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CICLO XXXIV

Investigations on a potential antimicrobial adjuvant as inhibitor of bacterial non-essential pathway in human and veterinary bacteria

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Investigational Studies on a Hit Compound Cyclopropane-Carboxylic Acid Derivative Targeting O-Acetylserine Sulfhydrilase as a Colistin Adjuvant

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Abstract

The threat of antimicrobial resistance (AMR) is critically worldwide widespread and, in recent years, the search for novel therapeutical strategies to counteract this phenomenon both in human and veterinary medicine is of great interest. Antibacterial adjuvants represent a valid alternative to the use of conventional single antimicrobial molecules, since their activity in combination allows to significantly reduce the therapeutical dosage of the latter, in reason to the synergistic effect of their combination. With this aim, in this PhD thesis was reported the antimicrobial activity of an inhibitor of bacterial non-essential pathway – that is O-acetylserine sulfhydrilase (OASS) inhibitor, UPAR415 – as colistin adjuvant towards six bacterial reference strains of veterinary and human interest, three Gram-negative (*Escherichia coli*; *Salmonella* Typhimurium and *Klebsiella pneumoniae*) and three Gram-positive (*Staphylococcus aureus*; Methicillin resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius*). UPAR415 represents the most potent OASS inhibitor so far, due to its nanomolar ranged dissociation constant (K_d) and, in addition to the already demonstrated inhibition of the enzyme, it showed a promising activity in combination with colistin, synergistic against almost all Gram-negatives and additive against Gram-positives. Furthermore, association activity was confirmed in a rich medium (Müeller Hinton broth), where cysteine (the biosynthetic product of reductive sulfate assimilation pathway using inorganic sulfur in procaryotes) is abundant. The intracellular target engagement of UPAR415 was confirmed using a *S.* Typhimurium strain inactivated for OASS-A, supporting the proposed mechanism of action. Moreover, to assess the absence of cell toxicity, UPAR415 and its association with colistin were tested on bovine kidney cells (MDBK) and sheep defibrinated blood, ensuring a good biocompatibility and a very low hemolytic effect. At last, the 3D structure of UPAR415 in complex with OASS was determined, providing the first structural insight about the interaction between the enzyme and this class of competitive

inhibitors. The obtained results showed in this PhD thesis could represent a good starting point for the development of antibiotic adjuvants operating on a bacterial non-essential target, such as cysteine biosynthetic pathway, and could pave the way for further research on adjuvant compounds able to overcome the current pressing issue of AMR, both in veterinary and human medicine.

1. Introduction

1.1. Origin and development of the antimicrobial resistance

The phenomenon of antimicrobial resistance threatens the use of antibiotics themselves since their first use as therapeutic agents (1). The first use of antibiotics was followed after few years by the development of bacterial resistance mechanisms: an example is represented by penicillin, discovered by Fleming in 1928, of which in 1940 the bacterial enzyme penicillinase was discovered capable to disrupt its beta-lactam ring (2). The number of beta-lactamase enzymes has increased exponentially over the years since the introduction of the first beta-lactam antibiotic (3) and, together with this antimicrobial class, each molecule able to interfere with an antimicrobial target inevitably led to the development over the years of a drug bacterial resistance.

Antibiotics, in their broadest meaning of compounds killing microorganisms, were overinterpreted: this term does not define a specific class of molecules/compounds able to hit a similar target or functions on the organism, but only an ability to kill or inhibit bacteria, viruses or parasites by specific interactions with their biochemical targets (1, 4).

Unfortunately, the massive use of antimicrobial compounds, from their introduction in the therapeutical clinical practice until today, accompanied with an incorrect and off-label use, nonprescription, not only in human, but also in veterinary medicine, contributed to the selection and spreading of resistance genes and resistant bacteria (4).

Indeed, antimicrobial resistance is a natural process, essentially related to the Darwinian selection: microorganisms developed an adaptive response able to counteract the effects of toxic compounds. In fact, penicillinase enzymes were identified before the use of penicillin and this finding can be appreciated since antibiotic resistance genes are components naturally present in microbial populations (1, 4). In the past 60-70 years the use of antimicrobials has determined an increasing of

selective pressure and competition between pathogenic bacteria, that caused the onset of protective mechanisms by bacteria like the prevention of entry or the export of drugs, the production of enzyme that destroy or modify the antimicrobial molecules (4). Moreover, Andersson et al. reports that the exposition of bacteria to sub-lethal or sub-inhibitory concentrations of antimicrobials can increase the phenotypic and genetic variability, as well as the antimicrobials themselves can act like signaling molecules that influence physiological activities, biofilm formation and gene expression, with the possibility to increase the virulence of bacteria (5).

Even though the natural production of antimicrobials in many microorganisms is widely recognized and widespread, this phenomenon is limited and contributes to the onset of antimicrobial-resistant bacteria; the introduction of antibiotics in agriculture, human and veterinary medicine has increased the selective pressure and should be considered the worldwide drivers of antimicrobial resistance (6).

In high-income countries the selective pressure caused by the high rate of antimicrobial use, has increased the prevalence of resistance strains and the transmission of resistance genes, inducing countries to shift towards new and expensive drugs (7, 8). In these countries where there are more economic possibilities and bacterial infectious diseases are not of primary importance, the decrease of effectiveness of first line antibiotics has been replaced by more expensive second and third choice antibiotics (8). Conversely, in low-income and middle-income countries where infectious disease has a great importance, the access to these drugs can be difficult and patients with a resistant infection may be unable to obtain a specific therapy (7, 8). Moreover, in developing countries the high rate of infections due to poor hygiene (diarrheal diseases, HIV, respiratory bacterial infections and nosocomial infections) facilitate the spread and evolution of resistant pathogens (8, 9).

In recent years, large differences in the frequency of resistant infections have been noted between European countries and different regions of USA (10, 11). In European countries has been identified a higher rate of antimicrobial resistance in countries with high consumption of antimicrobials, probably due to the higher consumption in southern and eastern countries than in northern (10). In USA, between 1999 and 2010, prescription of antibiotics is decreased of 17%, but in regions of southeast the consumption was twice than those consumed per person in the regions of Pacific northwest and in New England (12).

The spread of resistant bacteria and genes coding for their acquired resistance can follow a variety of routes: in particular the use of antimicrobial agents in animals can results in antimicrobial resistant bacteria and their resistant genes can reach the human population through different transmission ways and vice versa (Fig.1) (13). The degree of contribution of these distribution pathways of resistance genes are very complex and not well determined, because of the complexity of distribution on environment and expression of resistance in bacterial pathogens (13).

Mostly, the antimicrobial resistant pathogens come from antimicrobial use in human medicine, but resistant bacteria such *Enterococcus* spp., *Salmonella* spp. and *E. coli* of animal origin can colonize the intestinal human microbiota: for example people more exposed to contact with animals (farmers who use medicated feed, slaughter-house workers and food business operators) often have a higher incidence of resistant bacteria in feces than the general population (13). Not only pathogenic and zoonotic bacteria can be transmitted from animals to humans and carry resistance genes, but also commensal nonpathogenic bacteria: for the former the principal challenge is the treatment of the infection itself, for the seconds the acquired resistance is more insidious, because resistance genes can be integrated in the commensal intestinal flora and give treatment problems in the future (13).

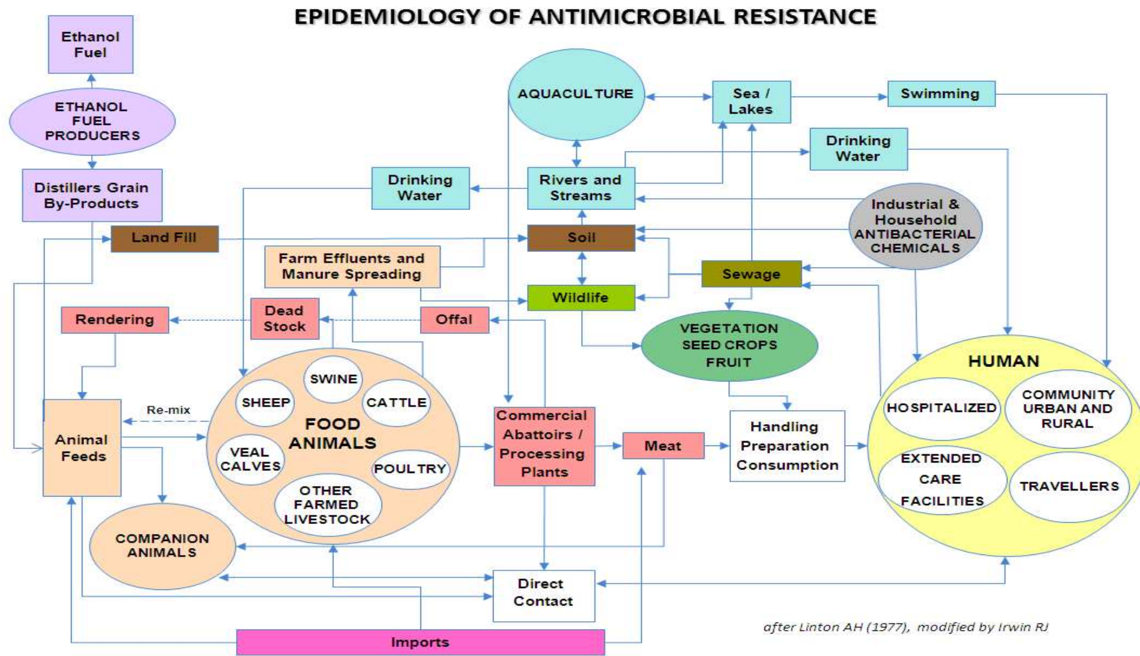

Figure 1: Epidemiology of antimicrobial resistance. McVey D, Kennedy M, Chengappa M. Veterinary microbiology, Wiley. Blackwell, Hoboken, NJ; 2013.

1.2. A "One Health" approach: perspective and reality

In the first two decades of 21st century, emerging zoonotic disease with high pandemic potential and mortality created several international crisis: several viruses have had an animal to human spillover (Sars-CoV; Avian Influenza H5N1) and in some cases (Sars-CoV-2) rapidly spread worldwide with a human to human transmission, although it has been also reported a blind bottom human to domestic animal (cats and dogs) transmission (14, 15). Most of the factors influencing the emerging rate of zoonotic disease in recent years include: the extensive international tourism and global trading with very quickly transport of pathogens in every part of the globe; the emergence of new pathogens because of high ecosystem disruption, due to human population pressures and the proximity with wildlife and the intensive agriculture with loss of biodiversity with potential viral amplification (15). Interdisciplinary collaboration is required for prevent and control zoonosis:

governments and scientists worldwide recognized that to reach this common effort is required the collaboration not only of physicians and veterinarians, but also of wildlife specialists, environmentalists, anthropologists, economists and sociologists, among others (16). To encourage this notable collaboration, the World Health Organization (WHO) proposed the term “One Health”, that was adopted with a great enthusiasm by veterinarian and international agencies charged with control of zoonosis: WHO, Food and Agriculture Organization (FAO) and the World Organization for Animal Health (OIE) (16). There is not a single definition of “One Health”, despite the main idea is the promotion of the health, through interdisciplinary study and actions, across all animal species (16). The “health”, according to WHO, takes the meaning of a completely physical, mental and social wellbeing, but must not to be confused with the absence of disease or infirmity (17): according to this conception the One Health Global Network suggests that the aim of One Health is to improve health through the prevention of risks and by reducing the impact of crisis originate from the contact between humans, animals and their environments (18).

The coordinated and multisectoral approach of “One Health” is optimal to manage the complexity and ecological implications of the antimicrobial resistance: this great problem is characterized by interactions between microbial populations affecting the health of animals, humans, and their environments (19,24). Antimicrobials are essential drugs; they are necessary to maintain health and wellness in populations and environments, both humans and animals, and for this reason the resistance to these drugs is a crucial point that needs a worldwide program for its mitigation, well represented by the “One Health” project (20, 23).

Is not a case that Dr. Calvin Schwabe, a veterinarian, coined the term “One Medicine” to denote that human health and medicine is strictly related with animal health, recognizing that the most veterinary activities benefit human health, directly or indirectly (for example control of food of

animal origin, antibiotic residues, etc.) (21). But “One Health” has a wider meaning: animal, human and environmental health are interconnected and with the increment of world human population, pollution, climate changes, evolution of antimicrobial resistance and reduction of earth’s resources, sciences of life must work together to achieve a sole purpose, the future worldwide health (19,22,23).

On the other hand, resistance issue must be addressed taking into account the complexity of ecology using a coordinated and multisectoral approach, such as the “One Health” approach (24). As reported by WHO “The areas of work in which a One Health approach is particularly relevant include food safety, the control of zoonoses and combatting antibiotic resistance” (25).

1.3. One Health approach on the antimicrobial resistance (AMR) issue

To understand the impact of AMR and its implications on health, it is necessary to approach the topic from a “One Health” point of view: causes and effects of resistance to antibiotics are closely related, even in the different use of these compounds that apparently do not seems to have a relationship (26).

1.3.1 Human medical practices and the development of antibiotic resistance

In human medicine, the development of antimicrobials in the last century led to the quickly decrease of mortality and morbidity rates of common infections, but at the same time led to the overuse and misuse of these molecules, not only in people but also in food animals (27). This overuse in human medicine was mostly due to unprescription in countries where antibiotics are available without prescription, but also in countries with more sophisticated healthcare system; furthermore, they are frequently used for mild viral infections, where the benefits are either nonexistent or marginal (27,28). Early signs of antibiotic misuse in Europe and United States showed up with the failure of patients to understand and make their own the rational use of antibiotics, possibly because of poor

adherence to prescriptions and to stocking antibiotics for later (28,29). Since even the optimal use of antibiotics often leads to development of resistance bacteria, the frequent uncertainty of diagnosis in human clinical practice ensures that also a little overuse of antibiotics will occur, even in regimen of proper use of antibiotics (28).

Furthermore, in human medicine, the impact of antibiotic resistance was already evident in the last years of 20th century, when nosocomial and community-acquired infections with antimicrobial resistant bacteria have progressively increased in frequency compared to infections caused by other microorganisms (30). The responsibility of these changes has been attributed to the reduction of mortality in hospital, with the consequent persistence in these structures of patients with impaired host defenses and increasing of susceptibility to infections with opportunistic pathogens, which has limited virulence but can be intrinsically resistant to common antimicrobials, as well as carrying acquired resistance genes (30). Also, the concurrent increased frequency of community-acquired AMR infections influences the models of nosocomial infections, leading to a potential reservoir in addition to the pathogens already present in the hospital setting (30).

As far as human medicine is concerned, the misuse of antibiotics in the past years has been well documented: rates between 40 and 75% in the United States have been reported for prescribing antibiotics for viral upper respiratory infections; from 1980 to 1991 the prescription of antibiotics in England and Scotland increased of 46% and in addition to the inappropriate prescription, physicians are more likely to prescribe broad spectrum antimicrobials, currently considered as second or third-line agents to treat common infections (31,32,33). To reduce the incidence of AMR infections due to AMR organisms is necessary to act against the inappropriate use of antibiotics in community and hospital settings, but to gain this result is also necessary changing physician behavior by overcoming the barriers to change in practice (34).

Consequences on human health of AMR could be different, in fact Barza M. (2002) identified at least five mechanisms by which AMR can have adverse effect on human health: the first he called "the attributable fraction", referring to a treatment with antimicrobial agents that reduce various components of microbiota, and in consequence to this treatment, the susceptibility of the patients to other infections increases (35). The second potential mechanism of increasing disease burden caused by AMR is a genetic linkage of resistance traits and virulence factors, resulting in increased intrinsic virulence of resistant bacteria compared to drug-susceptible strains, due to the co-transfer of resistance and virulence genes. The result is a potential reduction of the infective dose for multi-drug resistant bacteria, or an increasing number of potential infected subjects, compared to drug-susceptible strains (35). Another important consequence of increasing AMR, mostly against commonly used antimicrobial drugs, is the inefficacy of initial therapy or reduction of available molecules, often with the need to resort to more toxic or expensive molecules. The fourth and the fifth mechanisms identified by Barza M. were similar to the "attributable fraction" and the genetic linkage between virulence and resistance, but originated from food animals, which could be a potential source of infection for humans. These phenomena originate from increased exposition to antimicrobials in animals, in particular food animals, which can lead to increased colonization of the animals by antimicrobial resistant bacteria (35).

1.3.2 Veterinary practices and the development of antibiotic resistance

In recent years the use of antimicrobials in animals and agriculture is increased, especially in the twentieth century because of the intensive use antimicrobial growth promoters (AGP) in livestock animals: AGP were administered as feed additives at subtherapeutic amount with the aim to improve growth of food animals (36). The mechanisms of action of AGP are various, but in general involve an interaction with the intestinal microbiota; in literature were reported 4 principal mechanisms of action of AGP: (i) AGP inhibit endemic subclinical infection, reducing the organisms

use of innate immunity for this purpose; (ii) AGP reduce the production of catabolites like ammonia or bile, because of the reduction of bacteria in the organism; (iii) AGP reduce the use of nutrients by bacteria, and lastly (iii) AGP allow the uptake of nutrients because the intestinal wall in AGP-fed animals is thinner (37,38). Subsequently, other mechanisms of AGP mode of action were identified, in particular is involved a non-antibiotic anti-inflammatory effect of these antibiotics through the accumulation in phagocytes and the reduction of pro-inflammatory cytokines (36). If on one hand the use of AGPs has led to benefits, especially in improving the production efficiency of certain categories of farming (39,40), on the other hand the AGPs themselves have irreversibly increased the onset of antibiotic-resistant bacterial strains, and this increase it was reported after few years the beginning of their use (41,42). For this reason, the World Health Organization (WHO) in 1997 and the Social Committee of the European Union in 1998 included the use of antimicrobials in animal feed among public health issues (43). Already in 1985, the WHO had identified the problem of antibiotics in animal feed as a source of potential spread of antibiotic resistance for humans, as many molecules were used both in feed and for the treatment of human infections (44). For example, the increase of resistant strains is well documented in consequence to the use as AGP of tetracycline and penicillin in swine and poultry feed, mostly in the case of *Salmonella* infections, and at the same time the colonization of human intestine with antibiotic-resistant *E. coli* for long enough is able to pass on their resistance genes (45,46,47).

From the first report in 1976 to more recent years, there have been numerous articles in peer-reviewed journals and reports documenting the possibility of the spread of antibiotic-resistant bacteria from animals to humans via the food chain or directly (48,49,50). A recent report by the British government carried out a statistical evaluation on articles published up to that moment concerning the possibility of transmission from animals to humans of antibiotic resistant bacteria: 59% of these showed that the use of antimicrobials in agriculture increases the number of AMR

bacteria in humans (51). It should be noted that several infections in humans with probable foodborne origin have been reported, which have been pointed out on the use of antibiotics in animals, in order to reduce the possible sources of AMR bacterial infections in man. For example, Nordstrom et al. reported *E. coli* AMR urinary tract infections (UTI) derived from foodborne infections, noting that it could transiently colonize the human gastrointestinal tract and create a reservoir for possible infection (52,53).

A ban was introduced in 2006 in Europe from the European Commission (EC) on the use of growth promoters, limiting the use of antibiotics for direct administration and medicated feed only in the presence of a medical prescription (54). However, the consumption in agriculture of antibiotics is not decreased, because of the increasing of prophylactic and metaphylactic use: this is documented by the European Medicines Agency (EMA) report on sales of veterinary antimicrobials in 25 European Countries in 2011 (55). The administration through oral powder (medicated feed) represents the 48% of the total sales for the different pharmaceutical forms of veterinary antimicrobial agents for food-producing animals, followed by premix (36%) and oral solution (8%); only 7% is due to injection and 1% to intramammary preparations (56).

Although the spread of AMR from food of animal origin to humans has been widely described in the literature, there are few publications that highlight a real threat to human health deriving from the use of antimicrobials in farmed animals. Most of the studies and microbiological surveillance report does not define the actual direction or source of resistance genes between man and livestock (55). For example, a study of 2012 on carbapenem resistance identified the first carbapenem-resistant Enterobacteriaceae (CRE) on livestock, and from this year, numerous studies have succeeded it, but these antimicrobial molecules are banned in veterinary medicine, thus suggesting that these bacteria are of human origin rather than veterinary origin (57,58,59).

In any case, the need to reduce the use of antibiotics in animals remains essential, in order to reduce the selective pressure on bacteria in general, on a "One Health" point of view. For this purpose, to promote the responsible antimicrobial use and in consequence to the publication by WHO of the classification of Critically Important Antimicrobials (CIAs) in human medicine, the EMA published a categorization of antimicrobials in four classes according to the potential consequence to public health when used in animals: A (avoid – not authorized in veterinary medicine), B (restrict), C (caution) and D (prudence) (60,61). Furthermore, in this report, are indicated the route of administration of antibiotics that should be considered alongside the categorization, when prescribing antibiotic: these are listed by the worst in terms of AMR impact (oral administration via feed or premixes) to the best (local and parenteral individual treatment) (61).

1.4. Bacterial threat: the need for new antimicrobials

There are many bacterial species involved in the principal infections with AMR bacteria: their resistance targets have been identified starting from the last years of the twentieth century, as in the case of some Gram-positive bacteria, Methicillin and Vancomycin Resistant *Staphylococcus aureus* (MRSA; VRSA), Penicillin resistant *Streptococcus pneumoniae* and Multi-Drug Resistant *Clostridium difficile* (62).

WHO recently listed the 12 bacteria or bacterial family that pose the major threat for human health in terms of drug-resistance, and for which new antibiotics are desperately needed: the aim of WHO was to put the test on bacteria that urgently need novel effective antimicrobials (63). These bacteria are ranked according to the degree of threat to human health (63,64):

- <u>Critical priority</u>

1. *Acinetobacter baumannii* (carbapenem resistant)
2. *Pseudomonas aeruginosa* (carbapenem resistant)
3. *Enterobacteriaceae*, extended-spectrum-β-lactamase-producing (carbapenem resistant; 3rd generation cephalosporin-resistant)

- <u>High priority</u>

4. *Enterococcus faecium* (vancomycin resistant)
5. *Staphylococcus aureus* (methicillin, vancomycin resistant)
6. *Helicobacter pylori* (clarithromycin resistant)
7. *Campylobacter* spp. (fluoroquinolones resistant)
8. *Salmonella* spp. (fluoroquinolones resistant)
9. *Neisseria gonorrhoeae* (3rd generation cephalosporin-resistant, fluoroquinolones resistant)

- Medium priority

10. *Streptococcus pneumoniae* (penicillin-non-susceptible)

11. *Haemophilus influenzae* (ampicillin resistant)

12. *Shigella* spp. (fluoroquinolones resistant)

The serious threat posed by these resistant bacteria and others not listed is certainly accentuated by the lack of investment by large pharmaceutical companies in the development of new antimicrobials with new targets for action. The reasons for this disinvestment are to be found in the reduced economic return: new antibiotics would be used only to treat infections that broader spectrum antibiotics would not be able to treat; furthermore, antibiotics are provided for a limited treatment in time, as opposed to drugs intended to treat chronic diseases (62).

Furthermore, the major threat is represented by Gram-negative bacteria, due to their outer membrane (LPS), which confers greater protection than Gram-positive peptidoglycan, and for this reason, few new antimicrobials active against them have been authorized, the last of which are represented by quinolones, discovered in 1962 (64,65).

It should be noted that, in a 2009 EMA reported that from 2002 to 2007 in member countries the human blood infections caused by resistant Gram negative (*E. coli* and *K. pneumoniae* resistant to third generation cephalosporins; *P. aeruginosa* resistant to carbapenems) have proportionally equaled over time the Gram-positive infections, which until then were the most frequent, in particular those caused by MRSA (66).

1.4.1 Superbugs: Multi, Extensively and Pan-Drug Resistant bacteria (MDR, XDR, PDR)

Among bacteria that represent the greatest threat to health, in the broadest meaning of "One Health", there are **superbugs**, bacteria with high pathogenicity, both in terms of mortality and morbidity, for which resistance has not developed against an only antimicrobial, but to different

antimicrobials, even belonging to different classes (67). Superbugs are super-resistance vehicles and in certain cases they have developed an increased virulence and transmissibility, due to multiple mutations which confer high level of resistance to the recommended antibiotic classes and the therapeutic options for these bacteria are very low, as well as hospital care are longer and more expensive (1,67). Superbugs are both nosocomial and community-acquired bacteria, the species of which are mainly represented by *Acinetobacter baumannii, Burkholderia cepacia, Campylobacter jejuni, Citrobacter freundii, Clostridium difficile, Enterobacter* spp., *Enterococcus faecium*, *Enterococcus faecalis*, *Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Mycobacterium tuberculosis, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella* spp., *Serratia* spp., *Staphylococcus aureus, Staphylococcus epidermidis, Stenotrophomonas maltophilia*, and *Streptococcus pneumoniae* (1).

The exact definition of superbugs should be Multi-Drugs-Resistant Organisms (MDROs), as they are resistant in vitro to more than one antimicrobial agent: this precise definition is necessary above all to carry out an accurate epidemiological surveillance, in different places using the same criteria (68). MDROs infections are particularly difficult to treat because their treatment is often delayed, due to the lack of effectiveness of the first therapeutic approach; furthermore, these infections have a poor outcome for the patients (68,69). For example, MDR-non fermenting Gram-negative bacteria such as *Pseudomonas aeruginosa, Burkholderia cepacia* complex, *Acinetobacter* spp. *Stenotrophomonas* spp. are niche pathogens that mainly cause opportunistic infections in nosocomial patients that are immunocompromised or critically ill; rarely cause infection in healthy people (69,70). These microorganisms can lead to resistance to almost all antibiotics available on the market, being sensitive only to older molecules, such as polymyxins (polymyxin B, colistin), which are more toxic; and this phenomenon is potentially more dangerous because there are very limited numbers of new antimicrobials in development (68,70).

Magiorakos et al. focused on the use of the definitions of MDR, extensively drug resistant (XDR) and pan-drug resistant (PDR) bacteria, that characterize the resistance in MDROs: these definitions have a defined significate and in function to the classification of MDROs in epidemiology surveillance reports they must be used with a standard parameter (68). With this classification, resistant organisms were categorized as non-susceptible, intermediate, or resistant to an antimicrobial agent using **clinical breakpoints** and not epidemiological cut-offs (European Committee on Antimicrobial Susceptibility Testing, EUCAST; Clinical Laboratory Standard Institute, CLSI and/or USA FDA) and only acquired resistances were taken into consideration, but not intrinsic resistance (68). Definitions of MDR, XDR and PDR bacteria proposed by Magiorakos et al. are showed in Figure 2: for each bacterium taken into consideration, different classes of antimicrobials are considered to define MDR, XDR and PDR strains. Definitions are applied only to five species of bacteria because of their epidemiological significance, also in terms of diffusion of their resistances.

Bacterium	MDR	XDR	PDR
Staphylococcus aureus	The isolate is non-susceptible to at least 1 agent in ≥3 antimicrobial categories listed in Table 1[a]	The isolate is non-susceptible to at least 1 agent in all but 2 or fewer antimicrobial categories in Table 1.	Non-susceptibility to all agents in all antimicrobial categories for each bacterium in Tables 1–5
Enterococcus spp.	The isolate is non-susceptible to at least 1 agent in ≥3 antimicrobial categories listed in Table 2	The isolate is non-susceptible to at least 1 agent in all but 2 or fewer antimicrobial categories in Table 2.	
Enterobacteriaceae	The isolate is non-susceptible to at least 1 agent in ≥3 antimicrobial categories listed in Table 3	The isolate is non-susceptible to at least 1 agent in all but 2 or fewer antimicrobial categories in Table 3.	
Pseudomonas aeruginosa	The isolate is non-susceptible to at least 1 agent in ≥3 antimicrobial categories listed in Table 4	The isolate is non-susceptible to at least 1 agent in all but 2 or fewer antimicrobial categories in Table 4.	
Acinetobacter spp.	The isolate is non-susceptible to at least 1 agent in ≥3 antimicrobial categories listed in Table 5	The isolate is non-susceptible to at least 1 agent in all but 2 or fewer antimicrobial categories in Table 5.	

[a]All MRSA isolates are defined as MDR because resistance to oxacillin or cefoxitin predicts non-susceptibility to all categories of β-lactam antimicrobials listed in this document, with the exception of the anti-MRSA cephalosporins (i.e. all categories of penicillins, cephalosporins, β-lactamase inhibitors and carbapenems currently approved up until 25 January 2011).
http://www.ecdc.europa.eu/en/activities/diseaseprogrammes/ARHAI/Pages/public_consultation_clinical_microbiology_infection_article.aspx.

Figure 2: Definitions of MDR, XDR and PDR bacteria. Magiorakos, A. P., Srinivasan, A., Carey, R. T., Carmeli, Y., Falagas, M. T., Giske, C. T., S. Harbarth, J.F. Hindler, G. Kahlmeter, B. Olsson-Liljequist, D.L. Paterson, L.B. Rice, J. Stelling, M.J. Struelens, A. Vatopoulos, J.T. Weber, Monnet, D. T. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical microbiology and infection, 18(3), 268-281.

In recent years MDR pathogens were identified also in animals, not only food animals but also wildlife and companion animals (71,72,73): these bacteria could be transmitted from human to animals and vice versa, but above all they can persist in the environment for long time and the

chances of maintaining resistance genes are high (72). Of particular interest is what reported by Brennan et al and Zanardi et al, namely a high resistance by Gram negative MDR bacteria detected both in samples from cattle in Europe and in wild micro-mammals in Italy against colistin (72,73). This antibiotic has taken on relevance in recent years as a life-saving drug in humans, despite being a long-standing antibiotic, previously abandoned due to its high patient toxicity (74).

Due to its role as a last chance antibiotic, the detection of plasmid-mediated resistance to colistin (mdr-1) in animal-derived MDR bacteria is particularly relevant, especially for their zoonotic potential, and for this reason they represent a potential public health hazard (71,72,74).

1.4.2 ESKAPE pathogens (and others)

Among the superbugs, six bacteria - mainly nosocomial pathogens - have been identified, called ESKAPE, which represent the most serious threat among all MDROs: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (75,76). The WHO has included ESKAPE pathogens among the 12 bacteria for which new antibiotics are urgently needed, and among these bacteria, WHO listed in the critical and high priority list the carbapenem, vancomycin and methicillin resistant one (64,65). These bacteria are associated with higher mortality, especially in the hospital setting, thus resulting in an increase in healthcare costs, not only in developed, but also in developing countries (77).

During the SARS-CoV-2 pandemic it has also been reported that in COVID-19 patients admitted to intensive care units the clinical course is worsened in case of nosocomial bacterial superinfections, with a significant correlation between nosocomial infection and patient death or extension of time in the ICU. Furthermore, in the study reported by Bardi et al., it was seen that the bacteria mainly responsible for infection in COVID-19 patients in the Intensive Care Units (ICU) are represented by *Enterococcus faecium* and *Enterococcus faecalis* followed by Coagulase Negative Staphylococci

(CoNS) regarding Gram-positive, which also represent most of the causative agents responsible for infection, while *Pseudomonas aeruginosa* was mainly found among the Gram-negative, especially in lower respiratory tract infections (LRTI). Furthermore, 31% of these pathogens are MDR, among which the most represented pathogens were MRSA, *Enterococcus faecium* and *Pseudomonas aeruginosa* (78).

The most important mechanisms of multidrug resistance exhibited by ESKAPE pathogens are essentially three, conveyed principally by transposable elements with the horizontal gene transfer (HTG) (plasmids, transposons, integrons or gene cassettes) (79):

- Drug inactivation/alteration by an irreversible cleavage catalyzed by an enzyme;
- Modification of drug binding site or targets of antibiotics;
- Changes in cell permeability due to a reduced permeability or by increased efflux of the drug thanks to efflux pumps (75,80).

In addition to these three mechanisms, the ability to form biofilms should be mentioned, which not only allows bacteria to avoid contact with antibiotics, but also with the host's immune system (75,81). In fact, biofilm can create chemical and physical diffusion barriers to the penetration of antibiotics and, due to the slow penetration of antimicrobials, to organize the microbial population in such a way as to give a response to the stress caused by the antimicrobial agent with a biofilm-specific phenotype (81,82). Furthermore, some biofilms (such as that of *P. aeruginosa*), under stimulation of sub-MIC concentrations of some antibiotics, can release DNA, called extracellular DNA (eDNA), into the extracellular matrix, causing an alteration of the extracellular environment as eDNA can chelate cations such as magnesium ions, whose function is above all to regulate the intracellular survival of bacteria and their pathogenesis (83,84,85).

Enterococci are Gram-positive cocci, normally present in nature in the external environment and as intestinal flora of man and animals. They can survive under contrary environmental conditions because of their capacity to produce bacteriocins, called enterocins, which are small molecules or peptides with antimicrobial activity towards closely related Gram-positive bacteria, such as *Listeria* spp., and for this reason, they are also used as probiotics (86). Enterococci are opportunistic pathogens which cause severe infections, mostly in hospitalized patients: 80-90% of clinical isolates are *Enterococcus faecalis* and 5-10% are *Enterococcus faecium* (87). Enterococci exhibit intrinsically reduced susceptibility to penicillin and aminopenicillins and in parallel intrinsically low level of resistance to lincosamides and aminoglycosides; but this low resistance is overcome when these bacteria have an acquired resistance against these antibiotics (87). Enterococci can in fact obtain acquired resistance to different classes of antimicrobials including erythromycin, glycopeptides, tetracyclines, vancomycin (Vancomycin Resistant Enterococci, VRE), aminoglycosides (high level of resistance to gentamicin, HLRG) and streptomycin (high level of resistance to streptomycin, HLRS) (88). Due to the dualism between the use of enterococci in the dairy industry and their recognized multiple resistance to antibiotics, enterococci are often found in dairy production, but their presence can be very dangerous. The rapid acquisition of multiple antibiotic resistance by these bacteria has raised the question of how to treat food-borne Enterococci (89). Actually, a correlation has not yet been found between ingestion of foods containing enterococci and infection with this genus; furthermore, it seems that food acquired Enterococci are sensible to the most part of antibiotics, rather than Enterococci from nosocomial infections (89).

The Enterococci that most represent the problem related to ESKAPE are VRE and HLR, as they cause a persistent problem in the hospital setting. The main genes carrying vancomycin resistance are *Van A*, present in enterococcus faecium and *Van B*, which causes a high degree of resistance to vancomycin, but susceptible to glycopeptides and teicoplanin (88). Moreover, Enterococci can

transfer the vancomycin resistance to other microorganisms, mainly to *Staphylococcus aureus*, causing the insurgence of vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus* (VISA, VRSA) (88).

For what concern MDROs *Staphylococcus aureus*, the major threat is represented by MRSA, whose resistance is reported against methicillin, dicloxacillin, oxacillin, cloxacillin, nafcillin, and cephalosporins. In recent years MDR MRSA is becoming resistant also to glycopeptides antibiotics, such as vancomycin, reducing the therapeutic possibilities, mostly in hospital setting (88). In the veterinary field, the first nosocomial MRSA infections were found in horses in 1997 in the USA and Japan (90,91), followed by the first report of MRSA infection in horses and in the veterinary staff of a veterinary teaching hospital in North Carolina in 1999 (92). Thanks to the recent progresses in next generation sequency of whole genomes, the different evolutions and disseminations of MRSA have been described more precisely. MRSA was originally a hospital-associated MRSA (HA-MRSA); MRSA infections were subsequently identified in the community, not associated with the hospital environment, with evolution and origin of their own (community-associated MRSA, CA-MRSA). CA-MRSA infections can also be caused by livestock-associated MRSA (LA-MRSA), but they differ from these in several genomic traits (93). Horses and veterinary staff of equine clinics infection with MRSA can be of different origin, both LA-MRSA and CA-MRSA (93).

Not only MRSA is of particular interest in veterinary medicine, but also *Staphylococcus pseudintermedius* and Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP), they both cause skin infections, upper respiratory tract infections and otitis in companion animals and livestock, but only *S. aureus* can be zoonotic; while *S. pseudintermedius* is preferentially an animal pathogen (94). Resistance of MRSA and MRSP to methicillin is encoded by *mecA* gene and is frequently associated with MDR phenotype (95,96). MDR-MRSA and MDR-MRSP are emerging

worldwide, mostly in companion animals, both healthy and symptomatic, and is reported for these pathogens an increasing resistance to tetracycline and erythromycin, that could represent a potential threat for the health of animal handlers or veterinarians, due to close contact with animals, and in consequence to this, for the public health in general (94,97).

Klebsiella pneumoniae is another important microorganism of the *Enterobacteriaceae* family, for which is requested a particular attention: it represent an ESKAPE MDRO causing frequently lower respiratory tract infection and urinary tract infections (UTI) associated to catheters (88). In addition, recently also emerged as an Extended Spectrum Beta-lactamase (ESBL) strain, for which resistance to beta-lactams antibiotics is extended to cephalosporins and oxyimino-monobactams but generally remains sensible to clavulanic acid and tazobactam (98,99,49). Furthermore, *Klebsiella pneumoniae* emerged also as carbapenem resistant (*Klebsiella pneumoniae* Producing Carbapenemase, KPC), because of the KPC-1 beta-lactamase enzyme which is harbored by a plasmid that encode a multiple resistance to carbapenems, ES-cephalosporins and aztreonam (100). Besides KPC-1, KPC-2 is another plasmid-mediated enzyme that vehicles imipenem resistance among carbapenem resistant *K. pneumoniae* and these resistance targets represent a potential MDR mechanism for the future (101). Great attention should be paid to *K. pneumoniae* not only as responsible for foodborne and extraintestinal infections in humans, but also as responsible for extraintestinal infections in animals, in particular mastitis in dairy cows, in which the prevalence of antibiotic resistance is increasing (102,103). Taking this into account, it is very important to monitor the presence of MDR *K. pneumoniae* in products of animal origin, because the misuse and abuse of antibiotics in animals can lead to the selection of strains resistant to different antibiotics that can involve the consumer and therefore cause severe and difficult to treat infections. This is the case, for example, of the colistin resistance gene *mcr-1* detected in *E. coli* and coming from livestock (104). In *K. pneumoniae*

mcr-1 it has only been identified from clinical and non-foodborne isolates, but it is still possible that it may be isolated from these samples in the future (102,104).

As reported by Hirsch and Tam, *K. pneumoniae* KPC retains the capacity to obtain resistance against carbapenems and other antibiotic classes, and for this reason good therapeutical results have been obtained thanks to the combination of different antibiotics, such as tigecycline and aminoglycosides or polymyxins associated with other antibiotics (101).

Acinetobacter baumannii is an opportunistic pathogen, causing principally nosocomial infections both in humans and, as more recently evolved, in animals, with the ability to spread in healthcare settings with epidemic potential, because of its tolerance to desiccation and to retain antimicrobial resistance to numerous drug classes (fluoroquinolones, broad-spectrum beta lactams, carbapenems and aminoglycosides) (105,106). In this case, the zoonotic potential of MDR *Acinetobacter baumannii* does not derive from food producing animals, but more probably companion animals can play a role, because of their direct contact with humans (107). In particular MDR isolates of *Acinetobacter baumannii* causing infection in animals and humans in some cases have the same genetic lineage, but the epidemiological origin may differ. This possible link between MDR *A. baumannii* of human and animal origin must pay attention to the importance of avoiding the selection of such strains, practicing targeted therapy, based on in vitro susceptibility to antibiotics (105).

The ESKAPE pathogen that has the greatest intrinsic resistance characteristics is *Pseudomonas aeruginosa* (PA), because of its very low permeability of membranes, due to the high presence of efflux pumps and for its ability to change the antibiotic targets: all these mechanisms are due to the high capacity of PA to transfer genetic elements horizontally (88,108). Furthermore, PA has constitutive expression of *AmpC* beta-lactamase that confer it, together with the low

permeability and the expression of efflux pumps with a wide substrate specificity, an intrinsic resistance to beta-lactams (penicillin G, aminopenicillins included those combined with beta-lactamase inhibitors, first and second cephalosporins) and a constitutive facility to acquire resistance to other antimicrobial classes such as aminoglycosides, fluoroquinolones and carbapenems (108). PA can exhibit all the known mechanisms of resistance against most antimicrobials in use, even in the same strain (108):

- Activation of the chromosomal AmpC cephalosporinase;
- Production of beta-lactamases for different antimicrobial classes: carbenicillinases and ESBL of class A, oxacillinases and carbapenemases of class B;
- Reduced membrane permeability due to loss of OprD membrane porins and consequent reduced entry of some antibiotics such as imipenem;
- Overexpression of active efflux pumps with a wide substrate range;
- Synthesis of aminoglycosides-modifying enzymes (phosphoryltransferases, acetyltransferases and adenylyltransferases);
- Structural alteration of topoisomerases II and IV determining quinolones resistance.

The infection with MDR or PDR PA is linked to hospital settings and patients in critical conditions, particularly in humans it is associated with the genetic disease cystic fibrosis (CF) and in these patients can cause serious implications, with high morbidity and mortality (109). The infection in CF patients with PA is related to the ability of this microorganism to form biofilm in compromised lungs, because of impaired mucociliary clearance that does not moves away the microbes. Prolonged antibiotic therapies in these patients cause the selection of resistant strains like PA, that in turn may cause chronic infections with a high percentage of therapeutic failure (109). In the case of PA, several studies have not been able to demonstrate that a combination therapy than monotherapy

is more effective to counteract infections from this multi-drug resistant microorganism (110). In infection with MDR-PA the combination of beta-lactam and aminoglycosides is the most used, but the emergence of PDR-PA resistant to beta-lactams, aminoglycosides and fluoroquinolones is extremely problematic, and pays attention to the early recognition and control of these bacteria (110). Also, in veterinary medicine PA plays an important role, mainly in canine dermal and urinary tract infections and otitis as a secondary pathogen (111,112). The direct contact between dogs and their owners may transfer antimicrobial-resistant bacteria which could lead to zoonotic infections, in particular infection with MDR-PA, which have also been identified in canine otitis, such as strains of PA carrying carbapenemase of the metallo-beta-lactamase VIM-2 type have been found (113).

Enterobacter spp., of which the main pathogen is represented by *Enterobacter cloacae,* is a genus of the *Enterobacteriaceae* family, significantly responsible of nosocomial infections with MDR strains, both in human and in veterinary hospital settings (88,114,115). As with *K. pneumoniae* and other *Enterobacteriaceae,* resistance in *Enterobacter* spp. occurs through ESBLs enzymes, carbapenemases - including KPC – cephalosporinases and metallo-beta lactamases; while remains susceptible generally only to tigecycline and colistin and few others in the human setting (114). In veterinary medicine, together with ESBL genes, fluoroquinolones resistance plasmid mediated (PMQR) were found in MDR strains of companion animals in extraintestinal infections: these genes are often co-located in the same plasmid and this suggest that the fluoroquinolones resistance in these pathogens is more widespread than expected (115).

Escherichia coli, belonging to the *Enterobacteriaceae* family, is not included among the ESKAPE pathogens and not even among the 12 pathogens on the WHO list, but is of particular interest because it belongs to the MDROs that mostly present ESBL enzymes (116). In human settings, *E. coli* is a worldwide widespread nosocomial and community acquired pathogen which causes

bloodstream and UTI diseases and for the treatment of which carbapenems are generally used (117). The main ESBL enzyme found especially in recent years in *E. coli* is CTX-M, name that reflects the high capacity of this enzyme to hydrolyze cefotaxime: MIC of these microorganisms against cefotaxime is generally > 64 µg/ml (116,118). These strains have become the most prevalent ESBLs widespread and have also been found in animals, both in food producers and companions, and sewage (119,120). Not only ESBL are of interest in *E. coli,* but also the expression of *mcr-1* gene, which confers it the resistance against colistin, a last-resource drug for human MDR bacteria therapies (74,121). Colistin was largely used for human bacterial infections until the '70 and abandoned for its nephrotoxicity, but recently has been reintroduced for MDR infections, mostly in critically ill patients, for which all other antibiotics used in therapy have not been effective (121,122). In parallel, colistin has always been used in food animals, especially in swine, cattle, and poultry, administered via feed, and in some countries, it is still used as a growth promoter (China, India, Japan and Vietnam) (123). Although surveillance on the use of colistin in animals is poor, since 2015 the presence of the plasmid-mediated gene *mcr-1* in *E. coli,* which confers resistance to colistin, has also been identified in animals, as before this year only resistances mediated by chromosomal mutations were known (123,71). *E. coli* is frequently used as a sentinel or bioindicator of antimicrobial resistance in animals, because of its widespread diffusion in animal species and its ability to cause various types of infections, both intestinal and extra-intestinal in humans and animals (124). In animals, for example, cross-resistance to amoxicillin and tetracycline is often found in the MDR strains of *E. coli*, and a high level of resistance to quinolones has also been found in poultry (124,125).

1.4.3 *Salmonella* Typhimurium

One of the major threats for the public health and food safety is the *Enterobacteriaceae* *Salmonella enterica* and its numerous serovars (based on the surface antigens O and H): within the non-typhoidal *Salmonella* group, most antibiotic-resistant infections are caused by *Salmonella enterica* subsp. *enterica* serovar Typhimurium (126). *Salmonella* Typhimurium infection is generally foodborne, because food-producing animals (poultry, pigs, cattle, fish and seafood) are the main reservoir of this pathogen and the consumption of contaminated meat, eggs or animal derivatives undercooked or raw are the main mode of transmission and infection to human or animals (127). Because of its broader range of host tropism, *Salmonella* Typhimurium is one of the two main serovars (with *S.* Enteritidis) responsible of foodborne infections in humans and animals with an MDR strain and, in the last decades, a peculiar phage type evolved and disseminated rapidly worldwide: *S.* Typhimurium DT104. This phage type is commonly MDR and resistant to 5 antibiotics: ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline, but it easily acquires additional resistance traits to other critical antibiotics such as quinolones, trimethoprim and cephalosporins (126,128). Studies on epidemiology of *S.* Typhimurium antimicrobial resistance in humans and animals revealed that there are distinct source clusters of this pathogen: animals, animal meat and humans, and for the latter the diversity of antimicrobial resistance panel is greater than the other two, but this presents point in contact with the other two, standing to mean that both animal and meat sources could be involved in *Salmonella* infections in humans (126). A lower level of antibiotic resistance has been shown towards fluoroquinolones (ciprofloxacin), therefore it has been suggested the use of these antibiotics in case of *Salmonella* MDR infections, although the use of cephalosporins is still frequently prescribed, for which the level of resistance is noticeably rising (126).

The sources of infection of *Salmonella* in animals are different because this pathogen is ubiquitous and persistent in environment: in farm animals the infection could be symptomatic or subclinical, and the latter is very dangerous as it allows the pathogen to persist in the farm and spread among animals. Furthermore, rodents, wild birds and bugs can play an important role in the transmission of *Salmonella* between different farms and favor the perpetuation of the infection (129). Both horizontal and vertical transmission are possible for *Salmonella* infection: the former is expressed both in fecal-oral and aerogenic transmission and is particularly effective in swine and cattle; the second, on the other hand, is particularly important in poultry, but present also in dairy cattle, as transmission to the progeny can occur through the transovarial route when the parent birds have a systemic infection and transmit it to the embryo in the egg. However, transmission from the cloaca to the egg through manure is not excluded in poultry (129,130,131). In poultry farms, an important role for the dissemination or introduction of *Salmonella* is played by wild birds, which contaminate water, feed or directly environment; furthermore, the role of human visitors for the increasing prevalence of *Salmonella* in the farms is not excluded (130,132).

The importance of infection with MDR *Salmonella* Typhimurium is related to the worldwide distribution of this foodborne zoonotic infection: in Europe, human salmonellosis is the second zoonotic infection for number or confirmed cases (in an EFSA report of 2013 approximately 96.000 cases), despite the total number of cases decreasing compared to 2007, thanks to the success of the *Salmonella* control plans in poultry (133). As found in the aforementioned report, the highest risk of infection for humans is linked to the consumption of meat or meat preparations as the lowest levels of compliance with the control criteria for *Salmonella* have been identified in foods of meat origin. Moreover, *Salmonella* has often been found in fresh broiler meat, less often in pig meat and rarely in table eggs (133).

Recently a new antibiotic resistance of *Salmonella* was recognized: the resistance to colistin (134). This antibiotic has been widely used in food animals (swine, cattle, and poultry) for prevention and treatment of gastrointestinal infections with *Enterobacteriaceae* and as a growth promoter in different countries, but in recent years was limited (135). The European Medicine Agency (EMA) in 2016 published an advice for the reduction of colistin sales of 65%, to decrease the risk of developing antibiotic resistance: EMA "recommended that colistin-containing medicine should only be used as a second line treatment in animals and that their sales should be minimized across all European Union (EU) Member States to reduce the risk of antimicrobial resistance" (136). The resistance to colistin in *Salmonella enterica* is both chromosomal and plasmid-mediated, but the most important is related to *mcr-1,2,3,4,5* genes, identified in different serovars, mainly in *S.* Typhimurium, derived from food producing animals, food products and human samples (134). The main reservoir of S. Typhimurium *mcr-1* are food producing animals, in particular swine and poultry are the most reported sources of these isolates, but their isolation in human samples is well documented, also of different *mcr* genes (for example *mcr-4*), thus justifying the passage through the food chain (137,138).

The presence of antibiotic resistance plasmid-related in *Salmonella* spp. could pose a threat to public health because of the horizontal spread of resistance mobile elements, with the possible transfer of cross-resistance to different antibiotic classes (134).

1.5. The need for new antimicrobial strategies to counteract AMR

The phenomena of AMR in general and the massive spread of MDR bacteria, both in humans and in animals, are very threating for the public health and the numbers of this issue are emblematic: AMR bacteria caused in Europe only in 2015 about 670.000 human's infections of which about 33.000 patients died (139). For this reason, WHO consider the antibiotic resistance issue as one of the most

important threat for human health in the 21st century and promotes the search for new strategies to counter the advance of this phenomenon (64,65). To achieve this goal, it is not only important to reduce the use of existent antibiotics or improve their compliance, but it is essential to have a new arsenal of molecules (or alternatives to conventional antibiotics) capable of providing both a replacement for obsolete molecules, and an aid to these, to preserve the efficacy of pre-existing antibiotics that are important for the treatment of MDR bacterial infections (140,141).

Two main challenges for developing new antibiotics are represented by (7,8):

- pharmaceutical industries that poorly invest in this project because of the scarce profit and the limited application compared to other diseases;
- the practical difficulty, from a scientific point of view, of synthesizing molecules and/or identifying new bacterial targets capable of compensating for the lack of molecules already used.

In parallel, in veterinary medicine, limited number of investigations has been carried out aimed at identifying new antimicrobial strategies, focusing instead above all on reducing the use of antibiotics in animals, rather than on the development of new, more selective antimicrobials (142). Currently, many farmers adopted the reduction of antibiotic use, mainly in poultry farm, to reduce the impact on antimicrobial resistance, but this approach, if not used in a generalized way, is unable to make big changes, and only increases the production costs (142). Furthermore, the veterinary antibiotic arsenal is more limited compared to the human one, mainly for food animals, due to the possible risk of food contamination with resistant strains deriving from animals, and this further reduces the therapeutic possibilities for animals and leads to an even greater need for new antibiotics (143).

For these reasons the need of new therapeutic strategies against resistant bacteria, both in humans and in animals, are of primary interest, and much more, the research of new veterinary antibiotics

should be encouraged, to identify molecules that are different and more selective than those for human use (143).

1.5.1. New molecular strategies to fight resistant bacteria

Traditional antibiotics essentially work by killing (bactericides antibiotics) or inhibiting the growth (bacteriostatic antibiotics) of bacteria by inhibiting cellular mechanisms and functions (DNA replication, wall synthesis, protein synthesis or RNA transcription). These mechanisms, however, have the disadvantage of causing a high selective pressure on the bacteria, causing a serious increase in antibiotic resistance over the years, as is currently occurring (144).

Different approach should be used to achieve an antimicrobial effect, without further raising the selective pressure on bacteria; in recent years two new strategies are mainly being developed to counter this global threat (144, 145,146):

- Targeting bacterial virulence or bacterial behavior instead the bacterial viability;
- Using new antibiotic adjuvants that could preserve and prolong the lifespan of life-saving antibiotics.

1.5.2 Targeting virulence

Virulence factors of bacteria are essentially toxins, cytolysins, proteases or other excreted proteins and mechanisms that cause direct damage to the host. Therefore, targeting virulence determinants could disarm the pathogen by neutralizing the responsible of the most part of pathogenesis linked to the infection (144). The advantage of this approach is given by the selective pressure reduction caused by the antimicrobial treatment on the bacterial population, as it does not affect cell viability but only its excreted virulence factors. Different pathways of virulence were investigated as antimicrobial target: inhibition of toxin function, bacterial toxin delivery, regulation of virulence expression and the inhibition of bacterial adhesion (144).

Inhibition of toxin function:

Historically, the inhibition of toxin function is a direct effect of proteins such as antibodies (antitoxins) used against toxins of diphtheria, tetanus, botulinus and others severe disease; instead, recently were investigated small molecules as inhibitors of the three major toxins of *Bacillus anthracis*: lethal factor (LF), edema factor (EF) and protective antigen (PA) (147,148). One of these molecules, LFI (an hydroxymate), was able to prevent *Bacillus anthracis* spore formation when administered in combination with ciprofloxacin and confers complete protection in mice immunized with LF and PA (147).

Targeting bacterial toxin delivery:

This anti-virulence mechanism allows to interfere with the transfer of the toxin to the site of action in the host organism. The inhibition of the delivery of the toxin can be carried out both by the action of a molecule capable of binding the toxin before it reaches the site of action (for example cholestyramine in *Clostridium difficile* toxins), and by the inhibition of the excretion system of the toxin itself from the bacteria (for example the type III secretion system, T3SS, inhibited by acylated hydrazones of different salicytaldehydes in *Yersinia* spp., *Pseudomonas aeruginosa*, *E. coli*, *Salmonella* spp. and others) (149,150).

Targeting the regulation of virulence expression:

This virulence inhibition mechanism essentially aims to prevent the formation of the toxins themselves and is related to the interference with the quorum sensing (QS), an important communication system between bacteria of different species in a bacteria population used to regulate different process like biofilm formation, antibiotic synthesis, and virulence factors expression as a function of a population density (151,152). For example, in *S. aureus* the QS regulatory system is represented by *adr* (accessory gene regulator), responsible for the synthesis of

hemolysins, enterotoxins, exfoliative toxins, enzymes and surface proteins important in the pathogenesis of infections sustained by this bacterium in various animal species (152). Bacterial cells detect the concentration of signal molecules in the environment surrounding the colony, in order to determine the bacterial density: when these molecules reach a threshold concentration, there is the expression of specific genes that determine a synchronous change in total bacterial population behavior making up the biofilm (152). The mechanisms of quorum sensing are different, depending on the bacterial species from which they are produced: for example, Gram positive bacteria use modified post-translational oligopeptides called AIPs (autoinducing polypeptides) (153). In Gram negative bacteria, on the other hand, the signal molecules are represented by AHLs (N-acylhomoserine lactons) also called AI-1 (auto-inducer-1) which are synthetized and recognized by LuxI and LuxR homologs (151,154). There are also mixed QS systems, which allow communication between Gram positive and negative bacteria: the AI-2 or auto-inducer 2, represented by the furanosyl-borate diester (155). Therefore, the inhibition of the enzymes that regulate the QS system such as LuxI or LuxR allows to inhibit the AHL-mediated QS, but also the alteration of the AHL concentration through their degradation can inhibit the QS (156).

Not only QS is responsible of the regulation of virulence expression: there are others transcriptional regulators enzyme that allows adhesion or toxin production and secretion and could them represent a potential target for antimicrobial activity. An example is virstatin, a small inhibitor of the *Vibrio cholerae* cholera toxin promoter, which inhibit ToxT transcriptional regulator, preventing the expression of two virulence factors: the toxin coregulated-pilus and the cholera toxin itself (157).

Inhibition of adhesion

Adhesion bacterial proteins are a potential antimicrobial target because their prokaryotic specificity: they are called “pilicides” and are aimed to inhibit the formation of pili and fimbriae. Research on pilicides have been directed towards the inhibition of the periplasmic chaperone proteins of the chaperone usher pathway, a protein complex responsible for the assembly of pili: bicyclic 2-pyridones have shown an inhibition of *E. coli* adhesion on bladder carcinoma cells ex vivo (158,159).

1.5.3 Targeting behaviour

Often the lack of efficacy *in vivo* of traditional antimicrobial treatments is linked to certain behaviors of bacterial populations, such as the formation of biofilms and the ability to form subpopulations of persistent non-multiplying cells, in the host organism, which prevent the entry of molecules into the bacterial cells and confer a certain degree of resistance that is sometimes not found *in vitro* (160). Therefore, new antimicrobial strategies are directed towards the regulatory mechanisms of these behaviours which, to be coordinated between different cells, require communication and coordination systems that can be interfered and manipulated (160).

The persister cells are frequently present in chronic infections and can survive also at high doses of bactericidal antimicrobials, but this tolerance is different from resistance of multiplying bacteria: it essentially derives from the fact that these cells are metabolically inactive (161). Two potential strategies can be implemented against persister cells: kill these cells directly or induce them to revert from persister to multypling phenotype (162,163). One mechanism involved in the persistence state is the expression of chromosomal toxin-antitoxin (TA) protein genes of *E. coli* where toxin protein is involved in the induction of persistence state and antitoxin protein reverse its effect. This mechanism provides opportunities to develop drugs that promote the transition from

persister to actively growing states in bacteria causing infection, although this new strategy is still to be widely investigated, also since this TA system is not present in all bacteria (162,163).

The persister population, together with biofilm regulation and the production of virulence traits, is regulated by the QS system (164). As previously described, mechanisms of QS signaling like AHL in Gram negative and AIP in *S. aureus* have been investigated as targets for the disruption of cell to cell signaling (156). The process of interfering with the QS system is termed "Quorum quenching" (QQ) and numerous ways to disrupt QS system have been investigated and could be grouped into: (i) strategies that inhibit the generation of QS signaling; (ii) those that inhibit signal dissemination and (iii) reception (165). Furthermore, macrolide antibiotics have been shown that act as QS-inhibitors (QSI) at sub-MIC concentrations (166,167).

Another potential strategy to fight resistant bacteria is the bacteriophage therapy: this was used in past in Soviet Union to treat infections, both in animals and in sewage treatment (168). These phage viruses specific of bacteria have been modified to extend their natural host range and, in recent years, to mitigate the lysis effect on bacteria, because the massive lysis of bacteria in the host organism can cause toxic shock (169,170). In addition to the kill effect of lytic phage, the bacteriophage therapy could modify the bacterial behavior, increasing the efficacy of antibiotic therapy, by the overexpression of proteins that repress SOS response, a DNA bacterial repair system that confer antibiotic tolerance (171).

1.5.4 Targeting non-essential pathways: the importance of antibiotic adjuvants

In recent years, only two compounds have been synthetized with a different mechanism of action from traditional antimicrobials with bactericidal action: (a) Brilacidin, a synthetic mimetic of host defense protein and (b) ACHN-975, which is an inhibitor of LpxC, an enzyme involved in the Lipid A biosynthesis (172). All the other antibiotics approved in the last years are essentially modifications

of the pre-existent antimicrobial classes to make them more efficient and robust against the resistant bacteria (173,174). The scarcity of new antibiotics in the pre-clinical experimental phase is due to both scientific and commercial difficulties in this therapeutic field. As already mentioned above, pharmaceutical companies are not encouraged in the search for new antimicrobials due to the reduced cost of their production (cheap drugs administered for a short period of time) and at the same time also in the academic field the research of new antimicrobial strategies are long and expensive (175).

In response to the growing need both to have new molecules in the arsenal of antimicrobials active on resistant strains, and to preserve the activity of those still used, the search for adjuvant molecules is increasingly developing. These are molecules with reduced or absent antimicrobial activity per se, but when combined with other antimicrobials allow to enhance the activity of the antimicrobial compound, therefore determining a synergistic effect (175,176). The best-known combination of antimicrobial + adjuvant is given by amoxicillin (beta-lactam) + clavulanic acid (inhibitor of beta-lactamases). The latter has a very low antimicrobial activity *per se* but administered in association with amoxicillin allows to significantly increase the activity of this antimicrobial, as well as increasing its spectrum of action and lowering its minimal inhibitory concentration (MIC). The action of the two compounds is synergistic, therefore greater than the sum of the two compounds administered individually (177).

The antimicrobial combination has long been used in clinic to improve activity against particular pathogens or to achieve broad spectrum coverage when the nature of the infection is unknown (177). The effectiveness of the combination is evaluated in four possible outcomes: synergy, additivity, indifference, and antagonism: depending on the genetic networks in the specific bacterial species and strain tested (178). Synergy is the most desirable effect, when the two drugs work better

together than individually, whereas additive effect is simply the sum of the effect of each drug, but they do not interact with each other. Indifference occurs when a single component overrides the other, while antagonism is due to the interference of the two compounds which also reduce the effect of the single one (178).

To evaluate the activity of two molecules in combination the Fractional Inhibitory Concentration Index (FIC index) is determined in microbiological laboratories with the checkerboard assay (179). This procedure consists in serial dilutions above and below the MIC of the two molecules cross diluted in a 96-well plate. The FIC is determined at the border of inhibition, called the isobologram and the FIC index is mathematically determined using the following formula (179) where A and B are the two different compounds (Fig. 3):

$$FIC = \frac{MIC_{A\ in\ combination}}{MIC_A} + \frac{MIC_{B\ in\ combination}}{MIC_B}$$

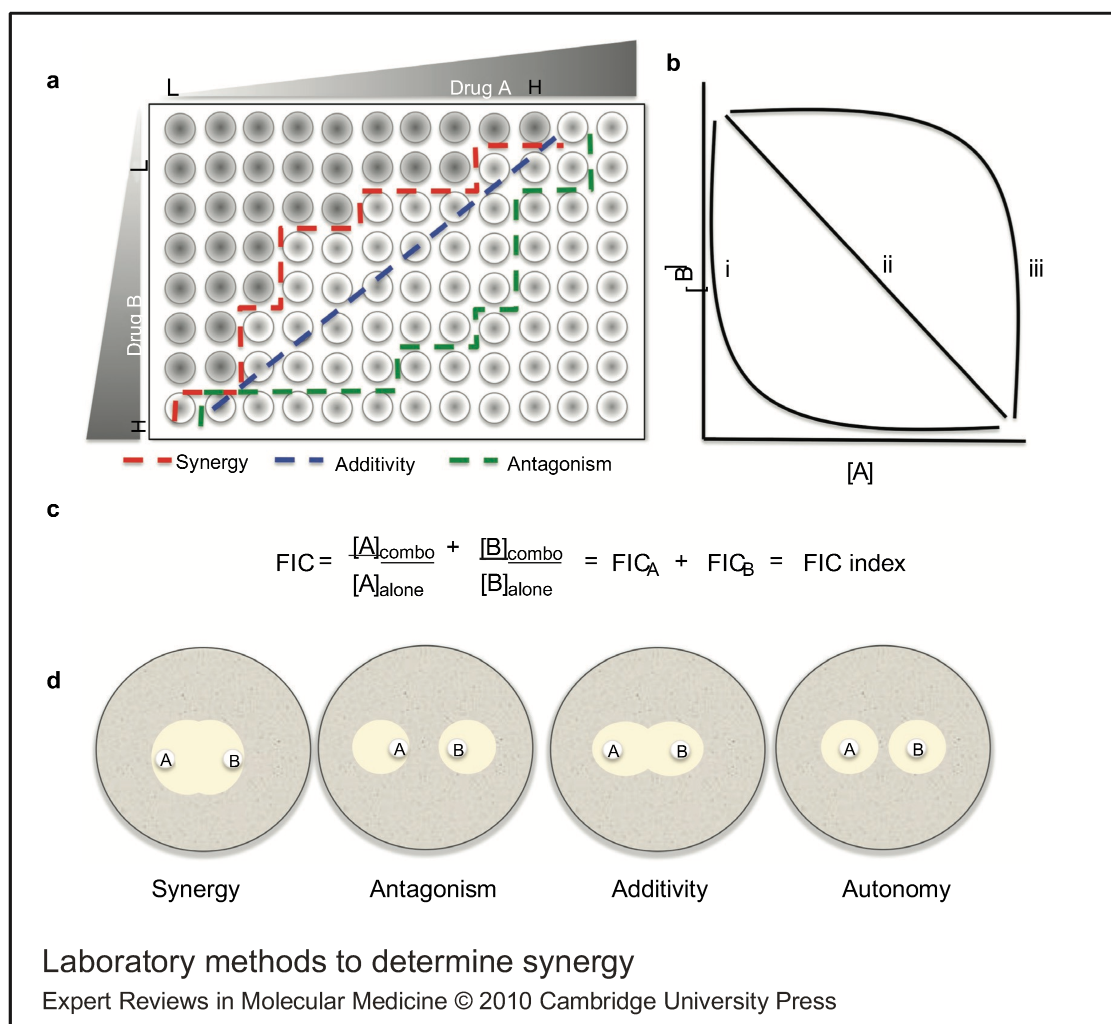

Figure 3:(177) "Kalan, L., & Wright, G. D. (2011). Antibiotic adjuvants: multicomponent anti-infective strategies. Expert reviews in molecular medicine, 13". Laboratory methods to determine synergy: (A) Checkerboard assay: two compounds are serially diluted in a microtiter plate perpendicularly. (B) Isobologram: each combination of drugs is scored for growth and drawn to create the graph. (C) The equation of FIC index to quantitatively assess the combination with MIC values of single drugs and associations. (D) Qualitative assessment of FIC: each drug is soaked onto a paper disc and placed near the other drug on a Petri plate inoculated with the tested strain. Zones of inhibition indicate the type of interaction.

The molecular basis of synergy between two different antibiotics or antibiotic and adjuvant is very important to understand the mechanism of the association action, not only in a microorganism model but also in pathogens implicated in *in vivo* resistant infections. Currently, biological systems applies this approach to understand the relationships between metabolic pathways in bacteria and to make a rational selection of antibiotic pairs (179).

In this sense, the activity of antibiotic adjuvants is not only linked to the increase of the effectiveness of an antibiotic, but it can also be useful for toxic antimicrobials at high concentrations, such as

colistin, for which the association with the adjuvant can lead to a reduction of the side effects related to the action of antimicrobial by significantly reducing its therapeutic dosage (141).

On the other hand, antibiotic adjuvants have not only the potential to reverse the resistance mechanisms in strains with acquired resistance (by reducing the MIC value of associated antimicrobial molecule), but also the promising ability of sensitizing intrinsic resistant strains (for example colistin against Gram positive strains) (176).

Another important advantage to use antibiotic adjuvants is linked to the efforts in the challenging and expensive identification of new essential bacterial targets, which are completely bypassed with the use of this strategy (172).

The peculiarity of antibiotic adjuvants to have little or no antibiotic activity *per se* is related to their different mechanisms of action, therefore, the antimicrobial effect is related to the association with the antibiotic, with which it interacts synergistically. The different mechanisms of adjuvants action were classified according to the target profile in (175) (Fig.4):

- (I): Adjuvants that work on the same bacterial target of antibiotic with two different mechanisms: (I-A) directly inhibiting antibiotic resistance systems (inactivating enzymes) and (I-B) enhancing antibiotic activity by inhibiting bacterial metabolic pathways which are not implied in the direct resistance elements but contributes to the intrinsic resistance mechanisms (efflux pumps systems, membrane permeability, QS inhibitors, biosynthetic pathways inhibitors, etc.).
- (II): Adjuvants that do not directly acts on bacterial resistance pathways, but on the host defense mechanisms to infections (enhancers of innate immune system).

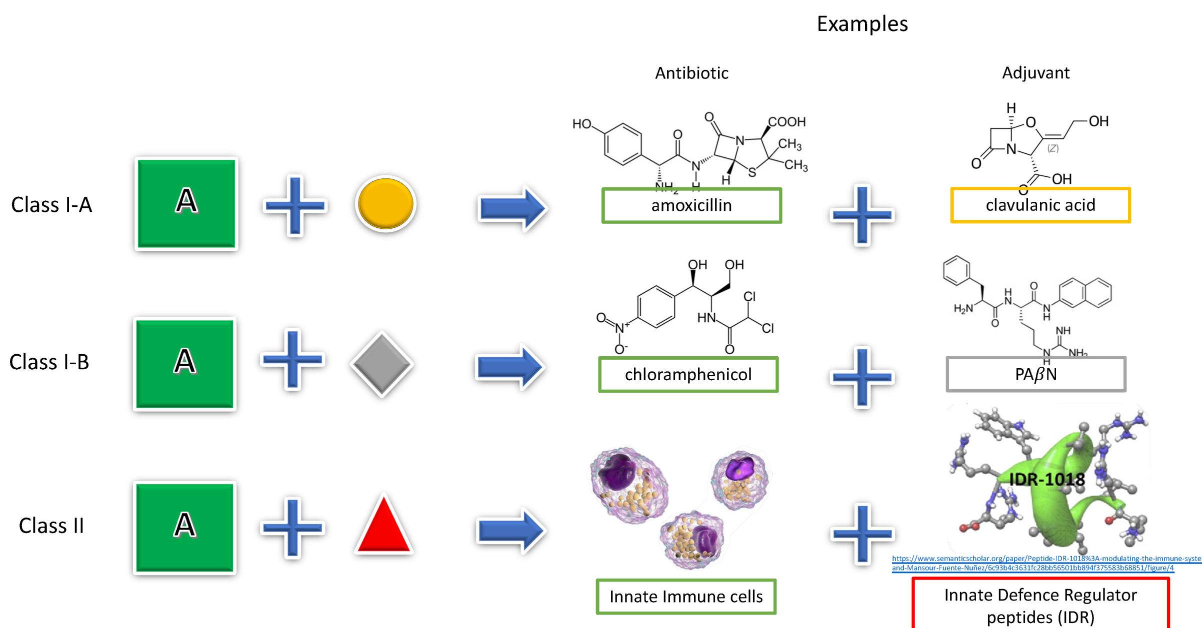

Figure 4: Classification of antibiotic adjuvants: the I-A class is given by antibiotic A (amoxicillin) and adjuvant (clavulanic acid) that act on the same target (in this example bacterial wall) and the action of the adjuvant is given by the enzymatic inactivation of an active resistance mechanism (beta lactamase) (141); class I-B is characterized by the adjuvant action on passive resistance mechanisms or non-essential metabolic pathways (in this case an efflux pump inhibitor -EPI – PAßN) which acts synergistically with the antibiotic (chloramphenicol) thanks to the intracellular accumulation of this molecule (180); class II differs from the previous one because the adjuvant target is not a bacterial pathway but an host defense mechanism of innate immunity to counteract infections, in this example the synthetic Innate Defense Regulatory peptides (IDR) acts both on innate immune receptors of different immunity cells and directly on microbial outer membranes disrupting them, to enhance the antimicrobial and anti-inflammatory properties of this immune system (181,182).

Therefore, the adjuvants activity on the non-essential targets can be carried out through different mechanisms of action, each linked to the activity on different targets, which can be responsible for the antimicrobial resistance capacity, both intrinsic and acquired, of the specific microorganism.

Of the three classes of adjuvants listed, mainly 3 categories of compounds belonging to these have been developed: beta-lactamase inhibitors, efflux pump inhibitors and outer membrane permeabilizers (141,172,175). In addition to these, as previously described, research on anti-virulence targets or metabolic pathways involved in the resistance mechanisms and compounds able to inhibit them in pathogenic bacteria are under development (but not yet in clinical trial) and can be considered together with the class of adjuvants targeting passive and/or intrinsic resistance factors (class 1-B) (144,172,175).

ß-lactamase inhibitors

One of the major resistance mechanisms of bacteria is given by the active production of inhibiting enzymes of antimicrobial molecules, such as ß-lactamase. These enzymes have been naturally produced by bacteria since the origins of the antibiotic era, that is the discovery of penicillin (1). The inactivation of ß-lactam antibiotics is carried out by hydrolysis of the ß-lactam ring, thus giving the bacterium a resistance towards them. ß-lactamases are widespread in pathogenic bacteria thanks to horizontal genetic transmission and can be classified both according to the antibiotics they inactivate (ESBL, carbapenemases, oxacillinases), and to their mechanism of antibiotic hydrolysis (Ser-ß-lactamases and metallo-ß-lactamases) (183,184,185). In general, ß-lactam antibiotics by means of ß-lactam ring induce acylation of Penicillin Binding Proteins (PBPs), responsible for the synthesis of the bacterial wall peptidoglycan: the role of ß-lactamase is to preserve the integrity of this bacterial barrier by disrupting the ß-lactam ring and generating an open circle, which is unable to generate the electrophilicity necessary to acylate the PBPs (185). Two mechanisms of hydrolysis are used by ß-lactamase to destroy the beta-lactam ring: Ser-ß-lactamase use the residue Ser- in the active site to covalently bind the antibiotic, which is released as hydrolyzed inactive compound; metallo-ß-lactamase use the Zn^{2+} atoms of its active site to activate a H_2O molecule for the hydrolysis of the antibiotic (175). Clavulanic acid is a ß -lactamase inhibitor adjuvant with poor antimicrobial activity *per se*, but able to make a strong and irreversible inactivation of the Ser-ß-lactamases, which confer a broad spectrum and potent antimicrobial activity to the association with amoxicillin (175,185).

Strengthened by the success of clavulanic acid, other ß-lactam adjuvants (all inhibitors of Ser-ß-lactamases) have been established and marketed: sulbactam and tazobactam have been combined with ampicillin and piperacillin respectively to increase the spectrum of action of these antibiotics towards resistant strains (186,187). More recently, another class of ß-lactamase inhibitors without

ß-lactam ring structure has been approved, the diazabicyclooctanes (DABCOs), of which the major molecule is avibactam, an adjuvant of cephalosporin ceftazidime, with an active site given by a reactive urea, that increase the efficacy against MDR Gram negative bacteria. The difference with other Ser-ß-lactamase inhibitors is given by the ability of avibactam to bind the ß-lactamase enzyme covalently but reversibly, which instead are irreversible bonds for the other adjuvants (188). Thanks to its ability to restore the hydrolysis of Ser-ß-lactamase in contrast to the formation of an acyl-enzyme intermediate by the ß-lactam-based ß-lactamase inhibitors, avibactam shows an extended spectrum of activity compared to the other three ß-lactamase inhibitors, which confers to the association ceftazidime-avibactam the property of inhibit ESBLs including CTX-M, type A carbapenemases including KPC, type C ß-lactamases, such as AmpCs and type D oxacillinases, such as OXA-48 (188,175).

Nevertheless, DABCOs lack efficacy against metallo-ß-lactamases (type B ß-lactamases), which in recent years have been increasing, of which NDM-1, New Delhi metallo-lactamase, represents the principal exponent, especially associated with elements of multiple resistance in MDR or even PDR bacteria (189). NDM-1 gives resistance to almost all lactams, except for aztreonam, including last resort carbapenems and frequently is bonded with resistance traits to aminoglycosides and colistin (175,189). For this reason, inhibitors of metallo-ß-lactamases (MBLs) should be important to increase the efficacy of penicillins, but also to reduce the presence of MDR and PDR bacteria, though few molecules are being studied and in none of them are clinical trials (190,191). The difficulty to synthetize effective NDM-1 inhibitors is essentially linked to the extreme variability of this enzyme (three different classes of MBLs and 20 variants of NDM-1 evolved in a few years) and to its active site, as the substrate-enzyme interactions involving two Zn ions (191). Aspergillomarasmine A (AMA), a natural fungal derivate, was investigated as NDM-1 and VIM-2 (Verona integrin-encoded MBL) inhibitor, associated with carbapenems, such as meropenem, to enhance the activity of these

antibiotics against resistant strains of *Enterobacteriaceae* and *Pseudomonas* sp. with these resistance alleles (190,192). AMA showed a restored activity of meropenem on murine models of infections with NDM-1 expressing *K. pneumoniae* thanks to the sequestration of the Zn^{2+} ion, essential for the catalysis of the reaction (175,190).

The search for enzymatic inhibitors towards antibiotic inactivating enzymes has not only developed towards ß-lactamases, but also inhibitors of aminoglycosides kinases, or other enzymes that inactivate aminoglycoside antibiotics, have recently been investigated. The resistance towards these antimicrobials is mainly due to an enzymatic modification of the antimicrobial molecule itself operated by Aminoglycosides Modifying Enzymes (AMEs) of three different families: Aminoglycosides Phosphotransferases (APHs), Acetyltransferases (AACs) and Nucleotidyltransferases (ANTs) (193). APHs catalyze the phosphorylation of hydroxyl groups in aminoglycosides antibiotics, thus introducing a negative charge into the antibiotic molecule, causing a loss of ability to bind the ribosome in the active site and therefore a loss of antimicrobial activity (193). APHs confer resistance to different aminoglycosides: tobramycin, amikacin, streptomycin, neomycin B and spectinomycin, but also AACs and ANTs can cause major changes in these antibiotics, such as to prevent their antimicrobial activity (193). A way to reverse resistance to aminoglycosides was found in the synthesis of APHs inhibitors resembling eukaryotic protein kinases (ePKs), given the similarity in folding between APHs and ePKs, although this similarity does not affect the selectivity of action on bacterial and non-eukaryotic kinases (194). An example of these APH-inhibitors is the pyridopyrimidines family, that selective inhibits APH(3’) bacterial enzymes, mainly present in Gram negative resistant strains, thanks to a different binding mode compared to ePKs (194,195). Also other APH-inhibitors were synthetized and tested as adjuvants of aminoglycosides, to restore the sensibility to these antibiotics: for example among different flavonoids, quercetin is able to inhibit the APH(2’’)-IVa in complex with kanamycin, allowing the rationalization of inhibitory

ability of other flavonoid molecules (194). Different inhibitors were also identified against ANTs, enzymes responsible of the inactivation of tobramycin, amikacin, and gentamicin: 7-hydroxytropolone and other tropolones have been recognized to be able to rescue the activity of gentamicin in bacteria expressing ANTs (196,197). The inhibition of AACs was instead obtained thanks to bovine-derived antimicrobial cationic peptides, such as indolicidin and analogues, capable of also inhibiting APHs (198).

However, these enzymatic inhibition strategies of aminoglycosides are still at an early stage of research, so the future application of these molecules as antibiotic adjuvants is still quite far away.

Efflux pump inhibitors (EPIs) and other inhibitors of passive resistance

In addition to the specific active mechanisms of resistance to antimicrobials, such as enzymatic inhibitors, bacteria can evade bactericidal action also thanks to more transversal nonspecific passive mechanisms, such as efflux pumps. The difference between the search for antibiotic adjuvants that act through active inhibiting mechanisms of a specific form of resistance and the development of inhibition systems of passive resistance to different antibiotics essentially lies in the difficulty of identifying such molecules: the former are easily screened using purified enzyme-substrate / adjuvant systems and specific association tests, while for the latter it is necessary to proceed in reverse, starting from a library of molecules, among which it is possible to find a particular molecule capable of inhibiting the passive resistance system interested. An already applied strategy is to test Previously Approved Drugs (PADs) as potential adjuvants of antibiotics already known and in use, choosing from a collection of molecules those that show synergistic activity with the antibiotic or various antibiotics (199). This strategy allows to identify more easily non-toxic molecules for the organism, capable to enhance the activity towards antibiotics already in use, without having their own antimicrobial activity. Furthermore, given the great variability of the possible passive resistance

mechanisms, it allows scientists to identify the mechanism of action starting from an already known adjuvant activity.

For example, Ejim et al. (199) screened more than 1 thousand PADs as adjuvants of minocycline, a semi-synthetic tetracycline antibiotic that inhibits bacterial protein synthesis and which is used both in Gram negative and positive infections, especially for the treatment of acne vulgaris and sexual transmitted diseases, but for which recently intrinsic and acquired resistance occurred (114,200). Of 69 nonantibiotic compounds that synergized with minocycline on *E. coli*, *P. aeruginosa* and *S. aureus* reference strains, six molecules were selected for their potentiation on growth inhibition of minocycline: loperamide resulted the best candidate, especially against Gram negative bacteria and with different tetracyclines other than minocycline (199). Further experiments revealed that loperamide (and other molecules) act as a disrupter of membrane proton motive force (PMF) decreasing the electrical component of this in several bacteria, causing an increase in the energy demand for ATP synthesis and triggering in the bacterium an increase in the pH gradient across the inner membrane, that is responsible for the increase uptake of tetracyclines and other antibiotics (cephalosporins), also in intrinsic resistant strains (199, 201).

This approach of screening phenotypically active compounds from a PADs library on bacterial cells *in vitro* in combination with known antibiotics was able to identify other adjuvants as well, including: (i) compounds targeting the wall teichoic acid biosynthesis for the resumption of ß-lactam activity on MRSA (ticlopidine, an antiplatelet drug, synergically potentiate cefuroxime on MRSA) (202); (ii) compounds which, in combination with novobiocin, an antibiotic that alone has activity exclusively on gram positive, on *E. coli* cause an altered cellular shape due to the inhibition of the cytoskeletal protein MreB (molecule A22), an alteration of the peptidoglycan (pivmecillinam) and impaired DNA synthesis (echinomycin) (203); (iii) compounds such as murgocil, a specific inhibitor of intracellular

membrane associated glycosyltranferase, MurG, which synthetizes the peptidoglycan component lipid II, that, in association with imipenem, potentiate its activity against MRSA (204); (iv) compounds targeting efflux pump systems as potentiators of Gram positive antibiotics through Gram negative bacteria (for example rifampicin), including an anthracycline isolated from the fermentation of a Streptomyces, which synergically acts with rifampicin and linezolid against *E. coli* and *A. baumannii* (205).

The phenomenon of active export of antibiotics through the EP systems is well established as a bacterial passive resistance mechanism, and potentially caused the decrease of efficacy of a variety of antibiotics from 1 to 64-fold (206). The efflux-mediated resistance is predominantly in Gram negative bacteria because of their complexity of cell membrane, which allows to express both a reduced drug intake thanks to the low outer membrane permeability, and to the active drug export, via EPs (206). In most cases EPs are chromosomally encoded, but in some cases, they may also be encoded in plasmids or other mobile genetic elements, thus favoring the transmission of passive resistance elements to other bacteria (207). Bacterial EPs are currently classified into 5 families (Fig.5) (206):

1. The major facilitator (MFS) superfamily
2. The adenosine triphosphate (ATP)-binding cassette (ABC) superfamily
3. The small multidrug resistance (SMR) family
4. The resistance-nodulation-cell division (RND) superfamily
5. The multidrug and toxic compound extrusion (MATE) family

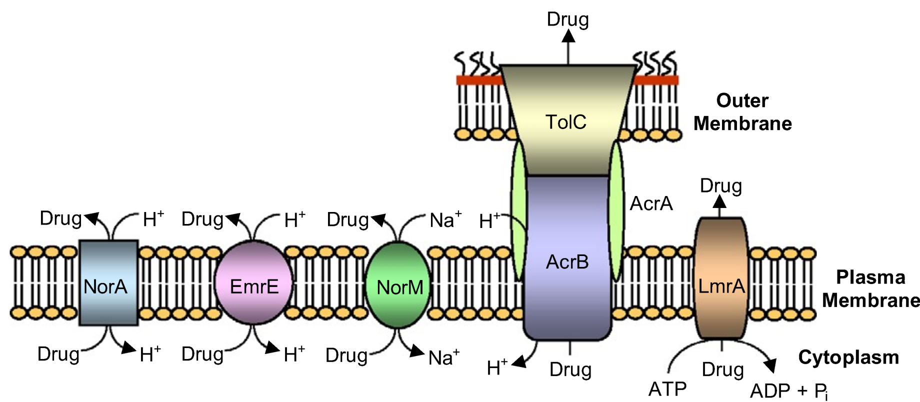

Figure 5: Kumar, A., & Schweizer, H. P. (2005). Bacterial resistance to antibiotics: active efflux and reduced uptake. Advanced drug delivery reviews, 57(10), 1486-1513. Schematic illustration of the main types of bacterial drugs EPs: NorA is a Staphylococcus aureus EP of the MFS type, EmrE is an E. coli SMR, NorM of Vibrio parahaemolyticus belongs to MATE superfamily, AcrAB-TolC of E. coli is a member of RND superfamily and LmrA of Lactococcus lactis belongs to ABC superfamily. All pumps are active ATP or ion gradient dependent (H^+ or Na^+). The RND superfamily can acquire drugs not only from the cytoplasm (such as for the other EPs) but also from the periplasm or from the outer left of the inner membrane.

The most common antibiotic efflux-systems in bacterial pathogens are the MFS and the RND superfamilies, of which the second is expressed by many Gram-negative bacteria (208). The RND tripartite pumps offer to Gram-negative bacteria the ability to synergize with the reduced outer membrane permeability thanks to the directly externalization of drugs in the external medium, so the reentry of the drugs is more difficult because require the traversal of outer membrane, that is an effective permeability barrier (208).

Due to the transmembrane location of these efflux pump systems, most antibiotics acting within the bacterial cytoplasm are affected by resistance mediated by these: for example, tetracyclines, macrolides, fluoroquinolones (206,209). Moreover, the main efflux systems of pathogenic bacteria are involved in the multiple resistance to several antibiotics of different classes, allowing not only the extrusion of these, but also of other substances such as dyes, detergents, and disinfectants (209). Among the most studied EPs systems capable of conferring cross-resistance in MDR pathogens are: (i) MexAB of *Pseudomonas aeruginosa* and AcrAB-TolC of *E. coli* (RND superfamily), which have a broad substrate specificity giving them the ability to expel molecules of various types included fluoroquinolones (210), (ii) NorA of *Staphylococcus aureus* (MFS superfamily) which also

confer resistance to fluoroquinolones (211), (iii) the DHA-3 family of the MFS superfamily, which is presents in both Gram negative and Gram positive bacteria and is responsible for the extrusion of macrolides and tetracycline (tetracycline efflux pumps are well-characterized members of this family) (206).

Therefore, the major concern related to the efflux pump resistance systems is the multiple extrusion of antibiotics and other biocidal compounds from the bacterial cell, which give the ability to the pathogen to express cross-resistance: for example, MexAB system of *P. aeruginosa* confers the ability of extrude the agent and in parallel to over-express the pump itself, with the consequent cross-resistance to all other substrates of the pump, including other relevant antibiotics (fluoroquinolones, ß-lactams, chloramphenicol and trimethoprim) and biocides (triclosan) (212). This mechanism of over-expression of a multidrug resistance efflux pump does not confer alone a high-level of resistance, but when a bacterium genome is equipped with this defense strategy, its capacity of resistance to the selective pressure given by antibiotics will be better and it will be able to develop successive mutations on the antibiotic targets (209).

For the same reason, a single efflux pump involved in the extrusion of multiple drugs could be an ideal target for specific drug inhibitors when administered in combination with antibiotics, to increase their susceptibility in MDR bacteria (141).

The RND EP superfamily, due to its importance in the intrinsic resistance of Gram negative to different classes of antibiotics, it has been mainly investigated as a target of efflux pump inhibitors (EPIs), mainly in *Enterobacteriaceae* and *Pseudomonas aeruginosa* (213,214). One of the first EPIs to be identified was an RND inhibitor, phenylalanine-arginine ß-naphthylamide, also called PAßN or MC-207,110: this acts as a substrate analog of EPs because induce a competition-like process during the recognition/transport of the antibiotic determining a preferentially efflux of PAßN out of the

cell and the consequently increase of antibiotic in the bacterial cytoplasm (214,215,216). Initially PAßN was investigated as a specific inhibitor of MexAB-OprM of *P. aeruginosa* as an adjuvant of levofloxacin, as it determines the accumulation of quinolones within the cell, but subsequently its activity was confirmed also in others Gram negative, including *E. coli*, *Enterobacter aerogenes*, *K. pneumoniae* and *S. enterica* (216). In addition, PAßN, thanks to its activity on RND pumps of numerous Gram negative pathogens, not only restore the activity of fluoroquinolones, but also of other antibiotic classes, such as chloramphenicol, macrolides/ketolides, oxazolidinones and rifampicin, but not ß-lactams and aminoglycosides (180,217). In reason of its mechanism of action, PAßN is placed in the category of peptidomimetic EPIs, as it fits in a binding pocket different from that of the antibiotic on the EP in respect to the involved amino acid residues (218). Furthermore, PAßN showed a dual mechanism of action: in addition to inhibiting RNDs, it also acts as a membrane permeabilizer at concentrations > 16 µg/ml, but only in *P. aeruginosa* mutants deficient for MexAB-OrpM (215).

Although the development of new efflux pump inhibitors is growing and even a PAßN derivative is in clinical trial phase in patients with cystic fibrosis or pneumonia ventilator-associated as an aerosol formulation (MP-601,205), the major challenge remains in finding non-toxic compounds capable of inhibiting only the efflux pumps of prokaryotes, as the extruding proteins antibiotics can be important for the physiological functions of the host organisms (216,217).

In addition to PAßN, other EPIs have been identified thanks to research among the PADs: reserpine, an alkaloid with antihypertensive and sedative properties, was one of the first drug to be identified as an inhibitor of specific efflux pumps, in particular of NorA of *Staphylococcus aureus*, a multi-drug transporter of MFS superfamily, similar to the mammalian multi-drug transporter P-glycoprotein (Pgp) and increasing the intracellular concentration of fluoroquinolones, leading to the lowering of

the MIC of these antibiotics (219,220). As well as reserpine, other inhibitors of EPs were identified searching for active molecules among the PADs:

- verapamil, a calcium channel antagonist used as antihypertensive, and its metabolite norverapamil, were documented to inhibits the macrophage-induced rifampicin tolerance in *M. tuberculosis*, due to the activity on Rv1258c, an efflux pump of MFS superfamily that induce multidrug tolerance and intra-macrophage growth of *M. tuberculosis* and *M. marinum* (221,222).
- biricodar (VX-710) and timcodar (VX-853), two previously molecules described as inhibitors of mammalian multiple drug resistance efflux pumps Pgp and MRP-1 with poor *in vivo* toxicity, were described as inhibitors of efflux pumps of several Gram positive pathogens (*S. aureus, E. faecalis, Streptococcus pneumoniae*) and *M. tuberculosis*, thanks to the intracellular accumulation of antibiotics (fluoroquinolones, rifampicin) and toxic compounds (ethidium bromide) (223,224).
- phenothiazines (chlorpromazine, prochlorpromazine and promazine), a drug class of dopamine receptor antagonists clinically used as antihistaminics and neuroleptics, were identified as inhibitors of the proton force-dependent efflux pumps of *S. aureus*, *M. tuberculosis* and *Burkholderia pseudomallei,* as well as omeprazole, a proton pump inhibitor, which provide augmentation of antimicrobial activites of ampicillin, amoxicillin, gentamicin and erythromycin (225,226,227).
- alkylaminoquinolines, a class of derivatives from natural alkaloyds of which quinoline is the principal compound (chloroquine, quinine, amodiaquine and primaquine are anti-malarian derivatives) have shown activity on the AcrAB-TolC pump of various Gram negative resistant pathogens, with a consequent intracellular accumulation of antibiotics like chloramphenicol (180,228).

- arylpiperazines, a class of compounds derived from piperazine, with several therapeutical but most of all non-therapeutical uses (anthelmintics, psychoactives with dopaminergic, noradrenergic and serotoninergic stimulant activities) which potentiate the activity of levofloxacin and increase the intracellular accumulation of toxic compounds (ethidium bromide) in *E.coli* overexpressing acrAB and acrEF (genes encoding the AcrAB and AcrEF pumps) through the interference with the functional assembly of these pumps (229,230). The main disadvantage for these compounds is the low level of potency compared to other EPIs and the toxicity, due to the similarity with serotonin agonists (217).
- thioridazine and its derivatives, a phenothiazine derived compound with antipsychotic properties, were evaluated as EPIs in combination with first line anti-tubercular drugs (rifampicin, isoniazid) and showed synergy with them against *M. tuberculosis*; furthermore, its derivatives were found to be less toxic towards host macrophages and, thanks to to their structure with a different scaffold compared to phenothiazines, their side effects on the CNS were reduced (231,232).

Membrane permeabilizers

A separate chapter must be dedicated to membrane permeabilizers, which, although they fall into the category of adjuvants that act on passive resistance patterns of bacteria, play an important role in their own right, especially as regards the strategies to counteract MDR Gram negative bacteria. The peculiar morphology of bacterial wall forces antibiotics which have a cytoplasmatic target to through it with a specific mechanism, and this mechanism could be hampered by intrinsic resistance factors of the wall (or the outer membrane in the Gram-negative bacteria). To through the cell wall, antibiotics use two different strategies, depending on the hydrophilicity of the molecule: they can overstep the wall by passive transport, thanks to an intrinsic hydrophobicity of the molecule, or they must be actively transported by porins, because of their hydrophilicity (233). The difficulty to cross

the membrane is higher for antibiotics in Gram-negative than in Gram-positive bacteria, and for that with a cytoplasmatic target, rather than ß-lactams. Furthermore, some antibiotics act directly on the outer membrane of Gram negative, such as polymyxins (polymyxin B, colistin, bacitracin) through their amphiphilic structure: for example colistin activity is due to the impairing of the lipopolysaccharides of the outer membrane through the displacing of cations Ca^{2+} and Mg^{2+} caused by the insertion of the hydrophobic terminal acyl fat chain and subsequently, the phospholipidic bilayer of the inner membrane is destabilized and destructed by the hydrophilic groups in the fatty acid chain of colistin (234).

A developing strategy uses permeabilizer molecules as adjuvants of traditional antimicrobials to disrupt bacterial wall or outer membrane both in metabolically active bacteria and in persisters cells in dormant phase, especially found in biofilms (235). One of the first adjuvant molecule found with a permeabilizing activity was Polymyxin B Nonapeptide (PMBN), a derivative from polymyxin B lacking the fatty acid tail, giving it a poor direct antimicrobial function, but retaining its membrane disrupting activity (236). PMBN was then investigated in association with other antibiotics and its activity as adjuvant was confirmed by the notable reduction of relatively MICs (particularly evident for hydrophobic antibiotics, which MICs on *E. coli* and *S.* Typhimurium decreases by a factor of 10 to 300 with PMBN at 3 µg/ml) (236). The structure of polymyxin B and E (colistin) is a pentacationic cyclic lipodecapeptide, which confer them the ability to permeabilize the outer membrane and to kill bacteria (bactericidal antibiotics); instead, the structure of PMBN not only give it a weak antibacterial activity, but also a reduced toxicity compared to polymyxins (15 times less toxic in mice in acute-toxicity assay) and, as opposed to polymyxin B, it does not interfere with the pro-inflammatory $P2X_7$ receptor (237,238). However, both polymyxin B and PMBN showed nephrotoxicity which often compromise their clinical application and is related to the strong positive charge of these molecules (present also in aminoglycosides) that damage renal proximal tubuli

(238). New polymyxin derivatives were then synthesized, NAB739, NAB7061 and NAB741, with the same cyclic peptide portion of polymyxin B, but without two of the five positive charges of this: this structure confers them a reduced renal affinity with an implemented activity towards carbapenem-resistant strains (for NAB739) or an adjuvant activity of many antibiotics against *E. coli*, *K. pneumoniae, E. cloacae* and other Gram negative (NAB7061) (239).

Similar to the structure of the cyclic peptide portion of polymyxins are Antimicrobial Peptides (AMPs), a broad class of ancient natural compounds widespread in animal and plant kingdoms with a fundamental amphipathic structure where the hydrophobic and cationic amino acids are spatially separated in different parts of the molecules. This structure confers to AMPs the ability to kill bacteria (without damage mammalian and plant cells) thanks to the strong negative charge of the membrane lipids, which interacts with AMPs in ways not yet so well established, but has been hypothesized that are included (i) a depolarization of the bacterial membrane, (ii) the creation of physical holes on the membrane (toroidal pore), (iii) the induction of hydrolase enzymes that degrade the cell wall, (iv) the remixing of the membrane lipids and (v) the damaging of intracellular targets after internalization of the AMP (240). A different mode of action is recognized for AMPs against Gram positive bacteria: they interact with the negatively charged teichoic and teichuronic acids of the cell peptidoglycan, but the disrupting activity on the bacterial wall is the main but not the only mechanism involved in their bactericidal activity (241). Once translocated through different strategies, many killing activities of AMPs were recognized also inside the bacterial cytoplasm: (i) they can alter the cytoplasmic septum formation, (ii) inhibit cell-wall synthesis and (iii) nucleic-acid synthesis, (iv) inhibit protein and (v) enzymatic activity (241). The broad spectrum of activity of these various compounds, together with their rapid action and potency, ensure them a multiple strategy to kill bacteria, which is hardly overcome by resistant mechanisms (242).

In parallel, AMPs revealed a poor efficacy when administered as antimicrobials alone because of their high production costs, the possible toxicity, and the liability in the host organisms, due to the excreted protease action of bacteria and the physiological conditions of the host (serum, salt and pH conditions) (243).

AMPs are synthetized in many tissues of vertebrates and invertebrates as part of the innate immunity (defensins) and in some cases they have been detected also in animal venoms acting as toxins or facilitators of neurotoxins action (244,245). For example, the cardiotoxin 1 (CTX-1) of the Chinese cobra (*Naja atra atra*) was investigated for its antimicrobial activity and derivatives of this (*Naja* Cardiotoxin Peptides, NCPs) were identified as antimicrobial, antifungal and antiviral peptides with a minimal cytotoxic and hemolytic effect on eucaryotic cells (245). Among NCPs the best results were obtained with NCP-3, which showed a strong bactericidal and fungicidal efficacy, a rapid action and activity on mycobacteria and enveloped virus, such as BoHV-1 (Bovine Herpesvirus-1, responsible of the bovine infective rhinotracheitis) (245).

Miming the AMPs, but with substantial improvements respect to these in toxicity and liability on the host organism, are a new class of potential adjuvants, the "ceragenins", initially referred as "cationic steroid antibiotics (CSAs)", a colic acid-based class of compounds which repeat the cationic amphiphilic-based structure of most AMPs (246). Ceragenins use a scaffold with a bile acid, the colic acid, and appended amine groups to mimic the facially amphipathic morphology of AMPs but with a nonpeptide structure. These compounds were recognized as bactericidal and fungicidal, as well as were able to interact with also certain viruses and protozoa thanks to their disrupting action on the membranes, with consequent cell death; they exhibited activity on Gram negative and Gram positive, including resistant strains (vancomycin resistant *S. aureus*): for example CSA-13 showed similar MIC values against both Gram negative and Gram positive (MIC of 1 µg/ml on vancomycin

resistant *S. aureus*), and activity on carbapenem-resistant *Acinetobacter baumannii* (246,247). Most CSAs show greater activity towards Gram positive, due to the presence in the membrane of Gram negative of phosphatidylethanolamine, which confers greater intrinsic resistance (248), but a potential application of CSAs on Gram negative could also be the association with traditional hydrophobic antibiotics, with scarce activity on these bacteria, mostly in MDR. Thanks to the lipid chain extended from the C24 residue of CSA-13 this molecule is active against *E. coli* and other Gram negative as it facilitates the crossing of the external membrane and, furthermore, CSA-8 for the same reason is able to associate with the outer membrane, sensitizing Gram negative bacteria to hydrophobic antibiotics such as erythromycin (246,249,250). Schmidt et al. reported that a concentration of 0,7 µg/ml of CSA-8 is enough to reduce the MIC of erythromycin from 70 µg/ml to 1 µg/ml on *K. pneumoniae* and for *E. coli* a concentration of 0,8 µg/ml is necessary to lower erythromycin MIC from >200 µg/ml to 1 µg/ml (FIC index < 0,025) (249).

Miming host defense mechanisms: immunomodulatory peptides

The enhancement of host defense immunomodulatory mechanisms offers an alternative target for antibiotic adjuvants. In this sense, several compounds have been proposed with an immune modulation activity and in parallel a limited inflammatory stimulation, among which the innate-defense regulatory peptides (IDRs), the agonists of Toll-like receptors and NOD-like receptors (181).

Several AMPs are present in nature, mostly in vertebrates, as part of innate immunity and named Host Defense Peptides, HDPs, with antimicrobial and anti-inflammatory properties (cathelicidins, defensins, histatins) (182). HDPs, as well as other AMPs, showed the same bactericidal activity, due to the disruption of bacteria membranes or translocation into the cell with a cytoplasmic target; but in the host organism this can only occur only within leukocyte phagolysosomes, due to the presence of divalent cations and anionic macromolecules in the serum, such as glycosaminoglycans, which

inactivate their antimicrobial function in the host biological liquids (251). This therefore suggests that HDPs have more immunomodulatory functions under physiological conditions, such as induction of many antimicrobial and immune modulators with a target on leukocytes, mucosal epithelial cells and vascular endothelial cells (182).

Synthetic IDRs have the same purpose of natural HDPs, to mediate in vivo immune defense without having a direct antimicrobial effect: the first IDR synthetized was IDR-1, its mechanism involve modulation of specific signaling pathways between cells to enhance chemokine production and a reduction of pro-inflammatory response mediated by cytokines such as TNF-α with a protective effect in many animal models of multi-drug resistant infections, demonstrating that the *in vivo* effect of cationic peptides can be mediated by immunomodulatory activities (181,252). Advantages of IDRs application are related to the low potential of increase the AMR and low level of toxicity to host cells, because of the low dose necessary for the treatment, but in future could be interesting increase their efficacy also as antimicrobials, including the ability to inhibit biofilm formation (182).

1.5.5 Cysteine biosynthesis in pathogenic bacteria: a non-essential target for new antimicrobial adjuvants

In prokaryotes it has been demonstrated that the biosynthesis of essential amino acids plays an important role in the response to metabolic stress (nutrient starvation, hypoxia or oxidative stress) or adaptation to difficult conditions in host organisms: for example in *Mycobacterium tuberculosis* procurement of cysteine (by *de novo* synthesis or external acquisition) is essential for the synthesis of mycothiol, a functional analogue of glutathione, which confers ~~it~~ the ability to counteract the reactive oxygen produced by host macrophage and therefore to survive into the host cells, thanks to a redox defense mechanisms (253). Cysteine is the main building block of many biomolecules and cofactors such as methionine, CoA, biotin, lipoic acid and thiamin pyrophosphate, and it is involved in molecules with detoxifying properties as glutathione and mycothiol of Mycobacteria (253-259).

The role of cysteine biosynthesis in bacteria is crucial in some metabolic phases, that not necessarily involves the active growth of the cells but are mostly implicated in the persistence into the host organisms or dormient phases, or, on the other hand, when an external source of oxidative stress, such as the action of an antimicrobial molecule, requires it (254). It has already been highlighted how the response to metabolic stress of several microorganisms is linked to the up regulation of cysteine biosynthetic pathways: ATP sulfurylase and APS kinase in *M. tuberculosis*; Serine acetyltransferase (SAT) and Cysteine Synthase (CS) in protozoa (*Leishmania* sp., *Trichomonas vaginalis* and *Entamoeba histolytica*) and bacteria (*E. coli, Salmonella* Typhimurium, *S. aureus,* etc.) (255-259). In some cases, it has also been seen as phenomena of antibiotic resistance are increased by the activation of the biosynthetic pathways of cysteine: for example, in *M. tuberculosis* in case of treatment with vancomycin the genes of the cysteine regulon are activated, while in *S.* Typhimurium the swarming phenotype, correlated with increased antibiotic resistance, recedes to a non-swarming phenotype when cysteine regulon genes are down-regulated (260,261).

For these reasons the search for compounds capable of inhibiting the enzymes involved in the cysteine pathways is of great interest as they may be able to enhance traditional antibiotics, especially in *in vivo* conditions in the host, by repressing adaptive mechanisms of pathogens in adverse conditions, such as oxidative stress or starvation (262). Furthermore, another important point for the development of antimicrobials or adjuvants with a microbial sulfur-metabolic pathway target is the exclusive ability of prokaryotes to synthetize cysteine through the incorporation of inorganic sulfur rather than mammalian cells, which instead can only reverse from methionine through a trans-sulphuration, minimizing the possible side effects of this antimicrobial strategy (262).

Cysteine biosynthesis in prokaryotes requires the reductive sulfur assimilation pathway (RSAP) to assimilate sulfur from an external source as sulfate. RSAP is a multistep reduction of sulfate that ends with incorporation of bisulfide into cysteine using an activated form of serine (Fig. 6) (263). In *E. coli* this is possible thanks to the internalization of sulfate operated by the ABC-type membrane permease thiosulfate (and sulfate) PBP, encoded by the operon CysPUWAM, then it is assimilated in the form of adenosine phosphosulfate (APS) by an ATP sulfurylase encoded by genes *CysD* and *CysN* (Fig.7) (257). APS is the substrate of APS kinase, encoded by the gene *CysC*, which phosphorylate APS in 3'OH position, transforming APS in phosphoadenosine phosphosulfate (PAPS), subsequently PAPS in the sulfur assimilation pathway is reduced into SO_3^{2-} (sulfite) by a PAPS reductase (encoded by *CysH*) producing adenosine 3'-5' diphosphate (PAP) as a by-product. The sulfite ion is reduced to bisulfite (HS^-) by a NADPH-sulfite reductase, encoded by the operon *CysJIH*, and this ion is converted into hydrogen sulfide H_2S by complex oxido-reduction systems that are currently poorly known in bacteria (257).

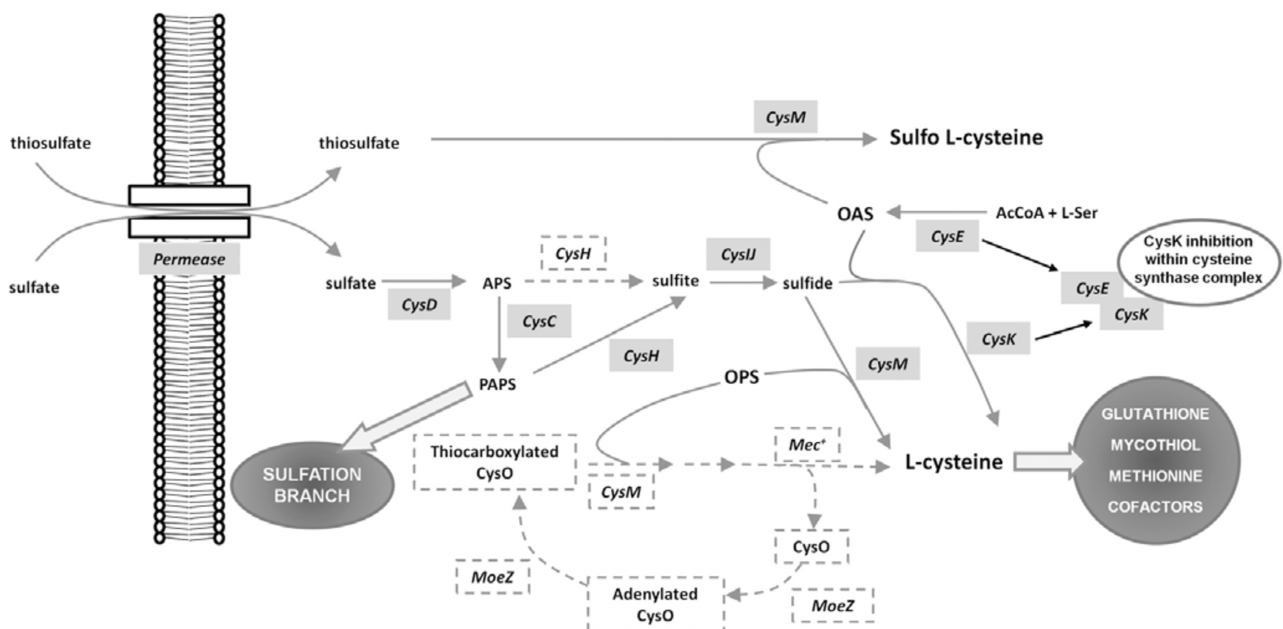

Figure 6: From: Campanini, B., Pieroni, M., Raboni, S., Bettati, S., Benoni, R., Pecchini, C., Costantino, G. & Mozzarelli, A. (2015). Inhibitors of the sulfur assimilation pathway in bacterial pathogens as enhancers of antibiotic therapy. Current medicinal chemistry, 22(2), 187-213. RSAP, reductive sulfur assimilation pathway in prokaryotes. Proteins and related operons are boxed and reaction are depicted whit an arrow. Reaction with a dashed line are presents only in actinomycetales.

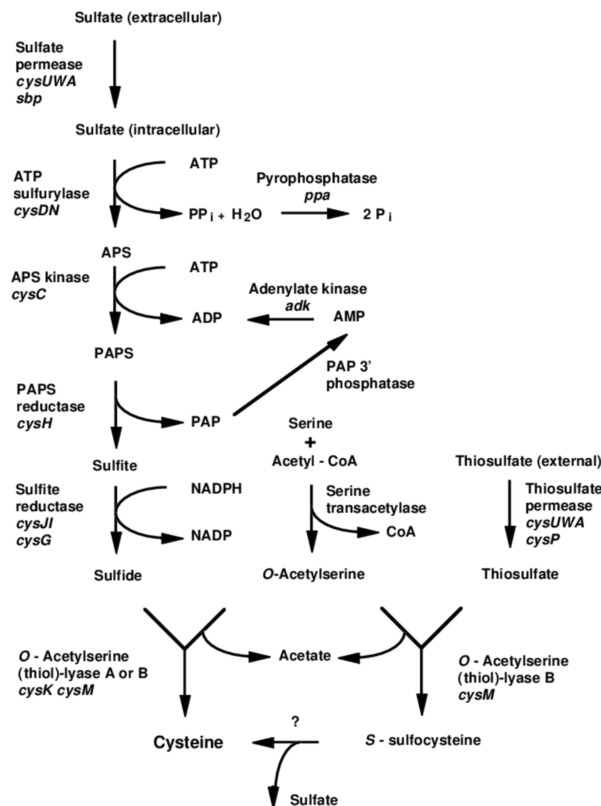

Figure 7: From: Sekowska, A., Kung, H. F., & Danchin, A. (2000). Sulfur metabolism in Escherichia coli and related bacteria: facts and fiction. Journal of molecular microbiology and biotechnology, 2(2), 145-177. Sulfur assimilation pathway and cysteine biosynthesis in E. coli.

The process of incorporation of sulfate inside the microbial cell and its reduction to bisulfide is extremely highly energy consuming and is driven by cellular needs, and because the bisulfide is a toxic compound, it is subsequently incorporated into cysteine by a cysteine-synthase complex (CSC) (264). The CSC is composed by two enzymes: the Serine Acetyltransferase (SAT) and O-Acetylserine Sulfhydrylase (OASS), respectively encoded by *CysE* and *CysK*/*CysM* the latter catalyze the final step of cysteine biosynthesis (257,265). SAT catalyze the condensation reaction of an acetyl group from acetyl-CoA on the hydroxyl group of serine, forming O-acetylserine (OAS), then the OASS catalyze the transformation of OAS in cysteine. Of this latter enzyme exists two isoforms: O-acetylserinesulfhydrylase-A (OASS-A) and O-phosphoserine sulfhydrylase-B (OASS-B), encoded respectively by *CysK* and *CysM,* which catalyze alternatively the final step of RSAP, depending on the organism and growth condition, that is aerobic and anaerobic growth condition respectively in

Salmonella Typhimurium (266). These two isozymes share a structure homology of 43% and a similar three-dimensional structure, but OASS-B has a less selectivity, as it can accept also thiosulfate as a sulfur donor and other ligands (267). Of the two isoforms of OASS, only OASS-A is capable of binding SAT to form the CSC, thanks to the complex with the lasts 5 amino acids of the C-terminal tail of SAT (268). Furthermore, the C-ter tail of SAT is fundamental for the function of CSC, because it is responsible of the intrasteric inhibition in presence of cysteine by binding OASS-A (141).

The OASS and SAT structure of several bacteria and protozoa was identified including *S.* Typhimurium, *E. coli*, *Haemophilus influenzae*, *E. histolitica*, *M. tuberculosis*, *Leishmania major*, *S. aureus*, *K. pneumoniae and Brucella melitensis* and its role in metabolic pathways is different depending on the type of microorganism (257-259,262-270). An important function of OASS in *S*. Typhimurium is related to the swarming motility and antibiotic resistance: Turnbull and Surette found that cysteine biosynthesis is fundamental for the complete swarm-cell differentiation of *S.* Typhimurium and this phenotype is correlated with a higher expression of antibiotic resistance. The demonstration of the phenomenon was made with mutant strains of *Salmonella* deleted for each *Cys* biosynthetic operons (*ΔCys* strains), in which the antibiotic susceptibility increased in swarm-state, but this decreased resistance could be restored by addition of cysteine in the swarm medium (261). Instead, in *M. tuberculosis*, was seen that *CysM* (OASS-B encoding operon) is up-regulated during oxidative stress and the deletion of *CysH* determine an attenuation of virulence, as well as in *B. melitensis* deleted of *CysI* and *CysK*. Similarly, *M. tuberculosis* mutants *CysM-CysO* shows a virulence attenuation in primary macrophage (271-273).

The investigation of deletion mutant bacteria, knocked-out or silenced for *Cys* enzymes of the cysteine biosynthetic pathway has been a landmark for the development of compounds capable of inhibiting this metabolic pathway, as blocking it impair the response of certain bacteria to oxidative

stress, causing for example in *S.* Typhimurium a reduction in its antibiotic resistance, both in planktonic and swarm state (254). Indeed, antibiotic activity on bacteria induce an oxidative stress, which could explain the reduced resistance rate of mutants with impaired cysteine biosynthesis (254).

For all these reasons a new strategy to fight antimicrobial resistance in pathogen bacteria was investigated searching for molecules capable of inhibiting the biosynthesis of cysteine, mainly through the inhibition of both OASS isoforms and the cysteine synthase complex (SAT-OASS) (141).

1.5.6 OASS inhibitors: state of the art

The rationale behind the synthesis of inhibitors of OASS enzymes (A or B) is given by the peculiar structure of the SAT-OASS complex (CSC), where the SAT decapeptide C-terminal tail act directly as inhibitor of OASS, triggering a transition from open to close conformation of the OASS active site and competing with the substrate O-acetylserine (OAS) on this (274). Furthermore, unlike OASS-A, SAT is unable to inhibit the activity of isoenzyme OASS-B (275). Therefore, these findings suggest that SAT can inhibit OASS-A with a double mechanism: the competition with OAS on the active site and stabilization in a closed conformation of the last, with a more difficult accessibility of the substrate (275). It was noted as in bacteria the conformation of CSC with SAT seems have a role in the protection of enzyme from proteolysis and cold inactivation (276). The three-dimensional structure and the molecular interaction of the bienzyme SAT-OASS-A was investigated in bacteria such as *S.* Typhimurium, *E. coli*, *Haemophilus influenzae, M. tuberculosis* and plants (*Arabidopsis thaliana*) and was identified, for example, that in *H. influenzae* only the last five amino acids (MNLNI) interact with the active site and that final isoleucine has an important role in the SAT-OASS-A bond not only in this bacteria but is conserved in other species (Fig. 8)(274,275).

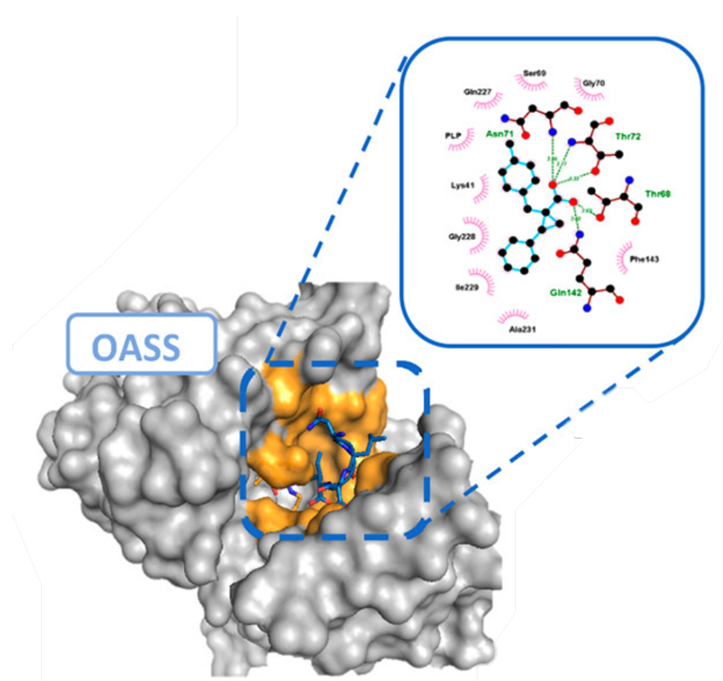

Figure 8: Annunziato, G., Spadini, C., Franko, N., Storici, P., Demitri, N., Pieroni, M., Flisi, S., Rosati, L., Iannarelli, M., Marchetti, M., Magalhaes, J., Bettati. S., Mozzarelli, A., Cabassi C. S., Campanini, B. & Costantino, G. (2021). Investigational Studies on a Hit Compound Cyclopropane–Carboxylic Acid Derivative Targeting O-Acetylserine Sulfhydrylase as a Colistin Adjuvant. ACS Infectious Diseases, 7(2), 281-292. 3D structure of OASS and its active site, where the interactions of the amino acidic residues of the active site with a potential synthetic inhibitor are highlighted in the dotted square.

In reason of this important role of the final pentapeptide of SAT to inhibit the activity of OASS-A, the synthesis of libraries of small molecules of oligopeptides was investigated, leading to the identification of molecules with a fair affinity to the enzyme (a dissociation constant - K_d - in the micromolar range) (274, 277). The interactions of final isoleucine (Ile267) of SAT with surrounding residues of *Hi*OASS-A inspired the synthesis of a library of 400 pentapeptides that were tested from Salsi et al. using a combined-docking scoring procedure, bringing to a final selection of 14 of these molecules showed an affinity in the micromolar range that well correlate with the K_d of the SAT-*Hi*OASS-A complex (274). This study was preliminary to identify the scaffold for the design of peptidomimetics inhibitors, which were investigated also by Spyrakis et al., both on HiOASS-A and *St*OASS-A/B, which found that the MNYDI pentapeptide is a nanomolar ligand of *St*OASS-A and also showed a good affinity towards *St*OASS-B, representing a good starting point for the design of potent non-peptidic *St*OASS-A/B inhibitors (277).

Due to the poor stability and liability on host organisms of small peptides, their use as potential OASS inhibitors was discarded, although their potential activity as antibiotics has been identified *in vitro* on *S.* Typhimurium and *H. influenzae* enzymes. In any case, thanks to the action of these compounds, the importance of cysteine biosynthesis as a potential antibacterial target has been confirmed, and how the inhibition of the enzymes of the CSC complex or the deletion of its coding genes are essential to achieve this goal (278).

To overcome limits imposed using peptides, Amori et al. decided to investigate the same properties of these molecules on *Hi*OASS-A also in non-natural synthetic molecules: the discriminant was the presence of four hydrogen bonds between the carboxylic acid moiety of Ile267 and the residues Asn72, Thr73, Thr79 and Gln143, together with the isobutyl lateral chain of Ile267, which is accommodated in the hydrophobic cleft of OASS-A active site, delimited by Phe144 (279). In the synthetic structure of OASS-inhibitors miming pentapeptides, the carboxylic acid moiety and the hydrophobic lateral chain of Ile267 were included, but the suitable spacer adopted was a cyclopropane ring, a three-carbon ring which can act as a blocker of the two anchoring arms of Ile267, in a favorable conformation to bind *Hi*OASS-A (Fig. 9) (279). To increase the lipophilicity of this structure, on carbon 2 of the cyclopropane ring different groups were added, in *trans* configuration, creating a series of 2-substituted cyclopropane carboxylic acids (279).

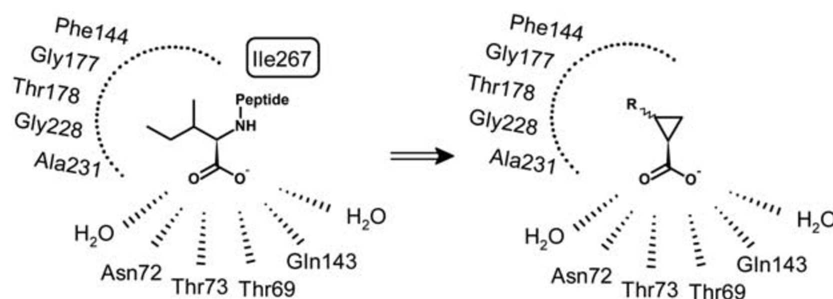

Figure 9: From Amori, L., Katkevica, S., Bruno, A., Campanini, B., Felici, P., Mozzarelli, A., & Costantino, G. (2012). Design and synthesis of trans-2-substituted-cyclopropane-1-carboxylic acids as the first non-natural small molecule inhibitors of O-acetylserine sulfhydrylase. MedChemComm, 3(9), 1111-1116. On the letf the interactions between Ile267 of a generic peptide with HiOASS-A active site residues; on the right the interactions of a cyclopropane carboxylic acid analogue with the same active site.

Subsequently, since 2016, the research group of Department of Food and Drugs of University of Parma started to investigate the design and synthesis of new compounds capable of inhibiting both OASS-A and OASS-B, by studying the best candidates with low dissociation constants on *S.* Typhimurium. Starting from the evidence that the cyclopropane is a good suitable spacer which could maintain the two anchoring arms of Ile267 into *trans* configuration and an alkyl moiety could mime the lateral chain of this residue, Pieroni et al. identified compounds which improved the stability, chemical feasibility and above all, the potency toward both the isozymes, compared to those identified by Amori et al. (278, 279). These compounds are 2-phenylcyclopropane carboxylic acids: the insertion of a phenyl ring into the molecule gave the possibility to better miming both the activity and the structure of the natural inhibitor on *St*OASS-A/B. Furthermore, considering both the isoforms of OASS, was investigated the possibility to functionalize the α-carbon of the cyclopropane ring, as it has been seen that a considerable portion of the binding pocket is empty and is characterized by a small lipophilic area surrounded by a polar area. The addition of a substituent in this position that can interact with this binding pocket can therefore increase the affinity of the molecule with the active site, both of *St*OASS-A and *St*OASS-B, as in the latter there is a pocket very similar, although slightly more polar. It was seen that the substitution in *para* position with bulky groups such as benzyl moieties give potent inhibitors and in particular a substituent such as chlorine at the para position of benzyl ring allows to a low selectivity towards the two isoforms, with a K_d of *St*OASS-B only 8 times higher than that of *St*OASS-A (278) (Fig. 10).

All these considerations led to the synthesis of 2-phenylcyclopropane carboxylic acids derivatives with a potency about 4000-fold and 7000-fold greater than the pentapeptide inhibitor MNLNI and a less selectivity towards the two isoenzymes (278).

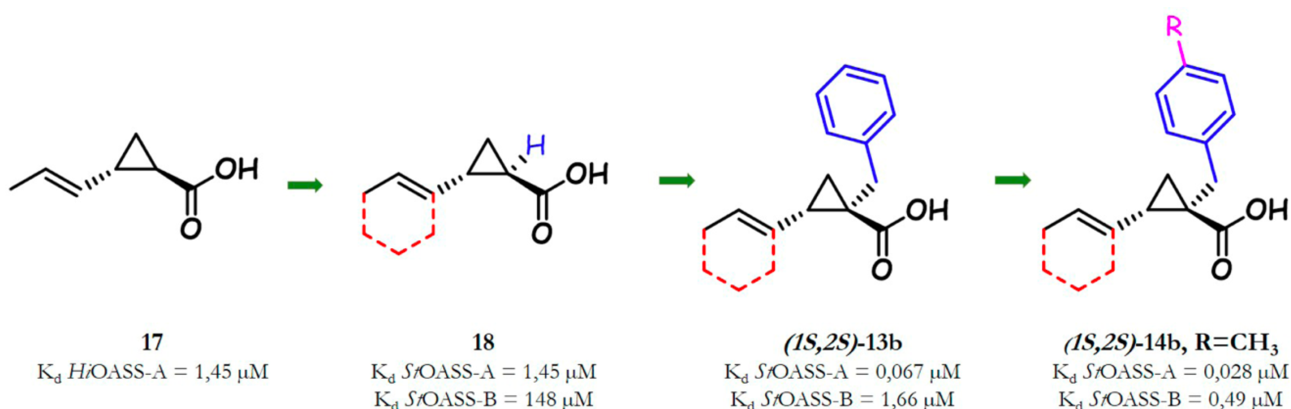

Figure 10: From Pieroni, M., Annunziato, G., Beato, C., Wouters, R., Benoni, R., Campanini, B., ... & Costantino, G. (2016). Rational Design, Synthesis, and Preliminary Structure–Activity Relationships of α-Substituted-2-Phenylcyclopropane Carboxylic Acids as Inhibitors of Salmonella typhimurium O-Acetylserine Sulfhydrylase. Journal of medicinal chemistry, 59(6), 2567-2578. Representation of the conceptual modification of HiOASS and StOASS inhibitors: compound 17 is a trans-2 (prop-1-enyl) -substituted cyclopropane carboxylic acid; compound 18 is 2-substituted with a phenyl, generating a phenylcyclopropane carboxylic acid, with a moderate affinity to StOASS-A, but still not satisfactory on StOASS-B; compound (1S,2S)-13b contains a benzyl group at the α-carbon which stabilize the binding mode in the most favorable pose, leading to a further reduction of K_d both on StOASS-A and StOASS-B; finally, compound 14b shows an electron withdrawing group, such as chlorine in para position which confers an high affinity towards both the isoenzymes.

The best candidate between synthetized 2-phenylcyclopropane carboxylic acids was the molecule (1S,2S)-1-(4-methylbenzyl)-2-phenylcyclopropane carboxylic acid (Fig. 9, molecule 1S,2S-14b), which showed a K_d on StOASS-A of 0,028 μM and on StOASS-B of 0,49 μM, both in the nanomolar range, though the affinity on StOASS-B was considerably higher (about 18 times) (278). Despite the molecule's high affinity for OASS, it should be noted that its physicochemical characteristics are not favorable, especially as regards its ability to penetrate membranes and in particular that of Gram-negative, which represents a challenge for many antimicrobial molecules (280). in fact, 1S2S-14b has a high ClogP (3,60), the calculated partition coefficient (logP), that is the ratio of its solubility in two immiscible solvents (octanole: water), indicating how this molecule is very hydrophobic. For example, quinolones have a negative ClogP and a polar character, so they are able to accumulate into Gram-negative cells, while macrolides have no activity on Gram negatives due to the large molecular size that does not allow passage through the membrane (281). For these reasons Annunziato et al. have tried to modify the structure of the compound 1S, 2S-14b in favor of a better physicochemical structure, such as to lead to a greater permeability inside the Gram-negative

membranes and to a more potent St-OASS inhibition (282). These investigations led to the identification of active substituted cyclopropane-1,2-dicarboxylic acids, using computational and spectroscopic approaches, such as saturation transfer difference (STD) and nuclear magnetic resonance (NMR). These compounds were synthetized with the aim of enhancing the polar character of molecules and showed a *cis* configuration with a cyclopropane carboxylic acid as pharmacophore and two small functional groups in position C1 and C2 (282). Unfortunately, also the best candidates for activity of these molecules did not show the desired activity on bacterial cells and, for this reason, further investigations were made on small molecules with low ClogP and published in two subsequent works from Magalhaes et al. (283,284). The carboxylic moiety was maintained, as its interactions with the OASS active site are fundamental for the molecular binding, but was removed the cyclopropane ring, due to stereochemical restrictions it implies, and a different scaffold was investigated. Two small molecules were identified: 1-H-pyrrole-2-carboxylic acid and thiazole-2-carboxylic acid with promising *St*OASS inhibitory characteristics, although their inhibition activity is lower than those of compound 1S,2S 14b (283). In a subsequently work, Magalhaes et al. found other compounds able to inhibit *St*OASS, albeit with less efficiency, with a sulphonamide instead of carboxylic acid to obtain compounds able to cross the Gram negative cells, thanks to the increased polarity given by this substituent. Furthermore, this substitution with sulphonamide showed a novel mechanism of partial inhibition which gave new information to be further investigated (284).

All these findings on novel potential OASS inhibitors conferred a proof of principle for the development of small molecules able to inhibit the biosynthetic pathway of cysteine, which could represent a potential newer antimicrobial target, mainly in response to the increasing problems of antibiotic resistance and bacterial virulence.

Other investigations were made by Brunner et al. aimed at searching for compounds capable of inhibiting the *CysM* enzyme of *M. tuberculosis*, analogue of OASS-B that uses O-phosphoserine, in dormant cells inside the host's macrophages (253). The active site binding capacity of small molecules was tested by a screening based on the fluorescence changes induced by the ligand of the PLP cofactor and subsequently the activity of these compounds was tested by means of an enzyme activity assay, also used for identifying a structure-activity relationship (SAR) (253). Compounds that showed the most potent *CysM* inhibition activities are based on a urea scaffold bringing two phenyl rings and with different substituents, thanks to the considerable flexibility of the active site of *CysM* to create suitable binding pockets to accommodate different substituted inhibitors (Fig. 11). Top hit compounds with higher affinity for *CysM* (all in the µM range and the best hit with 300 µM) were also tested in vitro on *M. tuberculosis* through MIC assay, and two of these showed a notable activity in conditions simulating the dormancy (nutrient starvation model), with a reduction of bacterial count of 3-log (1-log only for isoniazid). Furthermore, these compounds showed little or no cytotoxicity towards mammalian cells (253).

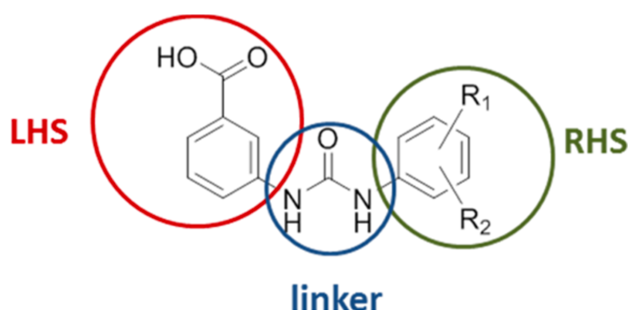

Figure 11: Brunner, K., Maric, S., Reshma, R. S., Almqvist, H., Seashore-Ludlow, B., Gustavsson, A. L., ... & Schneider, G. (2016). Inhibitors of the cysteine synthase CysM with antibacterial potency against dormant Mycobacterium tuberculosis. Journal of medicinal chemistry, 59(14), 6848-6859. The general structure of CysM inhibitors with a left-hand-side (LHS)-substituted phenyl group, a central linker part, and a right-hand-side (RHS) aryl group.

Magalhaes et al. in 2021, given the lack of activity in bacterial cells of compounds previously synthesized to inhibit StOASS due to their reduced permeability, shift their focus on the search for compounds that interfere with the activity of SAT of *Salmonella* Typhimurium, the enzyme that

catalyzes the rate-limiting step of cysteine synthesis, as its inhibition leads to reduced accumulation of OAS and induce the operon to convert it in N-acetylserine (NAS) (285). Previously, several efforts had been made to find compounds capable of inhibiting this enzyme, but with poor results in terms of efficacy (286, 287). Hit compounds were substituted (2-aminooxazol-4-yl) isoxazole-3-carboxylic acids, developed thanks to the virtual screening of their in-house library on *Ec*SAT (*E. coli*) and *Hi*SAT (*Haemophilus influenzae*): 7 compounds were selected and evaluated on *St*SAT, of which six were characterized by the presence of a carboxylic functional group, a group that was also found to be critical for OASS binding site activity (1S, 2S-14b, named also UPAR415, was the most active compound in that case) (285). Compound 5 showed the best data in terms of enzyme inhibition potency, as well as being a competitive inhibitor of *St*SAT towards acetyl-CoA. Furthermore, this molecule is part of a library of antitubercular molecules, but it was found to be inactive against *M. tuberculosis*, without therefore causing off-target problems. For these reasons, derivatives of compound 5 present in the in-house library were tested: all of these were more affine than the hit compound 5 on *St*SAT, and the carboxylic acid was confirmed as a fundamental moiety for the inhibition. Finally, the most potent compound in relation also to its stability was compound 22d and it was tested for its MIC against *E. coli* ATCC 25922 and *S.* Typhimurium ATCC 14028 both in rich medium (Müeller Hinton) with preformed cysteine and in minimum medium Middlebrook 9 (M9) in absence of cysteine, to evaluate the efficacy *in vitro* on bacterial cells. Unfortunately, this compound was unable to go through the bacterial outer membrane and cell wall of these Gram-negative bacteria, despite its potent activity on the target enzyme (285).

Given these considerable efforts in order to identify the most potent inhibitors of the last stages of the cysteine biosynthetic pathway, the direction of research must be aimed at identifying compounds capable not only of inhibiting the target enzyme *in vitro*, but of show activity on complex systems such as bacterial cells, both Gram positive and Gram negative, despite the penetration

difficulty of the latter due to the reduced outer membrane permeability. The action of such compounds may not even be directly antimicrobial, as investigated by Magalhaes et al. in the works of 2018 and 2021, but also adjuvant of other antimicrobials, given the role of the biosynthetic pathway of cysteine in *S.* Typhimurium and other bacterial species, i.e. the ability of this pathway to counteract oxidative phenomena due to the action of other antimicrobials and increase the antibiotic resistance towards them (141,261).

Aim of work

Antimicrobial resistance is a significant issue involving both humans and animals, then the search for new strategies capable of counteracting its increasing is of great interest in the scientific world. A promising strategy is given by using combinations of different compounds aimed at reaching the antimicrobial effect with a lower dosage of antimicrobial, thanks to adjuvant molecules. The antibiotic adjuvant led to the reduction of adverse events or side effects due to the low antibiotic concentration needed to be effective and, for the same reason, the reduction of resistance phenomena in bacteria. Furthermore, adjuvant shows a weak or absent intrinsic antibiotic activity, but can enhance the activity of antibiotic compounds by blocking or reduce antibiotic resistance mechanisms (141,172).

In this sense, this work is aimed to search novel antibiotic strategies using adjuvants, which can target bacterial pathways involved in the resistance towards antibiotics, but without interfering with their essential mechanisms.

The cysteine biosynthesis (also known as sulfur assimilation pathway) is a non-essential bacterial pathway – not possessed by mammalian cells – responsible for the synthesis of cysteine, an essential amino acid for all sulfur-containing biomacromolecules that is crucial for all living organisms for the response to metabolic stress (oxidative stress, hypoxia or starvation). In bacteria, the last step of cysteine biosynthesis is catalyzed by the isoenzymes O-acetylserine sulfhydrylase (OASS) A and B which convert O-acetylserine (OAS) in cysteine (254,255,258).

In the present study was evaluated the activity of a synthetic inhibitor of OASS-A/B of *Salmonella* Typhimurium – (1S,2S)-1-(4-methylbenzyl)-2-phenylcyclopropane carboxylic acid, named UPAR415 – as a potential colistin adjuvant against six different pathogenic bacteria of human and veterinary interest and, by using a *S.* Typhimurium deleted strain for *CysK* and *CysM* genes,

responsible for encoding OASS-A/B, was searched a proof of principle that UPAR415 exert in cell the inhibition of both the OASS isoforms. Furthermore, the structural details of the inhibition of OASS by UPAR415 were highlighted by performing x-ray crystallography.

Colistin was selected for its importance as life-saving molecule, both in human and veterinary medicine, but also with the aim to expand its therapeutic window (its active concentrations are too close to the toxic one), using an adjuvant that potentially increase its efficacy lowering its clinical dosage.

This work could represent a proof of principle for UPAR415, the hit compound in terms of enzyme inhibition potency (the K_d is in the low nanomolar range) among all known OASS inhibitors so far and could pave the way for further investigation of this antimicrobial target. UPAR415 and its possible derivatives could be used as adjuvants of traditional antimicrobials in the treatment of bacterial infections of both humans and animals, due to resistant bacteria to antibiotics, as they are able to act synergistically with these and significantly lower their MIC.

Further investigations will have to be carried out to evaluate other derivatives of UPAR415 and associations with antimicrobials other than those tested and their *in vivo* efficacy.

2. Materials and Methods

2.1. Laboratories and research groups

All the microbiological and cytological experiments were performed at the Laboratory of Bacteriology and Virology of the Operative Unit of Animals Infectious Diseases, at the Department of Veterinary Science of the University of Parma, 43126 Parma, Italy.

Biochemical assays were performed by:

1. Probes for Targets (P4T) research group at the Department of Food and Drugs of the University of Parma, 43124 Parma, Italy;
2. Laboratory of Biochemistry and Molecular Biology, at the Department of Food and Drugs of the University of Parma, 43124 Parma, Italy;
3. Biopharmanet-TEC, Interdepartmental Center of the University of Parma, 43124 Parma, Italy;
4. Elettra research group, at Sincrotrone Trieste S.C.p.A, 34149 Trieste, Italy.

2.2. Microbiological assays

To evaluate the antimicrobial activity alone and in association of UPAR415 were applicated two different *in vitro* assays: the Minimal Inhibitor Concentration (MIC) test and Checkerboard test, respectively following the Clinical and Laboratory Standard Institute guidelines (CLSI) and the protocols proposed by Meletiadis et al. (288,179).

2.2.1 Tested compounds

Subject of this work was the molecule (1S,2S)-1-(4-methylbenzyl)-2-phenylcyclopropane carboxylic acid, named UPAR415, which was synthetized and characterized as reported by Pieroni et al. (Fig.

12) (278). UPAR415 has a molecular weight of 266,34 (MW) and a K_d of 0,028 µM on *St*OASS-A and of 0,49 µM on *St*OASS-B (278).

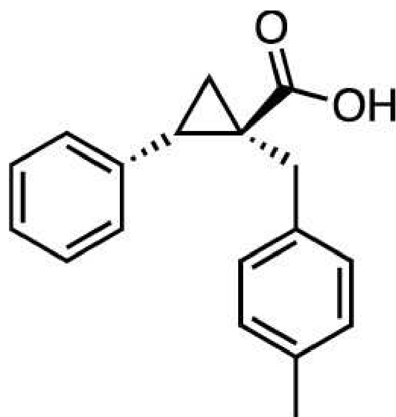

Figure 12: molecule (1S,2S)-1-(4-methylbenzyl)-2-phenylcyclopropane carboxylic acid, named also UPAR415.

UPAR415 was solubilized in DMSO to a stock solution of 25,6 mg/ml and subsequently, in the preparation phase of MIC and checkerboard assays was further diluted to a range of 25,6-0,05 mg/ml in DMSO. In order to carry out the microbiological tests, UPAR415 was then added to the microtiter plate wells at a maximum concentration of 1% of DMSO, diluting the compound in culture broth to a final range between 256-0,5 µg/ml.

Antibiotic compounds used in this work were:

- Colistin sulfate salt, purchased from Sigma-Aldrich, MO, USA, lot number 049M-4836V
- Polymyxin B nonapeptide hydrochloride (PMBN), purchased from Sigma-Aldrich, MO, USA, lot number 108M-4154V

All the antibiotics were purchased in powder form, then solubilized in sterile deionized water, following the CLSI guideline formula for the resuspension of antibiotic powders (288):

$$Volume\ (ml) = \frac{Weight\ (mg)\ x\ Potency\ \left(\frac{\mu g}{ml}\right)}{Concentration\ (\frac{\mu g}{ml})}$$

Where the antibiotic potency is calculated as following:

$$Potency\ (\frac{\mu g}{ml}) = (assay\ purity)x\ (Active\ fraction)x\ (1 - Water\ Content)$$

Stock solution of colistin was performed at 25,6 mg/ml in sterile water and PMBN at 2,56 mg/ml and these were stored in small aliquots at - 80°C until use.

2.2.2 Tested bacterial strains

Reference bacterial strains tested in this work were:

- *Escherichia coli* ATCC 25922
- *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028
- *Klebsiella pneumoniae* ATCC 13883
- *Staphylococcus aureus* ATCC 25923
- Methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300
- *Staphylococcus pseudintermedius* ATCC 21284

In addition to reference strains, a mutant strain of *Salmonella* Typhimurium was tested: DW378. This strain is defective for the expression of *Cys*K and *Cys*M genes, encoding for isozymes OASS A and B. In particular the genotype of this strain is *trpC109*, *cysK1772* and *cysM1770*. Due to its mutation, DW378 is auxotroph for cysteine and L-tryptophan and resistant to azaserine (TK181) (289). The strain was identified by Hulanicka et al. by isolation of strains lacking OASS-B in the genetic background of TK181 strain lacking OASS-A, and Hulanicka et al. report that DW378 completely lack of O-Acetylserine Sulfhydrylase activity, but no molecular evidence has been provided for the origin of this phenotype (289). In this thesis we demonstrated that OASS-A is expressed in comparable amounts both in *S.* Typhimurium ATCC 14028 and in DW378, while OASS-B expression is undetectable in both the strains (Chapter 3.4). Therefore, the cysteine auxotrophy

is probable due to the inactivation of the enzyme but not to the completely deletion of the encoding genes.

2.2.3 Minimal Inhibitory Concentration (MIC) test

The antimicrobial activity of UPAR415 was tested through broth microdilution assay to evaluate the MIC value of the compound. MIC value is the lowest concentration of the tested compound at which there was no bacterial growth (288). For each test, three independent experiments with three replicates each were performed. Two different culture media were used: the rich medium Müeller Hinton Broth (MHB) and the minimal medium Luria-Bertani broth (LB) at 20% in sterile water. In the firth medium cysteine is abundant, while in LB 20% cysteine is present at a lower concentration, as also other nutrients.

Inoculum preparation

Four or five bacterial colonies from solid fresh cultures of each tested strain were inoculated in sterile tubes with MHB or LB 20% and incubated at 37°C for 24 hours. After incubation, the bacterial suspension was centrifuged at 2000 rpm at 4°C for 20 minutes in order to separate the pellet from the supernatant. Then, the pellet was resuspended in phosphate buffer (PB) 10 mM at pH 7. The bacterial suspension was adjusted in PB to obtain an optical density (OD) value at 600 nm in a 1 cm light path cuvette in the range 0.08–0.13, approximately equivalent to a 10^8 CFU/ml suspension. This suspension was further diluted 1:100 in sterile MHB. Fifty microliters of the bacterial suspension containing 10^6 CFU/ml were inoculated into each well, to obtain a final concentration of $5x10^5$ CFU/ml. Bacterial suspensions were investigated with the aim of a Biophotometer plus (Eppendorf, Hamburg, Germany) spectrophotometer (λ = 600 nm). All the microbiological assays were performed within 30 min after the inoculum standardization.

MIC assays preparation and interpretation

MIC assays were evaluated following the CLSI guidelines with minor modifications (288). Twofold dilutions 25,6-0,05 mg/ml ranged of the UPAR415 stock solution were performed in DMSO in separate 96-well microtiter U-plates (Greiner, Milan, Italy). In a different 96-well microtiter U-plate, for each well of the replicates, 49 µl of culture medium (MHB or LB 20%) was added. Therefore, in each well of the plate, one microliter of each UPAR415 dilution in DMSO was added. Finally, fifty microliters of bacterial suspension containing 10^6 CFU/mL was added to obtain a microbial concentration of $5 \cdot 10^5$ CFU/ml. The final dilution range tested was 256-0,5 µg/ml. Growth and sterility controls were performed for each strain and for each tested compound. Plates were then incubated for 24 hours at 37°C in aerobic atmosphere. After incubation, plates were read by unaided eye with a microtiter reading mirror and then the optical density (OD) of each well of the plates was measured with the aim of a spectrophotometer at λ of 620 nm.

MIC values were calculated as the arithmetic media $\pm$ standard deviation (SD) of the unaided eye reading and the inhibition of growth for each tested dilution was calculated from the OD values (media $\pm$ SD).

A quality control microorganism (*E. coli* ATCC 25922) was tested periodically to validate the accuracy of the procedure.

MIC test was performed also with colistin alone on the seven bacterial strains used in this work. MIC evaluation of colistin was performed following the CLSI guidelines for microdilution broth assays, similarly to that of UPAR415 (288). The main difference from UPAR415 is given by the dilution medium of colistin: this antibiotic was diluted in broth medium (MHB or LB 20%) at a stock concentration of 512 µg/ml, then 100 µl of this solution were added to the first column of microtiter plates; subsequently colistin was serial diluted taking 50 µl from the first well and inserting it in the

following ones, making a twofold dilution in the following wells containing 50 ml of medium. After the addition of 50 µl of bacterial inoculum at the test wells, the colistin reaches the desired concentration, ie the range 256-0.5 µg/ml.

2.2.4 Checkerboard assay

Antimicrobial activity of UPAR415 in association with two different antibiotics – colistin and PMBN – was tested through checkerboard assay with minor modification on reference strains and *S.* Typhimurium DW378 (179). For each assay, three experiments with three replicates each were assessed, and each experiment was performed in two different culture media: MHB and LB 20% broth (as mentioned above).

Inoculum preparation

Bacterial suspensions for checkerboard assays were prepared at the same way as those of MIC tests.

Colistin and UPAR415 checkerboard plates preparation and interpretation

In the checkerboard assays with colistin and UPAR415, the latter was tested as adjuvant of colistin. 96-wells microtiter U-plates of the same replicate were prepared with twofold serial dilutions of colistin in MHB and LB 20% starting from the MIC value (µg/ml) for ten consecutive dilutions in 50 µl of broth. In each well of the same replicate, 1 µl of UPAR415 in DMSO was added at a fixed concentration, 100 times higher than the final desired concentration. Subsequently, 49 µl of the bacterial suspension at a concentration of 10^6 CFU/ml, adjusted spectrophotometrically as reported above, was added to each well, reaching the final bacterial concentration of $5x10^5$ CFU/ml. Growth and sterility controls were performed for each experiment and for each replicate. Finally, the plates were incubated at 37°C in aerobic atmosphere for 24 hours. After incubation, plates were read by the unaided eye with a microtiter reading mirror and then the optical density (OD) of each well of the plates was measured with the aim of a spectrophotometer at λ of 620 nm.

MIC values were calculated as the arithmetic media $\pm$ standard deviation (SD) of the unaided eye reading and the inhibition of growth for each tested dilution was calculated from the OD values (media $\pm$ SD).

To evaluate the antimicrobial effect of the two molecules in association, the FIC Index was calculated as follow. The MICs of each of the two molecules tested individually and in combination with each other were evaluated and the results have been included in the following formula reported by Meletiadis et al (179):

$$FIC = \frac{MIC_{A\ in\ combination}}{MIC_A} + \frac{MIC_{B\ in\ combination}}{MIC_B}$$

Where MIC_A was the MIC of the compound UPAR415 and $MIC_{A\ in\ combination}$ was the MIC of UPAR415 in combination with colistin. MIC_B was the MIC of colistin and $MIC_{B\ in\ combination}$ was the MIC of colistin in combination with UPAR415.

Since the result of the FIC index formula, the antimicrobial activity in combination of the two molecules can be considered: synergistic, additive, indifferent of antagonistic. If the FIC index is $\leq$ 0,5 the association is synergic, additive if FIC is between 0,5 and 1, indifferent if FIC is between 1 and 4 and antagonistic if FIC is $\geq$ 4 (179).

Statistical analysis of checkerboard results in comparison with MIC values of colistin alone were performed using Analysis of Variance (ANOVA) test.

PMBN and UPAR415 association assays

This MIC test was performed using broth microdilution assay as recommended by the CLSI guidelines with minor modifications (288). For this assay the bacterial strain tested was *S.* Typhimurium ATCC 14028, which was prepared for the inoculum as mentioned above (inoculum preparation). In a sterile tube, PMBN was diluted from the stock solution at the different desired

concentrations, i.e 1-3-5-10 µg/ml in MHB and subsequently, 50 µl of these solutions were inoculated in the U-plate wells. Growth and sterility controls were prepared respectively with 50-50 µl of sterile MHB and bacterial suspension and 100 µl of sterile MHB.

Serial twofold dilutions of UPAR415 in DMSO were performed in a separate sterile 96-well microtiter plate from 25,6 to 0,05 mg/ml and then 1 µl of each concentration was inoculated in the plate, to obtain a serial dilution from 256 to 0,5 µg/ml. 50 µl of bacterial suspension were inoculated in each well and subsequently plates were incubated at 37°C for 24 hours in aerobic atmosphere. For this assay, three experiments with three replicates each were assessed.

After incubation, plates were read by unaided eye with a microtiter reading mirror and then the optical density (OD) of each well of the plates was measured with the aim of a spectrophotometer at λ of 620 nm.

2.3. Cytotoxicity and hemolysis assays

Cytotoxicity and hemolysis assay were respectively performed on Madin-Darby Bovine Kidney Cells MDBK ATCC CCL-22 and sheep defibrinated blood (Thermofisher Diagnostic, lot number 36889400).

2.3.1 Cytotoxicity assay

Cytotoxicity assay was performed using the MTT test, following the procedure used by Donofrio et al. (290).

For the cytotoxicity assay, MDBK cells were cultured in microtiter tissue culture plates in DMEM medium for 24 hours at 37°C in the presence of 5% of CO_2. After incubation, when the cell monolayer is at confluence, an aliquot of 1 µl of UPAR415 in DMSO at different concentrations was added in each well, containing a volume of 100 µl of DMEM medium and MDBK cells, then the plates were reincubated at the same conditions. After incubation, 10 µl of MTT at 200 mg/ml (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well and incubated at

37°C for 6 hours. At the end of the incubation, 100 µl of the solubilization solution (10% SDS in 0,01 M of HCl) was added to each well and then incubated overnight. The MTT compound, a yellow tetrazolium salt is reduced by mitochondrial enzymes (succinate dehydrogenase) of metabolically active eucaryotic cells to insoluble formazan crystals. In the presence of metabolically active cells, after the addition of a detergent solution (SDS) that allows the formazan to be released from the cells, a violet color is seen in the medium. Instead, in the presence of non-viable cells, MTT is not reduced to formazan and therefore the solution will remain yellow.

After incubation, plates were read with a spectrophotometer at a λ of 620 nm. Positive controls (PC) - without any compounds – and PCs with 1% of DMSO were performed for each plate and three replicates for two independent experiments were performed for each assay.

2.3.2 Hemolysis assay

For the hemolysis test, in a U-bottomed microtiter 96-well sterile plate, 50 µl of sheep defibrinated blood were inoculated in each well with 49 µl of sterile saline and 1 µl of different concentrations of UPAR415 and then incubated at room temperature for 24 hours. Positive and negative controls were prepared respectively with 50 µl of sterile water and 49 µl of sterile saline, in contact with 50 µl of blood to obtain a completely hemoglobin release (100%) and absence of hemoglobin release (0%). A negative control with 1 µl of sterile DMSO and 49 µl of sterile saline was also prepared. Three replicates for three independent experiments were performed for each assay.

After incubation, plates were centrifuged at 1400 rpm for 15 minutes, then the supernatant of each well was transferred in sterile plates and their OD were read with a spectrophotometer at a λ of 540 nm. Finally, the hemolysis percentage of each experimental point was calculated as follow:

$$\left[1 - (A_{comp} - A_{NC})/(A_{PC} - A_{NC})\right] x\ 100$$

Where A_{comp} represents the OD of the samples at 540 nm, A_{PC} the OD of the positive control and A_{NC} the OD of the negative control.

2.4. Biochemical assays

Biochemical analysis performed in this work were addressed to:

- Identify the amino acid homology of *Cys*K of the tested bacterial strains with those of *S.* Typhimurium, which represent the model for computational studies;
- Identify the expression of OASS-A and OASS-B respectively in S. Typhimurium ATCC 14028 and DW378 (*trpC109*, *cysK1772* and *cysM1770*);
- Identify the crystal structure of UPAR415 in complex with OASS-A.

2.4.1 Amino acid alignment of CysK of the tested bacterial strains

For what concern the determination of amino acid alignment of CysK of the tested bacterial strains with those of *S.* Typhimurium was applied the Cluster Omega program running on UniProtKB and Kegg Pathways databases (291,292). To calculate the similarity scores was used the ESPript program using the Blosum62 matrix set at default global score (293).

2.4.2 Evaluation of expression of OASS-A and OASS-B in reference and mutant *Salmonella* Typhimurium strains

Salmonella enterica subsp. enterica serovar Typhimurium ATCC 14028 and strain DW378 were inoculated in MHB and statically grown overnight at 37°C. 0,25 ml of culture was used to inoculate 25 ml of fresh MHB and the culture grown under shaking until OD 600 of 0,4 was reached. Bacteria were collected by centrifugation at 2000 rpm for 15 minutes at 4°C and the pellets were resuspended in phosphate buffered saline and centrifuged again to remove the supernatant. The pellets were then precipitated in acetone at -80°C overnight. After centrifugation and acetone removal, cells were resuspended and lysed in 40 mM Tris at 0,25% SDS, pH 7,4. A total protein

amount of 4 µg was loaded in each lane of a Criterion stain free gel (Biorad, Hercules, CA, USA). 15 ng of purified OASS-A and OASS-B43 were loaded as a control. Proteins were transferred on a nitrocellulose membrane using the Biorad RTA transfer kit and the Trans-Blot TurboTM transfer system. Rabbit primary polyclonal antibodies were specific for *Cys*K and *Cys*M from *S.* Typhimurium and were purchased from Preclinics (Potsdam, Germany). Secondary anti-rabbit antibody (A0545) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Blot was developed by adding chemiluminescent substrate (Chemiluminate-HRP PicoDetect, Applichem, Chicago, IL, USA) and the image was acquired by a ChemiDocTM gel imaging system (Biorad).

2.4.3 Crystallization of *Salmonella* OASS-A in combination with UPAR415 and data collection

*St*OASS-A was produced from recombinant *E. coli* BL21(DE3) expression and purified as described by Franko et al. and Tian et al. (294,295). Purification of His-tagged protein was performed using ion metal affinity chromatography on immobilized Co^{2+} ions (Talon Technology, Clontech Laboratories, Inc., Mountain View, CA, USA). His-tag was removed at 37°C by factor Xa in a 1:200 ratio with protein in 20 mM HEPES, 100 mM NaCl and 4 mM $CaCl_2$ at pH 7,5. Crystallization was performed by hanging drop vapor diffusion at similar conditions as described by Burkhard et al. (296). The drops contained 1 µl of 20 mg/mL of *St*OASS-A mixed with 1 µL of reservoir solution containing 30−31% PEG4000 (w/v), 130−180 mM Li2SO4 (Fluka), and 100 mM Tris base pH 7.0. Crystals grew within five days at 25°C as monoclinic plates of 1,1 x 0,4 x 0,1 mm. Then, crystals were soaked for 2 hours at RT in a solution containing 1 mM of UPAR415, 32% PEG 4000 (w/v), 150 mM $LiSO_4$, 100 mM Tris at pH 7,0 and 5% of glycerol as a cryoprotectant agent. Subsequently, the crystal was flash frozen in liquid nitrogen to be measured.

Diffraction data were collected at the Elettra XRD1 beamline (Trieste, Italy) using a Pilatus 6 M (Dectris) detector and processed by the XDS program (297).

2.4.4 Structure determination and refinement

The crystal structure of *St*OASS-A and UPAR415 was solved by molecular replacement direct fft using the structure of 1OAS by a rigid body procedure, implemented in PHASER of the CCP4 software suite (298). Flexible loops of the protein, PLP factor and water molecules were removed from the initial model to exclude model bias during the first round of refinement. The UPAR415 molecule [DRG] manually fitted in the FoFc electron density map. The model was improved using manual rebuilding with COOT and maximum likelihood refinement using REFMAC5 (299,300). The final step of the structure refinement was performed to 1,2 Å with an R_{work} of 15,9% and R_{free} of 18,2%. Structural alignments were calculated using the GESAMT algorithm and structure analysis were performed using COOT and PyMOL™ 2.0.6 (The PyMOL Molecular Graphics System, Version 2.0, Schrödinger, LLC) (301). The structure has been submitted to the Protein Data Bank with PDB id 6Z4N.

3 Results

3.1 Identification of amino acid homology between tested bacterial strains

Since in this thesis project it is tried to establish the activity as antimicrobial adjuvant of a OASS inhibitor, we primarily wanted to establish the presence of the target in the tested bacterial strains and their degree of homology with the *Cys*K amino acid sequence of *S.* Typhimurium, that is the bacterium whose structure of OASS-A and OASS-B has been thoroughly examined and towards which the structure of UPAR415 has been studied as its best inhibitor (278). Although the presence of *Cys* genes encoding for enzymes of sulfur assimilation pathway has been confirmed for all the six tested strains (257-259,269), both thanks to published studies, and by homology in the UniProtKB and Kegg Pathways databases (292), the possibility to have different substrates of the isozymes OASS-A and OASS-B was not confirmed, especially in Gram positive strains. For example, in *S. aureus* was identified an OASS enzyme which showed a high sequence identity to *Cys*K and capable of using thiosulfate as alternative substrate (258).

Therefore, it was investigated the amino acid alignments of *Cys*K of the six bacterial pathogens tested, three Gram negative (StCysK; EcCysK; KpCysK) and three Gram positive (SaCysK; MRSACysK and SpCysK) (Fig. 13). As highlighted in the figure 13, the active site residues are strictly conserved in all the tested pathogens, except for the Ile230 that in *Staphylococcus* spp. (*S. aureus*, MRSA and *S. pseudintermedius*) underwent a conservative substitution to leucine. Therefore, the differences between the six different *Cys*Ks have not proved so high as to justify a different action between the molecule designed towards the *Salmonella* enzyme and the pathogens of other species that share the binding site.

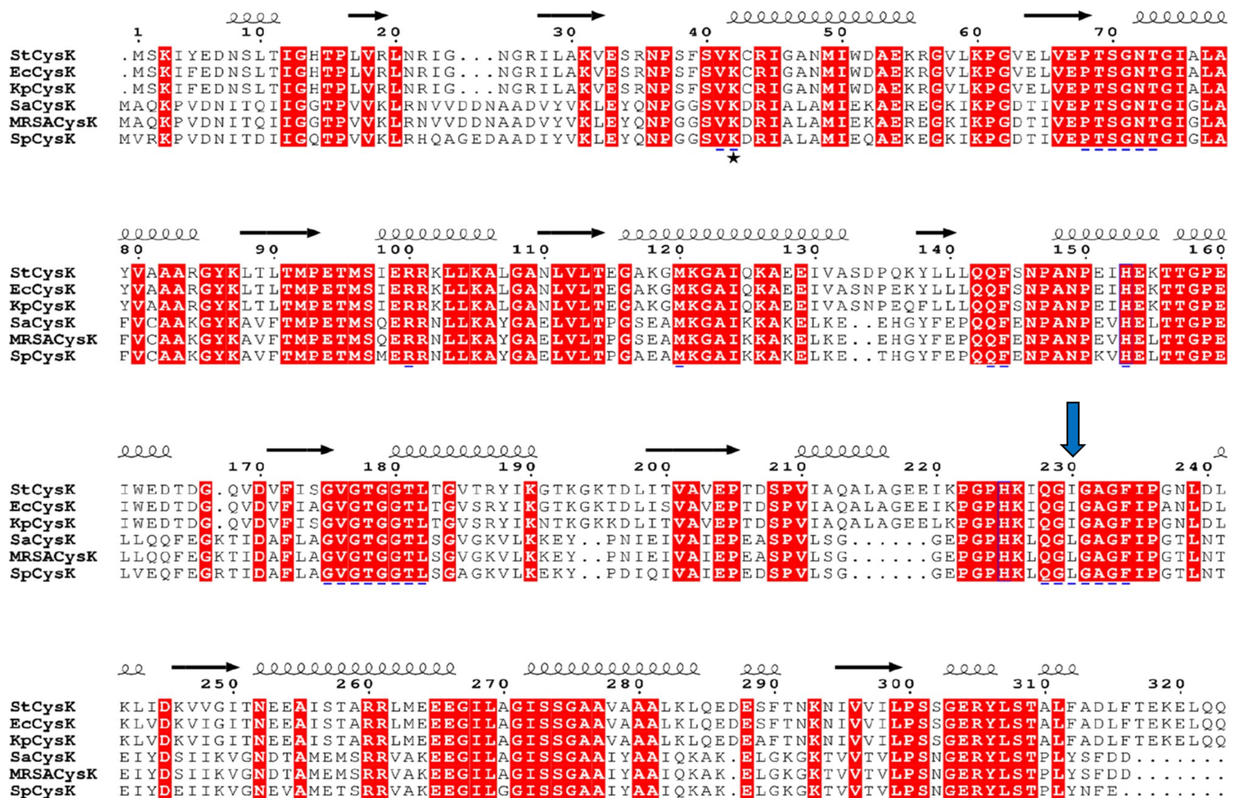

Figure 13: Alignment of the amino acid sequences of CysK from Gram negative (Salmonella Typhimurium StCysK, E. coli EcCysK and Klebsiella pneumoniae KpCysK) and Gram positive (Staphylococcus aureus SaCysK; MRSA MRSACysK and Staphylococcus pseudintermedius SpCysK) bacteria. Programs used are described in section 2.4.1. Residues of the active site are marked with a blue dash below the alignment. The active site lysine is marked with a star and the difference in the Ile230 between Gram positive and Gram negative CysK is highlighted by a blue arrow. The secondary structure elements depicted on the top of the alignment are taken from PDB entry 1OAS.

3.2 Microbiological assays on bacteria of interest

3.2.1 MIC determination of UPAR415 alone

To evaluate the efficacy of UPAR415 to inhibit the growth of the six tested reference pathogens, both under physiological condition of nutrients and in "starvation", broth microdilution assays in rich (MHB) and minimal media (LB diluted in sterile water at 20%) were performed. In LB at 20% cysteine is present at very low concentration and this can lead to a growth inhibition of bacteria when UPAR415 inhibit the OASS, final enzyme of the biosynthetic way of this amino acid.

Results of the MIC evaluation of UPAR415 alone are represented in Table 1 and showed absence of antimicrobial activity both in rich and in poor media on the Gram-negative strains (*E. coli*,

S. Typhimurium and *K. pneumoniae*). Similarly, on Gram positive strains UPAR415 showed a negligible (on *S. aureus* and *S. pseudintermedius*) or absent (on MRSA) antimicrobial effect both in rich and poor media.

Bacterial strains	**MIC value (μg/ml)**	
	Müeller Hinton Broth (MHB)	LB 20%
E. coli ATCC 25922	>256	>256
S. Typhimurium ATCC 14028	>256	>256
K. pneumoniae ATCC 13883	>256	>256
S. aureus ATCC 25923	128	107
MRSA ATCC 43300	>256	>256
S. pseudintermedius ATCC 21284	111	136

Table 1: MIC values of UPAR415 alone against six reference bacterial pathogens of human and veterinary interest. No activity was detected against Gram negative strains and only a negligible activity against two of the three Gram positive tested bacteria, both in rich and poor media.

3.2.2 MIC determination of UPAR415 associated with polymyxin B nonapeptide (PMBN)

To evaluate the activity of UPAR415 on S. Typhimurium ATCC 14028 in presence of a membrane destabilized without antibacterial activity, broth microdilution assays with fixed concentration of PMBN, chosen from literature for their ability to destabilize the outer membrane without causing cell death (236), and scalar concentrations of UPAR415 were performed in rich media (MHB). Results showed an absent antimicrobial activity of UPAR415 also in association with a non-antibiotic membrane permeabilizer, demonstrating a non-antibiotic activity *per se* of this compound (Table 2).

Bacterial strain	**PMBN (μg/ml)**	**MIC value (μg/ml)**
S. Typhimurium ATCC 14028	1	512 ± 0
	3	398,2 ± 134,9
	5	369,8 ± 134,9
	10	369,8 ± 134,9

Table 2: MIC values of UPAR415 in association with fixed concentration of PMBN as membrane permeabilizer against S. Typhimurium ATCC 14028. No antimicrobial activity was detected.

3.2.3 Checkerboard assays between UPAR415 and colistin against six bacterial pathogens

To assess the antimicrobial adjuvant efficacy of UPAR415 in combination with colistin, checkerboard assays were performed in both rich and poor media (MHB and LB 20%). Before setting up the checkerboard assay for each tested bacterium, MIC tests of colistin alone were prepared with the two different medium and results were showed at table 3 (Table 3). Subsequently, checkerboards were prepared with ten scalar concentrations of colistin, starting from the twofold dilution of one well above the MIC value for each tested bacterium, and for each experiment three different concentrations of UPAR415 were added to each well: 64, 32 and 16 µg/ml. Checkerboard MIC values and FIC index for each tested bacteria and combinations were reported at table 4 (Table 4). For each experiment, to calculate the FIC index was chosen the most potent UPAR415 concentration (generally the higher concentration, that is 64 µg/ml). All results from checkerboard assays were calculated from both the unaided eye and the spectrophotometer reading, and the inhibition percentages of each experiment at each concentration tested were reported at table 5 (Table 5).

Little differences were found in the two considered media between MIC values of colistin alone and MIC values of the association with UPAR415 at the three tested concentrations on the six tested bacterial pathogens.

For what concern the checkerboard assays, the association of colistin and UPAR415 showed for all tested bacteria a synergy or additivity of action.

In particular, on Gram-positive the association was additive, and MIC of colistin dropped 4-times for *S. aureus*, 3-times for MRSA and 34-times for *S. pseudintermedius* on MHB, and 9-times, 3-times and 17-times respectively on LB 20% when 64 µg/ml of UPAR415 was administered. At the same time, on Gram-negative the association was additive only for *E. coli* on MHB, in which the MIC of colistin dropped only 2,3-times when UPAR415 at 64 µg/ml is co-administered, while for the other

associations the FIC index revealed a synergy of action. In particular, in LB 20%, *E. coli* showed a MIC in association 6-times lower than colistin alone, and for *S.* Typhimurium and K. pneumoniae MIC lowered respectively 18,3-times and 25-times. In MHB MIC in association of *S.* Typhimurium and *K. pneumoniae* lowered respectively 2,78-times and 7-times when associated with UPAR415 at 64 µg/ml, and for *S.* Typhimurium the addition of 32 µg/ml of UPAR415 lowered the MIC of colistin 4-times.

Furthermore, from the checkerboard assays is possible to notice that generally MIC values of colistin and associations UPAR415-colistin are much higher in LB 20% medium than MHB for Gram-negative strains, while for Gram-positive the opposite is true, that is MIC values on LB 20% are lower than MHB, but generally higher than those of Gram-negative strains.

Statistical differences between MIC values of colistin and association colistin/UPAR415-64 µg/ml were calculated for each tested bacterial strain, and results are indicated in figure 14 (Figure 14). Significant statistically differences have been observed for all tested strains in at least one of the two media tested, except for *S. pseudintermedius,* which does not show any significant difference. For Gram negative, MIC of *E. coli* and *S.* Typhimurium showed significant differences on both the media, while *K. pneumoniae* only in LB 20%. MIC values of *S. aureus* and MRSA showed significant differences on both the media, with a $p < 0,001$ in LB 20%.

Bacterial strains	MIC colistin (µg/ml)	
	MHB	LB 20%
E. coli ATCC 25922	0,389 ± 0,13	1,278 ± 0,565
S. Typhimurium ATCC 14028	0,64 ± 0,00	2,56 ± 0,00
K. pneumoniae ATCC 13883	0,21 ± 0,13	2,00 ± 0,00
S. aureus ATCC 25923	64,00 ± 0,00	64,00 ± 0,00
MRSA ATCC 43300	64,00 ± 0,00	32,00 ± 0,00
S. pseudintermedius ATCC 21284	8,00 ± 0,00	4,00 ± 0,00

Table 3: MIC values of colistin against six reference bacterial pathogens of human and veterinary interest both on MHB and LB 20% media.

Bacterial strains	MHB			LB 20%		
	UPAR415 (µg/ml)	MIC colistin (µg/ml)	FIC index	UPAR415 (µg/ml)	MIC colistin (µg/ml)	FIC index
E. coli ATCC 25922	0	0,39 ± 0,13	0,56 Additivity	0	1,28 ± 0,56	0,28 Synergy
	64	0,17 ± 0,07		64	0,21 ± 0,06	
	32	0,47 ± 0,08		32	0,94 ± 0,17	
	16	0,47 ± 0,08		16	0,83 ± 0,25	
S. Typhimurium ATCC 14028	0	0,64 ± 0,00	0,48 Synergy	0	2,56 ± 0,00	0,17 Synergy
	64	0,23 ± 0,08		64	0,14 ± 0,01	
	32	0,16 ± 0,07		32	0,21 ± 0,16	
	16	0,24 ± 0,11		16	0,46 ± 0,17	
K. pneumoniae ATCC 13883	0	0,21 ± 0,13	0,26 Synergy	0	2,00 ± 0,00	0,16 Synergy
	64	0,03 ± 0,01		64	0,08 ± 0,04	
	32	0,26 ± 0,19		32	0,19 ± 0,30	
	16	0,75 ± 0,57		16	1,78 ± 0,44	
S. aureus ATCC 25923	0	64,00 ± 0,00	0,75 Additivity	0	64,00 ± 0,00	0,61 Additivity
	64	16,00 ± 10,45		64	7,00 ± 1,85	
	32	35,56 ± 10,67		32	35,56 ± 10,67	
	16	56,89 ± 14,11		16	64,00 ± 0,00	
MRSA ATCC 43300	0	64,00 ± 0,00	0,58 Additivity	0	32,00 ± 0,00	0,52 Additivity
	64	21,33 ± 8,00		64	8,57 ± 3,60	
	32	64,00 ± 0,00		32	42,67 ± 16,00	
	16	120,89 ± 21,33		16	64,00 ± 0,00	
S. pseudintermedius ATCC 21284	0	8,00 ± 0,00	0,58 Additivity	0	4,00 ± 0,00	0,62 Additivity
	64	0,23 ± 0,04		64	0,23 ± 0,04	
	32	0,22 ± 0,30		32	0,29 ± 0,30	
	16	8,00 ± 0,00		16	1,59 ± 1,55	

Table 4: MIC values and FIC index of the checkerboard assays performed on the six reference bacterial pathogens. For each bacterium were reported the MIC values of colistin alone and in combination with three different concentrations of UPAR415: 64,32 and 16 µg/ml. For each bacterium was also reported the FIC index of the association between colistin and UPAR415 calculated using the MIC value of the most potent association.

Bacterial strains	**UPAR415 64 µg/ml**	**→ Ten scalar concentrations of colistin from 1 well above its MIC value→** Growth inhibition (%)									
E. coli ATCC 25922	MHB	100%	100%	100%	84%	47%	32%	28%	28%	25%	25%
	LB20%	100%	100%	100%	74%	61%	41%	29%	20%	17%	20%
S. Typhimurium ATCC 14028	MHB	100%	100%	100%	96%	84%	54%	23%	18%	19%	17%
	LB20%	100%	100%	100%	100%	98%	65%	34%	32%	18%	20%
K. pneumoniae ATCC 13883	MHB	100%	100%	100%	100%	100%	99%	100%	84%	66%	52%
	LB20%	100%	100%	100%	100%	100%	100%	94%	66%	49%	36%
S. aureus ATCC 25923	MHB	100%	100%	100%	91%	99%	98%	95%	90%	83%	66%
	LB20%	100%	100%	100%	100%	100%	88%	73%	87%	76%	72%
MRSA ATCC 43300	MHB	100%	100%	97%	86%	81%	80%	77%	77%	79%	79%
	LB20%	100%	100%	100%	100%	74%	64%	71%	68%	80%	78%
S. pseudintermedius ATCC 21284	MHB	100%	100%	100%	100%	100%	100%	100%	99%	85%	62%
	LB20%	100%	100%	100%	100%	100%	100%	96%	47%	37%	28%

Table 5: Growth inhibition percentages of the associations with ten scalar concentrations of colistin, starting from one well above the MIC value of colistin for each tested bacteria, and UPAR415 at 64 µg/ml. OD values were converted in inhibition percentages using the formula: 1- (average OD of test wells – average sterility controls)/(average growth controls).

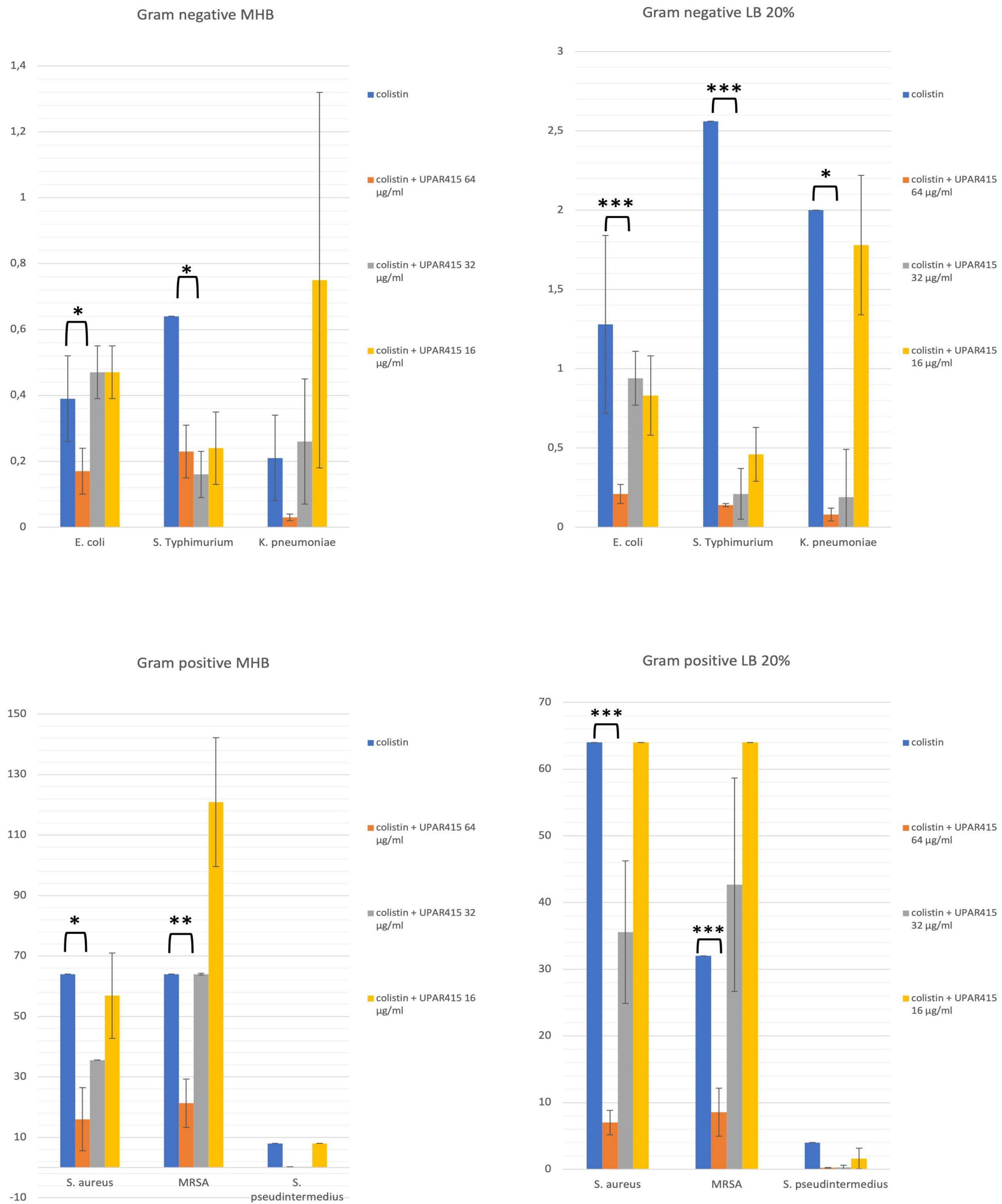

*Figure 14: Y-axis: MIC (µg/ml) values of colistin alone and in combination with UPAR415 as adjuvant, in Gram negative and Gram positive bacteria and both MHB and LB 20% media. X-axis: tested bacterial strains. Results are presented as the average of three independent experiments, performed in triplicate each, ± standard deviation (SD). With *; **; *** are indicated the comparisons between colistin alone and colistin + UPAR415 64 µg/ml with a statistical significance (calculated with ANOVA test) at * p < 0,05; ** p < 0,01; *** p < 0,001.*

3.2.4 Target engagement experiments with mutant and wild-type strains of *Salmonella* Typhimurium

To establish the target engagement of UPAR415 inside the bacterial cell of *Salmonella* Typhimurium, MIC assays with the mutant strain DW378 (with inactivated OASS-A and OASS-B (289)) were performed in MHB, comparing them with those performed with the wild-type (WT) strain (ATCC 14028). These assays were performed with the aim to prove that the phenotypic observations of UPAR415~~’s~~ growth inhibition were linked to the chemical inhibition of *St*OASS-A and /or *St*OASS-B inside the cell.

MIC tests of UPAR415 and colistin alone and in association were performed on DW378 strain and compared with the same evaluations performed on the WT strain ATCC 14028 (Table 6).

Furthermore, to confirm and support the UPAR415’s mechanism of action, MIC assays of UPAR415 against ATCC 14028 were performed in M9 minimal medium, a medium deprived of relevant metabolites and cysteine but containing $MgSO_4$ as sulfur source, also with the addition of two different concentration of cysteine (Table 7, Figure 15).

UPAR415 alone does not exert any antimicrobial activity on mutant strain DW378, as observed with WT strain. No differences between MIC alone (0,14 µg/ml) and in combination with UPAR415 (0,14 µg/ml) have been detected for colistin on mutant strain DW378, showing a FIC index of 1,16 which corresponds to indifference. Furthermore, the MIC value of colistin alone on DW378 is about 5-fold lower (0,14 µg/ml) than that on WT strain (0,71 µg/ml) and similar to the MIC value of the association with 64 µg/ml of UPAR415 (0,23 µg/ml) on WT strain.

In M9 medium UPAR415 alone does not show again any antimicrobial activity on WT strain, while colistin shows a MIC of 16 µg/ml, which becomes 64-fold lower when UPAR415 is administered in association at 64 µg/ml (0,25 µg/ml). The addition of cysteine in the medium slightly counteracts

the synergistic effect of UPAR415/colistin, in particular after adding 0,02 mM of cysteine in the medium, MIC value of association increases at 0,4 µg/ml (1,6-fold). The addition of a higher concentration of cysteine (0,2 mM) does not influence this result (MIC of 0,25 µg/ml of UPAR415-64/colistin) but lower the MIC of colistin alone (8 µg/ml).

Bacterial strains	**MIC values (µg/ml) individuals**		**MIC values (µg/ml) in combination**		**FIC index**
	UPAR415	colistin	UPAR415	colistin	
***S.* Typhimurium ATCC 14028 (WT)**	>256 ± 0	0,71 ± 0,21	64	0,23 ± 0,08	0,48 Synergy
***S.* Typhimurium DW378**	>256 ± 0	0,14 ± 0,09	64	0,14 ± 0,07	1,16 Indifference

Table 6: MIC values of UPAR415 and colistin alone and in combination on MHB against WT and mutant S. Typhimurium.

Media	**UPAR415 (µg/ml)**	**colistin (µg/ml)**	**UPAR415 64 (µg/ml)**	**UPAR415 32 (µg/ml)**	**UPAR415 16 (µg/ml)**	**FIC index**
M9	>256 ± 0	16 ± 0	0,25 ± 0	0,25 ± 0	0,22 ± 0,06	0,05 Synergy
M9 + cysteine 0,02 mM	>256 ± 0	16 ± 0	0,4 ± 0,15	0,31 ± 0,11	0,39 ± 0,13	0,08 Synergy
M9 + cysteine 0,2 mM	>256 ± 0	8 ± 0	0,25 ± 0	0,25 ± 0	0,44 ± 0,24	0,09 Synergy

Table 7: MIC values of UPAR415 and colistin alone and in combination on M9, M9 + cysteine 0,02 mM and M9 + cysteine 0,2 mM against S. Typhimurium ATCC 14028 (WT) and relatives FIC index.

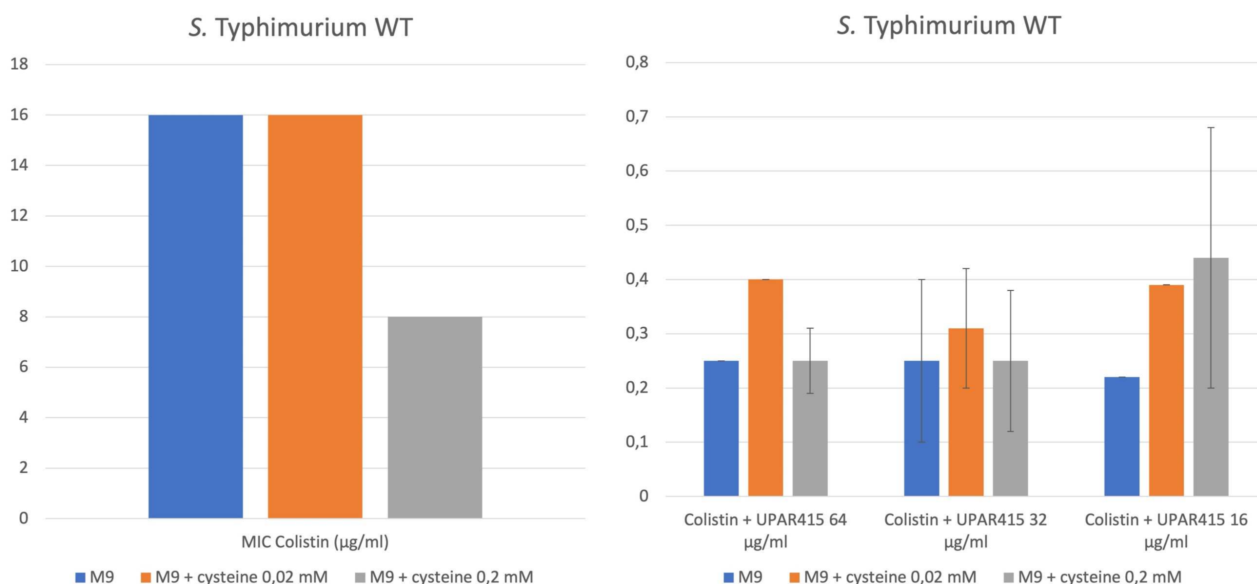

Figure 15: Left: Y-axis: MIC values of colistin in (X-axis) M9, M9 + cysteine 0,02 mM and M9 + cysteine 0,2 mM against S. Typhimurium WT. Right: Y-axis: MIC values of colistin in association with (X-axis) UPAR415 at 64,32 and 16 µg/ml in M9, M9 + cysteine 0,02 mM and M9 + cysteine 0,2 mM.

3.3 Evaluation of toxicity of UPAR415 and its associations with colistin

3.3.1 Cytotoxicity assay on MDBK cells

To evaluate the potential toxicity of UPAR415 on eukaryotic cells, the molecule, and its combinations with colistin were tested on MDBK cells (bovine adult kidney cells) at different concentrations and evaluated with MTT assay. UPAR415 tested concentrations were 256, 64, 32, 16 µg/ml, while 64, 32, and 16 µg/ml of UPAR415 were tested also in association with 256 and 0,125 µg/ml of colistin, which was tested at these concentrations alone also. The positive control (PC) of the test was assessed without adding compounds and results were normalized also using a PC with 1% of DMSO.

Results showed generally low levels of toxicity on MDBK cells for the compound tested and their associations (Table 8, Figure 16).

Worst result was showed by colistin at the maximum concentration tested (52,95% viability), while the associations of UPAR415 and colistin remain far above this level (about 65-92%). UPAR415 alone

showed a low toxicity range on MDBK cells at 16, 32 and 64 µg/ml, with 70-86 % of viability, while the higher concentration (256 µg/ml) showed the best result in terms of viability of MDBK cells, 93%.

UPAR415 (µg/ml)	Colistin (µg/ml)	Viability % of MDBK cells
16	-	71,76 ± 9,78
16	0,125	74,85 ± 10,39
16	256	67,13 ± 16,82
32	-	85,97 ± 6,88
32	0,125	81,74 ± 9,80
32	256	66,86 ± 11,87
64	-	80,14 ± 9,68
64	0,125	91,53 ± 12,50
64	256	73,52 ± 7,24
256	-	93,67 ± 9,75
-	0,125	87,99 ± 7,29
-	256	52,95 ± 16,77
PC + 1% DMSO		117 ± 18,38
PC		100

Table 8: Evaluation of the toxicity of UPAR415 alone and in association with colistin as viability of MDBK cells in vitro by MTT assay. Results are expressed as % of viable cells on the total present in the wells of the PCs with 1% of DMSO. Viability of the PCs +1% of DMSO were respectively calculated based on PCs wells.

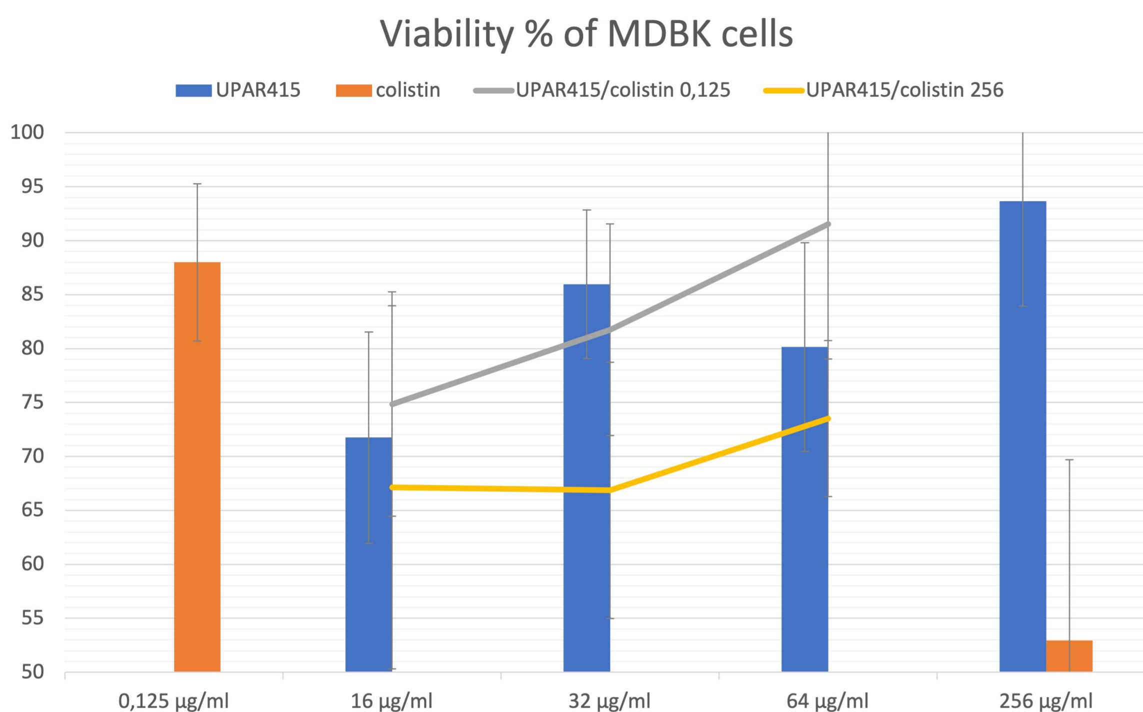

Figure 16: Y-axis: percentages of viable MDBK cells after 16 hours 37°C + 5% CO_2 of incubation with (X-axis) UPAR415, colistin and UPAR415/colistin at different concentration. Histograms represent the viability percentages of MDBK incubated with UPAR415 and colistin alone at respectively 16,32,64,256 µg/ml and 0,125,256 µg/ml. Lines represent the viability percentages of MDBK incubated with the association of UPAR415/colistin at 0,125 µg/ml and UPAR415/colistin at 256 µg/ml.

3.3.2 Hemolysis assay on sheep defibrinated blood

Toxicity evaluation was also performed with hemolysis assay on sheep defibrinated blood, to evaluate the damage caused by UPAR415 on eukaryotic cell membranes. UPAR415 was tested at different concentrations alone and in combination with colistin, as tested on MDBK cells. After incubation of 24 hours at room temperature hemolysis percentages were calculated for each tested concentration.

UPAR415 showed a negligible hemolytic activity alone at 16, 32 and 64 µg/ml concentrations, where the hemolytic percentage stands below the 2% (higher value is 1,2% of 64 µg/ml), while the higher concentration tested (256 µg/ml) resulted in a 5% total hemolysis (Table 9). For what concern the associations between UPAR415 and colistin none of these resulted in a hemolytic activity higher than 0,9%, as the highest result was found for UPAR415 at 64 µg/ml + colistin at 0,125% (Table 9).

Altogether, the tested compound, and its associations with colistin did not show cellular toxicity results such as to give rise to concern for possible clinical use, in particular on blood cells, for which critical toxicity values are considered those exceeding 10% hemolysis (302).

UPAR415 (µg/ml)	Colistin (µg/ml)	Average OD ± SD	Hemolysis %
16	-	0,058 ± 0,02	0,255
	0,125	0,052 ± 0,01	0,151
	256	0,059 ± 0,02	0,288
32	-	0,070 ± 0,01	0,514
	0,125	0,067 ± 0,01	0,442
	256	0,072 ± 0,01	0,546
64	-	0,103 ± 0,04	1,206
	0,125	0,086 ± 0,02	**0,837**
	256	0,081 ± 0,01	0,748
256	-	0,266 ± 0,10	**5,07**
NC		0,052 ± 0,03	0,00
NC + 1% DMSO		0,045 ± 0,03	0,00
PC		4,861 ± 1,05	100

Table 9: Hemolysis percentages on sheep defibrinated blood of UPAR415 alone at 16, 32, 64, 256 µg/ml and in combination with 0,125 and 256 µg/ml of colistin. Average optical density (OD) of each concentration tested ± its standard deviation (SD) was reported. Negative control (NC) was assessed without addition of compounds and with 50 µl of sterile saline, NC + 1% DMSO with sterile saline and 1 µl of DMSO, and positive control (PC) with 50 µl of sterile deionized water.

3.4 Expression of OASS-A and OASS-B in DW378 and ATCC 14028 *Salmonella* Typhimurium strains

To evaluate the effective expression of isoenzymes OASS-A and OASS-B in both *S.* Typhimurium tested strains, that are wild type strain ATCC 14028 and mutant strain DW378, with genotype *trpC109*, *cysK1772* and *cysM1770*, protein extraction and purification were performed from bacterial lysates, followed by the execution of a western blot with rabbit primary antibodies specific for CysK and CysM of *S.* Typhimurium (Figure 17). Experiment was performed in collaboration with the Laboratory of Biochemistry and Molecular Biology, at the Department of Food and Drugs of the University of Parma.

Western blot showed expression of comparable amount of OASS-A both in ATCC 14028 and DW378 (at 0,4 OD and at overnight incubation), whereas OASS-B expression is undetectable in both strains.

The genotype of the mutant bacterium was previously identified as completely lacking the activity of OASS, but for this purpose no molecular tests attesting this absence have been carried out (289). This western blotting test confirmed the presence of OASS-A in DW378, indicating that the actual auxotrophy for the cysteine may result from secondary inactivation of the enzyme and not from complete gene deletion.

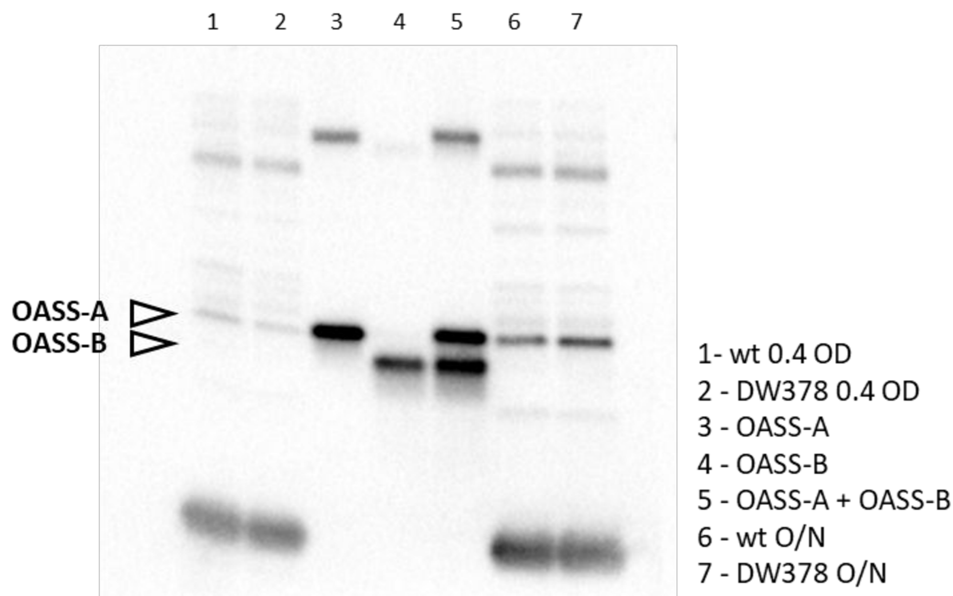

Figure 17: Western blot of bacterial lysates of WT (ATCC 14028) and mutant (DW378) S. Typhimurium strains grown in MHB at 37 °C under shaking either until 0.4 OD was reached or O/N. Purified OASS-A and OASS-B of Salmonella Typhimurium were loaded as control. Arrows indicate the bands specific for OASS-A and OASS-B. Some aspecific bands at high and low molecular weight are visible both in the samples and in the OASS-A control.

3.5 Crystal structure of *S.* Typhimurium OASS-A in complex with UPAR415

With the aim to confirm the functional data suggesting a competitive mechanism of UPAR415 on *St*OASS respect to the physiological substrate, OAS (270), the crystal structure of *St*OASS-A in complex with UPAR415 was determined, at 1,2 Å resolution. Experiment was performed in collaboration with the Elettra research group, at Sincrotrone Trieste S.C.p.A, 34149 Trieste, Italy.

The only *Salmonella* OASS-A structure previously detected in the literature and complexed with a small organic molecule was obtained in 1999, in which in a K41A mutant strain a purification-derived methionine was found bound in the active site of the enzyme (PDB id: 1D6S) (303). While the structure of the unligated (PDB id; 1OAS) and inhibited forms of *St*OASS-A were previously determined, the latter had a sulfate bound in the active site and a chloride bound in an allosteric site (PDB id: 1FCJ) (296, 304). For the crystallization of OASS-A with UPAR415 was used a protocol similar to that of Burkhard et al. (303) and obtained crystals belonged to the space group $P2_12_12_1$ with unit cell parameters a = 56,67 Å, b = 122,87 Å, c = 127,52 Å and $\alpha = b = \gamma$ = 90° and contained one dimer per asymmetric unit. Detailed data collection statistics are shown in Table 10 and 3D structure of the dimer is represented in Figure 18, panel A (Table 10, Figure 18).

The final model structure contains a well-defined electron density for the main chain of each polypeptide in the dimer, except for the last C-terminal residues. OASS adopts the fold of a type II pyridoxal 5'-phophate (PLP)-dependent enzyme with protomers having the PLP cofactor linked as an internal aldimine to Lys41 (residues are numbered as in 1OAS). No significant differences were observed among the dimer chains, which superimpose well with a root-mean-square deviation (RMSD) of 0,12 (aligned in Pymol). Density of UPAR415 was identified near the PLP cofactor (Figure 18, panel B), with the two aromatic substituents on the cyclopropane ring pointing toward the active site entrance (Figure 18, panel A) and forming hydrophobic interactions with residues lining the pocket (Phe143, Ile229, Ala231, Figure 18, panel C). The carboxylate group of UPAR415 forms hydrogen bonds with the side chains of Thr68, Thr72 and Glu142, with the main chains of Asp71 and Thr72, and with a water molecule; furthermore, it inserts deeply into the active site occupying a position close to the one occupied by the carboxylate group of methionine in the 1D6S structure (Figure 18, panel D).

Of interest is the almost complete overlap between the carboxylate group of UPAR415 and the C-terminal carboxylate of the NLNI peptide (four C-terminal amino acids of a decapeptide that mimics the C-ter of SAT; 274,275,306). The observation that the carboxylate of UPAR415 occupies the same position of the carboxylate group of both peptidic and nonpeptidic ligands of OASS suggests that it should bind to the same carboxylate subsite of the active site that was identified as essential for anchoring the ligand to the enzyme (303). This subsite was identified on the complex of OASS-A and methionine (1D6S) and the trigger key to induce a movement of the so-called asparagine loop (or substrate-binding loop, SB loop) is given by its occupation. This movement pulls an entire N-terminal subdomain of the protein, leading to the closure of the active site entrance. In the presence of methionine, OASS-A structure is a closed state, in which the N-terminal subdomain has undergone a rotation by moving 7 Å towards the entrance of the active site.

On the other hand, the OASS-A structure in presence of NLNI peptide is in open form, demonstrating that, in this case, binding with the ligand does not trigger the closure of the active site. We then superimposed the UPAR415/OASS-A structure with the open (1OAS), intermediate/inhibited (1FCJ) and closed (1D6S) structures in their dimeric form. Structural alignments resulted in RMSDs of 0.576, 0.607, and 1.081 for the open, intermediate, and closed structures, respectively. The superpositions are shown in Figure 19.

Based on these results, it was seen that UPAR415 induce only a partial closure of the active site, and thus blocks the enzyme in an almost open conformation, reminiscent of the unligated 1OAS structure, as well as the allosterically inhibited structure of 1FCJ. For this reason, was further investigated this feature and compared with the position of SB loop and near regions in the present and closed structure (Figure 20). The SB loop moves towards the cofactor in the closed conformation and contributes to the narrowing of the active site. This large movement is not triggered by

UPAR415, probably because the loop would clearly collide with the totyl substituent of cyclopropane in the closed conformation. In particular, the electron density for some residues belonging to the SB ring and to the helix 4 can be adapted to two alternative conformations, with about 50% occupancy each, suggesting that this segment of the sequence is not stabilized by the ligand in a defined conformation.

	OASS-UPAR415
Wavelength	1
Resolution range	46.6-1.2 (1.243-1.2)
Space group	$P2_12_12_1$
Unit cell	53.261; 96.275; 140.835; 90; 90; 90
Total reflections	409172 (22602)
Unique reflections	214538 (14933)
Multiplicity	1.9 (1.5)
Completeness (%)	94.35 (65.85)
Mean I/sigma (I)	10.89 (0.72)
Wilson B-factor	10.08
R-merge	0.0286 (0.5601)
R-meas	0.04044 (0.7921)
R-pim	0.0286 (0.5601)
$CC_{1/2}$	0.999 (0.569)
CC*	1 (0.852)
Reflections used in refinement	213161 (14736)
Reflections used for R-free	10571 (747)
R-work	0.1596 (0.2977)
R-free	0.1824 (0.3058)
CC (work)	0.942 (0.402)
CC (free)	0.918 (0.466)
Number of non-hydrogen atoms	6208
Macromolecules	5156
Ligands	103
Solvent	949
Protein residues	639
RMS (bonds)	0.016
RMS (angles)	1.95
Ramachandran favored (%)	97.48
Ramachandran allowed (%)	2.52
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	1.40
Clashscore	10.02
Average B-factor	17.90

Macromolecules	16.91
Ligands	18.19
Solvent	23.22

Table 10: Data collection and refinement statistics: values in parentheses are for the highest-resolution shell. $CC_{1/2}$ is the Pearson's correlation coefficient calculated for I_{mean} by splitting the data randomly in half by AIMLESS/SCALA (305). Statistics for the highest-resolution shell are shown in parentheses.

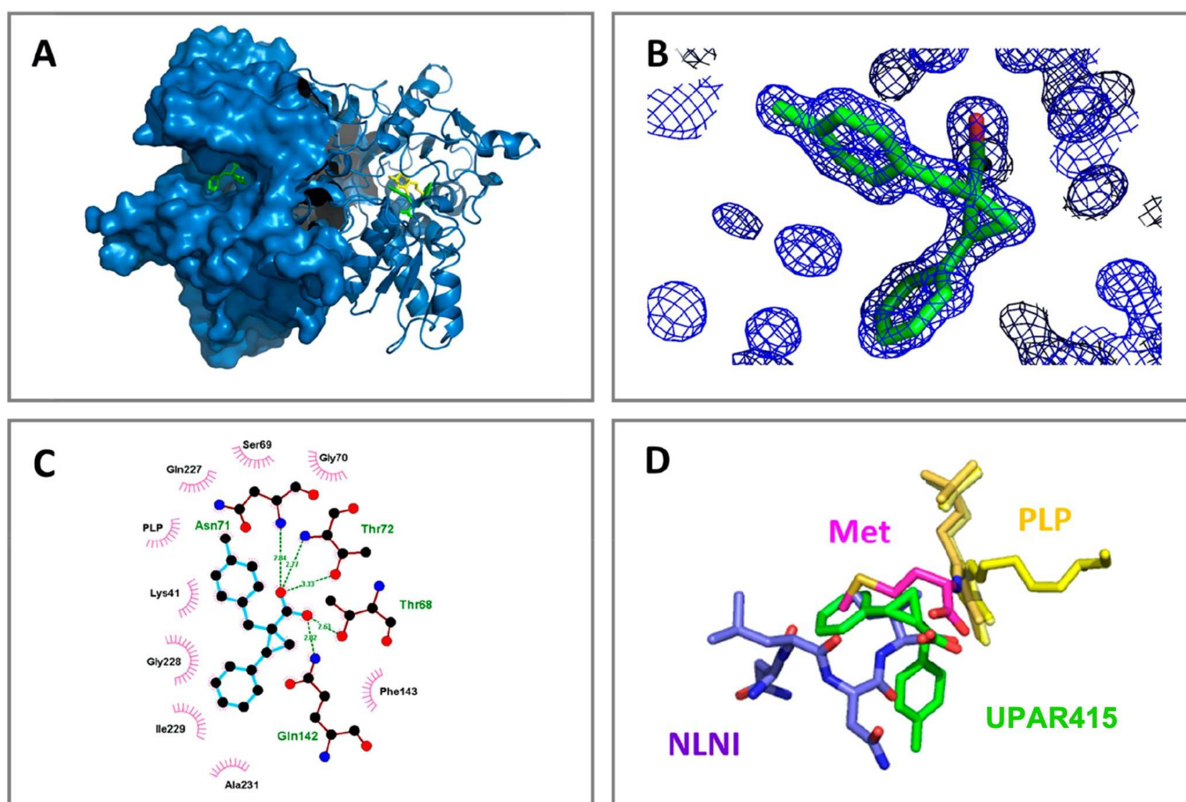

Figure 18: Three-dimensional structure of the complex between OASS-A and UPAR415. Panel A: The OASS-A dimer is shown, with one monomer represented on tape and one monomer represented in surface mode. The PLP cofactor is shown in yellow and UPAR415 is shown in green. Both ligands are shown in stick mode. Panel B: UPAR415 modeled within the electron density is represented in stick mode. Panel C: LigPlot showing the residues involved in the interaction with UPAR415 in the active site of OASS-A. Panel D: Superimposition of 6Z4N (PDB id of this entry) with 1D6S and 1Y7L. The protein structure has been removed from the representation and only PLP and ligands are shown. The ligands are the following: Met (pink) bound as an external aldimine to the PLP (from 1D6S); NLNI tetrapeptide (purple) (from 1Y7L); UPAR415 (UPAR415, green) (from 6Z4N).

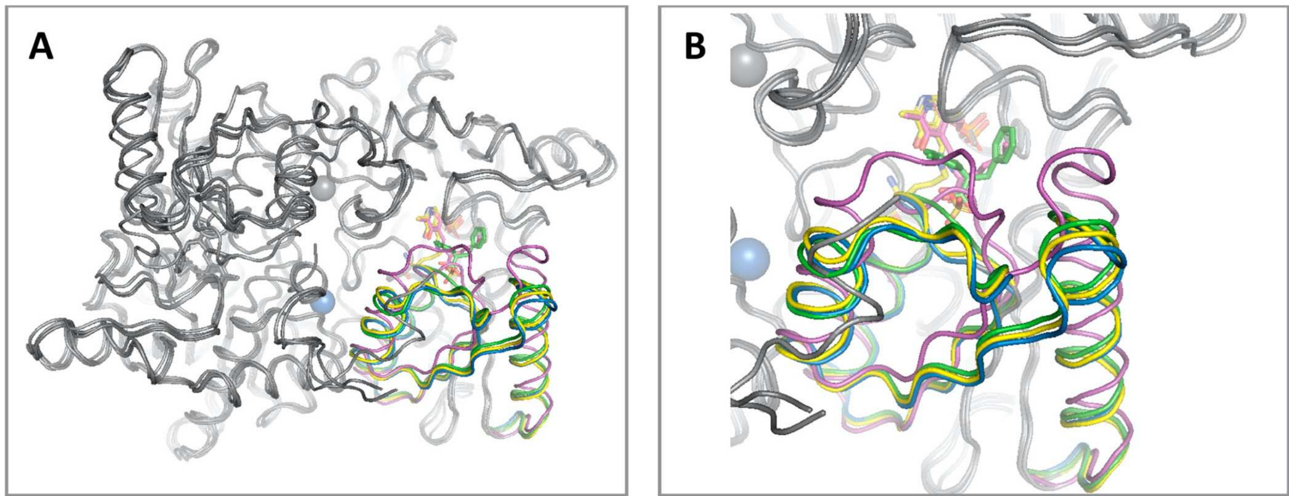

Figure 19: Superimposition of the open, inhibited, and closed structures of OASS-A in complex with UPAR415. Panel A: Structural alignment of the UPAR415 inhibited OASS-A (6Z4N, green) to the open unbound form (1OAS, yellow), the allosterically inhibited intermediate form (1FCJ, blue) and the closed methionine-bound form (1D6S, magenta). Its cofactor and ligands are represented as a stick and bond, while the Cl⁻ ions are as blue spheres. Panel B: Close-up view of the binding site region, where the 3−SB loop− helix 4 helix movement is visible.

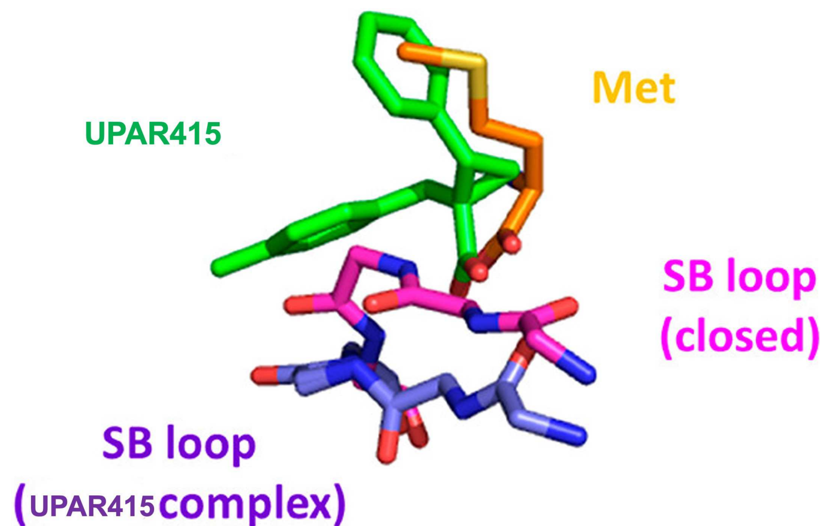

Figure 20: Detail of the superposition of the structure of OASS-A in complex with UPAR415 with the closed structure of OASS-A in complex with methionine. SB loop: substrate-binding loop

4 Discussion

Since the antimicrobial resistance has assumed an extreme importance both in human and veterinary field, so much so that in 2021 it has led to legislation in the EU such as the motion "*Criteria for the designation of antimicrobials to be reserved for the treatment of certain infections in humans*" proposed by the German MEP Hausling, which would drastically reduce the use in the veterinary field of critical molecules (CIA) used in humans, the research and development of new antimicrobial strategies that can also be used in the veterinary field is of utmost importance (307). With this purpose, an effective alternative to the use of traditional antimicrobials alone, is to use adjuvant molecules that allow to obtain a synergistic effect with them, such as to significantly lower the effective concentrations to obtain the MICs on resistant bacteria (141, 175-179).

In veterinary medicine, the use of antimicrobial molecules is much more limited than in humans, due to the importance to safeguard the efficacy of life-saving molecules for these, and for this reason in certain cases last therapeutical chances may be ineffective, due to the strong resistance to antibiotics of different veterinary strains (*S. aureus*, MRSA, MRSP, *Salmonella* Typhimurium, *E. coli*, etc.) (45,71-73). In these cases, the possibility to potentiate the efficacy of current antibiotics with adjuvants of new generation is very interesting.

Therefore, the identification of bacterial non-essential targets as O-acetylserine sulfhydrilase and its inhibitors allowed to discover a new potential antimicrobial target that is not directly microbicidal, as the biosynthetic pathway of cysteine is not an essential pathway for bacterial survival, but which is very important for survival in some stressful situations for the cell, such as oxidative stress or starvation (254,262). These stressful conditions can occur, for example, during antimicrobial treatment with traditional antimicrobials, such as colistin or other polypeptide antibiotics, due to the breakdown of the bacterial outer membrane (234).

For this reason, the use of cysteine biosynthesis inhibitors such as UPAR415, associated with colistin, can prove to be a winning strategy, as they act through two separate mechanisms, but together they are able to obtain a result that is greater than the sum of the two single compounds.

The choice to investigate a potential colistin adjuvant is related to the recent reintroduction of this antibiotic as life-saving molecule, especially against MDRO, both in humans and animals, despite of its toxic effects (nephro/neurotoxicity) (234,308). The use of colistin in association with an adjuvant, such as UPAR415, allows to decrease the antimicrobial concentration necessary to obtain the MIC of colistin and therefore to considerably reduce the possible therapeutic dosage, finally reducing the side effects.

In addition to the choice of the antibiotic molecule to associate with the tested compound, the bacterial strains tested are also of veterinary and human relevance. it was chosen to evaluate both Gram negative and positive bacteria with a significant impact on global health, as they represent some of the strains with the greatest resistance to the most used antibiotics (ESKAPE pathogens) (76). In veterinary medicine, the six tested bacteria in this work are opportunistic pathogens, responsible of many infections of different nature (enteritis, cutaneous/auricular infections, genitourinary and iatrogenic infections) and, in addition, they can cause foodborne infections both in humans and animals (90-97,99-104,124-138). For this reason, they can fully represent a good spectrum of activity of the tested compound UPAR415 on bacteria that potentially possess a wide range of resistance towards commonly used antibiotics, but also to CIAs.

First, it was decided to evaluate, both through the present literature and through the evaluation of the homology of the amino acid sequences of *CysK* gene encoding for OASS isozymes, the presence of the UPAR415 target in the tested bacteria (the biosynthetic cysteine pathway and the OASS enzyme) (265,276,309,310). In fact, the presence of this enzyme has also been correlated to

antibiotic resistance mechanisms such as swarming motility (261). For all the tested pathogens was confirmed the presence of genes encoding for the sulfur assimilation pathway thanks to the alignment of amino acid sequences of *CysK* with Clustal Omega program, as explained in figure 13 (chapter 3.1). Thus, for all Gram-positive and Gram-negative tested strains has been established a high sequency homology with St*CysK*, due to the conservation of active site residues, demonstrating that *Salmonella* Typhimurium was a good model for target engagement and crystallography studies, representing well other pathogens of great interest as those investigated.

The evaluation of presence and homology of compound target in the different bacteria is fundamental to start *in vitro* investigations on the UPAR415 antimicrobial activity, in reason of the correspondence between activity on target and cell viability.

The evaluation of *in vitro* activity of UPAR415 against the six bacteria tested was performed both in rich (MHB) and poor (LB 20%) media, with the aim to determine the ability of the compound to exert its activity also in presence of cysteine (great and limited amount, respectively), that is the product of the biosynthesis which is inhibited by UPAR415. The effect of inhibition of cysteine biosynthesis caused by UPAR415 would lead to the actual growth inhibition in absence of cysteine in the growth medium, but the same is not so true when the medium is enriched with cysteine. In fact, in this case, the external administration of cysteine leads to the reduced activity of the biosynthetic pathway and therefore of OASS enzyme, thus reversing the sense of the inhibition of its biosynthesis. This is clearly showed by the MIC tests performed on UPAR415 in both media (Table 1, chapter 3.1.2), in which the compound did not show relevant activities in either MHB or 20% LB medium, both containing cysteine even if in different percentages.

An alternative to this behavior is given by the possibility of UPAR415 to fail to penetrate through the external membranes of Gram negatives and partially through that of Gram positives. To exclude

this possibility, UPAR415 was tested in combination with a permeabilizer of membranes, PMBN, lacking antimicrobial activity *per se* (Table 2, paragraph 3.2.2). As expected, no relevant antimicrobial activity was detected also in this case, supporting the thesis that UPAR415 has no antimicrobial activity *per se.* Furthermore, lack of antimicrobial activity of UPAR415 is not due to a problem of penetration through the outer membrane or defect of accumulation in the cell.

These findings are in agreement with the assumptions made previously: the biosynthetic pathway of cysteine is not essential for the survival of bacteria in normal conditions, i.e., in the absence of stressful phenomena. For this reason, UPAR415 as cysteine inhibitor biosynthesis used individually does not have a bactericidal action.

In reason of the negligible antibacterial activity of UPAR415 alone, it was decided to further evaluate the potential adjuvant activity of this compound in association with colistin, a traditional antimicrobial with Gram negative outer membrane target. The association was also tested on Gram positive bacteria, to eventually enhance the antimicrobial activity of colistin through these microorganisms. Results obtained from checkerboard assay for the association UPAR415 – colistin on the six bacterial pathogens showed no indifference or antagonism of action between the two compounds and additivity on all the Gram-positive strains (Table 4, paragraph 3.2.3). Synergy of action were obtained for all the Gram-negative strains in LB 20% medium and for *S.* Typhimurium and *K. pneumoniae* in MHB, only on *E. coli* the combination showed additivity of action. Of notice, is the strong activity of the association towards all tested bacteria in poor medium (LB 20%), together with the higher values of colistin MICs (Table 3), especially on Gram-negatives in this medium compared to rich medium. These results support the thesis that bacteria under stress conditions given by the reduced supply of nutrients and similar to the conditions of natural infection, tend to develop defensive strategies that increase their resistance to antibiotics (increased MIC of

colistin). Among these bacterial strategies is the activation of the cysteine pathway, whereby the final enzyme of the cascade (OASS) is blocked by UPAR415, the bacteria are more susceptible to the colistin action.

Focusing on Gram-positive strains, it is possible to notice that on these bacteria MIC values of colistin alone are very high, due to the intrinsic resistance to this molecule given by the absence of outer membrane target. Contrariwise, when colistin is administered in association with UPAR415 both in the two media, MIC values decrease, and FIC index shows additivity for each association. For example, MIC of colistin on *S. pseudintermedius* decreases 34 and 17 times respectively on MHB and LB 20% when UPAR415 at 64 µg/ml is co-administered. These data are interesting to obtain a broader spectrum of action for colistin, and for this reason decrease the impact of colistin on the development of further resistance to antimicrobials. In fact, the use of the adjuvant strategy allows to reduce the administered concentrations of the single antibiotics and enhancing their effectiveness by using their synergistic property.

To further confirm that UPAR415 *in vitro* activity on bacterial cell is indeed on the OASS target, was chosen to perform MIC assay on S. Typhimurium mutant strain DW378, in which OASS-A is presents but secondary inactivated (Table 6; Figure 17). As expected, UPAR415 did not exert any antibacterial activity on mutant strain, due to the absence of bacterial target and the administration of colistin resulted in a lowering of 5-times of MIC compared to the wild type *S.* Typhimurium ATCC 14028. The last is in line with the same activity of the association UPAR415/colistin on the other strains. Furthermore, the coadministration of UPAR415 at 64 µg/ml to colistin did not alter in any way the MIC of this last.

Other experiments were performed to further support the mechanism of action of UPAR415: in minimal media M9, deprived of any metabolites - cysteine included - MIC assays of colistin and

association UPAR415/colistin on *S.* Typhimurium WT were performed with and without cysteine at different concentrations (0,02 and 0,2 mM) (Table 7, Figure 15). As previously confirmed, UPAR415 does not exert antimicrobial activity alone, also in these conditions, but colistin showed a MIC value in combination with this compound reduced by 64 times respect to its MIC value alone in M9 and FIC index is much lower than the same value on MHB (0,05 respect to 0,48). When cysteine is added to the medium at 0,02 and 0,2 mM, the MIC of combination slightly increases, especially when UPAR415 at 16 µg/ml was added, showing that the compound, as expected, acts on the OASS target and the association operate on bacterial viability with a strong synergy of action.

Since the strong antimicrobial activity of colistin adjuvated by UPAR415 is evident, to further confirm the potential of the association as a new class of antimicrobial compounds, it was evaluated the effect on eukaryotic cell viability, both on nucleated and enucleated cells: MDBK cells from bovine kidney and defibrinated sheep blood. In particular, effect on erythrocytes is indicatives of a cell membrane damage (Table 8,9; Figure 16). Results showed a good biocompatibility of UPAR415 both on MDBK and blood cells, particularly in terms of reduced hemolytic activity, which remained well below the 10% threshold value for membrane damage. On MDBK, UPAR415 showed low toxicity, also when associated to colistin, demonstrating a safe profile in both cell types. This reduced toxicity could allow a possible application of the association, especially if it is destined to become a potential association that can be used in the therapeutic field. Its extremely good compatibility with erythrocytes could indicate reduced toxicity when applied *in vivo*, both topically and systemically.

Finally, the promising results obtained by microbiological assays, encouraged further investigations to understand the molecular details of the interaction of the inhibitor with the enzyme of *S.* Typhimurium. Indeed, functional results from microbiology tests suggested a competitive binding

mechanism of UPAR415 respect to the main substrate of OASS: O-acetyl serine (OAS). With the determination of 3D structure of UPAR415 in complex with OASS-A by X-ray diffraction studies, it was confirmed the link between the enzyme and UPAR415 in the active site, competing with the amino acid substrate (Figure 18). With the crystallographic studies of this complex was further highlighted as UPAR415 is able to arouse only a modest closure of the active site, probably due to steric clashes between the totyl substituent and the substrate-binding loop in the fully closed conformation. This result is very interesting to further design derivatives of UPAR415, which shows better properties as ligands of OASS with medicinal chemistry optimization, to further evaluate for their eventually antimicrobial activity *in vitro*.

5 Conclusions

In this PhD thesis, it was evaluated a potential antimicrobial adjuvant molecule, UPAR415, with a novel microbial target, the O-acetylserine sulfhydrylase (OASS) enzyme, presents in different bacterial species, both of veterinary and human interest, and catalyzing the biosynthesis of cysteine in procaryotes (274). The peculiar non-essential activity of OASS for the bacterial viability, characterize UPAR415 for its activity as adjuvant of other antimicrobial molecules, which exert an oxidative stress on bacterial cell that interact with UPAR415 synergistically and/or with additivity of action (267).

It was evaluated UPAR415 as adjuvant of colistin, a polymyxin of relevant interest for its life-saving properties against multi-drug resistant organisms (MDROs), which assumed particular relevance in recent years for the decreasing of efficacy of other important antimicrobial classes, such as beta-lactams, aminoglycosides, macrolides, etc. Colistin is frequently used in human medicine when no other antimicrobial molecule is effective in the treatment of infections, often of nosocomial origin in immunocompromised patients (121-123). In veterinary medicine, colistin was included by EMA in B category of antimicrobials: its use is not avoided but restricted to use only when other antibiotics in lower categories C and D are not effective to threat the infection, and its choice should be based preferentially on antimicrobial susceptibility testing (60,61). Aim of this restriction is essentially due to the critical importance of this molecule in human medicine, as previously mentioned. In parallel, in human medicine, colistin is a critically important antibiotic, but of limited administration options as its therapeutic window is narrow: its active concentration is also the most neuro/nephrotoxic concentration. For this reason, the possibility to increase efficacy of colistin reducing its dosage by using an adjuvant molecule could be given by UPAR415 or other inhibitors of cysteine biosynthesis in bacteria. With this strategy, colistin should be effective at lower

concentration than its single administration and the impact on bacterial selective pressure against this molecule may be less than for its single use. This could lead even to a controlled and prudent use, also in veterinary medicine, both for the treatment of pet single cases of infection with resistant bacteria, and for the treatment of zootechnical animals in the cases assessed/foreseed by EU and national guidelines (60-61).

Results obtained with UPAR415 as adjuvant of colistin are very promising, because of its activity resulted synergistic with colistin on Gram negative bacteria tested (*E. coli*, *S.* Typhimurium, *K. pneumoniae*), also in media enriched with cysteine, essential amino acid that is present in almost all body districts. Furthermore, UPAR415 was able to improve the efficacy of colistin on Gram positive bacteria tested (*S. aureus*, MRSA, *S. pseudintermedius*), against which alone is normally not effective. The confirmation of the UPAR415 activity on the OASS target of *S.* Typhimurium, on which the molecule was conceived, gave a further strength to the molecule due to the great potential of its mechanism of action as a new antimicrobial pattern.

The specific mechanism by which UPAR415 synergistically act with colistin was not determined in this work, but a recent study reported how in *Acinetobacter baumannii* after only 15 minutes of treatment with polymyxins profound changes occur in the transcriptome of the microorganism, which also affects the synthesis of amino acids and their metabolism (311). This adaptive response in *A. baumannii* could also occur in other bacteria and may be altered by the treatment with non-essential metabolic pathways inhibitors, such as OASS inhibitors. The condition in which UPAR415 acts as inhibitor of cysteine synthesis during treatment with colistin, might cause a stressful state for bacteria, in which the efficacy of colistin appears at lower concentrations respect to MIC values of colistin alone.

Furthermore, the good biocompatibility of UPAR415 alone and in combination with colistin on eukaryotic cells, especially the nontoxic level of hemolysis on blood cell, bodes well for a possible *in vivo* application, after carrying out the necessary experiments. Further experiments will certainly be carried out to verify the antimicrobial efficacy of UPAR415 associated with other antimicrobials and to test other molecules derived from UPAR415 as colistin adjuvants, presenting a better activity as ligands of the OASS enzyme, given the steric clashes at the active site level for this molecule.

In conclusion, targeting non-essential metabolic pathways as synthesis of cysteine in bacteria could represent a good strategy to overcome bacterial resistance, mostly when this strategy is used in association with other well established and synergistic antimicrobial mechanisms. The administration of OASS inhibitors as a new adjuvant class of traditional antimicrobial can thus be considered an effective method for restoring the activity of molecules already known and which have lost their activity against MDROs.

Results obtained and showed in this PhD thesis could be considered a good starting point to develop new class of antimicrobial adjuvants, acting on non-essential bacterial pathways that can be used both in veterinary and human medicine.

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