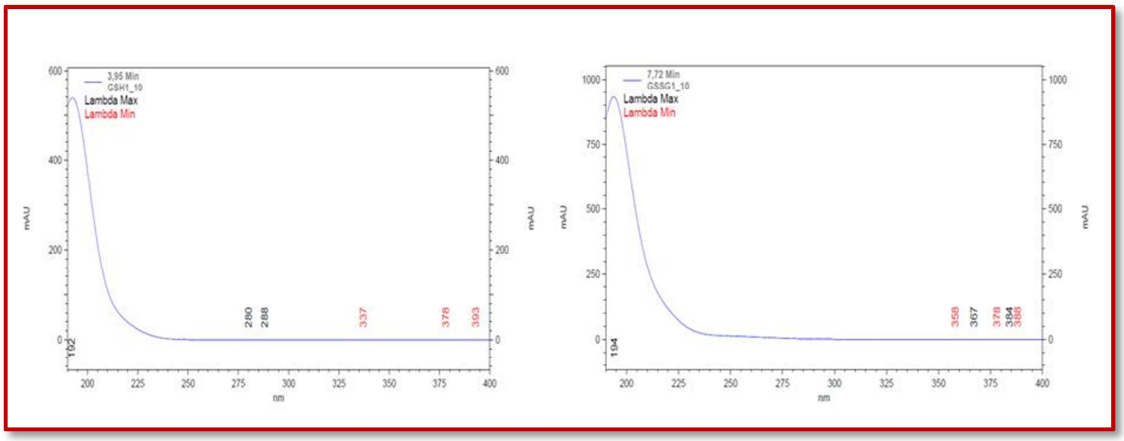


Figure 7.2: Absorption spectrum for GSH (*left*) and GSSG (*right*) forms.

Samples were filtered by membrane filters from Pall Life Sciences® (GHP Acrodisc 13 mm Ø Syringe filter 0.45 µm GHP membrane; GHPolypro 47 mm Ø 0.45 µm hydrophilic Polypropylene Membrane filters). After filtration samples were transferred to 2 mL vials.

7.4.2 CHROMATOGRAPHIC METHODS

Reduced and oxidized standard solutions, at 2.77 mM and 7.18 mM respectively, as well as the mixture of the two forms with [GSH] = 1.38 mM and [GSSG] = 3.59 mM, were prepared using 0.085% ortho-phosphoric acid. Diluted standard solutions were prepared by adding 0.085% ortho-phosphoric acid to the previous mentioned standards, ranging dilutions from 1:10 to 1:80.

Membrane filters from Pall Life Sciences® filtered both standards and samples (GHP Acrodisc 13 mm Ø Syringe filter 0.45 μm GHP membrane; GHPolypro 47 mm Ø 0.45 μm hydrophilic Polypropylene Membrane filters). After filtration standard and sample solutions were transferred to 2 mL vials. Glutathione separation was developed on a Purospher® STAR C18 chromatographic collumn, with 5 μm particle size and 4.6 x 250 mm internal dimensions. Autosampler tray was maintained at 4 ºC and the column temperature was maintained constant at 40 ºC. Isocratic elution was achieved with ortho-phosphoric acid at 0.085% with a 1.0 mL/min flux. The mobile phase was filtered with a filtration system by Millipore vacuum, equipped with pore sized 0.45 mm and degasified by ultra-sonication. The wavelengths of detection elected were 200 nm, 215 nm e 225 nm, initially. The chromatograms obtained were analysed by the software incorporated in the chromatographic system. For the wavelengths established, 200 nm was the one that presented the optimum relation noise/area and less interference in sample analysis.

7.4.3 LIVER EXTRACT SAMPLES

10 g of liver tissue was immersed in saline (NaCl, Carlo Erba). Tissue was cut into small pieces and washed in KME buffer (1 mM Tris, pH 8.0, with 50 mM KCl), and then homogeneized for 5 minutes, with KME buffer, in ice bath. Tthe homogenate was filtered through cheesecloth and centrifuged for 10 minutes, at 4 ºC and 1000 g. The supernatant was decanted and the pellet discharged. The supernatant was centrifuged, in millipore falcon tubes for 20 minutes, at 4 ºC and 4000 g. The obtained supernatant was then centrifuged for 10 minutes, at 4 ºC and 10000 g. The pellet obtained was resuspended in 15 mL of STKME buffer and centrifuged for 10 minutes, at 4 ºC and 10000 g. Finally, the pellet obtained is resuspended in KME buffer.

Sample is divided into three aliquots, a negative control, a positive control and the test sample. Negative control corresponds to the extract obtained, alone. To the positive control tBOOH, an oxidative stress inductor was added. To the test sample, solutions of five quinoxaline derivatives, with confirmed biological activity were added at concentrations corresponding to the MIC values determined in previous works [411]. The compounds tested were: quinoxaline-1,4-dioxide (QNX), 2-methylquinoxaline-1,4-dioxide (2MQNX), 2-amino-3-cyanoquinoxaline-1,4-dioxide (2A3CQNX), 2-hydroxiphenazine-*N*-dioxide (2HF), 3-methyl-2-quinoxalinecarboxamide-1,4-dioxide (3M2QNXC). Samples were filtered, transferred to 2 mL vials and injected in the HPLC-DAD system. Analyses were performed accordingly to the developed chromatographic method presented in 8.2. Data was analyzed using EZChrom Elite software.

7.4.4 GLUTATHIONE KIT ASSAY

For the glutathione kit assay, the standard glutathione curve and blood samples were treated according to the method's specifications (Sigma-Aldrich, Co., USA,

CS0260). Briefly, for the standard curve, glutathione standard solutions were prepared by serial dilutions in 5% 5-sulfosalicyclic acid (SSA), with concentrations ranging from 3.125 μM and 50.00 μM and absorbance was measured at 405 nm. The biological samples were first deproteinized with 5% SSA and centrifuged to remove the precipitated protein.

The measurement of GSH followed a kinetic principle in which the GSH present in the sample caused a continuous reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to 2-nitro-5-thiobenzoate (TNB) and the GSSG formed was recycled by glutathione reductase and NADPH.

7.4.5 BLOOD SAMPLES

Human blood samples were collected from volunteers with erectile dysfunction and/or diabetes only and non-diabetic volunteers, with the same range of age that have given their informed consent. Moreover, all the directives of the ethical councils of the institutions involved were followed. Venous blood was collected by antecubital puncture into tubes containing EDTA. Half of each sample was frozen at -20 ºC and half centrifuged at 5900 rpm for 10 minutes to obtain the supernatant plasma and frozen at -20 ºC. All samples are properly identified.

For the analysis of samples precipitation of proteins was necessary, using trifluoroacetic acid (TFA). The optimal concentration found was 15% of TFA. Freshly prepared acid solutions provided better results. After addition of TFA, samples were stirred by vortex for 20 seconds and centrifuged at 8000 rpm for 10 minutes. The resultant supernatant was then filtered and transferred to 2 mL vials.

7.4.6 STATISTICAL ANALYSIS

It was used a Mann-Whitney test for independent samples (glutathione measurements in two different methods), for a significance level of 0.05 (SPSS 19.0 for Windows).

7.5 RESULTS

The reduced and the oxidized forms are both measurable by the chromatographic method. Standard solutions, ranging from 3.59 mM to 19.75 μM were injected in order to determine calibration curves to GSH and GSSG. A chromatogram of the both forms is presented in Figure 7.3.

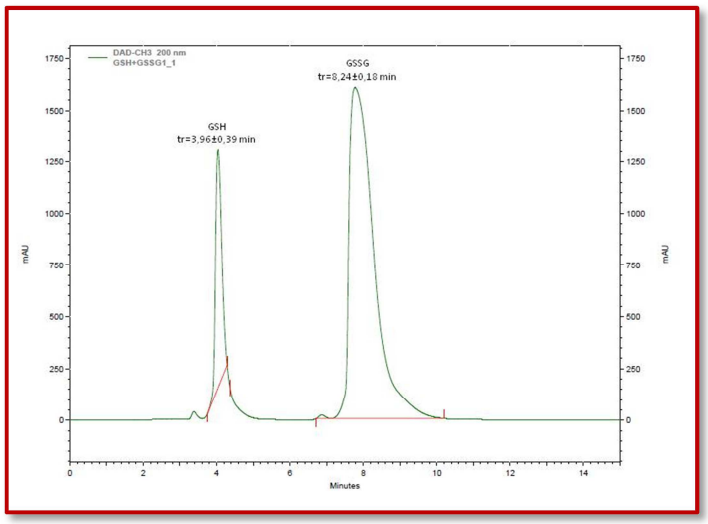


Figure 7.3: GSH and GSSH Chromatogram: GSH form corresponds to the left peak, with a time of retention (tr) of 3.96 min; GSSG corresponds to the righ peak, with a tr = 8.24 min.

Results obtained for the calibration of the chromatographic method are represented in Figure 7.4. It was verified a linear relationship between peak area and the concentration of both glutathione forms in the interval of concentrations studied. The lowest concentration level studied is included in the linearity of the method and has a peak area above the limit of detection.

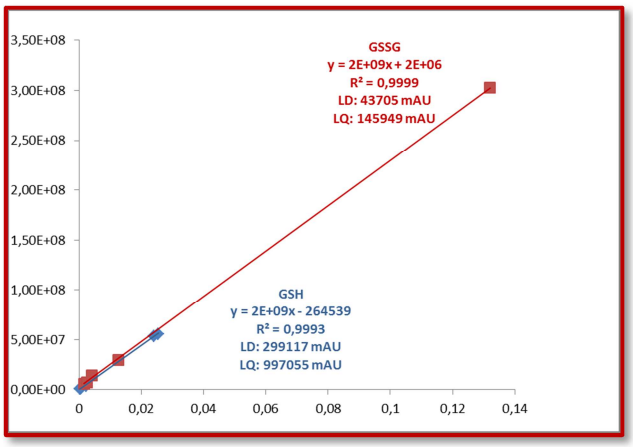


Figure 7.4: Calibration curves for Glutathione (oxidized and reduced forms) by HPLC-DAD method.

Due to technical problems, only three of the five available compounds were studied. The obtained results are, however, quite surprising. The studied compounds were 2HF, 2MQNX and 3M2QNXC. It was observed that all the compounds induced a lower oxidative stress than the tBOOH, used as positive control. It was also observed that at 24 hours, the GSH/GSSG was increasing, relatively to GSH/GSSG at 3 hours. tBOOH positive control keeps inducing disturbance in the GSH/GSSG equilibrium at 24 hours. Results are presented in Table 7.1.

The ratio determinations presented in Table 7.1 were obtained through peak integrations of absorbance recorded at 215 nm. Convertion of peak area data to mass of

GSH and GSSG was made by application of the analytical curves determined in method development.

Table 7.1: Results for [GSH]/[GSSG] analysis in liver tissue exposed to quinoxaline derivative compounds with biological activity.

	2 HF		2MQNX		3M2QNXC		tBOOH		Negative Control
	3 hours	24 hours	3 hours	24 hours	3 hours	24 hours	3 hours	24 hours	24 hours
[GSH]/[GSSG]	No data	1.43	0.68	1.23	1.44	1.53	0.70	0.20	2.71
%GSSG		43	78	54	49	52	62	83	25

The treatment applied to the liver tissue, previous to HPLC-DAD analysis, was found to be very effective for evaluating the oxidative stress caused by quinoxaline derivatives studied. GSH and GSSG peaks were well resolved in the obtained chromatograms, as shown in Figure 7.5. Time of retention for the two glutathione forms in complex liver tissue samples were in accordance with those obtained for the standard solutions.

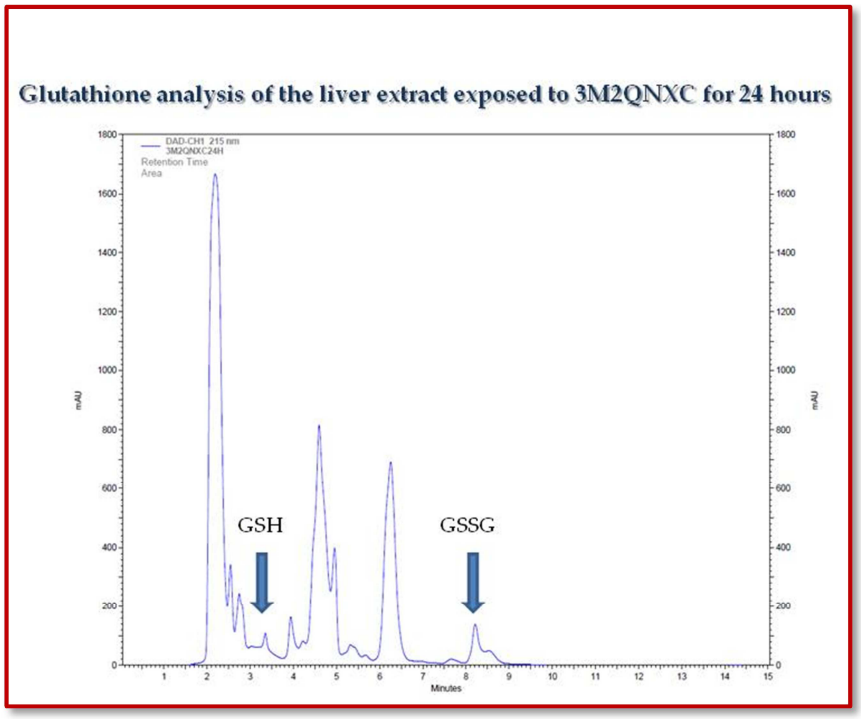


Figure 7.5: Chromatogram for oxidative stress analysis, in liver tissue, after 24 hours exposed to 3M2QNXC

In order to evaluate the applicability of the developed method to other matrices, blood samples were used, in order to determine GSH/GSSG ratio. To validate the results obtained through the new chromatographic methodology, a glutathione kit assay available in the market was used (Sigma-Aldrich, Co., USA CS0260). Blood samples were analyzed by the two methods and the results were compared.

The results obtained are consistent for the two methods. In order to be doable to compare the two quantification methods, the results obtained for the amount of GSSG through the chromatographic method were converted into amounts of GSH.

The mean values obtained were $7.51 \cdot 10^{-01} \pm 2.81 \cdot 10^{-01}$ μM for the GSH total amount by the kit assay method and $7.11 \cdot 10^{-01} \pm 1.19 \cdot 10^{-01}$ μM for the GSH total amount by the developed chromatographic method.

For the glutathione kit used, the output of the method corresponds to the total amount of the two forms (oxidized and reduced) of glutathione measured at 405 nm. The results obtained for the kit calibration, through standard solutions measurements, are presented in Table 7.2. The calibration curve is represented in Figure 7.6.

Table 7.2: Data for the kit assay calibration, according to the producer's guidelines (Sigma-Aldrich, Co., USA CS0260)

Standard (μM GSH)	mmol (10 μL GSH)	ΔAbs 405 nm/min
3.125	0.03125	0.00360
6.250	0.06250	0.00873
12.5	0.12500	0.01653
25	0.25000	0.03193
50	0.50000	0.05313

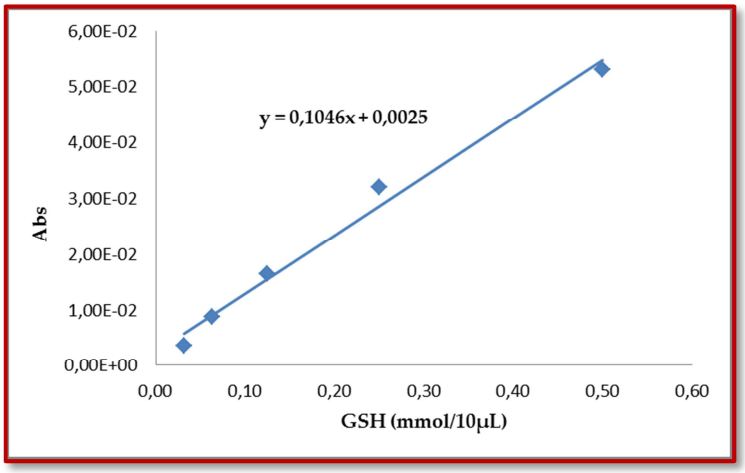


Figure 7.6: Calibration curve for glutathione kit assay, at 405 nm (Sigma-Aldrich, Co., USA CS0260).

By analyzing the results, it appears that there are no significant differences between the two methods. In fact, the values obtained for the total amount of GSH in the analyzed samples are statistically identical ($p > 0.05$). The results obtained allowed us to apply the developed method to other investigation projects and had a great impact on clinical samples evaluation.

7.6 DISCUSSION / CONCLUSION

The method developed in the present study has the advantage of allowing a simultaneous quantification of the oxidized and reduced forms of glutathione and so the ratio between the two forms can be determined, which is an important factor in the evaluation of cell oxidative stress.

Measurments of glutathione reduced (GSH) and oxidized (GSSG) forms concentration in liver extract samples showed efficient and the sample treatment proved to be appropriate.

The analysis of the [GSH]/[GSSG] suggest that exposure to the quinoxaline derivatives studied has influence on the intracellular equilibrium, probably due to drug biotransformation. Results indicate, however, that reestablishment of the natural equilibrium may be underway after 24 hours, for 2MQNX, since there is an increase in the ratio, when compared with measurments after 3 hours of exposure. The exposure to 3M2QNXC seems to continue affecting [GSH]/[GSSG] at 24 hours measurments. The difference between ratios for samples subjected to the presence of the compound and

the negative control suggest a significant change in equilibrium between the two forms of glutathione. GSSG percentage in negative control (25%) is in accordance with literature [457], which supports the accuracy of [GSH]/[GSSG] measurments presented in this work.

The developed chromatographic method proved to be reliable, since the values obtained for total amount of glutathione were in agreement with the same values obtained by the kit assay method. Moreover, the new method is quick, and a run takes 15 minutes to perform. Also, sample preparation is fast and the cost of the analysis is significantly less than the amount expended in an analysis using a kit assay. The cost of a single sample analysis by the developed chromatographic method is about forty times less than the same analysis by the kit assay.

We emphasize the fact that this method runs in an acidic aqueous isocratic eluent, organic solvent free, which carries less environmental issues and allows the mobile phase recycling.

In conclusion, the new method developed is presented as extremely advantageous from every point of view and can be expected to have a major impact on the study, diagnosis and treatment of various diseases oxidative stress related.

Chapter 8: General Discussion and Conclusions

8 GENERAL DISCUSSION AND CONCLUSIONS

8.1 GENERAL DISCUSSION

The use of antimicrobial agents in humans and animals allowed the appearance and spread of resistance among several species of bacteria. Recent studies report, in the European Union, multi-resistance and co-resistance to critically important antimicrobials in both human, animal and food isolates [6]. This fact means that the detection and quantification of antimicrobial agents on food from animal origin is an important factor in food quality control. The study of the influence of these contaminants in the environment is nowadays an important concern, since the bacterial population disturbance is yet not well known [362, 365]. The presence of this kind of contaminants appears to influence the appearance and spread of resistant strains of bacteria, by exposure to these drugs (WHO) [13].

In the present work, it was intended, in a first approach, to determine the presence of antimicrobial residues in foods and evaluate their influence in the environmental scene.

As reported by previous studies [351, 363, 374], HPLC associated to a DAD detector technique proved to be appropriate and reliable in the detection of antimicrobial and has application, namely, in food quality control The chromatographic method developed in the present work allows the simultaneous quantification of five antimicrobial agents in milk matrices and the quantification limits established are below the MRLs regulated in the European Union (Commission Regulation (EU) No 37/2010, OJ L15, 20.1.2010, pl1).

Effects of sub-inhibitory concentrations of antimicrobial agents have been reported, especially on adhesiveness alterations[460-462], but also in virulence factors

expression [463] and MDR bacteria gene expression [464]; resistance development [465] and bacterial diversification [466]. In order to contribute to the understanding of bacteria populations under selective pressure, the present work reports a study on the survival of *E. coli* resistant and susceptible to ciprofloxacin strains. Results obtained suggest that the presence of ciprofloxacin, even in sub-inhibitory concentrations, has influence on bacterial populations' performance and that the bacterial populations' equilibrium is unequally perturbed by the presence of this antimicrobial agent. These data are in accordance with the findings described in the previously reported studies.

The ability of bacteria to quickly adapt to different conditions, such as exposure to antimicrobial agents, makes the emergence of resistance faster than the appearance of new drugs able to combat these strains. Several studies indicate the heterocyclic organic family of quinoxalines as potential new antimicrobial drugs [200, 205, 209, 244, 382, 411]. In the present study, antimicrobial activity of quinoxaline derivatives was assessed and the results obtained indicate selective antimicrobial activity between both eukaryotic and prokaryotic strains. Antimicrobial activity was observed only in the Gram-negative strains studied for some of the compounds used.

Since there was no inhibition on eukaryotic strains used, toxicity of the quinoxaline derivatives was evaluated on different cell lines. Quinoxaline derivatives studied in the present work revealed to apparently have no significant toxic effects on primary cell lines and on osteoblast promoting cells. There was also no significant proliferative influence observed in tumor cell lines tested. On contrary, results show a negative influence on cell growth, suggesting some anti-tumoral effect. Although there are also studies pointing to no proliferative influence of quinoxaline derivatives [244, 418], the results obtained in the present work are in accordance with other data found in literature, where anti-tumoral activity of quinoxalines has been observed in several tumor cell lines [107, 218, 414-417].

Since the data collected for the quinoxaline derivative compounds studied seemed promising, we tried to evaluate their effect on oxidative stress in the liver, by exposing an extract of this organ to solutions with sub-MIC concentrations of quinoxaline derivatives. In order to be able to assess this effect, an analytical method (HPLC-DAD) was developed for the determination of the quantities of oxidized and reduced forms of glutathione present, in order to determine the ratio GSH/GSSG, an important factor in the evaluation of cell oxidative stress. Although there are many methods for the determination of the ratio of both reduced (GSH) and oxidized (GSSG) forms of glutathione, they are expensive, time-consuming and do not determinate individualy [GSH] and [GSSG]. The method developed allows the simultaneous quantification of each form, individually, and was already applied in other matrices (blood). Results of these studies indicate it as an appropriate aid in the diagnosis and treatment of some diseases, such as erectile dysfunction (Avaliação do Stress Oxidativo em Doentes Diabéticos com Disfunção Erétil, Almeida, Fábio; Almeida, Mónica; Louro, Nuno; LaFuente, José; Ribeiro, Severino; Fraga, Avelino, Sociedade Portuguesa de Andrologia, 2012).

8.2 CONCLUSIONS

The quantification of antimicrobial residues in food of animal origin is an important quality control aspect in order to avoid consumers' contamination and health issues due to ingestion of this kind of drugs.

In the present study, a new chromatographic method for multiple antimicrobial agents' detection was developed and proved to be efficient, providing monitorization of five (AMOX, CIP, CLOR, SULFO and CLOX) antimicrobial agents. The limits of detection and quantification of this method allows a quantification bellow the

European directives for antibiotic Maximum Residue Limits (MRL's) and, thus, can be used as a food quality control method.

The studies on the survival of *E. coli* resistant and susceptible to ciprofloxacin strains performed suggest influence of sub-inhibitory concentrations on bacterial popullations. The susceptible strain studied (*E. coli* S3R9) demonstrated high vulnerability to sub-inhibitory concentrations and resistant strain (*E. coli* S3R22) demonstrated survival in the same conditions. Alterations in equilibrium were observed and prevalence of resistant strains was suggested, when selective pressure is present, for the four strain combinations studied. Without the stimulus of selective pressure, results point to susceptible strain longer survival. A chromatographic method for CIP time-course quantification was developed and CIP determinations indicate stability of the antimicrobial agent, with non significant concentrations alteration through time, continuing to promote selective pressure and, maybe, promoting resistant bacterial strains prevalence on contaminated environments, even at concentration s of 0.05 μg/mL.

Due to the antimicrobial abusive and misuse, growing contamination of natural environments and presence of antimicrobial residues in food stuff, resistance to antimicrobial agents is spreading [359, 364, 367, 368]. Looking ahead to an antibacterial fighting substitute quinoxaline derivatives have been tested as a therapeutic alternative to infection diseases [202, 384, 411].

In the present study, eight quinoxaline derivatives (QNX, 2MQNX, 2A3CQNX, 2M3BenzoylQNX, 2M3BQNX, 3M2QNXC, 2HF and 3MN(2MF)QNXC were tested for their antimicrobial activity. Results indicate that the studied compounds present no activity against the Gram-positive bacterial strains tested (*S. aureus* ATCC 6538, *S. aureus* ATCC 6538P and *S. aureus* ATCC 29213) and against eukaryotic yeast strains *C. albicans* and *S. cerevisiae*. Antimicrobial activity was observed against the Gram-negative bacterial strains *E. coli* ATCC 25922, *E. coli* S3R9, *E. coli* S3R22, *E.coli* TEM-1, *E.*

coli AmpC MOX-2 and *E. coli* TEM-1 CTX M9. Some of these strains present antibiotic resistance profiles, and *E. coli* S3R22 is a MDR bacterial strain. These results indicate selective antimicrobial activity of the compounds studied to Gram-negative bacterial strains, revealing high inhibition of bacterial growth, including against resistant strains. The results obtained are in accordance with other studies on this family of compounds [396-398]. The MIC values determined for the quinoxaline derivatives are lower than those indicated by CSLI for two antimicrobial agents used (CIP and CEFO), suggesting a more effective activity of quinoxaline derivatives with respect to those antibiotics.

These results encouraged further study of the biological activity of these quinoxaline derivatives.

Cell toxicity effects of the quinoxaline derivatives with antibacterial activity were tested in a panel of seven cell lines. The results indicate no proliferative influence on primary cell lines (GF) and on osteoblast promoting cells, but proliferative effect on 3T3-L1 fibroblast-like cell line. No proliferative influence was observed on the four tumoral cell lines tested. In fact, a negative influence on cell growth was observed, indicating a possible anti-tumoral effect of these compounds. The outcome of the assays performed is corroborated by studies of other investigation groups [107, 414-417], and point to a low toxicity of these compounds, and have shown a potential anti-tumor action.

In order to evaluate the cellular oxidative-stress promoted by exposure to the quinoxaline derivatives, an original chromatographic method, for [GSH]/[GSSG] determination was developed. This method is distinctive in simultaneous quantification of both glutathione forms (reduced and oxidized) and reliability was verified by comparison with a commercial kit assay. This method was applied on liver tissue extract, after exposure to the quinoxaline derivatives, for a total period of 24 hours, and compared with a negative and a positive control. Results suggest influence

of this compounds on [GSH]/[GSSG] equilibrium, suggesting biotransformation occurence. Nevertheless, data indicate, also, a reestablishment of the [GSH]/[GSSG] after a 24 hours period. Results' confidence was supported by the comparison of negative control with literature data [457], indicating that the values obtained are in agreement with other studies performed with dofferent methods.

The bioassays performed in order to evaluate quinoxaline derivatives biological activity suggest their ability to become a therapeutic alternative as antibacterial agent. Further studies may be conducted to appraise their potential anti-tumoral activity likewise.

Chapter 9: Future perspectives

9 FUTURE PERSPECTIVES

The present study intended to be a contribution to the quantification of residues of antimicrobial agents in food and assess their impact on the environment.

With the perspective to develop alternatives to the current situation regarding the emergence of resistance and loss of efficacy of certain antimicrobial agents, the quinoxalines are presented as a possible alternative treatment, and some bioassays were conducted in order to elucidate their potential and application.

It is expected to apply the developed chromatographic method for antimicrobial residues in food stuffs to other matrices, as cheese, yogurt, meat and eggs. The method may also be applied in water samples, in order to contribute to determination of water contamination by antimicrobial agents.

The survival of resistant and susceptible *E. coli* strains performed pointed to selective pressure influence on bacterial survival. Further studies will be performed, in order to evaluate other variables observed in natural environmentas, as well as bacterial strains and combinations.

Further studies on antimicrobial activity, toxicity and anti-tumoral activity of quinoxaline derivatives and other heterocyclic organic compounds are intended to be performed, so that additional data on potencial therapeutic agents is accessible.

The oxidative-stress chromatographic method developed, for quantification of glutathione forms, revealed to be an important tool on investigation works of our group and collaborations, on disease diagnosis and evaluation of several disease conditions. Other methods, including nitrotyrosine quantification are being developed so that we have more data to assist the evaluation / interpretation of oxidative damage in disease conditions or exposure to biological active compounds.

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