**Significance**

**Susceptibility to nosocomial infection and the prevalence of bacteria-resistant infection.** There is a growing number of immunocompromised populations at risk for severe infections. This includes the elderly over the age of 65, newborns, diabetics, chemotherapy patients, and surgical patients, both in hospitals worldwide and at home. These populations are most susceptible to contract severe infections. The most common hospital-acquired infections are pneumonia and urinary tract infections (UTIs), which can be caused by viral infection or infection by many different bacterial species and strains. Pneumoniae infection represents 15% of all nosocomial infections and 24% of intensive care units acquired infections. Of the most prevalent nosocomial infections, severe pneumonia has the highest mortality rate observed in hospital acquired-infections and is the 6th leading cause of death, and the leading cause of sepsis\(^2\,^3\).

**Antibiotic resistance and the need for novel therapeutic approaches for hospital-acquired infection.** The current state of affairs for severe nosocomial infections is alarming, as some have become untreatable with current antibiotic drugs as a result of new emergent pathogens with a heightened and insurmountable antibiotic resistance. Multidrug resistant strains (MDR) of bacteria, such as the so-called "ESKAPE Pathogens" represent a grouping of antibiotic-resistant Gram-positive and Gram-negative bacteria\(^4\,^5\). The group is so-named because these bacteria include: *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, ESBL producing bacteria, such as E. coli* and the *Enterobacter* species, effectively “escape” the effects of most approved antibacterial drugs. These resistant strains have become particularly prevalent and deadly in the EU, US and Asia, contributing to the significant incidence and mortality rates of Hospital-acquired infections (HAIs). To address these nosocomial infections and improve the likelihood of adequate antibiotic coverage, current hospital guidelines typically recommend treating the pneumonia with a combination of antibiotics. While treating infection with more than one antibiotic is effective in targeting several different bacterial mechanisms, these guidelines provide only a temporary solution to the problem of the development of antibiotic resistance and will ultimately cause more harm, as increased antibiotic usage will only fuel the evolution of more widespread antibiotic resistance. The increasingly relevant problem of nosocomial infection and antibiotic resistance, particularly in Gram-negatives, remains a daunting and highly critical unmet medical need, as both R&D and federal approval of antibiotics are on the decline.

**New treatment directions for antibiotic resistance infection.** New treatment solutions are needed to improve antibiotic efficiency and/or reduce the mechanism of resistance. National and international health agencies have established a series of measures to encourage the control of drug usage and the development of new molecules specifically for treatment of Gram-negative bacteria. The low permeability of these bacteria, which are surrounded by an outer membrane that decreases the diffusion of compounds, and the constitutive expression of efflux pumps (protein complexes that actively transport toxic molecules out of the cell) by these bacteria contribute to the small number of new active molecules. Moreover, it is well documented that they can overproduce these efflux pumps in response to extra-cellular compounds, including drugs.

**We propose a combination therapy of conventional antimicrobial agents/antibiotics with small molecules that block multidrug efflux pumps.** Historically, improving the activity of antibiotics has been widely achieved by varying the design of pioneer bioactive molecules\(^6\,^7\). Since the 1980s, new classes of antibiotics have emerged, although they have mainly been active against Gram-positive bacteria\(^8\). Recent target-based high-throughput screening programs, along with in silico studies, have led to the identification of hits with high potential. Although this strategy appears attractive, the major drawback of target-based assays is that they fail to consider the membrane translocation barriers, which include bacterial permeation and efflux pump issues\(^9\,^12\). We thus hypothesized that a cell-based assay must be a suitable method for the discovery of new compounds, efficient against Gram negatives. This was first applied using natural products such as ianthelliformisamines isolated from the marine sponge *Suberea ianthelliformis*\(^13\). Chemical modifications, which we conducted later, demonstrated that some synthetic ianthelliformisamine derivatives could be more efficient and more water soluble. Thus, in a continuation of this research we want to develop an innovative approach consisting in the synthesis of bimodal molecules (typically a ianthelliformisamine derivative covalently linked to an antibiotic (doxycycline or chloramphenicol for example) to decrease antibiotic resistance of typically *Pseudomonas aeruginosa* strains and perform in vivo proof of concept on *Galleria mellonella* animal model.

**Innovation**

Alternative and complementary therapeutic approaches to antimicrobials are eagerly awaited to face the challenges of MDR bacteria and rampant nosocomial infections\(^14\). An analysis of the drug pipeline showed 39 antibiotics in development, fewer than half of which have the potential to address difficult-to-treat Gram-negative infections\(^15\). Nearly all of these drugs are modifications of existing classes of antibiotics.

Our strategy of identifying compounds that are able to circumvent the MDR phenotype is most appealing, as one single molecule could restore activity of many antibiotics. We aim to develop chemosensitizer agents, which are able to promote an increase in the intra-bacterial concentration of well-known antibiotics in resistant strains. We took the opportunity of the
use of natural products that are able to restore activity of ineffective, well-known antibiotics. We previously showed that ianthelliformisamines found in the marine sponge *Suberea ianthelliformis*, acted in a synergistic manner with several antibiotics\(^2\) in Gram-negative bacterial species, including *E. coli*, *E. aerogenes* and *P. aeruginosa*. In this context, we investigated the antibacterial activity of various ianthelliformisamine molecules, against *E. aerogenes* (a clinical MDR isolate EA289) and *P. aeruginosa* PAO1 bacterial strain\(^3\). This work allowed us to selectively generate and modify chemical compounds tailored for the discovery of a new enhancer of antibiotic efficacy.

**Approach**

**Preliminary Studies:** The emergence of multidrug resistance in bacteria results conjointly from the acquisition of genes involved in antibiotic inactivation by bacteria, accumulation of efficient target mutations and activation of general mechanisms of transport actively expelling molecules from the bacterial cell. These efflux mechanisms decrease the intracellular concentration of antibiotics under the threshold required for target inhibition. Additionally, downregulation of porins involved in the diffusion of hydrophilic compounds through the outer membrane to the periplasm also contribute to the decrease of antibiotic concentration in the bacteria. In *Escherichia coli*, the AcrAB–ToIC efflux pump is the major contributor to multidrug resistance.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>FICindice</th>
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<tbody>
<tr>
<td>Doxycycline</td>
<td>PA01 Kp CIP 82.91</td>
</tr>
<tr>
<td>Piperacillin</td>
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</tr>
<tr>
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<td>Ceftiraxone</td>
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<tr>
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<td>Colistin</td>
<td>0.25 0.34</td>
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<tr>
<td>Polymyxine B</td>
<td>3.42 5.16</td>
</tr>
<tr>
<td>Polymyxine B</td>
<td>1.49 1.09</td>
</tr>
</tbody>
</table>

*Table 1. Results of isobolograms assays on P. aeruginosa PA01.* The FIC index values (mean of 3 MIC independent experiments) are indicated for each association of compound 3 with antibiotics. Colors correspond to: green, synergism; blue, additive; orange, no effect; red, antagonism.

Homologues of this pump are found in *Enterobacteriaceae*, including *Salmonella*, *Enterobacter*, *Klebsiella*, and share homologies with other drug transporters, such as MexAB-OprM from *Pseudomonas*. Based on that, our on-going project has been dedicated to the synthesis of new biologically active molecules derived from ianthelliformisamines extracted from the marine sponge *Suberea ianthelliformis*. Several polyamino derivatives were identified to be able to decrease chloramphenicol- nalidixic acid-resistance levels of multi-drug-resistant *Enterobacterial* strains. The derivatives improved Chloramphenicol Minimum Inhibitory Concentration (MIC) to a ratio of 8 on *E. aerogenes* at doses from 15 to 2.5 µM. We also studied the question of the natural resistance of *Pseudomonas* to doxycycline. The aim was to select an antibiotic for which the bacterium is naturally resistant and to use an escort molecule to restore susceptibility, similar to the model of β-lactam/ β-lactamase inhibitors. This strategy constitutes an opportunity for an old neglected molecule to be rejuvenated by using an adjuvant to improve its activity. A high-content screening was performed on *P. aeruginosa* PA01, the reference strain, allowing the selection of four ianthelliformisamine compounds 1–4 that acted synergistically with doxycycline (figure 1). These 4 compounds were also assayed against clinical isolates and MDR strains; at a dose of 10 µM they were able to improve the susceptibility of *P. aeruginosa*, *A. baumannii*, *S. Maltophilia* and *P. putida* clinical isolates to doxycycline (2µg/mL) (figure 2). Compound 3 was also able to decrease the MIC of doxycycline on the reference strain, efflux pump overproducer of *P. aeruginosa*, to the susceptibility level.

In addition, compound 3 was tested for its capability to improve efficacy of other antibiotic families including β-Lactams, fluoroquinolones and aminoglycosides, that are representative of the main classes of anti-pseudomonas agents. Experiments were performed with the β-lactams ceftazidime, ticarcillin, ciprofloxacin and others on PA01 and efflux pump overproducer *P. aeruginosa* strain. Compound 3 (10 µM) displayed synergistic or additive effect with several antibiotics (table 1) and showed a significant synergy or additive effect with ceftazidime on all *P. aeruginosa* strains tested. The first experiment of compound 3 mechanism of action (MOA) was performed on *P. aeruginosa*. The results demonstrated that compound 3 improved membrane permeation and inhibited pump efflux on *P. aeruginosa* (figure 3). Thus, compound 3 improved antibiotic activities by increasing intra bacterial antibiotic concentration in *P. aeruginosa*.

![Graph showing percentage of bacteria (%) in each strain and compound](image)

Doxycycline (2µg/ml) on various isolates from patients in each species (0 indicate no additive treatment).
Similar assays need to be performed in other Gram negative strains. These preliminary results demonstrated that ianthelliformisamine derivatives can enhance doxycycline efficiency against *P. aeruginosa* and *A. baumannii* laboratory and clinical strains, by increasing bacteria permeability to the antibiotics. Furthermore, compound 3 enhances efficiency of different class of antibiotics on *P. aeruginosa*. In the current proposal, we aim to design and select the most effective linked ianthelliformisamine-antibiotic (doxycycline, chloramphenicol…) as a tool to fight bacterial resistance against both Gram-positive and Gram-negative bacterial strains. Compound 3 appears to be a good candidate. However, existing and newly synthesized derivatives during the project will be assessed for their chemo sensitizer properties in aim #1, then the MOA of the selected compound will be confirmed on different bacterial strains (aim #2) and, finally, the proof of concept in *Galleria mellonella* larvae infection model will be performed (aim #3).

**Specific Aim #1: Selection of the most efficient covalently linked ianthelliformisamine-antibiotic compound for each pathogen: *S. aureus, P. aeruginosa, K. pneumoniae, A. baumannii* and *E. coli.*

**Introduction:** Preliminary studies selected four compounds in the ianthelliformisamine derivatives series (figure 1) which improved doxycycline efficacy on *P. aeruginosa*, with compound 3 as the most active, by improving the efficacy of different classes of antibiotics on this bacterium (figure 2). In this aim, we will identify the most active compound from the existing polyamino-isoprenic derivatives library, modified to improve activity or solubility, on the most prevalent bacterial strains in nosocomial infection, in addition to *P. aeruginosa*, as the companion drug to doxycycline and antibiotics used in the clinic.

1.1 **Linked ianthelliformisamine-antibiotic derivatives library optimization and synthesis.** We will optimize and synthesize chemical entities. Synthesis of various salt derivative combinations using hydrochloride salt will be performed under various experimental conditions to optimize the encountered yield, as well as the selectivity of the process. The optimization of the synthesis process will be performed in order to further proceed with *in vitro* and *in vivo* assays.

**Methods:** The compound will be synthesized under a methodology developed in the methodology (currently patented). From a chemical point of view and according to the structure-activity relationships encountered, some modifications could be envisioned to enhance the potency of the selected compound. These modifications could occur at the level of the nature of the amine used and in the nature of the considered antibiotic (figure 4).

**Figure 4.** chemical strategy for the envisioned derivatives

1.2 **Selection of the most efficient polyamino-isoprenic derivatives for each pathogen: *S. aureus, P. aeruginosa, K. pneumoniae, A. baumannii* and *E. coli.*** We will assess compound antimicrobial efficacy on each pathogen (listed above) by *in vitro* growth assay.

**Methods:** A liquid bacteria culture (separate for each pathogen) will be incubated with 12 concentrations of the designed derivatives in MW96 plates. The MIC will be determined for each compound. All experiments will be performed in triplicate for statistical significance. Data will be analyzed by the Student t-test analysis to determine significance of the results.

**Specific Aim #2: Determine Mechanism of Action**

**Introduction:** First experiments of MOA have shown that compound 3 improves antibiotic efficacy by increasing membrane permeation and inhibiting efflux pumps. After the selection of the most active compound, three experiments will be performed to determine the MOA of compounds on *P. aeruginosa, K. pneumoniae, A. baumannii* and *E. coli* according to published methods.16

2.1 **Efflux inhibition: Method (brief description):** A single colony of each strain, from an overnight plate, will be grown
in Cation Adjusted Mueller Hinton Broth (CAMHB). Bacteria will be collected by centrifugation and re-suspended in Potassium Phosphate buffer (PPB), supplemented with CCCP 5 μM, and incubated with 1,2'-dianaphthylamine at 37°C. After overnight incubation, the bacteria will be washed in PPB and incubated in wells pre-loaded with compounds, antibiotic alone or controls. Membrane incorporated 1,2'-diNA will be followed by monitoring the fluorescence ($\lambda_{ex}$ = 370 nm; $\lambda_{em}$ = 420 nm). 50 mM of Glucose will be added at 300 s to initiate bacterial energization. Additionally, efflux will be measured by monitoring fluorescence decrease every 30 s at 37°C. To normalize the results, experiments will be performed with bacteria treated with chlorhexidine and/or benzalkonium chloride lysis solution, as positive control, and with no glucose addition as negative control. Compounds able to inhibit the dye transport in a concentration-dependent manner will be considered efflux inhibitors.

2.2 Membrane permeation: Method (brief description): A single colony of each strain, from an overnight plate, will be grown in CAMHB. Bacteria will be collected by centrifugation and re-suspended in PPB, supplemented with CCCP 5 μM. Bacteria will be mixed with nitrocefin before addition of compounds, antibiotic alone, or controls. Nitrocefin hydrolysis will be followed by measuring the absorbance ($\lambda_{abs}$= 490 nm) every 30 s at 37°C. To normalize the results, experiments will be performed with bacteria treated with chlorhexidine and/or benzalkonium chloride lysis solution, as positive control, and no chemical addition as negative control. Compounds able to increase the kinetic of nitrocefin hydrolysis in a concentration-dependent manner will be considered outer membrane permeabilizers.

2.3 Transmembrane potential disruption: Method (brief description): Bacteria will be grown in the same conditions as above and re-suspended in Hepes 5 mM pH 7.0 with 3-3’-Dipropylthiadicarbocyanine iodide (diSC3(5)). The membrane potential-sensitive cyanine dye diSC3(5) distinguishes between cells and the medium, depending on the cytoplasmic membrane potential gradient. Released diSC3(5) will be quantified by measuring the fluorescence ($\lambda_{ex}$= 622 nm; $\lambda_{em}$= 690 nm), every 30 s at 37°C. 300 s after the addition of compounds, antibiotic alone or controls. To normalize the results, experiments will be performed with bacteria treated with chlorhexidine and/or benzalkonium lysis solution, as positive control, and no chemical addition as negative control. Compounds able to favor the dye release in a concentration-dependent manner will be considered disruptors of the membrane potential.

Interpretation of results: A synergistic compound could have different effects on bacteria, including: (i) outer membrane permeation, that will increase antibiotic diffusion through the outer membrane and will make the Gram-negative envelope similarly permeable to the Gram-positives; (ii) competitive inhibition of efflux pumps, thus decreasing efflux of antibiotics and consequently increase the intra-cellular concentration of the drug; (iii) inhibition of efflux pumps by collapsing the proton motive force, which is the energy source for the majority of efflux pumps, particularly the RND type efflux pumps such as the AcrAB-ToIC and the MexAB-OprM of Enterobacteria and Pseudomonades respectively. For each set of experiments, compound alone will be compared to the antibiotic alone to determine the specific action of the considered compound. We will then consider each compound at a concentration of 100μM and compare its behavior in the three experiments with the following criteria: (i) Inhibition of efflux ≥ 20% = efflux inhibitor (ii) Permeabilization ≥ 20% = permeabilizer (iii) Membrane potential disruption ≥ 20% = Membrane potential disruptor. Inhibition of efflux associated with membrane potential disruption activity will demonstrate that the compound blocks the efflux pump at the level of energy.

2.4 Bacteria / Mammalian cells specificity: The effect of the selected derivative will be assessed on mammalian cells growth. The potential cytotoxicity effect on liver and lung mammalian cells.

Methods: Cells will be incubated with different concentration of derivative for 3 days. Then, the viable cells will be quantified with Cell titer Glo kits. The EC$_{50}$ on cell growth will be determined.

Interpretation of results: The MIC/EC$_{50}$ ratio will give the specificity of activity on bacteria.

Milestone: These experiments will give clear insights on the mechanism of action of how the compound enhances different antibiotic activity on different Gram-negative bacteria strains. Furthermore, the ratio between the effective dose on bacteria and the cytotoxicity dose on mammalian cells will give the bacteria activity specificity. The resulting ratio needs to be higher than 10. If it is not the case, different derivatives will be selected using this parameter and in parallel to FIC determination.

| Specific Aim #3: In vivo POC |

**Introduction:** The objective of this aim is to demonstrate *in vivo* the proof of concept for the use of the combination of a selected compound on bacteria responsible for nosocomial infection, such as ESAPKE by using *Galleria mellonella* larvae animal model.

3.1 Salt formulation and batch production: To perform *in vivo* experiments, it is important to have the best bioavailability in the animal. This bioavailability depends mostly on the chemical entities. Nevertheless, formulation with different soluble salts impacts solubility, stability and thus bioavailability. The derivate will be formulated with different salts, such as: hydrochloride, tartrate, citrate salts.

Methods: Synthesis of various salt derivatives combination such as: tartrate salt and hydrochloride salt will be performed.
under various conditions to optimize the encountered yield as well as the selectivity of the process.

3.2 **Assess exposure of the best compound for each pathogen:** It is important to know the tolerance of the compounds before moving forward to pharmacology experiment.  

**Methods:** The compound will be administered to the larvae by injection in the body and the survival larvae will be determined after 24 hours.

**Milestone:** Determine the optimal dose of the chosen compounds.

3.3 **Assess combination efficacy in Galleria mellonella larvae infection model:** As there are several strains of bacteria responsible for causing HAP, it is typical to treat patients with broad-spectrum antibiotics before identifying the responsible bacteria. This can be problematic as several of these strains are more likely to develop MDR. Once the efficacy and tolerance of selected previously designed compounds (Aim #1 and 3.2) will be determined in *S. aureus*, *P. aeruginosa*, *A baumannii* and *K pneumoniae* Galleria mellonella larvae infection model. The following experiment will be performed per bacteria strain and for each selected compound.

**Methods:** *Galleria mellonella larvae* will be infected with either *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, or *A. baumannii*. Subsequently, the derivative will be administered as previously determined. Selected derivative activity will be determined bacteria burden and larvae survival. The parameters analyzed will be: bacteria burden (CFU/tissue) measured early after infection (18h or 24h after infection) and larvae survival tested during the experiment (for at least 5 days). Each experiment includes a control treated group with the corresponding vehicle.

**Interpretation of results:** Effective treatment is determined by statistically significant (p<0.05) differences in decreased bacteria burden and increased larvae survival as compared to the treatment with the same dose of a standard antibiotic alone.  

**Milestone:** Demonstrate that the derivatives tested are effective in treating infection caused by *S. aureus*, *P. aeruginosa*, *K. pneumoniae* or *A. baumannii*.

**Future Directions:**

In this current project, we first select the most effective compound *in vitro* on *S. aureus*, *P. aeruginosa*, *A. baumannii*, *K. pneumoniae* and *E. coli*; then demonstrate proof of concept of the selected compound in acute models, by either: efflux pump inhibition, membrane permeabilization and membrane potential disruption. Once proof of concept is determined, an early safety profile will be performed, as well as determination of efficacy in *Galleria mellonella larvae infection models*. The aim of this proposal is to transition the selected leads from *in vitro* activity to proof-of-concept in animal models and to position it as a strong preclinical candidate for the treatment of MDR nosocomial infections.

**REFERENCES**


   [http://apps.who.int/iris/bitstream/10665/112738/1/9789240692671_eng.pdf](http://apps.who.int/iris/bitstream/10665/112738/1/9789240692671_eng.pdf)


10. Tommasi, R., Brown, D. G., Walkup, G. K., Manchester, J. I. & Miller, A. A. ESKAPEing the labyrinth of


