Rafael Fernando Maurício Lopes

New target on bactericidal compounds: lipid II Synthesis inhibitor

Monografia realizada no âmbito da unidade de Estágio Curricular do Mestrado Integrado em Ciências Farmacêuticas, orientada pela Professora Doutora Maria Manuel Cruz Silva e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro 2016

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Coimbra, 14 de setembro de 2016

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Aqueles que por aqui passaram… para todo o sempre!

Abstract

Recently, a new antibiotic discovered through a pioneering microorganism cultivation method - IChip technology - produced an enthusiastic response by the international science community as a consequence of its classification as a new class of bactericidal compound. This antibiotic candidate, defined by Losee L. Ling's research team as possible replacer of Vancomycin, exposed an excellent activity against gram-positive chains, especially those with multi-resistant factor. Moreover, its undifferentiated mode of action even allowed the structure investigation and optimization as a possible biosynthesis capsides inhibitor and some gram-negative inhibitor as well.

Teixobactin, as was named, represent the first lipid II inhibitor able to bind with the pyrophosphate section of the lipid carrier, showing an unique mode of action. The properties described demonstrate a huge improvement on the correct pathway that must be followed in order to hurry the antibiotics research.

Resumo

Recentemente, um novo antibiótico descoberto através de um pioneiro método de cultivo de microrganismos – Isolation chip – produziu uma resposta entusiástica pela comunidade científica internacional, devido à sua classificação como pertencente a uma nova classe de bactericidas.

Este candidato a antibiótico, definido pela equipa de investigação de Losee L. Ling como um possível substituinte da vancomicina, apresentou uma excelente atividade contra bactérias gram-positivas, em particular para as mais resistentes. Demonstrou também um mecanismo de ação indiferenciado que permitiu o seu estudo para potencial otimização e aplicabilidade como inibidor de algumas bactérias gram-negativas e da biossíntese da cápide.

O teixobactin, como foi definido, apresenta-se como o primeiro inibidor do "Lipid II" capaz de se ligar à porção pirofosfato do transportador, apresentando assim um inovador mecanismo de ação. As propriedades descritas demonstram um enorme avanço no correto caminho a seguir para a investigação de novos antibióticos.

Indice

List of Abbreviations

Abreviations	Explanation
EMA	European Medicine Agency
ESBL	Extended spectrum beta-lactamase
FDA	Food and Drug Administration
GlcNAc	N-acetylglucosamine
IChip	Isolation Chip
L/D-ala	L/D-alanine
L-arg	L -arginine
ManNAc	N-acetylmannosamine
MRSA	Methicillin-resistant *Staphylococcus aureus*
MurNAc	N-acetylmuramic acid
USA	United State of America
WTA	Wall teichoic acid

Introduction

Since the widespread of antibiotics via penicillin's discovery during World War II, infectologists have been rethinking the perfect pathway to follow in order to reach the multifaceted compound able to treat a large number of species without having resistance signals.

From 1940s to 1960s, the remarkable focus of pharmaceutical companies initiated the golden years of antibiotic's market, (Sengupta *et al.,* 2013) being a result of screening cultivable soil microorganism technique. (Sherpa *et al,* 2015) This mechanism, as the first researching method, allowed the medical community to treat basic infections while they were increasing the population average life expectancy. (Sengupta et al. 2013)

The vital influence of the introduction of bactericidal products in the current medicine practices promoted the idea of a post antibiotic era. However, the extensive use of antibiotics and the decreasing discovery of new molecules after 1980s, promoted a dissemination of resistant bacteria, (Ventola 2015a) yielding an overwhelming threat upon human life and a throwback to pre-antibiotic era.

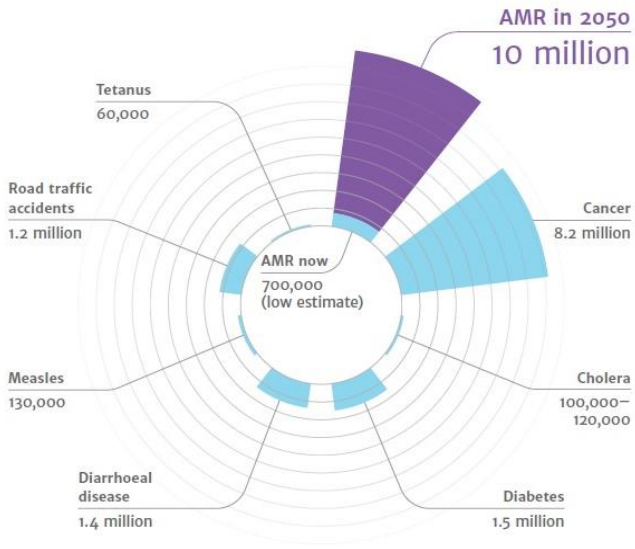


Fig.1- Number of expected deaths in 2050 from resistant infections. (O'Neill 2016)

It is mainly unknown the precise numbers of infected people by resistant bacteria and its costs to global economy, however there are some countries such as USA predicting an increase of yearly expenses going around the 21 to 34 billion dollars. These expenses are highly correlated with an increased time spent by patients on hospital facilities. (O'Neill 2016; Yewale 2014) In may 2016, UM government also released another report where they described more than one million deaths in the previous 19 month which represent more than 700 thousand casualties by drug-resistant infections. (O'Neill 2016)

This huge threat not only created a necessity to stop the incorrect use of antibiotics in order to increase their lifespan but also highlighted the emergent necessity of new investments by governments and pharmaceutical companies in antibiotic research area.

Background of Antibiotic's Resistance

In our days it is consensual that all antibiotics have resistant bacterial strains, which turn the decision's process a lot harder when the doctors need to prescribe an antibiotherapy. The penicillin was the first to see is effectiveness reduced after an outbreak in a Germany hospital by resistance strains of *Klebsiella pneumonia* in 1982 and ever since large number of other resistances strains emerged as a threat to human beings. (Yewale 2014)

The gram-negative match to the main concern because it is the continuous source of new a ruthless resistant species, becoming resistant to nearly all available drugs. (Ventola 2015a) In USA the most common resistant pathogens are *Pseudomonas aeruginosa, Acinetobacter and Enterobacteriaceae* which represent the daily concern on healthcare facilities. These microorganisms were considered the main priority for US department of Health and Human services after being detected resistant strains to almost every single β-lactam antibiotic. (CDC 2013a)

The prevalence of extended spectrum beta-lactamase (ESBL) pathogens, especially the *Enterobacteriaceae (E. coli and klebsiella),* represent a warning example of β-lactam resistance, corresponding to a 26 000 infections and 17000 deaths caused by resistant strains each year in USA. (CDC 2013a; Ventola 2015a) It is noteworthy the capability of these species to easily mutate small portions of their genome in order to successfully produce an ESBL able to hydrolyze more than one broad spectrum β-lactam. (Davies & Davies 2010) Not surprisingly, carbapenem resistant strains was also found, which normally represent a total failure of marketed antibiotics. However some studies attested that novel combinations of β-lactam/β-lactamase inhibitor, e.g. Ceftolozene/Tazobactam, should be a good alternative to the traditional carbapenem used as last resource in ESBL pathogens. (Karam et al. 2016)

Although gram-negative appeared as an imperative problem to solve, there are a few gram-positive resistant strains also important. As gram-negative, β-lactamase-producing bacteria still remain one of the major resistance mechanism used by these cells to inhibit penicillin-like antibiotics. (Karam et al. 2016) Fortunately, during the early years of antibiotic research period new antibiotics had been released to gradual solve these resistance problems. (Yewale 2014)

The Methicillin was approved as the main candidate to control the penicillin-resistant species in 1959, however only a few years was enough to observe resistances to this antimicrobial agent. Methicillin-resistant *Staphylococcus aureus* (MRSA) was the name given to

resistant infections, which were mainly observe on healthcare facilities. (Davies & Davies 2010) Nevertheless, today it is also detected outside from hospital facilities be responsible for more than 20% of *S. aureus* infections observed all over the world, reaching values close to 80% in some countries (Yewale 2014). Despite the large distribution, these infections still have some drugs available to use, e.g Daptomycin, Linezolid and as last resource Vancomycin.

Another example of gram-positive challenges is the Vancomycin-resistant *Enterococcus*, which represent 30% of healthcare facilities infections in USA (CDC 2013a). Although the mortality number is higher in MRSA, the lack of drug alternative makes this infection a warning disease all over the world, which could lead to new research points to overcome this remaining problem.

Antibiotic Resistance

The resistance to antibiotics is one of the most imperative topics in modern medicine representing an enormous warning to all human life during his cohabitation with microorganisms (CDC 2013a).

Since the discovery of the first antibiotic, resistances to them were identified representing an adaptation mechanism that turns ineffective or less effective an antibiotic treatment. (O'Neill 2016) Despite being studied after the introduction of antibiotics on the market, several resistance mechanisms were identified as a natural response from prokariots to survive in their own environment, creating a propitius natural selection to thoose which had the correct encoding genes. (Sengupta et al. 2013)

The first intrinsic mechanism was defined as the cell capability to become impermeable to antibiotics. This survival function that could act symbiotically to a lack of targets on the cell membrane was classified as a non-specific resistance mechanism and it is the result to a varied phylogenetic evolution. Another intrinsic means used by primitive bacteria were efflux pumps, protein target protecting and enzymatic inactivation method which represent the selective advantage to environmental antibiotics, justifying the existence of those bacteria today. (Sengupta et al. 2013; Davies & Davies 2010)

Although resistance mechanisms could be intrinsic from a specific cell, a several number of them are the result of gene modifications promoted by a gene mutation or horizontal gene transference. The horizontal transmission is responsible for the enzyme gene diffusion and cell permeability reduction, occurring by three different mechanisms

defined as transformation, transduction and conjugation. (Ventola 2015a) These methods allow the transmission trough different species even in a powerful hostile environment caused by an intensive use of antibiotics like it is observed today. (Davies & Davies 2010)

Causes of Resistance Strains

The therapeutic levels observed during a therapeutic course, allow the survival of resistant bacteria, requiring them a fast adaptation method. These mechanisms that invalidate antibiotic's effect are emphasized after the treatments due to a low drug concentrations exposure. (O'Neill 2016) This perfect environment, allied with a massive proliferation rate observed on stress-induced bacteria, enhances the mutation rate. Once, genes containing some resistance's degree are obtained and they can be easily transferred to other cells by horizontal transference mechanisms. (Ventola 2015a)

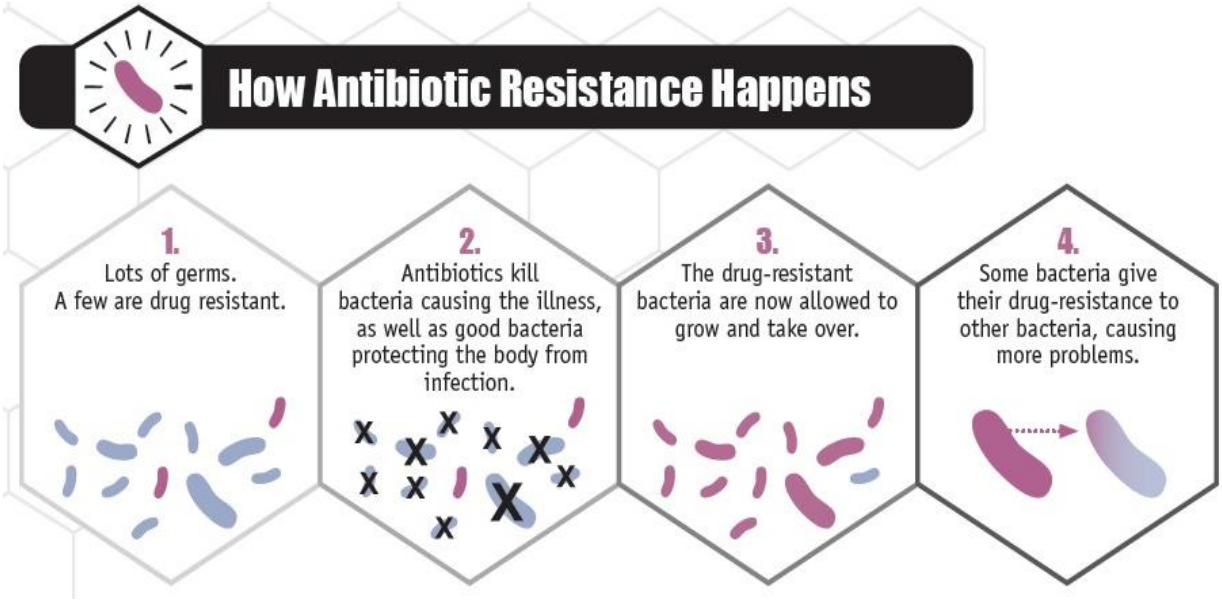


Fig.2- How antibiotic resistance happens. (Yewale 2014)

During a drug course used to treat a specific infection, several antibiotic agents are used in order to minimize the bacteria proliferation. The continuous misuse of drug prescriptions before the correct diagnosis has been determined as a common mistake observed in unregulated countries. (Ventola 2015a) In United States of America, an absence on limited antibiotic guidelines and the patient's demand for antibiotic prescriptions, (CDC 2013a; O'Neill 2016) led to a 67, 5% of misused antibiotics in respiratory issues. As the incorrect prescriptions grow in some countries, others have a minimal arsenal of drugs to treat resistant strains becoming more susceptible. (O'Neill 2016)

In order to manage the resistance strains proliferation, an investment on faster and cheaper diagnostic allied with the patient's instruction on how to prevent transmissions of bacterial infections and when do they need antibiotics, could be a useful resource to reduce antibiotic misuse. Moreover, the possible optimization of therapeutic regiments, adjusting them to a shorter therapeutic course, could also be a useful approach to reduce the lower drug concentrations exposure. (Ventola 2015b)

Another incorrect use, is frequently detected when livestock productions apply sub-therapeutic levels of antibiotics as a prophylactic action against microorganism as well as promoting growth rate on animals. (Yewale 2014)

The first reference to antimicrobial subtherapeutic levels as grow promoter was reported by Stokstad and Jukes in 1949 and since then this supplements have become the nourishment of cattle world. (Economou & Gousia 2015) However the numerous studies released in the past decades sensitized the European Union assembly, which defined these substances as forbidden since 2006. (O'Neill 2016)

Unlike Europe, the USA still use this mechanism as a traditional source for livestock grow and according to FDA, there are more kilograms of antibiotics sold for food-producing animals rather than human diseases, in USA. (CDC 2013a; Economou & Gousia 2015) Several recommendations were made to USA about this matter by WHO advisory group during the past decades in order to increase the regulatory surveillance on livestock production however only recently mandatory reducing regulation on antibiotics consumption had been suggested. (Yewale 2014)

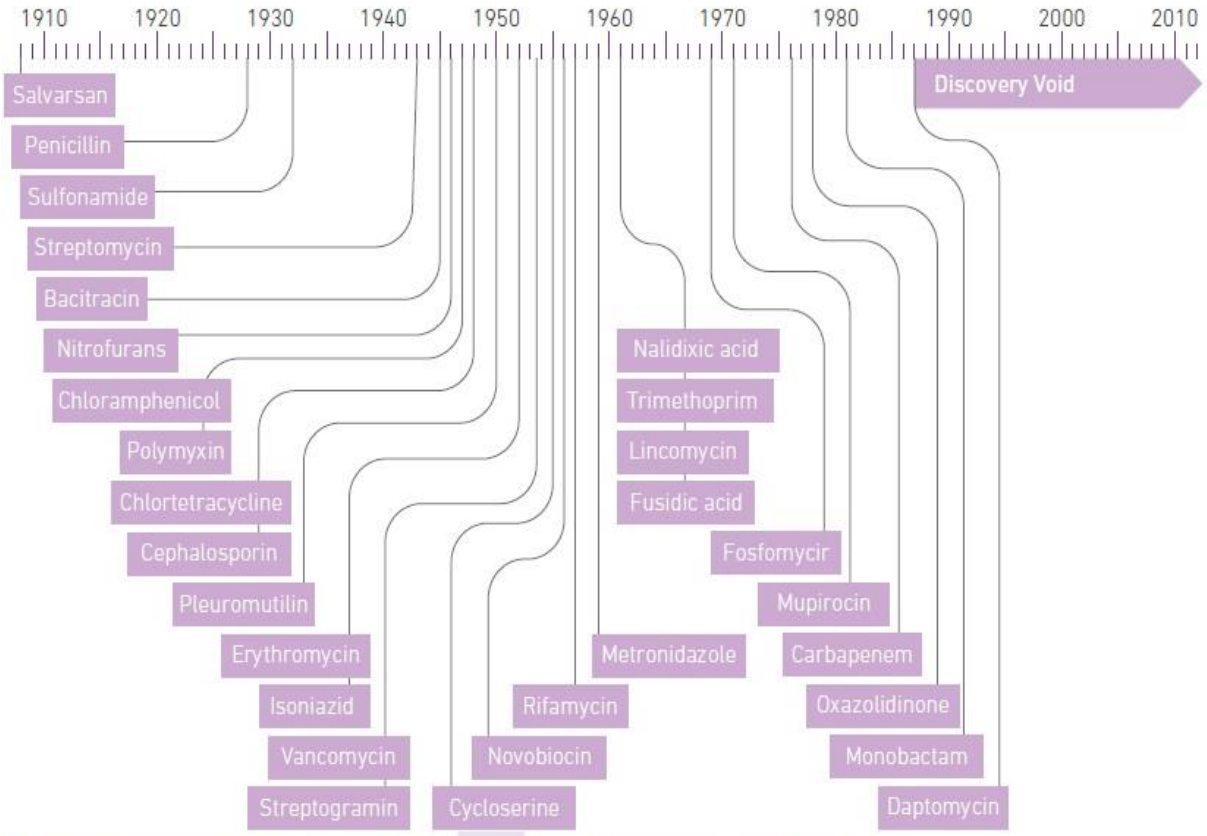
In order to decrease the risks associated to animal-human resistance transmissions, the use of antimicrobial as a grow factor should be considered irresponsible, increasing the international surveillance even in developing countries. This strategies supported by international regulatory institution, allows the prohibition of specific antibiotics more vulnerable to develop risky resistance in the next years.(Ventola 2015b)

Tackling the resistance crises through international reduction of antimicrobial agents use, could represent the first step in this problem, however unlike it was observed in beginning of post-antibiotic era, the development of bacterial resistances is radically superior to antibiotic market introduction, promoting a lack of solutions for actual resistance strains. (Davies & Davies 2010) Hence, this first measures should be balanced with an active pursuit of new chemical elements.

Lack of New Drug Discovery

After 1980 an under-investment by pharmaceutical companies and the governments decreased the number of antibiotics reaching the market. (O'Neill 2016) The void observed on drug discovery pipeline until today generated an emergence absence of new chemical entities capable to treat the new resistances, which are the result to last shelf drugs bacterial exposure. (CDC 2013a)

The main problem was supported by commercially-unattractive market, which gives to generic antimicrobial drugs a priority on the infection chain treatments. (O'Neill 2016) This pressure applied to medical care teams to only use the patented new antibiotics as a last resource, caused a difficult background for industries to profit, requiring them a competition with the low prices established to generic products. (Servick 2015) Usually, in other researching areas the new products with better characteristics are chosen as the first line and reach the maximum sales period on the beginning of patent time. (O'Neill 2016)



Adapted from Silver 2011 (1) with permission of the American Society of Microbiology Journals Department.

Fig.3 – Discovery void observed after 1980. (Yewale 2014)

The lack of a shielding period led some companies to adapt their approach to antibiotic's research using new technologies as genomics, high-throughput screening and

other high-tech chemical approaches. However these new investments were expensive or fruitless promoting a lower investment at the same time as new resistance crisis arrived. (Servick 2015; Phimister et al. 2015)

Developing New Strategies

An adjustment to current sales-model was found as a major pathway to approach this problem which allowed an intervention by governments and international infection associations in order to create a financial motivation for new discoveries. (TATFAR 2014; Boucher et al. 2013) As a result, several approaches have been made in the last decade in order to pump up the economic attention to this researching area.

The "10x'20 initiative" make their first move in 2010 trying a more political approach that simplifies the acceptance of 10 newest Infection products by 2020. This program also demanded a rising on government's investment to companies that center their attention in product designed for treating gram-negative bacillus especially the β – lactamase and carbapenemase resistance mechanism. (TATFAR 2014; Boucher et al. 2013)

In both sides of the Atlantic measures have also been adopted to motivate research efforts. In 2012 the FDA approved the "Generating Antibiotic Incentives Now" as an assisting method to evaluate the importance of new chemical entities on researching process. The antimicrobial products, which act in life-threatening infections, have special category that allow them to have five more years on drug-patent processes. These initiatives also classify the new antibiotic as a first priority product to be review and analyze during the FDA approval. (Ventola 2015b)

At the same time, the EMA was promoting some researching areas and preventing misuse of antibiotics. Furthermore, an upgrade on guidelines intended to boost the development of new antibiotics as also been done, showing us the modeling and simulation method that could analyze the pharmacokinetics and pharmacodynamics before the clinical trials. (CDC 2013b; Agreed et al. 2014) According to several reviews, more than 80% of development's cost of a new antibiotic occurs on clinical trials. (O'Neill 2016) This process phase can also lead to failure products exposing the company's investments. The reduction on the time spent on that phases by generating a faster way to discover the correct dose and frequency of administration is an useful tool to decrease the costs and to hurry the market introduction process. (CDC 2013b; Agreed et al. 2014)

Although the governments have only recently started these interventions, the results are already being classified as successful. (Lewis 2012; Harbarth et al. 2015) From 2014 to

2015, the number of antibiotics approved was equal to the last decade being observed a slight increase on big companies investment. (Lewis 2012; Ventola 2015b) However, the main investment continuous to be a Small Companies and University Department's responsibility which are a major role model that could lead us to new discoveries in the near future by proposing new innovative approaches. (Servick 2015)

The Original Method

"The ability of man to domesticate microorganisms, including those living below ground and those living above it, those that are able to control diseases and those that bring about useful process, may be looked as one of the greatest triumph of modern civilization."

Selman Waksman in "Soil Microbiology"
1952

Since the discovery of Streptomycin by Selman Waksman in 1943 trough screening methods, a lot of new approaches were tested in order to find out the Holy Grail method on antibiotics research, creating a false impression of improvement in this research area. However, promises like genomics and high-tech chemical reveled unsuccessful and economically unattractive promoting a reduced number of investments as a huge gap on lead compounds discovery. (Sherpa et al. 2015)

The absent antibiotic discovery exposed a potential return to the original method based on the study of different microorganism species that are already known by their natural antibiotic's production. Make use of the natural antibiotics diversity and create an environment for the proliferation of these species is a necessary planning phase. (Ling et al. 2015)

The conventional cultivation technique starts by isolating the potential microorganism from environmental particles, which is guaranteed by the dissolution of the soil sample in purified water. After the particles had settled, a small portion of the liquid is used as the concentrated cell sample, continuing with successive dilutions. (Nichols et al. 2010) After obtaining a reduced cell concentration in the sample, the bacteria is introduced in the liquid or solid agar culture created specifically for the bacteria. Subsequently the sample is incubated according to its perfect temperature and CO_2/O_2 concentration. (Sherpa et al. 2015)

During the cultivation process trough the classical method, the optimal conditions of the artificial growth medium represents the hardest objective to achieve, be responsible for numerous failures of complex cell cultivation. (Sherpa et al. 2015; Kaeberlein et al. 2002)

Consequently, a different approach to the cultivation medium used, involving the natural environment, might be an useful approach to improve the growth ratio.

Isolation Chip Method - IChip

A few years ago a new paradigm was proposed on microbiology research area becoming the first step in the "in situ "initiative studies. This new approach exposed the first result in 2002 when a new device was present for cultivating microorganism which was considered "uncultivable". Although this technique was proposed in 2002, only in 2010 the same research team reached its goal by producing an high-throughput device efficiently enough. (Nichols et al. 2010)

This method consists in incubating the potential microorganisms in their natural environments by gather the different soil samples for the respective microbial species e.g seawater and soil suspensions. These samples not only could provide every nutrient needed for the species development but also some specific growth factors only present on familiar environments. (Kaeberlein et al. 2002)

As the original method, the Ichip technique starts by isolating microorganism from the sample. This process occurs by diluting them to acquire a concentration of one cell per 20 μl. (Kaeberlein et al. 2002) Subsequently, the diffusion chamber is inoculated with 20 μl of the dilution mixture, ensuring that only one cell is placed by chamber. (Sherpa et al. 2015)

The IChip device starts to assemble the four nutrient permeable membranes in the top and bottom side of a central plate with two arrays of through-holes. Then, the cell present in each hole is isolated by agar's solidification process assuring no cell migration between the liquid soil sample and the chamber. (Nichols et al. 2010)

To assure that every membrane and the central plate remains in the correct place, two large symmetrical plates are applied on both sides. The arrays of through-holes on the plate match with the central plate assuring a small distance between the bacteria and the environmental factors. The transference to the natural liquid or solid environment is then required, remaining in contact with it for one month. Following, the IChip chamber is dissembled and the produced colony tested for is ability to grow outside. (Nichols et al. 2010)

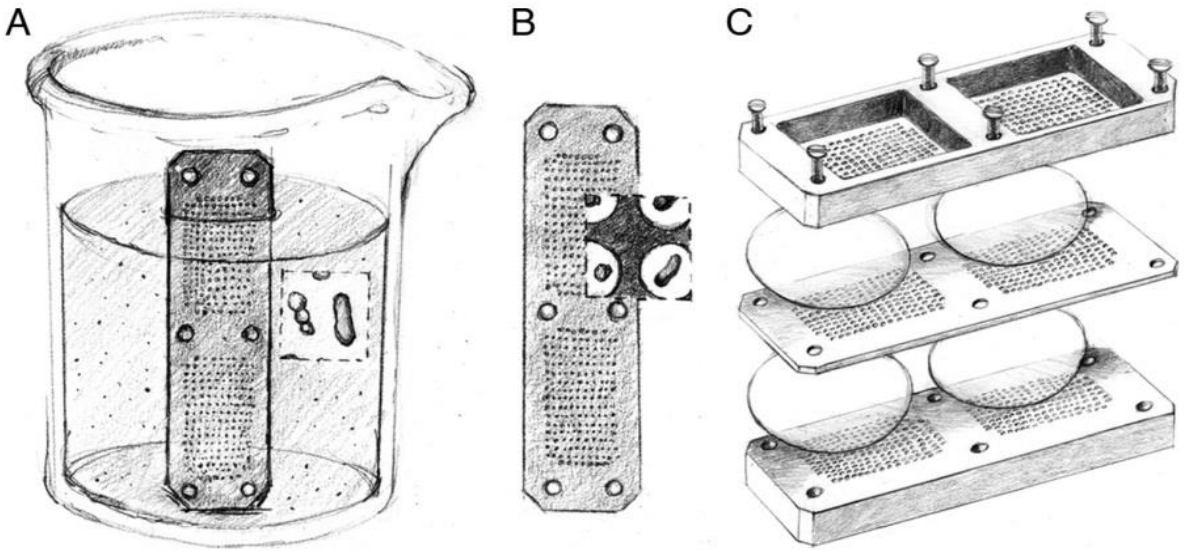


Fig.4 – High-throughput ICHIP device. (A) Central plate immerged on diluted soil samples. (B) Central plate cultivated with one per through hole. (C) ICHIP design for cell proliferation. (Nichols et al. 2010)

The transferring problem observed on traditional Petri-dishes still remains, however the colonies tend to respond better after a few incubations on the natural habitat, becoming more suitable for laboratory manipulation. (Kaeberlein et al. 2002) Indeed, the recovery ratio tend to be 50% comparatively with 1% when the Petri dish is the only used technique. (Ling et al. 2015)

Therefore the bacteria obtain from IChip method allow the identification of previously uncultivable ones and its DNA sequencing process, becoming a potential technique to find new lead compounds as forming a large number of colonies to produce antibiotics – functional for industrial production.

Cell Wall Synthesis: Role of Lipid II

The cell-wall is an organelle with a major role on cell's survival becoming responsible for structural rigidity that gives the cell a protected surround against dangerous molecules and full water environments. For this reason, it has been studied over the time for potential synthesis targets that turn the cell more susceptible for killing agents and avoid the cell development.

On bacteria, the cell wall is constituted by peptidoglycan chains, which are glican monomers of alternating N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) linked to other by peptide bridges. (Breukink & de Kruijff 2006) The rigid structure is similarly formed between different species, however small differences could be detected in chemical composition, according to the peptide linked with MurNAc. Therefore, singular interaction points and compound's permeability might be observed in different species. (Scheffers & Pinho 2005)

The peptidoglycan synthesis occurs in three stages, starting with the biosyntheses of the two sugar precursor's on the cytoplasm. This primordial steps allow the formation of UDP-GlcNAc from frotose-6-P and UDP-MurNAc from a transferring process of enolpyruvate to the third hydroxyl carbon of UDP-GlcNAc. Then, the lactyl ether previously formed, provide the perfect platform to include the three fist amino acids – D-alanine (D-ala), D-glutamic acid and meso-diaminopimelic acid. These peptide bonds are catalyzed by three different enzymes and a last one is needed to connect the UDP-MurNAc-tripeptide with a dipeptide previously formed between two D-analine – D-alaninyl-D-alanine. (Barreteau et al. 2008)

The second phase takes place in the cytosolic side of cytoplasmatic membrane, where UDP-NAM-pentapetide is connected with undecaprenyl-pyrophosphate, which is a carrier lipid embedded on the bacterial membrane. The N-acetylmuramyl-pentapetide-undecaprenyl-pyrophosphate complex, called lipid I, will be linked to the NAG creating a second complex identified as lipid II. Then lipid II is flipped to the outer side of the cell membrane in order to connect with the nascent peptidoglycan. (Breukink & de Kruijff 2006)

The last phase is mainly penicillin-target-enzymes dependent, which allow the transglycosylation and transpeptidation of the new monomer to the main glycan chain. (Seltmann & Holst 2013) The lipid carrier is then recycled by undecaprenyl-pyrophosphate phosphatase forming the undecaprenyl-phosphate. (Ling et al. 2015)

Another component of bacterial cell wall matrix is wall teichoic acid (WTA), which also starts is biosyntheses in the cytoplasm. As peptidoglycan, the teichoic acid start is synthesis by a previous formation of is precursors, which are GlcNAc, two glycerol-pyrophosphate residues and N-acetylmannosamine (ManNAc), a GlcNAc derivate sugar. (Seltmann & Holst 2013)

Secondly, in the inner side of the cell wall membrane the GlcNAc is linked with the undecaprenyl-pyrophosphate forming the lipid III, which will be connected with the ManNac and the two glycerol-3-P residues. This main structure will allow the elongation process of forty ribitol-5-P molecules in the cell membrane. (Seltmann & Holst 2013)

The lipid carrier is now able to flip the WTA to the external side of the membrane, exposing the GlcNAc sugar to the nascent peptidoglycan chain. With a catalysis enzyme, the transglycosylation of GlcNAc residue from the WTA and MurNAc residue from a peptidoglycan occurs. (Scheffers & Pinho 2005)

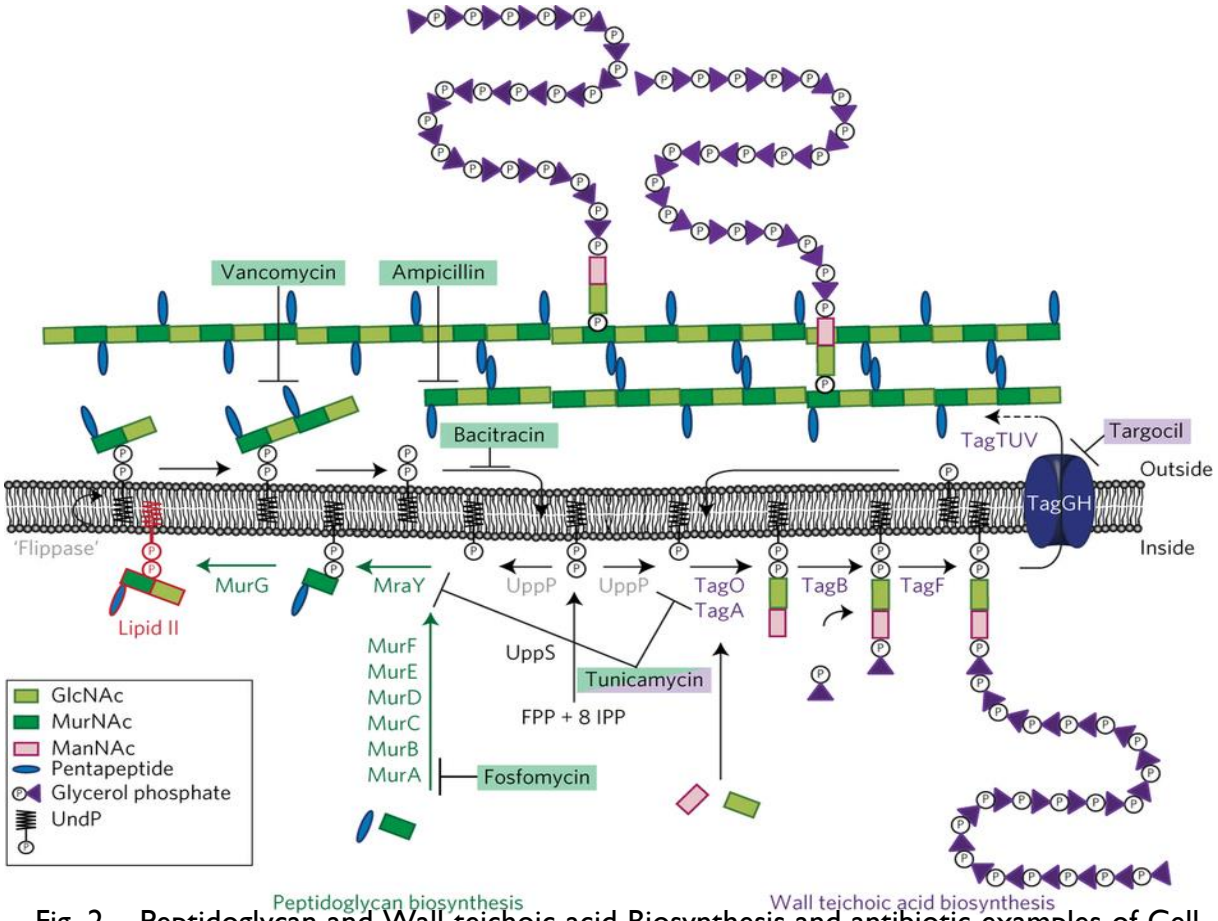


Fig. 2 – Peptidoglycan and Wall teichoic acid Biosynthesis and antibiotic examples of Cell Wall inhibitors. (Schirner et al. 2015)

In spite of the large number of peptidoglycan monomers, it was reported in some studies a small number of carrier lipids, which create a perfect biosynthetic step for antibiotic's inhibition. However, a transformation on lipid II molecule allows the cell to resist against harmful agents, (Breukink & de Kruijff 2006) which is observed in resistance Vancomycin bacteria. These strains have other operons that encode different terminal pentapetide sequences as D-lactate or D-serine that replace the D-ala promoting the low affinity to mutated molecules. (Courvalin 2006)

As the last therapy for many multi resistant strains of *Streptococci, Staphylococci* and *enterococci*, Vancomycin resistance developments have been much studied in order to hold them back. Furthermore, the absence of a barrier on transference genes between gram-positive cocci as heterospecific gene expression for different bacteria emphasize the hazard

potential to resistant species without new candidates to treat this infections. (Courvalin 2006)

Potential New Antibiotic Lead Compound

In 2015, a potentially useful molecule that was discovered by Ichip technique represented the twist on the antibiotic research.

The new lead compound was entitled as "Teixobactin" and was obtained from *Elepheria terrae*, which is a new genus of *β-proteobacteria* correlated with *Aquabacteria*. This depsipeptide containing methylphenylalanine the uncommon amino-acid L-allo-enduracidin, and three D-amino-acids was determined by mass spectrometry analyses as a 1242 Da molecule and it was characterized as a non-ribossomal peptide encode by txo1 and txo2 gene also named by the research team (Ling et al. 2015).

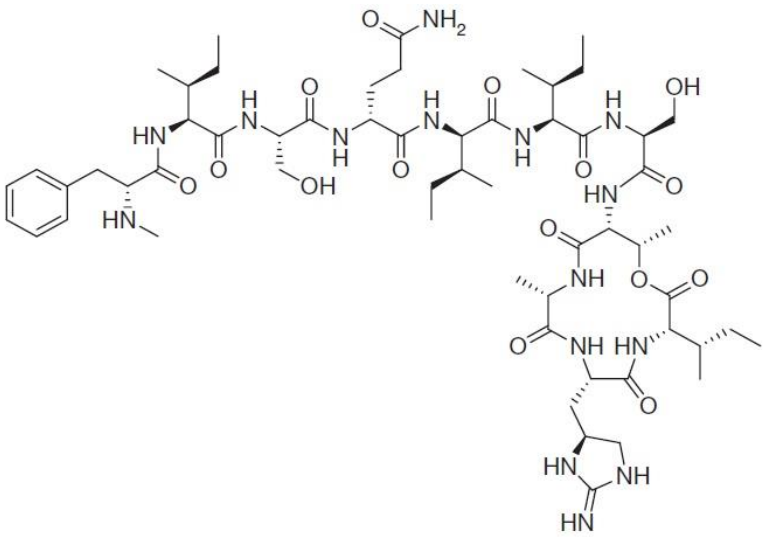


Fig.5 – Chemical structure of Teixobactin. (Ling et al. 2015)

The high molecular mass of teixobactin indicates a peptidoglycan biosynthesis as a potential target, (Von Nussbaum & Süssmuth 2015) which has confirmed by Ling et al. In spite of already exist similar modes of action based on inhibition of enzymes and interaction to lipid I and lipid II precursors it was identified as a new target after being confirmed the teixobactin activity against modified forms of lipid II. As a consequence, a different interaction site was proposed for the molecule by Ling et al. that could be present in the six different types of modified Vancomycin resistant bacteria. (Ling et al. 2015)

The research team proposed the first half of pyrophosphate lipid and the first sugar, MurNAc, as the linking site of teixobactin. Besides, the absent of the sugar could also be used to establish the inhibition process, nevertheless the teixobactin concentration need to

increase significantly. As a consequence to this discovery researchers also proposed potential inhibition significance to this linking site as a target used to treat *Mycobacterium tuberculosis* and some *staphylococci* and *streptococci* species. All of them differ on the sugar that is used to form peptidoglycan or polysaccharide capsular but have the pyrophosphate site. (Sherpa et al. 2015; Ling et al. 2015)

Another site proposed with a significant highlight in the process was the WTA. The inhibition of this secondary target not only allows the accumulation of toxic intermediates but also helps the liberation of autolysins responsible for wild peptidoglycan hydrolysis.

The analysis to identify the correct mode of action occurs by the addiction of teixobactin to cultures of *S. aureus*, which allowed the differentiation between a blockade on a biosynthetic pathway and is possible incorporation with DNA or proteins. After being confirmed an inhibition of peptidoglycan formation step, by observation of undecaprenyl-N-acetylmuramic acid pentapetide accumulation, a potential interaction with enzymes was discharged subsequent to radiolabelled substrate studies. Consequently, these studies confirmed a new inhibition manner that form stable complex with peptidoglycan precursors as lipid II and also with lipid III. (Ling et al. 2015)

A second test was also performed in a MRSA septicemia model by intraperitoneal infection of a mouse with a dose that leads to 90% of death. (Sherpa et al. 2015) After one hour, teixobactin was also injected in different concentrations in order to determine the PD50 (protective dose at which half of the animals survive), which showed a lower dose comparatively with Vancomycin, the most common antibiotic used to treat this septicemia. (Ling et al. 2015)

The Ling et al. study also performed several test in order to observe the resistance development to teixobactin. This test was able to create a continuous low concentration teixobactin environment for *S. aureus* e *M. tuberculosis* cells during 28 days without developing resistant strains. (Ling et al. 2015)

In summary, the potential characteristics of teixobactin as the first candidate to a new class of antibiotics showed a wake up in this researching area. This new class with new a chemical target represents a studding model used to optimize is effectiveness as preserve is low toxicity profile against gram-positive bacteria.

Lead Compound Development

Latest on 2015, a partnership between a few University Chemistry departments and research institutes reported an upgrade on the teixobactin molecule, which indicates a promising start on this group of antibiotics.

The initial idea was to replace the L-allo- enduracidine, the unusual amino acid, for L-arginine (L-arg), which is a natural one also with the group δ-guanino. This group is very useful to macrocyclization process with L-ala, however is propone to form a lactamic ring requires a protection of the group. L-ala as the second crucial amino acid used for cyclization was the first to be attached to a protection resin, which was connected with the carboxyl group and in the amino side another bond was formed with Fluorenylmethyloxycarbonyl chloride. (Jad et al. 2015; Verlander 2001) This compound is added to all amino-acids used to expand the peptide chain as a protection group in the first step of the process. However after reacting with the base *N*,*N*-Disopropylethylamine the amino group become available to react with the activated carboxyl site in the next amino-acid. (Verlander 2001)

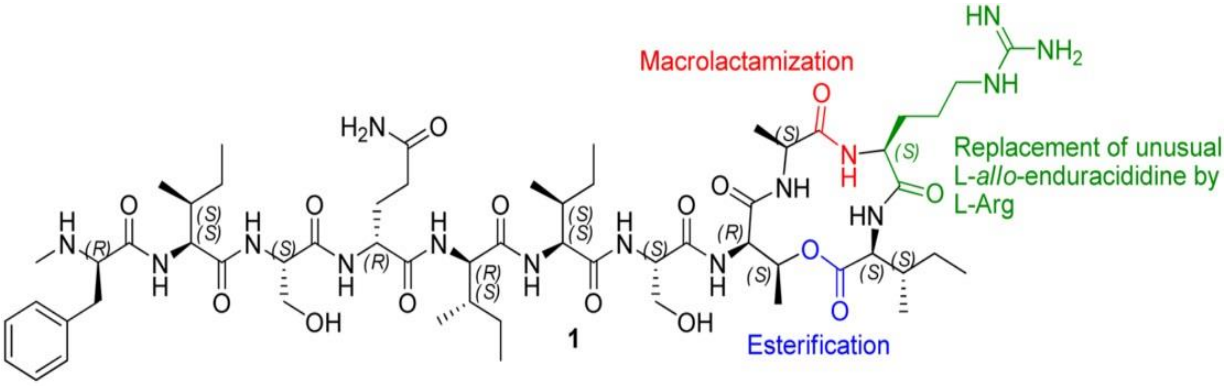


Fig. 3 – Teixobactin analogue – Replacement of L-allo-enduracidine by L-arg. Macrolactamization of C-terminus of D-ala with N-terminus of L-arg. Esterification was the hardest step to obtain the dipepsipeptide formation. (Jad et al. 2015)

All process is called "Solid phase synthesis of Peptide" and it was used by this team to synthesize the main chain of the peptide until L-isoleucine and L-arg are incorporated in order to end the cyclization process. These amino-acids were protected as a result of the esterification process preparation that forms the dipepsipeptide. A reaction cocktails investigation was then required with the objective to increase the income on this step. (Jad et al. 2015; Verlander 2001)

After obtaining the analogue 1, several studies was performed in order to evaluate is chemical activity as a bactericidal compound. In spite of the similar activity against gram-positive strains it was showed up a potential synthetic process that could be upgraded to industry production.

Conclusions

The emergent resistance crisis observed in our days exposed the modern society to an undetected threat in the last decades. The investment declination experienced on antibiotics researching areas coupled with misuse promoted a "last resource line use" as an intensification of resistance strains consequence.

Despite the efforts applied to optimize new synthetic developments, the new approaches adopted proved fruitless and economic unattractive triggering a new exploration of the previous researching method. As a result, the Isolation Chamber Method was optimized to a multichannel device - ICHIP becoming a vital instrument to take advantage of the numerous species that wasn't possible to study until our days.

As the owner of ICHIP technology, Novobiotic® pharmaceuticals proposed teixobactin as a new candidate to future clinical trials representing a massive advance on new antimicrobial chemical class. (Ling et al. 2015) This class linked with Vancomycin, trough is similar mode of action, emerge as a potential new resource for multi-resistant strains especially on gram-positive. Besides, the results presented by this researching team revealed an efficient *in vivo* activity against *M. tuberculosis*, which could represent a new therapeutic solution for this disease. (Piddock 2015)

As a new molecule discovered from the soil sample cultivation method, the possible resistance development manners were evaluated, which has been determined as possible but unlikely to occur in the next years. Like Vancomycin took 30 years since its market introduction for resistance being detected, it is expected a similar or even longer period until the same problem occur for teixobactin. (Ling et al. 2015)

The difficult resistance scenarios noticed in our days created a mobilization and awareness necessity to shape the antibiotics consumption. The adaptations programs used by EMA and FDA were the beginning of a chain of events which provided evidences to be usefully to minimize the collateral damage induced by the misuse of these compounds. However, the late intervention in this area left us with no bactericidal weaponry to use in multi-resistant strains, which engendered a new potential era for antibiotics research. As teixobactin, new discoveries are expected by ICHIP researching method establishing this compound as the first of a new class of antibiotics.

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