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Research review paper

Antimicrobial peptides as novel anti-tuberculosis therapeutics

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ABSTRACT

Tuberculosis (TB), a disease caused by the human pathogen *Mycobacterium tuberculosis*, has recently joined HIV/AIDS as the world's deadliest infectious disease, affecting around 9.6 million people worldwide in 2014. Of those, about 1.2 million died from the disease.

Resistance acquisition to existing antibiotics, with the subsequent emergence of Multi-Drug Resistant mycobacteria strains, together with an increasing economic burden, has urged the development of new anti-TB drugs. In this scope, antimicrobial peptides (AMPs), which are small, cationic and amphipathic peptides that make part of the innate immune system, now arise as promising candidates for TB treatment. In this review, we analyze the potential of AMPs for this application. We address the mechanisms of action, advantages and disadvantages over conventional antibiotics and how problems associated with its use may be overcome to boost their therapeutic potential. Additionally, we address the challenges of translational development from benchside to bedside, evaluate the current development pipeline and analyze the expected global impact from a socio-economic standpoint.

The quest for more efficient and more compliant anti-TB drugs, associated with the great therapeutic potential of emerging AMPs and the rising peptide market, provide an optimal environment for the emergence of AMPs as promising therapies. Still, their pharmacological properties need to be enhanced and manufacturing-associated issues need to be addressed.

Contents

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1. Introduction: tuberculosis - a global emergency

Tuberculosis (TB) recently joined HIV/AIDS on the top rank of the deadliest infectious diseases, being actually responsible for one fourth of HIV-related deaths. According to the latest data from World Health Organization, around 9.6 million people were diagnosed with TB in 2014, having about 1.2 million of those died from the disease (WHO, 2015a). Globally, TB incidence remains highest in Africa, in terms of new cases per inhabitants, but new TB occurrences are also increasing in Southeast Asia and Western Pacific regions.

As a result of the implementation of the *Millennium Development Goals* in 2000 (WHO, 2015b), which particularly focused on reducing TB incidence, around 37 million lives were saved between 2000 and 2013 due to effective diagnosis and treatment and since 2007 the treatment success rate has been at or above 85%. Despite all efforts to fight this disease, its death toll remains elevated and multi-drug resistant TB (MDR-TB) strains are emerging mostly as a result of overuse or misuse of antimicrobial agents (*e.g.* antibiotics). By definition, MDR-TB is resistant to, at least, isoniazid and rifampicin, the two most powerful, first-line (or standard) anti-TB drugs (Onyebujoh et al., 2005). Also, extensively drug-resistant TB (XDR-TB), an even more severe form of MDR-TB, resistant to even more available medicines, has emerged. XDR-TB strains are usually resistant to at least isoniazid, rifampicin or any fluoroquinolone, and to any of the three second-line injectables (amikacin, capreomycin, and kanamycin). Noteworthy, about 480,000 people developed MDR-TB in 2013, being estimated that around 9% of those cases were XDR-TB. Nonetheless, the term XDR-TB, as well as totally drug-resistant TB (TDR-TB), have not been clearly defined by WHO due to technical challenges and limitations of *in vitro* drug susceptibility testing.

The approval of the *Beijing Call for Action* in 2009 and the *World Health Assembly Resolution 62.15* by UN Member States represented a major commitment towards MDR-TB treatment and control (WHO, 2009). Still, MDR-TB represents a major public health concern within the European Union (EU), as only a third of MDR-TB patients are successfully treated in the EU, one of the lowest rates in the world. This has led EU members to implement an Action Plan against antimicrobial resistance, which started in 2011 (European Commission, 2011).

Within this context of multi-drug resistance strain emergence, a new class of drugs – antimicrobial peptides (AMPs) – arises as promising candidates for TB treatment.

2. Mycobacteria: made to resist

Although over 170 species and subspecies of mycobacteria have been reported (http://www.bacterio.cict.fr/m/mycobacterium.html) only a few are described as pathogenic, namely *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Mycobacterium ulcerans* (Gaspar et al., 2008). Mycobacterial species are Gram-positive, non spore-forming, aerobic bacteria, which feature a characteristic thick cell wall that confers them a unique impermeability to many molecules, namely antimicrobials, and comprising several distinct layers (Jarlier and Nikaido, 1994; Neyrolles and Guilhot, 2011). The innermost is composed of peptidoglycan. External to the peptidoglycan is a covalently linked polymer of sugars, arabinogalactan, to which mycolic acids are esterified. Finally, a variable mixture of glycolipids and lipoglycans are thought to interact *via* their acyl groups with the mycolic acids through hydrophobic interactions. Fig. 1 schematizes this unique cell wall and shows how it compares with the cell walls of Gram-negative and Gram-positive bacteria. A capsule composed of non-covalently linked loosely associated glycans, lipids and proteins has been shown to decorate the outer surface of the mycobacterial envelope. Noteworthy, the prevalence of mycolic acid molecules covalently linked to arabinogalactan in the intermediate layer confers its high hydrophobicity and decreased permeability to external compounds (Gaspar et al., 2008; Jarlier and Nikaido, 1994; Neyrolles and Guilhot, 2011).

In addition to the intrinsic basis of antimicrobial resistance of mycobacteria related to their peculiar cell wall, both life-style and pathological consequences of infection dictate additional levels of difficulty in obtaining effective chemotherapeutical drugs. Mycobacteria are able to replicate inside the macrophage.

In the case of lung infections by *M. tuberculosis*, mycobacteria are first phagocytized by alveolar macrophages and quickly spread locally in the lungs and eventually to other organs *via* lymphatic and blood circulation (Guirado et al., 2013). Once inside phagosomes, mycobacteria impair the recruitment of proteins and phosphoinositides, required for intracellular trafficking, to the phagosomal membrane, which results in phagosome maturation arrest (Guirado et al., 2013; Hmama et al., 2015). Through this process, mycobacteria avoid the subsequent phagosomal fusion with lysosomes and the contact with potent hydrolytic enzymes and antigen-presenting organelles within the host macrophage (Fratti et al., 2004). At tissue level, both infected and non-infected macrophages will be organized within granulomas, which frequently undergo central necrosis (caseous necrosis) or may be found scattered in the alveolar spaces in pneumonic forms (Hunter, 2011). The heterogeneity of the lesions in human tuberculosis will certainly impact on the bioavailability of anti-tubercular drugs, as recently observed by Prideaux et al. (2015). Finally, freely replicating mycobacteria have been found in biofilms lining the aerial side of cavities (Orme, 2014), further complicating the issue of the access of the drugs to their targets.

3. The TB drugs pipeline

3.1. Standard treatments

Mycobacterial infections are very difficult to treat. Bacille Calmette-Guérin (BCG) vaccine, a live attenuated strain of *Mycobacterium bovis*, is the only vaccine available. Although quite effective in the prevention of childhood TB, adults can have new infections (Roy et al., 2014; WHO, 2012).

Current therapeutics rely mostly on the use of antibiotics (antimicrobials, by definition) of natural or chemical origin, that kill or inhibit the growth of infectious agents (O'Toole, 2003). Indeed, the discovery of streptomycin in 1944 (Bugie and Waksman, 1944) brought forth the first anti-tuberculosis drug. Soon after, many other drugs have been developed, including para-aminosalicylic acid, thiacetazone and isoniazid (Fox et al., 1999). Together with streptomycin, these drugs constituted the first TB treatment regimen (Stehr et al., 2014). However, long-lasting treatment (18–24 months), along with painful injections and toxic effects deterred the use of this regimen, until rifampicin appeared around 1959, reducing therapy length to 6 months (Sensi et al., 1959).

Current standard treatments for non-resistant TB are based on an intensive 2-month administration of a multi-drug cocktail consisting of isoniazid, pyrazinamide, rifampicin and ethambutol, followed by a second 4-month treatment of rifampicin and isoniazid (first-line therapy). These four drugs combine different actions: both isoniazid and ethambutol inhibit cell wall synthesis, rifampicin causes the inhibition of RNA synthesis and pyrazinamide disrupts the plasma membrane and energy metabolism (Somoskovi et al., 2001). However, despite being highly active against replicating mycobacteria, these drugs (especially isoniazid) are ineffective against mycobacteria in stationary phase or with very low proliferation rates (Onyebujoh et al., 2005; Sosnik et al., 2010). In addition, lack of patient compliance with the 6-month treatment, along with adverse drug reactions and interactions, resulted in the emergence of MDR-TB (Gaspar et al., 2008). Treatment of MDR-TB is based on the administration of pyrazinamide together with second-line drugs, such as ethionamide, prothionamide, cycloserine, capreomycin or fluoroquinolones (Mukherjee et al., 2004). Standard recommendations for TB therapy, including the treatment duration, according to the resistance pattern of each strain, are summarized in

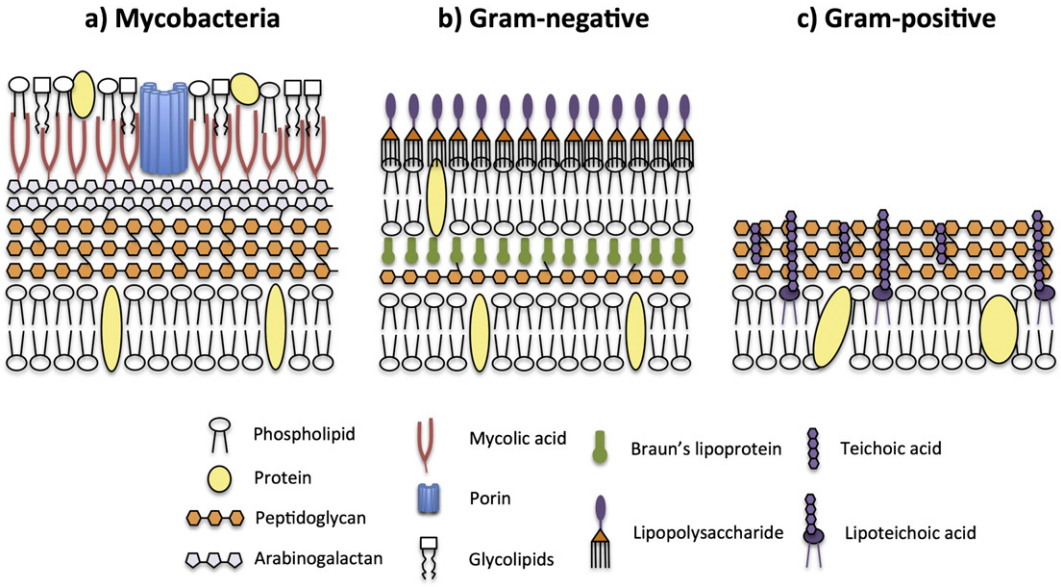

Fig. 1. Comparison between cell envelopes of mycobacteria and other bacteria. a) The innermost layer of the mycobacterial cell envelope is composed of peptidoglycan and is lined by a layer of arabinogalactan. The presence of mycolic acids covalently bound to arabinogalactan, as well as the interaction of glycolipids and lipoglycans with mycolic acids in the outer layer, confers high hydrophobicity to the mycobacterial cell wall; b) Gram-negative cell walls contain a thin peptidoglycan layer that lines the plasma membrane and an outer membrane composed of lipopolysaccharides, responsible for their antigenic properties; c) the cell walls of Gram-positive bacteria are thick and mainly composed of a peptidoglycan layer adjacent to the plasma membrane.

Table 1. MDR-TB treatment still poses a great challenge due to the high toxicity, as well as elevated costs and reduced activity, of second-line drugs (Sosnik et al., 2010). Also, their bioavailability can be reduced under certain clinical conditions (*e.g.* HIV) or due to interactions with other drugs: for example, the intestinal absorption rate of rifampicin is greatly reduced in the presence of isoniazid (Mariappan and Singh, 2003).

3.2. New developments in anti-TB therapy

Due to the growing global concern over multidrug-resistant bacterial strains, efforts have been made in recent years to develop new drugs against TB (WHO, 2014). Also, this concern has already resulted in the creation of the consortium *More Medicines for Tuberculosis* (www.mm4tb.org) and the *Working Group on New TB Drugs* (http://www.newtbdrugs.org) (Zumla et al., 2012).

Some older drugs, like fluoroquinolones and rifamycins have been re-purposed to obtain higher efficiencies and, among the novel drugs, diarylquinolines and nitroimidazoles seem the most promising (Gaspar et al., 2008; Zumla et al., 2013).

Fluoroquinolones act by inhibiting DNA topoisomerase IV and DNA gyrase, and show favorable pharmacokinetics, easily penetrating into tissues and host macrophages (Tomioka, 2006). One such example is moxifloxacin (Avelox®), which has shown a high *in vitro* activity against *M. tuberculosis*. However, the high level of resistance to fluoroquinolones limits their use (Wang et al., 2007). A combined administration of rifapentine (a rifamycin derivative modified from, but more potent than, rifampicin) with isoniazid is highly effective against latent TB (Sterling et al., 2011).

A promising new drug, already in phase III of clinical trials, is bedaquiline (also known as TMC-207 or R207910), a diarylquinoline developed by Janssen Pharmaceuticals found to inhibit ATP synthase (Andries et al., 2005). Bedaquiline was shown highly effective *in vitro* against *M. tuberculosis* and results so far indicate bactericidal activity in patients suffering from drug-susceptible TB (Stehr et al., 2014). Clinical trials against MDR-TB were recently approved by FDA, although adverse effects (*e.g.* arrhythmia induction and even mortality) have been described (Cohen, 2013).

Nitroimidazoles are a class of compounds described as active against tuberculosis (Zumla et al., 2013). PA-824 and the more potent delamanid (OPC67683) are two examples of nitroimidazoles that are currently in Phase III clinical trials for the treatment of MDR-TB (Gler et al., 2012; Tasneen et al., 2015). Delamanid (Otsuka Pharmaceutical Co., Ltd), in particular, acts by inhibiting the synthesis of the mycobacteria cell wall components, namely mycolic acid. (Gler et al., 2012).

Table 1
Recommended strategies for TB therapy.

	Regimen	Total duration (months)	Nr of drugs	Costs per patient
Susceptible TB	2 months INH + RIF + PZA + EMB, followed by 4 months INH + RIF	6	4	US$ 19–22
Multidrug-resistant TB				
Resistance pattern				
INH, RIF	PZA + EMB + FQN + 1 SLD (entire course) + INJ (first 6 months)	18–24	5–6	US$ 4000–6000 (*ex works*)
INH, RIF, (EMB or PZA)	(PZA or EMB) + FQN + 2 SLD (entire course) + INJ (first 6 months)	18–24	5–6	
INH, RIF, EMB, PZA	FQN + 3 SLD (entire course) + INJ (first 6–12 months)	18–24	5–7	
INH, RIF, EMB, PZA, (FQN or INJ)	(INJ or FQN) + 3 SLD + TLD (entire course)	> 24	5–7	
INH, RIF, EMB, PZA, FQN, INJ	INJ + all available SLD + TLD (entire course)	> 24	5–7	

Resistance pattern is based on the results of Drug Susceptibility Tests. INH: isoniazid; RIF: rifampicin; PZA: pyrazinamide; EMB: ethambutol; FQN: fluoroquinolones; INJ: injectable drugs (*e.g.* streptomycin, kanamycin); SLD: second-line drugs; TLD: third-line drugs (*e.g.* clarithromycin, amoxicillin, linezolid). All treatment administrations are performed on a daily basis.

Thousands of molecules were synthesized based on the 1.2-ethylenediamine core of ethambutol (considered the weakest link in the standard regimen) and screened for activity against *M. tuberculosis*. (Lee et al., 2003). The diamine SQ109 (Sequella, Inc.) was selected due to its high efficiency after *in vitro* and *in vivo* testing against strains resistant to standard drugs, such as ethambutol, isoniazid and rifampicin (Protopopova et al., 2005). Although the exact mechanism of SQ109's action remains unclear, it is believed to inhibit cell wall synthesis (Tahlan et al., 2012). Phase II trials are currently ongoing (Zumla et al., 2013).

Oxazolidinones (*e.g.* linezolid) are a novel class of compounds that inhibit protein synthesis in bacteria, targeting the 23S rRNA in the 50S ribosome subunit (Shaw and Barbachyn, 2011). Although promising candidates for TB therapy, many of these drugs present adverse side effects (*e.g.* thrombocytopenia, peripheral neuropathy) and inadequate pharmacokinetics profiles that may actually hinder their use (Lee et al., 2012). However, two of these compounds, AZD5847 and Sutezolid (PNU-100480) are currently in Phase II clinical trials, having shown improved mycobactericidal activity compared to other oxazolidinones (Balasubramanian et al., 2014; Wallis et al., 2014).

Q203 is a promising new imidazopyridine amide, which targets the respiratory cytochrome bc1 complex in *M. tuberculosis*, preventing its growth, and has shown activity against MDR *M. tuberculosis* in clinical isolates at a nanomolar range and in a mouse tuberculosis model at a dose lower than 1 mg/kg body weight (Pethe et al., 2013). This drug has recently entered Phase I clinical trials.

There are other promising drugs aiming TB therapy currently in preclinical trials: SQ609, a dipiperidine that interferes with cell wall synthesis showed the highest *in vitro* and *in vivo* anti-tubercular activity in a screening study (Bogatcheva et al., 2011); SQ641, a capuramycin analogue that inhibits translocase I (involved in cell wall synthesis), showed a remarkable *in vitro* activity against *M. tuberculosis*, but its poor hydrosolubility and poor intracellular activity stand as major drawbacks (Nikonenko et al., 2009); TBI-166 is a clofazimine analogue showing higher activity against intracellular and non-replicating *M. tuberculosis*, being less lipophilic and presenting reduced plasma half-life compared with clofazimine, thus resulting in decreased accumulation (Li et al., 2014); CPZEN-45 is a caprazamycin isolated from an actinomycete strain, which targets the biosynthesis of mycobacterial cell wall constituents in *M. tuberculosis* (Ishizaki et al., 2013); PBTZ169 is a piperazine-containing benzothiazinone that binds to, and inhibits, the essential flavo-enzyme DprE1 (deca-prenylphosphoryl-beta-D-ribose-2-epimerase), responsible for the biosynthesis of key cell wall components in mycobacteria and has shown additive activity against TB, when combined with other anti-TB drugs (except bedaquiline) (Makarov et al., 2014).

Regardless of the latter listing of new drugs, summarized in Table 2, the pipeline remains very short and other major challenges still need to be addressed such as the duration of therapies and how to prevent drug resistance (Zumla et al., 2012).

3.3. Economic burden of the disease

Calculation of total treatment costs is highly difficult to perform, since each country has its own health system, as well as its own methods for monitoring and registering costs. Moreover, pharmaceutical companies charge different prices for identical drugs, depending on the country's gross domestic product (GDP) and the degree of occasional sponsoring by non-profit organizations. As such, data regarding total treatment costs is heterogeneous (Diel et al., 2014).

In 2001, the *Stop TB Partnership Global Drug Facility* (GDF) was established, functioning as a one-stop mechanism to provide grants and procurement services to countries in need (Global Drug Facility, 2014). Regarding medicines alone, GDF has estimated a six-month first-line treatment against susceptible TB to cost around $19–22 per patient (WHO, 2014). On the other hand, the cost of a 24-month second-line treatment comprising four drugs (capreomycin, moxifloxacin, 4-aminosalicylic acid and cycloserine) ranges between US$4000–6000 *ex works* (Médecins sans Frontières, 2012). It should be noted that the exact price also varies among individuals, due to different patient's drug resistance profiles. In general, GDF announced that the total value of orders in 2013 was US$226.4 million (a 56% increase compared to 2012), of which US$128 million concerned second-line treatment (83% more than in 2012) (Global Drug Facility, 2014).

The *Tuberculosis Network European Trials Group* (TBNET) is an European network that promotes clinically-oriented research in the field of TB, through exchange of ideas and protocols among its members (Giehl et al., 2012). A study carried on behalf of TBNET in 37 European countries evaluated the availability and cost of *anti*-TB drugs in Europe (Gunther et al., 2014). Costs of standard treatments for either susceptible, MDR or XDR TB were compared using a purchasing power analysis and affordability was evaluated relatively to monthly GDP *per capita*. This study demonstrated that at least one second-line injectable and either moxifloxacin or levofloxacin were available in all countries. More importantly, it revealed that treatment for drug-susceptible TB represents an average of 8.5% of the monthly GDP across countries, increasing to 30% or even to more than 100% for MDR and XDR TB, respectively.

Expenses with infection control, laboratory support and psychosocial care and counseling add up to the cost of production and distribution of anti-TB medicines (Médecins sans Frontières, 2012). In a 2009 study, Kik et al. (2009) calculated the average costs of a household

Table 2
Pipeline of new anti-TB drugs.

Drug	Class	Sponsor	Target	Stage	Clinical trial ID
AZD5847	Oxazolidinones	Astra Zeneca	Protein synthesis	Phase II (on hold)	NCT01516203
Bedaquiline (TMC-207, R207910)	Diarylquinoline	Janssen Pharmaceuticals	ATP synthase	Phase III	NCT01600963
Clofazimine (TBI-166)	Riminophenazine	Institute of Materia Medica (Shanghai, China)	DNA synthesis	Preclinical	n.a.
CPZEN-45	Caprazamycin	Institute of Microbial Chemistry (BIKAKEN, Tokyo, Japan)	Cell wall synthesis	Preclinical	n.a.
Delamanid (OPC6768)	Nitroimidazole	Otsuka Pharmaceutical Co., Ltd.	Cell wall synthesis	Phase III	NCT01424670
PBTZ-169	Benzothiazinone	Innovative Medicines for Tuberculosis (iM4TB)	Cell wall synthesis	Preclinical	n.a.
Pretomanid (PA-824)	Nitroimidazole	TB Alliance	Cell wall synthesis	Phase III	NCT02342886
Q203	Imidazopyridine amide	Qurient Technologies	Cytochrome bc1 complex	Phase I	NCT02530710
SQ109	Ethylenediamine	Sequella, Inc.	Cell wall synthesis	Phase II	NCT01218217
SQ641	Capuramycin	Sequella, Inc.	Cell wall synthesis	Preclinical	n.a.
Sutezolid (PNU-100480)	Oxazolidinones	Sequella, Inc.	Protein synthesis	Phase II	NCT01225640

n.a. – non-applicable.

with a TB patient treatment, in the Netherlands, as being €2603. Of those, only €353 resulted from direct costs. The gross remainder was due to hospitalization and time loss (about 2.7 months), indicating that the highest burden was mainly due to indirect costs, namely loss of productivity.

Recently, Diel et al. (2014) performed a cost-assessment analysis of TB treatment in Europe, to build a case for investing in a new vaccine development. These authors analyzed the cost of TB treatment throughout the different European Union (EU) members, considering direct and indirect costs. Loss of productivity was considered in the total sum of TB treatment costs. As such, the total average cost of TB per case for the first 18 EU members was €10,282, €57,213 and €170,744 for the treatment of susceptible, MDR and XDR TB, respectively. In the other nine recent EU members, the corresponding total average cost was determined to be €3427 (susceptible) and €24,166 (MDR and XDR TB) (Diel et al., 2014).

Overall, and according to recent data, considering direct and indirect costs, TB and MDR-TB together cost the EU €5.9 billion per year (http://www.fighttb2015.eu). Moreover, it should be noted that the burden related with TB treatment is also relevant in high-income countries, as demonstrated by Blaas et al. (2008), who analyzed four XDR-TB cases in Germany and concluded that even in this developed country, the disease setting had a tremendous impact on life quality and total cost of health resources.

4. Antimicrobial peptides (AMPs)

The emergence of multi-drug resistant strains, together with ineffective and expensive therapeutics has paved the way to the development of new antimicrobial compounds able to act through different mechanisms (Khara et al., 2014). Among those, antimicrobial peptides (AMPs) show particular interest, either for administration as a monotherapy or combined with other drugs (Padhi et al., 2014). AMPs are a diverse group of molecules found in most living organisms and recognized for their relevant role in the innate immune response (Giuliani et al., 2007). They are usually short in length (20–60 amino acid residues), cationic, amphipathic and have a broad spectrum of activity against bacteria, fungi and viruses. The amphipathic and cationic nature of AMPs enables them to interact in both aqueous and lipid-rich environments, and bind the negatively charged membranes of bacteria (Hancock and Lehrer, 1998; Yamasaki and Gallo, 2008). Overall, AMPs can be classified into four major classes, according to their secondary structure: α-helix, β-hairpin structure or loop (formed by a single disulfide bond at the carboxy end and/or cyclization of peptide chain), antiparallel β-sheet (restrained by two or more disulfide bonds), and linear, non α-helical peptides (Zasloff, 2002). Some examples of AMP representative of these classes are given in Table 3. The ultimate proof of AMPs involvement in mammalian innate immunity was achieved after observing that the deletion of the gene *Cnlp* (which expresses CRAMP, a murine cathelicidin) in mice resulted in a decreased ability of isolated mast cells to kill the pathogen *S. pyogenes* (Nizet et al., 2001). The diversity of these peptides is evident in the different AMP collections, such as the multifunctional Antimicrobial Peptide Database (APD), established in 2003 with the aim of promoting research and information exchange in the field (Wang et al., 2009). An update to this database was performed in 2012 with the Dragon Antimicrobial Peptide Database (DAMPD) (Sundararajan et al., 2012). Over 1200 AMPs were manually selected from a wider peptide list retrieved from UniProt and GenBank databases. Only peptides with experimentally validated antimicrobial activity were included in DAMPD. More recently, a comprehensive database linking AMPs (LAMP), describing the antimicrobial activity and cytotoxicity of over 5500 entries of both natural and synthetic AMPs, was developed to aid in the design and discovery of new peptides (Zhao et al., 2013).

4.1. Mechanisms of action

Different mechanisms have been described to explain the killing of bacteria by antimicrobial peptides (Yeaman and Yount, 2003). Some of these are summarized in Fig. 2. Positively charged AMPs associate with the anionic lipopolysaccharides and phospholipids of the bacterial membrane through electrostatic interactions, resulting in their displacement or in the modification of membrane structure due to alterations in surface tension (Aoki and Ueda, 2013; Giuliani et al., 2007). This further results in membrane disruption that causes leakage of cellular contents or the translocation of the peptide through the outer membrane in gram-negative bacteria, as proposed in the Shai-Matsuzaki-Huang model (Matsuzaki, 1999; Shai, 1999; Yang et al., 2000). Three different main models have been further proposed, depending on the peptide insertion state: i) in the “carpet” model the

Table 3
Representative AMP families and sub-families of the major structural AMP classes.

Structural classes	Family/sub-family	Examples	Origin	Length (a.a.)	Activity
α-Helix	Bacteriocin[a]	Nisin, pediocin, lactococcin G	Bacteria	22–70	Bacteria (Gram + and −)
	Bombinin	Maximin 1, Maximin H1	Frog	20–27	Broad-spectrum
	Buforin	Buforin I	Frog	21–39	Broad-spectrum
	Cecropin	Cecropin A, Cecropin B	Insect	31–37	Bacteria (Gram + and −)
	Cathelicidin	LL37	Mammals	23–37	Broad-spectrum
	Dermaseptin	Dermaseptin S4	Frog	27–34	Bacteria, Fungi
	Magainin	Magainin 2	Frog	21–26	Broad-spectrum
β-Hairpin/loop (one disulfide bond or cyclic peptide chain)	Brevinin	Brevinin-1, Brevinin-2	Frog	24–31	Bacteria (Gram + and −)
	Cathelicidin	Bactenecin	Mammals	12–18	Broad-spectrum
	Cyclotide	Circulin-A, Cycloviolacin-1	Plant	28–37	Broad-spectrum
	Esculentin	Esculentin-1	Frog	18–46	Bacteria, Fungi
	Ranateurin	Ranateurin-2	Frog	27–32	Broad-spectrum
β-sheet (2 or more disulfide bonds)	Defensin	α-Defensin 1, β-Defensin 1, θ-Defensin	Vertebrate and invertebrates	18–45	Broad-spectrum
	Diptericin	Diptericin A, Diptericin B	Insect	82	Bacteria (Gram + and −)
	Penaeidins	Penaeidin-1, -2, -3	Crustaceans	23–31	Bacteria, Fungi
	Protegrin	Protegrin-1, Protegrin-2	Pig	16–18	Broad-spectrum
	Tachyplesins	Tachyplesin-1, Polyphemusin-1	Horseshoe crab	17–18	Bacteria, fungi
	Thionin	α-Thionin, β-thionin	Plant	45–48	Bacteria, fungi
Linear, non α-helical	Apidaecin	Apidaecin IB	Insect	18–20	Bacteria (Gram −)
	Cathelicidin	Bac5, PR-39	Mammals	39–80	Broad-spectrum
	Histatin	Histatin-1, -3, -5	Animal	24–38	Bacteria, fungi

Some families, like cathelicidins, are very heterogeneous and peptides with different structures can be found within the same family.
[a] Exceptions to the α-helical structure of bacteriocins comprise, for example, bovicin HJ50, which contains a rare disulfide bond, or the cyclic peptide AS-48.

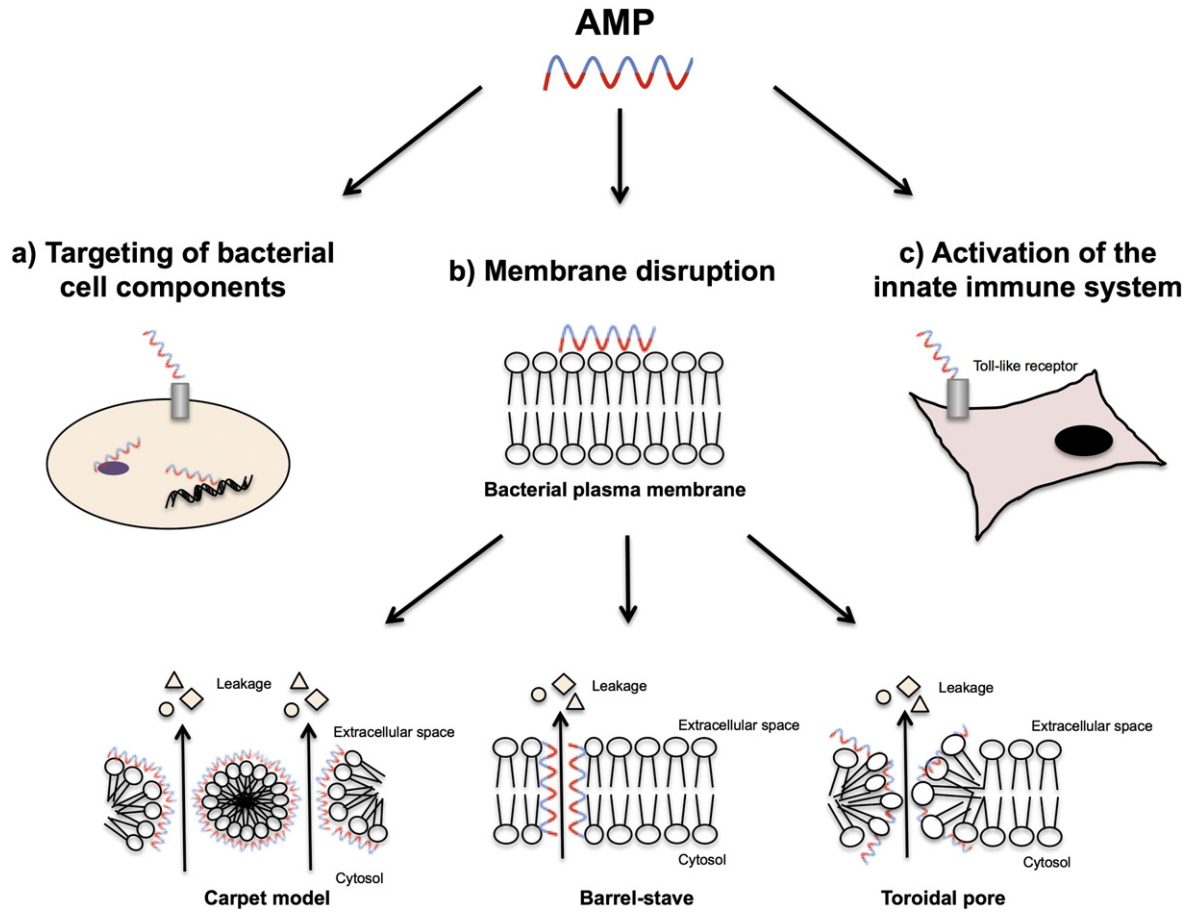

Fig. 2. Examples of the different mechanisms used by antimicrobial peptides to induce killing of bacteria: a) targeting of key intracellular processes (*e.g.* inhibition of protein or DNA synthesis) that lead to bacteria cell death without membrane disruption; b) positively charged AMPs bind to the anionic bacterial membrane through electrostatic interactions, resulting in bacterial membrane disruption. Three models (carpet, barrel-stave and toroidal pore models) help explain the different mechanisms that AMPs use to create pores on the membrane; c) AMPs can bind the host cells' toll-like receptors, inducing an immune response.
Modified from Duplantier and van Hoek (2013)).

peptide aligns parallel to the surface of the membrane, forming an extensive layer (or carpet) that eventually increase the surface tension of the membrane to a point of disruption; ii) in the "barrel-stave" model, the peptides (referred as staves) are inserted in a barrel-like ring inside the membrane, forming pores with their hydrophobic surfaces facing towards the membrane and the hydrophilic surfaces forming the pore lining; iii) in the "toroidal pore" model, the attached peptides intercalate with the membrane lipids, bending the lipid monolayer through the pore, so that the head groups of both peptides and lipids face towards the center of the pore (Brogden, 2005; Duplantier and van Hoek, 2013; Yeaman and Yount, 2003). Wenzel et al. (2014) recently described a complementary model for the peptides RWRWRW-NH_2 (also referred as MP196) and gramicidin S. In this model, AMPs integrate the membrane and delocalize peripheral membrane proteins essential for respiration and cell wall biosynthesis (cytochrome *c* and MurG, respectively), thus affecting energy metabolism and cell wall integrity.

Other models have also been proposed to help explain the AMP-induced membrane disruption. In 1999, Miteva and co-workers (Miteva et al., 1999) suggested the involvement of a molecular electroporation mechanism. Using NK-lysin (a 78 amino acid peptide secreted by porcine natural killer cells) as a model, the authors observed that the presence of a highly charged α-helix in a peptide was responsible for creating an electric field upon peptide binding to the bacterial membrane. The high electrostatic potential thus formed further resulted in pore formation. Pokorny et al. (2002) described a model in which an amphipathic α-helical peptide aggregates into a trimer and rapidly translocates across the membrane, similarly to a sinking raft. During this process, efflux of lipid vesicle contents and lipid flip-flop occur due to the transient, peptide-induced membrane instability. Some peptides have been described by Wimley (Rathinakumar et al., 2009) as having the ability to promote membrane destabilization by causing rearrangements in lipid organization upon partitioning through the interfacial zone of the bilayer. This phenomenon, described as interfacial activity, is usually associated to peptides that bind well enough to the membranes and are imperfectly amphipathic, meaning that their polar and nonpolar groups are segregated in an imperfect way. This implies that lipids must deform and disrupt their hydrocarbon core to accommodate the peptide's polar and nonpolar groups (Wimley, 2010).

Pore formation has also been suggested by Fuertes et al. (2011) as an intrinsic property of lipid bilayers. According to the authors, phase coexistence, as well as different internal and external sources of tension (including the binding of nonlipidic molecules), increase the probability of pore formation. Binding of amphipathic peptides to membranes acts as a tension factor and reduces the activation energy barrier, thus enabling pore opening. Peptides further act by reducing the line tension, which results in pore stabilization. In this lipocentric pore model, peptides are best described as pore-inducers rather than pore-forming molecules.

All the previous models induce bacterial killing through membrane disruption. However, AMPs can target key intracellular processes that lead to bacterial death without necessarily disrupting the membrane.

Such processes include the inhibition of cell wall components, DNA or protein synthesis, protein folding and metabolic turnover (Aoki and Ueda, 2013).

Lantibiotics are a class of AMPs containing the cyclic thioether amino acids lanthionine and/or methyllanthionine, which are produced by, and act against, Gram-positive bacteria (Bierbaum and Sahl, 2009). These peptides divide into two main groups based on their structures and modes of action - type A and type B lantibiotics - often combining different killing mechanisms in the same molecule. For example, type A lantibiotics (*e.g.* nisin, Pep5, epidermin) affect both cell wall biosynthesis and form pores in the lipid membranes through interactions with lipid II, a precursor of the cell wall (Asaduzzaman and Sonomoto, 2009; Wiedemann et al., 2001). Type B lantibiotics also inhibit cell wall biosynthesis but unlike type A molecules, they do not form pores. Duramycin, for example, binds membrane-bound phospholipids, thus inhibiting phospholipase A2, whereas mersacidin and actagardine directly complex lipid II (Brötz and Sahl, 2000).

Lipid II is also the main target of glycopeptide antibiotics, a class of actinomycete-derived AMPs composed of tri- or tetracyclic heptapeptides cores, which are usually glycosylated and may also comprise lipophilic fatty acid side chains. Examples of glycopeptide antibiotics include vancomycin, teicoplanin or bleomycin (Butler et al., 2014). Contrarily to lantibiotics, which bind the head group of lipid II, glycopeptide antibiotics complex the acyl-D-alanyl-D-alanine side chain of the peptidoglycan (Bierbaum and Sahl, 2009).

At a different level, buforin II, a 21-amino acid, broad-spectrum AMP derived from the stomach tissue of the Asian toad, inhibits DNA and RNA function (Park et al., 1998). Suppression of heat shock proteins, specifically DnaK and GroEL results in the inhibition of protein folding and has been reported to be the target of insect-derived drosomycin, apidaecin and pyrrhocoricin (Otvos et al., 2000). Pleurocidin, a α-helical peptide isolated from winter flounder, has been reported to inhibit the synthesis of both proteins and nucleic acids (Patrzykat et al., 2002), mechanisms also shared by human neutrophil protein-1 and -2 (HNP-1 and HNP-2) (Lehrer et al., 1989).

Recently, Scheinpflug et al. (2015), while studying a cyclic R-,W- rich hexapeptide cWFW, described a novel mechanism of action, based on preferential partitioning into particular lipid domains containing both phosphatidylethanolamine and cardiolipin. However, it is not clear how exactly this mechanism affects lipid function irreversibly. Alternatively, magainins, a class of AMPs isolated from the skin of African clawed frog *Xenopus laevis* (Zasloff, 1987), or LL37, a human cathelicidin (Agerberth et al., 1995), prevent the binding of lipopolysaccharide (LPS) to macrophages, thus avoiding the secretion of pro-inflammatory cytokines by those cells (Rosenfeld et al., 2006). Some AMPs (*e.g.* LL37) can also indirectly induce bacterial cell death through the regulation of the host's innate and adaptive immunity, due to their chemokine-like and immunomodulatory properties, including the chemotaxis of leukocytes (Durr and Peschel, 2002; Torres-Juarez et al., 2015).

Although mycobacteria present a different composition of the cell wall, there are studies reporting activity of natural AMPs, such as LL37 and human neutrophil defensin against *M. tuberculosis* at higher concentrations (Jiang et al., 2011; Kisich et al., 2002).

4.2. Antimicrobial resistance

The ability to resist against AMPs may be regarded as an impressive challenge for bacterial evolution. However, the few bacteria that exhibit AMP resistance, such as *Staphylococcus aureus* and *Salmonella* spp. have a survival advantage and are recognized as important human pathogens (Nizet, 2006).

One of the most common approaches developed by bacterial species to attain AMP resistance is the modification of the anionic constituents of their cell surfaces, thus avoiding the attraction of cationic AMPs (Ernst et al., 2001; Nizet, 2006). For example, modifications of teichoic acid composition, namely the incorporation of significant amounts of D-alanine, in *S. aureus* cell wall, reduces its negative charge (Peschel, 2002). Also, some species (*e.g.* from the genera *Morganella* and *Serratia*) reduce the amount of peptide-binding sites by establishing an inappropriate density of acidic lipids on cell surface; another resistance mechanism includes the extracellular proteolytic degradation or neutralization of AMPs (Nawrocki et al., 2014), like for example in the case of *Porphyromonas gingivalis* (Zasloff, 2002). Alternatively, this can be achieved either directly through bacterial surface-associated or secreted proteins or indirectly by inducing the release of molecules that bind and inactivate AMPs before they reach the cell membrane (Nizet, 2006). *S. aureus* produces staphylokinase, an enzyme that in addition to its proteolytic activity can bind and inactivate human neutrophil-derived α-defensins (HNPs) (Jin et al., 2004). However, it should be noted that some AMPs, like the ones that include proline-rich sequences, possess a relative resistance to proteolytic degradation, since proline prevents the cleavage of the scissile bond by serine proteases (Shinnar et al., 2003). Proteolytic degradation can also be prevented by the introduction of D-amino acid substitutions and by head-to-tail cyclization, as demonstrated by Molhoek et al. (2011) using chicken cathelicidin-2 as a model. Such modifications also reduce the peptides' cytotoxicity and do not alter their antimicrobial activity.

Moreover, some bacteria can actively extrude AMPs from the bacterial membrane, a process achieved through energy (ATP or proton motive force)-driven pumps (Levy, 2002). *Pseudomonas aeruginosa*, for example, possesses several multidrug efflux pumps able to export a broad range of molecules. Many human pathogens are also able to up- or downregulate their virulence factors according to environmental cues found in the host. Some examples are the PhoP/PhoQ components of *Salmonella typhimurium* (Ernst et al., 2001), the response regulator ArcA of *Vibrio cholerae* or the covRS locus of Group A Streptococci (GAS) (Nizet, 2006). Interestingly, some bacteria have also been found to regulate the production of AMPs by the host. Indeed, the DNA plasmid from *Shigella* spp. was reported to mediate the downregulation of LL37 and β-defensin-1 in epithelia surfaces upon infection, thus promoting bacterial adherence and invasion of the host epithelium (Islam et al., 2001). A schematic representation of the different resistance mechanisms employed by bacteria against AMP is shown in Fig. 3.

Acquisition of resistance has been reported to have a fitness cost, affecting the bacteria capability to survive and reproduce, which typically reflects in a reduced growth rate. In this sense, Andersson and Hughes (2010) suggested that a decrease in the use of antimicrobials would theoretically result in a reduced frequency of resistant bacteria, in a natural selection-mediated process, since susceptible bacteria (displaying higher growth rates) would outmatch resistant ones upon a decrease in the selective pressure. However, bacteria can ameliorate the costs of resistance through acquisition of additional fitness-compensatory mutations. In fact, acquisition of specific mutations is a mechanism that cross-features bacterial resistance against AMPs and conventional antibiotics. For example, mycobacterial mechanism of resistance to isoniazid and ethambutol is obtained by mutating the *katG* and *ethA* genes, respectively, which encode the expression of enzymes responsible for the activation of those two antibiotics. By preventing their activation, *M. tuberculosis* avoids the inhibition of InhA, an enoyl-acyl carrier protein reductase, thus securing the biosynthesis of mycolic acids (Vilcheze and Jacobs, 2014). Resistance to rifampicin is usually related with a mutation within the *rpoB* gene, which encodes the β subunit of bacterial RNA polymerase, the target of rifampicin (Goldstein, 2014). Similar mechanisms have also been described in AMP resistance. Vancomycin resistance, for example, occurs due to mutations in genes that encode enzymes involved in peptidoglycan biosynthesis, ultimately resulting either in modification or removal of vancomycin's binding target (Courvalin, 2006). Also, an accumulation of single nucleotide polymorphisms has been identified in *mprF*, a multipeptide resistance factor gene involved in the synthesis of cell membrane in daptomycin-resistant *S. aureus* strains. Overexpression of the *dlt* operon, responsible

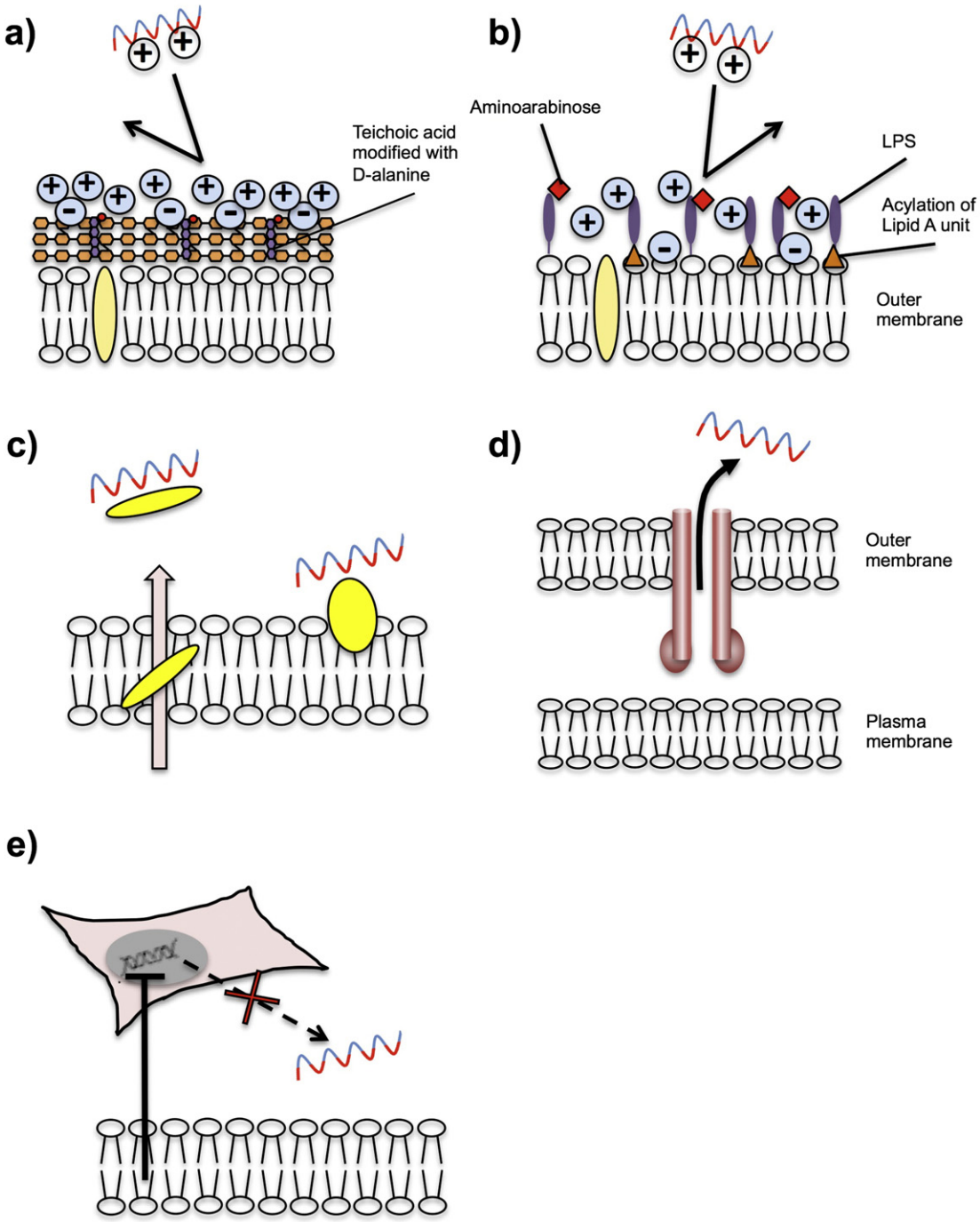

Fig. 3. Mechanisms of bacterial resistance against AMPs. One of the most common approaches involves the modification of cell surface charge, either by: a) adding D-alanine to teichoic acid composition (*e.g.* Gram-positive bacteria like *S. aureus*); b) altering the density of acidic lipids on cell surface by the incorporation of aminoarabinose in LPS molecules or by the acylation of the lipid A unit, also in LPS (*e.g. Morganella* sp., *Serratia* sp.). c) Extracellular proteolytic degradation of AMPs, which occurs after protein secretion and binding to AMPs in the extracellular environment or by binding of cell surface proteins to AMPs (*e.g. P. gingivalis*). d) Active efflux of AMPs (*e.g. P. aeruginosa*). e) Induction of the downregulation of AMPs expression by host cells (*e.g. Shigella* spp.).

for the D-alanylation of teichoic acids present in the cell wall, was also reported in the same resistant strains (Bayer et al., 2013). Studies also performed in *S. aureus* by Pietiainen et al. (2009) showed the involvement of vraDE (an ABC transporter) overexpression in resistance to bacicatrin.

Still, acquisition of resistance against AMPs may be considered very rare. This may be attributed in part to the non-specificity of these peptides' mode of killing, as well as the combination of different killing mechanisms in the same molecule, and the fact that mutations that grant bacteria increased AMP resistance involve metabolically expensive biochemical modifications. Moreover, these mutations may not be advantageous to the organisms upon epithelial colonization or host-to-host transmission (Kapoor et al., 2011; Nizet, 2006; Peschel and Sahl, 2006). A summary of these mechanisms, as well as the most suitable models for their study were recently described by Bauer and Shafer (2015). Development of resistance by *M. tuberculosis* occurs exclusively through spontaneous mutations in the chromosomes (rather than through plasmid-mediated mechanisms) that affect the drug target or bacterial enzymes that activate the prodrug. These mutations were selected by mycobacteria based on reduced fitness costs (Bottger

and Springer, 2008). To date, many natural AMPs, including LL37 and human neutrophil peptides (HNPs) have been reported to kill mycobacteria, although at higher concentrations than the ones used to kill other microorganisms (Fattorini et al., 2004; Jiang et al., 2011; Ramon-Garcia et al., 2013; Santos et al., 2014). Interestingly, Limoli et al. (2014) reported that sub-inhibitory levels of LL37 induced mutations in the DNA of *P. aeruginosa*, encouraging it to overproduce a protective coating (a process referred as mucoid conversion), which ultimately results in resistance to higher concentrations of LL37 or even rifampicin.

5. AMPs: the road to market and clinic

The road to market new AMPs can be long and costly. Following the identification of an AMP as a drug candidate, this has to be produced in large-scale by Good Manufacturing Practices (GMP) (Uhlig et al., 2014).

5.1. Manufacturing

Different processes, including chemical synthesis, recombinant DNA technology, cell-free expression systems and transgenic plants or animals have been used to produce AMPs in a cost-effective fashion (Li and Vederas, 2009). Indeed, high manufacturing costs can be a major problem, as the large-scale production of a cationic AMP may reach US$50–400 per gram (depending on the production method, peptide length and purification requirements), in comparison with less than US$1 per gram of antibiotic (Marr et al., 2006). However, strategies like recombinant technology may help circumvent this problem. Indeed, a recombinant version of LL37 was previously produced in *Escherichia coli* using a cost-effective method that allowed the maintenance of the peptide's antimicrobial and pro-angiogenic activities (Ramos et al., 2010; Ramos et al., 2011). Bacterial systems, in particular *E. coli*, are the most commonly used for heterologous AMP expression. These are expressed as fusion proteins, masking their lethal effects to the host while protecting them from proteolytic degradation (Li, 2011). Nevertheless, AMP expression in other systems has been explored as a means of obtaining higher yields and lower costs. In this scope, Novozymes Inc. described the production of plectasin in a fungal system at large scale and high purity through recombinant technology (Mygind et al., 2005). Moreover, production of recombinant peptides in plants is viewed as safe, efficient and cost-effective, yielding biomass at 10- to 50-fold lower costs as compared to the production in *E. coli* (Kusnadi et al., 1997). These peptides are synthesized with the correct folding and plant cells direct them to environments that reduce degradation, thus increasing stability (Horn et al., 2004). Recently, Zeitler et al. (2013) successfully produced the peptide SP1–1 as a fusion protein in *Nicotiana benthamiana*, using the tobacco mosaic virus strategy. The use of strains (usually bacteria or fungi) specifically mutated to enhance protein synthesis, secretion and folding may further increase the yield of a recombinant peptide production up to 1000-fold, compared to non-modified strains (Li and Vederas, 2009). On the down side, recombinant peptides require extensive design and development, as well as a rigorous quality control to meet the regulatory requirements. As such, even if the costs of the starting material are negligible, all the downstream processing to produce a large-scale GMP-grade lot of recombinant peptide can reach US$1 million with a lead-time of over 1 year (Lax, 2010). Noteworthy, recombinant technology changed the mindset of the peptide manufacturing industry. Before that, peptide production was considered expensive and complicated, and most of all, the industry had few or no interest in producing molecules lacking oral bioavailability, as is the case of peptides (most of them are degraded in the upper gut). Indeed, oral alternatives were favored in relation to peptides, in order to increase patient compliance. After the introduction of long-acting therapies based on the release of peptides encapsulated in biodegradable polymers, requiring only alternate injections, interest on non-orally administered drugs resurfaced and recombinant expression emerged as a cost-effective method for large-scale peptide production (Lax, 2010).

Chemical synthesis processes, which include solid-phase, solution-phase and hybrid, provide an interesting alternative for large-scale production of AMPs (Vlieghe et al., 2010). These are faster and allow the production of much higher peptide quantities. In addition, they use generic chemical and purification procedures and require less personnel for production, quality and regulatory issues management (Lax, 2010). As a result, production costs are highly reduced compared to other peptide production methods. If this process is set at a plant with a capacity to produce ~100 kg peptide per year, costs should be around US$7.5–10 per gram per amino acid residue. But if the plant is able to produce >1 ton per year, the cost may drop to less than US$1 per gram per amino acid residue (Bray, 2003). Also, chemical synthesis provides more flexibility in terms of peptide design, allowing the incorporation of unnatural amino acids, for example. Small to medium sized peptides are preferably synthetized through solution-phase, providing significant advantages in terms of isolation, characterization and purification of the intermediate products, while solid-phase is usually preferred for larger peptides (Lax, 2010).

A major challenge in peptide manufacturing is matching equipment and other resources with the customer needs. Usually small annual amounts (10 g–10 kg) are required for clinical trials or other research purposes, being larger quantities (100 kg–1 ton) needed only sporadically. This represents a problem, since larger-scale production requires larger equipment, whose acquisition and maintenance costs are difficult to justify if it stands idle. Moreover, it exposes the manufacturer to increased expense risks should this equipment fail to operate. Also, large-scale production is often associated with longer hold times between intermediate processes, which favor degradation and/or aggregation of the peptide. In this sense, a solution considering the use of smaller equipment organized in tandem or parallel configuration is favored to diminish risks associated with large-scale peptide production (Lax, 2010).

5.2. Quality control

Quality control of active pharmaceutical ingredients (APIs) is essential. Guidelines defining specific testing and validation criteria for APIs in general, are included in the *Code of Federal Regulations* from Food and Drug Administration (FDA) (2015), the *Q7A-Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients* of the International Conference on Harmonization (ICH) (2000) or in the *Rules Governing Medicinal Products in the European Union* from the Directorate-General of the European Commission (2014). However, there is only one FDA guideline that specifically addresses peptides, issued in 1994, entitled *Guidance for Industry for the Submission of Chemistry, Manufacturing and Controls Information for Synthetic Peptide Substances* Center for Drug Evaluation and Research (CDER) and Center for Biologics Evaluation and Research (CBER), (1994). This guideline determines the lot-release information that should be provided to guarantee the identity, purity, strength and quality of the peptide, as well as to show lot-to-lot consistency, by defining a set of different methods and criteria for peptide control testing. Purity identity assessment, in particular, constitutes a major additional cost for companies. Currently there are no clear guidelines that define a threshold for the amount of impurities allowed, so manufacturers usually adopt very narrow limits to prevent any regulatory issues. However, if one considers the low doses used, setting the impurity threshold to very low amounts can be catastrophic regarding the final cost of the product, as further unnecessary costs would result from additional purification steps (Lax, 2010). Moreover, higher purity implies increased manufacturing costs, as it requires more equipment, chemicals and time to achieve the same amount of peptide with less purity (Swietlow and Lax, 2004). On the other hand, the identity of the peptide is established by the amino acid composition, sequence and chirality, usually combining the use of expensive mass

spectroscopy, amino acid analysis and High-Performing Liquid Chromatography (HPLC) (Bartolomeo and Maisano, 2006; Rutherfurd and Gilani, 2009; Sherman et al., 2009). The proper selection of the purification method is essential to obtain high-purity peptides (Andersson et al., 2000). The assessment of the purity profile of these chemically synthetized peptides assumes extreme relevance. Indeed, the presence of an elevated amount of impurities may result in a cocktail of more than one pharmaceutically active substance, which may alter the main peptide's interaction with biological systems (Lax and Verlander, 2006).

5.3. Regulation

Regulation of new peptides represents a great challenge for companies, in part due to some ambiguity in their classification by regulatory entities. For the FDA, distinction between protein and peptide is solely based on size, the defined upper size threshold being set at 40 amino acids, according to the literature. In this sense, any polymer composed of 40 or less amino acids is considered a peptide, while larger ones falls within the definition of a protein. This difference in definition has implications for protein and peptide classification under the Federal Food, Drug & Cosmetic (FD&C) Act. Indeed, the term "protein (except any chemically synthesized polypeptide)" has been included in the definition of a "biological product". According to the FD&C Act, this definition comprises sugars, proteins, nucleic acids (or any combination of these), or living entities (*e.g.* cells, tissues) used for the treatment or prevention of diseases in human beings. Peptides, which fall within the definition of "chemically synthesized polypeptide" (meaning any alpha amino acid polymer made entirely by chemical synthesis and containing <100 aa), are commonly regulated as "drugs", which are usually defined as pure chemical substances of small size and known structure. Thus, consistent with data from the literature, describing peptides as smaller, less complex (absence of a 3D structure), able to perform fewer functions, of easier characterization and compared to proteins, FDA excluded peptides from the term "protein" in the statutory definition of "biological product". Nevertheless, exceptions, such as peptide vaccines, meet the requirements to be defined as "biological product" (Food and Drug Administration, FDA, 2009).

In Europe, the EMA defines peptides according to their origin: if of natural sources or produced using recombinant technology they are regulated as biological products; if chemically synthetized, they are treated as conventional small molecular chemical substances (European Commission, 2001, 2003). Nevertheless, a peptide may be considered as a significant therapeutic innovation, thus accelerating the process for obtaining marketing authorization (European Medicines Agency, 2011).

In terms of clinical trials regulations, the recognition of the difficulties to approve new antimicrobials led to the creation by FDA of the *Antibacterial Drug Development Task Force*, in 2012. This task force aimed at facilitating the design and performance of clinical trials for this class of medicines, particularly by dropping the requirement of the demonstration of its superiority as compared to existing ones. Moreover, by incorporating parts of the *Generating Antibiotic Incentives Now (GAIN)* Act in that task force, new antibiotics of interest can be nominated as Qualified Infectious Disease Products (QIDPs), which allows them to have priority review and fast-track status, along with five-year exclusivity if they are licensed (Fox, 2012). Also in 2012, the European Medicines Agency (EMA) released new guidelines with clearly defined criteria for the evaluation of new antimicrobials in clinical trials. Moreover, COMBACTE (Combating Bacterial Resistance in Europe), a consortium of different European universities and corporations with a budget of nearly €195 million, was launched to promote innovative trials for new antimicrobials, as well as design better diagnosis systems that allow a more suitable monitoring of treatment responses, thus identifying best performing treatments (Fox, 2013).

Approval of new drugs is usually based on their therapeutic efficacy, safety and product quality. However, due to the limited global financial resources, a drug approval step concerning pricing and reimbursement, dubbed "the fourth hurdle", was introduced. This criterion involves the analysis of the product cost-effectiveness and is required even if all the other criteria are met (McGhan, 2010).

5.4. AMPs in clinical trials

Since the approval of daptomycin (Cubicin®, Cubist Pharmaceuticals) by FDA in 2003, several companies have been forced to abandon the development of new AMPs, mostly due to reduced antimicrobial activity (in comparison to existing treatments), safety problems and/or or lack of funding (Eckert, 2011; Fox, 2013). For example, iseganan (Intrabiotics Pharmaceuticals, Inc.) reached Phase III trials for the treatment of pneumonia but was withdrawn due to toxicity issues (Eckert, 2011). Pexiganan® (also known as MSI-78), a magainin variant isolated from an amphibian and developed by Magainin Pharmaceuticals (Gottler and Ramamoorthy, 2009), was also removed from Phase III trials after manufacturing costs proved too high and demands to change the direction of the clinical study ensued. Nevertheless, this same peptide recently re-entered clinical trials for the treatment of diabetic foot ulcers-associated infections, under the name of Locilex® (Dipexium Pharmaceuticals). The development of Omiganan®, also referred to as MX-226 (Migenix Pharmaceuticals), a peptide designed to prevent bacterial colonization of catheters (Rubinchik et al., 2009), also came to a halt after failing to reach important regulatory endpoints during Phase III clinical trials.

A boost in the number of clinical trials exploring the therapeutic potential of AMPs followed after the onset of the Antibacterial Drug Development Task Force by FDA, in 2012. Indeed, there are currently several AMPs undergoing clinical trials for the treatment of bacterial and fungal infections. After the FDA approval of daptomycin for clinical use, other AMPs have followed. Some examples include polymyxins, gramicidins, bacitracin, vancomycin (as well as its derivatives dalbavancin and oritavancin) and telavancin (data obtained from http://www.fda.gov). Plectasin®, a defensin obtained from the fungus *Pseudoplectania nigrella* that proved quite effective in an *in vivo* model of endovascular infection with methicillin-resistant *S. aureus* (MRSA) (Xiong et al., 2011), was shelved by Sanofi-Aventis a few years after having it licensed from Novozymes. Other promising AMPs under clinical development include: surotomycin (CB-315), a lipopetide developed by Cubist Pharmaceuticals, is in Phase III trials also for the treatment of *Clostridium difficile* infections (Fox, 2013); NovaBiotics' lead compound Novexatin® (NP213), a cyclic cationic peptide, is in Phase II trials against fungal infections of the toenail (O'Neil, 2010); Lytixar® (also referred as LTX-109), a synthetic, membrane-degrading peptide developed by Lytix Biopharma currently undergoes Phase II trials for the treatment of MRSA nasal infections (Saravolatz et al., 2012); C16G2, a synthetic peptide designed to specifically target *Streptococcus mutans* (Kaplan et al., 2011), is currently being tested for the treatment of dental caries (Phase II); hLF1–11, which corresponds to an 11 amino acid sequence derived from human lactoferrin (van der Does et al., 2012) has reached the Phase II stage of clinical trials for the treatment of both bacterial and fungal infections; LL37 is currently in the Phase I stage of a clinical trial to evaluate the efficacy of its intra-tumoral administration in cutaneous or subcutaneous tumors. Moreover, lantibiotics, which are peptide antibiotics derived from lactococcal bacteria and containing lanthionine (polycyclic thioether) amino acids, showed quite promising results at pre-clinical level and are currently in clinical trials. The lantibiotic NAI-107, developed by Sentinella Pharmaceutics, Inc., showed great efficacy against MRSA, as well as vancomycin- and penicillin-resistant pathogens. Table 4 provides a list of AMPs that are currently under development (in preclinical trials) or already in clinical trials. This table also includes some examples of AMPs that were withdrawn at later stages of clinical trials, with the respective reason for the withdrawal.

So far, the only AMP that concluded pre-clinical trials for the treatment of tuberculosis, after showing promising activity against both

Table 4
Past and current clinical trials (CTs) involving AMPs.

Drug	Description	Indication	Stage/outcome	Sponsor	Clinical trial ID
Iseganan	Protegrin	Pneumonia	Phase III (2005)/withdrawn due to toxicity issues	Intrabiotics Pharmaceutical, Inc.	–
Pexiganan (MSI-78)	Magainin analogue	Diabetic foot ulcers	Phase III (1999)/withdrawn due to high manufacturing costs	Magainin Pharmaceuticals	–
Pexiganan (Locilex®, MSI-78)	Magainin analogue	Diabetic foot ulcers	Phase III	Dipexium Pharma/MacroChem/Genaera	NCT00563394
Omiganan (MX-226)	Synthetic peptide derived from indolicidin	Bacterial colonization of catheters	Phase III (2009)/withdrawn after failing regulatory endpoints	Migenix Pharmaceuticals	–
Omiganan (CLS001)	Synthetic peptide derived from indolicidin	Rosacea	Phase II	BioWest	NCT00608959
Plectasin	Defensin isolated from *Pseudoplectania nigrella*	Treatment of Gram-positive infections	Preclinical (2010)/withdrawn for commercial reasons	Novozymes, lic. to Sanofi-Aventis	–
Surotomycin (CB-315)	Lipopetide	Treatment of *C. difficile* infections	Phase III	Cubist Pharmaceuticals	NCT01598311 and NCT01597505
Novexatin (NP213)	Cyclic cationic peptide	Fungal infections of the toenail	Phase II	NovaBiotics	NCT02343627
Lytixar® (LTX-109)	Synthetic, membrane-degrading peptide	Treatment of MRSA nasal infections	Phase II	Lytix Biopharma	NCT01223222 and NCT01158235
NAI-107	Lantibiotic	Treatment of Gram-positive infections	Preclinical	Sentinella Pharmaceutics, Inc.	–
MU1140	**Lantibiotic**	***M. tuberculosis* infections**	**Preclinical**	**Oragenics Inc.**	–
OP-145	Synthetic 24-mer LL37 analogue	Chronic otitis	Phase II (completed)	OctoPlus, lic. to Dr. Reddys Laboratories	ISRCTN84220089
LL37	Cathelicidin	Melanoma	Phase I	M.D. Anderson Cancer Center	NCT02225366
C16G2	Specifically targeted antimicrobial peptide (STAMP)	Dental caries (specific for *Streptococcus mutans*)	Phase II	C3 Jian, Inc.	NCT02254993
hLF1-11	Human lactoferrin-derived peptide	Bacterial and fungal infections	Phase II	AM-Pharma	NCT00509938
Ghrelin	Peptide hormone	Airway inflammation, cystic fibrosis	Phase II	Papworth Hospital	NCT00763477
PMX-30063	Arylamide oligomer mimetic of a defensin	Acute bacterial skin infections caused by *Staphylococcus* spp.	Phase II	PolyMedix, Inc.	NCT01211470
PAC-113	Synthetic 12-mer peptide derived from histatin 3 and histatin 5	Oral candidiasis	Phase II	Pacgen Biopharmaceuticals	NCT00659971

To date, there is only one AMP in the pipeline to enter clinical trials against *M. tuberculosis* infections (labeled in bold).

active and dormant *M. tuberculosis*, is Oragenics Inc.'s lead compound MU1140 (a lantibiotic, derived from *S. mutans*). In addition, this peptide has shown activity against MRSA and *Bacillus anthracis* (responsible for anthrax) (Ghobrial et al., 2010; Padhi et al., 2014). Nevertheless, companies that have promising candidates in clinical trials still struggle to find funding to support the late and more expensive stages of those studies. Moreover, the synthetic nature of some of these new AMPs, together with the fact that they present a similar mode of action as biological molecules, may lead to regulatory issues that delay their development (Fox, 2013).

5.5. The anti-TB drug market: time for AMPs?

It is very difficult to define the market value for anti-TB drugs, since companies usually do not report reliable sales data for the markets most affected by TB. Nevertheless, it is estimated that in the US, sales of rifampicin reached near US$14.5 million in 2005. Additionally, the market is fragmented, due to existence of several local manufacturers and large generics pharmaceutical companies (Harper, 2007). Further market fragmentation derives from the fact that the *Global Drug Facility* (GDF) supplies anti-TB drugs for low-income countries, contributing to a decrease in the overall cost of treatments. A report from the TB Alliance in 2000 estimated the TB market value in US$412.5–$470.5 million per year. Of that, only US$12.5 million was for the treatment of MDR-TB (Global Alliance for TB Drug Development, 2001).

The comprehensive work and focus on new peptide development over the past few years has led to the approval of several new peptides for different therapeutic applications. Indeed, there are currently about 100 therapeutic peptides on the market worldwide, being cancer therapy the major application, holding a 21% share of the peptide market (Kaspar and Reichert, 2013). According to a recent report (Research, 2015), the peptide market was worth around US$14.1 billion in 2011 (corresponding to a 1.5% share of a global market worth US$956 billion) and is expected to reach US$25.4 billion by 2018, growing at a Compound Annual Growth Rate (CAGR) of 8.7%. Moreover, considering the possibility of patent expiration in the near future, it is expected that the segment comprising generic peptides will grow substantially, resulting in the overall growth of the peptide market.

Of note is the challenge most biotech and emerging pharmaceutical companies face today, as they try to reach the perfect balance between expediency and due diligence. High quality, expedited delivery and a low unit cost for the product are usually the main expected goals. However, the absence of proper quality control and/or regulatory departments at smaller companies often compromises the simultaneous achievement of all those goals (Lax, 2010). In this sense, analysts foresee a great potential may rise from the collaboration between small peptide companies and major pharmaceutical companies (Transparency Market Research, 2015).

An incremental analysis, a technique that compares one therapy with another, should help new companies deal with cost and decision analyses for the introduction of a new peptide in the market. This information is best displayed in quadrants, formed by two axes crossed perpendicularly, one relating to cost and the other to effectiveness (the center point being the comparison or standard therapy). Cheaper and more effective drugs will be considered as “dominant”, whereas the more expensive, less effective ones, would be “dominated”. Usually,

therapies with incremental cost-effectiveness ratios between US$20,000 and US$100,000 per life year saved are considered acceptable (McGhan, 2010).

With the rise of AMPs development and with a few of them already in Phases II/III clinical trials, it is only reasonable to think that these will also hold a significant share of the market in the near future.

Interestingly, to date there is no AMP in clinical trials for the treatment of tuberculosis. However, considering the conclusion of pre-clinical trials for MU1140 (Oragenics, Inc.) and other recent advances, it may be anticipated that AMPs will play an important role in the fight against TB. Indeed, it has already been demonstrated that LL37 expression is induced in macrophages in response to mycobacterial infections (Rivas-Santiago et al., 2008; Santos et al., 2014). Mohanty and colleagues recently suggested an additive effect *in vitro* of a LL37 analogue incorporated into silver nanoparticles against two strains of mycobacteria, the non-pathogenic *Mycobacterium smegmatis* and the pathogenic *Mycobacterium marinum* (Mohanty et al., 2013). Results from a clinical trial (NCT01580007) performed on adults with active pulmonary TB, described by Mily et al. (2015), demonstrated the ability of an orally-administered combination of phenylbutyrate and vitamin D_3 to induce LL37 expression in macrophages and lymphocytes, thus enhancing the intracellular killing of *M. tuberculosis*. Also, intra-tracheal administration of the innate defense regulator (IDR)- 1018, a modified version of the bovine neutrophil host-defense peptide bactenecin, reduced mycobacterial load in the lungs of animals infected with the virulent H37Rv laboratory strain and also with a *M. tuberculosis* clinical isolate (Rivas-Santiago et al., 2013a). Some examples of AMPs showing activity against *M. tuberculosis*, with respective mechanisms of action and activities are listed in Table 5. As observed, the plethora of AMPs currently being studied against this pathogen display diverse action mechanisms and high antimycobacterial activities, further reinforcing the huge potential of AMPs as promising candidates for TB treatment.

Additionally, many studies have shown the *in vitro* and *in vivo* efficacy of different AMPs against multidrug-resistant *Mycobacterium* strains. For example, PR-39, a proline-arginine-rich antibacterial peptide from porcine leucocytes, proved effective against multidrug-resistant clinical isolates of *M. tuberculosis* (Linde et al., 2001). Fattorini et al. (2004) showed the inhibition of MDR *M. tuberculosis* growth by protegrin-1 and human beta-defensin-1 (hBD-1). Jiang and co-workers (Jiang et al., 2011) tested the Minimum Inhibitory Concentrations (MICs) of five different synthetic peptides (derived from a previously described hybrid of cecropin A + melittin B) against a MDR TB strain. The majority of these peptides successfully reduced the growth of the MDR strain, with MICs similar to the ones obtained with the H37Rv (susceptible) strain. Recently, a group of AMPs containing D-amino acids (belonging to the D-LAK family) was also reported to inhibit the growth of MDR and XDR strains of *M. tuberculosis* both *in vitro* and *ex vivo*, although they were not able to eradicate the mycobacteria (Lan et al., 2014). The cathelicidin LL37 proved effective in reducing the mycobacterial growth of either susceptible (H37Rv) or MDR-resistant *M. tuberculosis*

Table 5
Examples of AMPs showing *in vitro* or *in vivo* activity against *M. tuberculosis* and respective mechanisms of action.

AMP	Mechanisms of action	Activity	Refs
1-$C13_{4mer}$	Pore formation	MIC (H37Rv): 6.6 μM	Kapoor et al. (2011)
Azurocidin	Bacterial envelope	H37Rv: 55% killing at 100 μg/ml	Jena et al. (2012)
Bacteriocins (Bcn1-Bcn5)	Pore formation	MIC (H37Rv): 0.01–1 μg/ml	Sosunov et al. (2007)
D-LAK analogues	Pore-formation, Inhibition of protein synthesis	MIC (H37Rv): 35.2–>200 μg/ml MIC (Vertulo): 49–100 μg/ml or inactive	Jiang et al. (2011); Lan et al. (2014)
Granulysin	Alteration of membrane integrity	H37Rv: 90% killing at 30 μM	Stenger et al. (1998); Toro et al. (2006)
Human Beta Defensins (hBDs) variants	Pore formation	MIC (H37Rv): 12–80 μg/ml MIC (MDR clinical isolate): 2.7–13.7 μg/ml	Corrales-Garcia et al. (2013)
Human Neutrophil Peptide-1 (HNP-1)	Pore formation; Immunomodulatory activity	MIC (H37Rv): 2.5–50 μg/ml *In vitro* (H37Rv-infected J774A.1 macrophages): 50% killing after 3 days treatment with 5 μg/ml; *In vivo* (H37Rv-infected mice): 1-log decrease with 5 μg per mouse	Kalita et al. (2004); Sharma and Morgan (2001); Sharma et al. (2000)
Innate Defense Regulators (IDR-1002, -HH2, -1018)	Immunomodulatory activity	MIC (H37Rv): 15–30 μg/ml *In vivo* (H37Rv-infected mice): 10–71% killing at 32 μg/mouse (3× per week, 30-day treatment) *In vivo* (MDR-infected mice): 10–71% killing at 32 μg/mouse (3× per week, 30-day treatment)	Mansour et al. (2015); Rivas-Santiago et al. (2013a)
Lactoferrin	Iron sequestration; Membrane damage through binding to LPS	*In vivo*: 1 $\log_{10}$ reduction after 3 weeks of oral administration of 0.5% lactoferrin, 7-days treatment	Welsh et al. (2011)
LL37	Pore formation; Immunomodulatory activity	MIC (H37Rv): ~5 μg/ml *In vivo* (H37Rv-infected mice): ~53% killing at 32 μg/mouse (3× per week, 28-day treatment) *In vivo* (MDR-infected mice): ~45% killing at 32 μg/mouse (3× per week, 28-day treatment)	Rivas-Santiago et al. (2013b)
LLKKK18	Pore formation; Immunomodulatory activity	*In vivo* (H37Rv-infected mice): 1.2-log reduction at 100 μM (10 every other day administrations)	Silva et al. (2016)
Magainin-1	Pore formation; Immunomodulatory activity	MIC (H37Ra): 1200 μg/ml	Santos et al. (2012)
MIAP	Inhibition of ATPase	MIC (H37Ra): 300 μg/ml	Santos et al. (2012)
M($LLKK)_2$M	Pore formation	MIC (H37Rv): 125 μg/ml MIC (CSU87): 62.5 μg/ml	Khara et al. (2014)
Nisin A	Inhibition of cell wall biosynthesis, pore formation (interactions with lipid II)	MIC (H37Ra): 60 μg/ml	Carroll et al. (2010)
PR-39	Inhibition of DNA and protein synthesis	H37Rv: 80% killing at 50 μg/ml E1380/94: 39% killing at 50 μg/ml P34/95: 49% killing at 50 μg/ml	Linde et al. (2001)
Protegrin-1	Formation of cation-selective channels on bacterial membrane	H37Rv: 68.4% killing at 64 μg/ml, 96.7% killing at 128 μg/ml; RM22: 45.1% killing at 128 μg/ml	Fattorini et al. (2004)
W- and R- rich peptides	Pore formation	MIC (H37Rv): 1.1–141 μM	Ramon-Garcia et al. (2013)

MIC (Minimum Inhibitory Concentration) represents the lowest concentration at which the peptide inhibits the growth of *M. tuberculosis*, after overnight incubation.
Vertulo, CSU, RM22, E1380/94 and P34/95 are all multidrug-resistant *M. tuberculosis* strains.

strains in the lungs of infected mice (Rivas-Santiago et al., 2013b). Around 53% and 45% reductions in mycobacterial levels were achieved after a 1 month treatment (3 times a week) with 32 μg LL37 per mouse.

To our knowledge, studies considering the use of AMPs against drug-resistant tuberculosis strains have still not reached preclinical trials. However, considering the major advances in the field, it is reasonable to expect that such studies will occur in the near future.

6. Boosting AMP potential

Promising prospects regarding AMPs arise from the clinical success of daptomycin and vancomycin (as well as its derivatives, like dalbavancin and oritavancin) in the treatment of bacterial infections. However, whether AMPs can make good candidates to fight mycobacterial infections still remains under discussion.

Their broad-spectrum activity, together with the potential to enhance the effect of other antimicrobials (namely antibiotics) by facilitating their access to the intracellular milieu (Zasloff, 2002) is regarded as their main advantages over antibiotics. Nevertheless, the combination of AMPs with conventional antibiotics may represent a very interesting approach to improve TB treatment. Kalita et al. (2004) observed that combining HNP-1 with isoniazid, rifampicin or both, decreased the MIC of isoniazid/rifampicin against *M. tuberculosis* H37Rv and significantly reduced mycobacterial load *in vitro* and *in vivo*. Such results were suggested to occur due to HNP-1-induced increased permeability of the cell membranes. Similarly, it has been reported that protegrin-1 or human beta-defensin-1 combined with isoniazid resulted in a significant reduction of both susceptible and multidrug-resistant *M. tuberculosis* strains, compared to the peptides or the antibiotic alone (Fattorini et al., 2004). Recently, Khara et al. (2014) reported that the combination of peptide $M(LLKK)_2M$ synergistically interacted with rifampicin against *M. smegmatis* and BCG (most likely due to the enhanced permeabilization of rifampicin promoted by the peptide), and additively against *M. tuberculosis*.

In addition, AMPs are mainly bactericidal (which is highly desirable), induce a more rapid killing of pathogens and an increase in concentration is not required against resistant strains, in comparison with antibiotics (Marr et al., 2006). Moreover, as previously mentioned, although acquisition of antimicrobial resistance to AMPs has been observed, this is very rare since reconfiguring the bacterial membrane involves high metabolic costs and compromises its functionality (Nizet, 2006). Another advantage is that some AMPs are able to stimulate the innate immune response, thus helping in the activation or enhancement of the innate response, while reducing associated harmful inflammatory responses (Brown and Hancock, 2006).

Some authors have claimed that the large size of natural AMPs lead to high production costs and that the potential for microorganisms to develop resistance against AMPs may compromise our own natural defenses against infection (Bell and Gouyon, 2003). Nevertheless, different strategies have been described to address this issue, including the use of production methods that render higher yields, while decreasing manufacturing costs, discussed in Section 5.1. An alternative approach to reduce production costs is the synthesis of smaller, thus less expensive, peptides through conventional or solution-phase synthesis (Zhang and Falla, 2006). In this regard, the synthesis of smaller analogues of known natural AMPs, such as in the case of LL37, has been reported (Ciornei et al., 2005; Nagaoka et al., 2005). Indeed, several companies have adopted such approach over the past years.

AMPs are cell selective, a term related to the ability of a molecule to selectively kill microorganisms without causing toxicity to host cells, which in the case of these peptides is mainly a dose-dependent effect (Matsuzaki, 2009). Nevertheless, this cell selectivity may be improved: 1) an increased net positive charge of the peptide up to ~10 has been found to improve antimicrobial activity without affecting hemolytic activity (Zelezetsky and Tossi, 2006); 2) the replacement of amino acid residues in hemolytic peptides by its D-analogues has been reported by Shai and Oren, in studies performed with paradaxin, to improve selective toxicity (Shai and Oren, 1996); 3) the same authors also found that selective toxicity could be increased by cyclization of linear peptides (Oren and Shai, 2000); and 4) PEGylation, which consists in the attachment of a polyethylene glycol (PEG) moiety to peptides, was also found to enhance *in vivo* efficacy (Harris and Chess, 2003). In this sense, short synthetic AMPs have been designed to target *M. tuberculosis*, and *in vitro* selectivity indices (ratio of *in vitro* toxicity against microorganisms and THP-1 cells – a monocytic cell line) close to 250 were described (Ramon-Garcia et al., 2013).

Moreover, AMPs usually present low bioavailability, due to their vulnerability to protease activity, as well as systemic toxicity (Hancock and Sahl, 2006; Sanborn et al., 2002). Also, partly as a result of this limited bioavailability, it is difficult to target the AMPs to specific sites of infection. Nanoparticle-based drug delivery systems can be used to overcome these problems and their potential has actually been recognized for TB treatment (Gaspar et al., 2008; Gelperina et al., 2005). Nanoparticles provide enhanced stability, adaptation to different administration routes, ability to encapsulate either hydrophobic or hydrophilic drugs and sustained drug release. Therefore, they can increase bioavailability, while reducing dosing frequency, which can also be beneficial to improve patient compliance to the treatment (Gelperina et al., 2005). Currently, several drug delivery systems have been used as carriers for anti-TB drugs. For example, *in vivo* studies have demonstrated the improvement of antibiotic action when incorporated in liposomes (Gaspar et al., 2008). Also, Pandey et al. (2003) provided evidence for the sustained release of different antibiotics encapsulated in poly-(lactic-*co*-glycolic) acid (PLGA) nanoparticles after administration *via* inhalation to guinea pigs. As such, it is only reasonable to expect that loading of AMPs in these delivery systems will improve the therapeutic potential of AMPs. Furthermore, drug delivery systems-associated parameters like particle size, shape, surface chemistry or mechanical properties have been described to influence their uptake by macrophages (Ahsan et al., 2002; Champion et al., 2008). Thus, the delivery of AMPs loaded within these systems can be further optimized. Using polystyrene particles of different sizes and shapes, Champion and Mitragotri (2006) reported that particle shape at the point of initial contact with macrophages is crucial to determine if the particle is phagocytized. Particle size and charge also largely impact the efficiency of internalization by macrophages. Studies performed by Tabata and Ikada (1988) with polystyrene microspheres of various sizes showed that maximal uptake by macrophages occurred for particles within 1.0–2.0 μm. Moreover, it has been reported that macrophages phagocytize anionic particles more easily, which is most likely associated with their ability to recognize negatively-charged bacterial membranes (Lunov et al., 2011). On a different approach, coating of particles with opsonic materials and amphiphiles, such as gamma-globulin, fibronectin or gelatin, may significantly enhance phagocytosis (Ahsan et al., 2002), thus being able to facilitate AMP targeting to the intracellular milieu.

Proper choice of the administration route also impacts the efficiency and bioavailability of AMPs. Oral administration, usually associated to standard anti-TB drugs, provides a more systemic drug distribution, limiting its action on the actual target cells and tissues. Also, orally administered drugs often display limited bioavailability and poor absorption (Florence, 2004; Zumla et al., 2014). Systemic distribution is also attained after intravenous administration. In this case, drug-loaded particles are endocytosed by macrophages belonging to the mononuclear phagocyte system and by monocytes in circulation. This method may be particularly interesting to address TB treatment in the liver, since the Kupffer cells of the liver preferentially uptake these particles from the blood stream (Kayser et al., 2003). Given the existence of niches of *M. tuberculosis* where diffusion of antimicrobials from the blood stream is difficult to reach (*e.g.* biofilms in cavities, pneumonic forms in alveoli), aerogenic strategies for AMP administration may represent powerful and perhaps the most suitable approaches to TB treatment, provided adequate particle shape and size parameters are met. In addition, this

method allows attaining a higher concentration at the lungs. Recently, Kwok et al. (2015) developed inhalable dry powders containing two D-enantiomeric D-LAK peptides. Spray drying the AMPs with mannitol, which was used as a bulking agent, produced spherical particles with particle size adequate for inhalation. Most important, the AMPs preserved their secondary structures and the particles showed a good aerosol performance, suggesting the potential of this formulation for TB treatment *via* the aerogenic route.

Interestingly, a single subcutaneous treatment of *M. tuberculosis*-infected mice with three standard antibiotics loaded into PLG nanoparticles resulted in an improved anti-mycobacterial effect, compared to 35 daily oral administrations (Pandey and Khuller, 2004). These results evidence the subcutaneous route as a promising alternative for AMP delivery, as greater efficacy may be achieved with fewer doses.

Other approaches may also rise, as for example Steinstraesser et al. (2014) recently developed a new technique aiming at wound treatment based on the delivery of an AMP (in this case LL37) through skin electroporation. However, this approach may not be as adequate for TB treatment, since peptide targeting to the main sites of infection.

7. Concluding remarks

The declaration of TB as a global emergency by the WHO in 1993, followed by a worldwide increase in MDR-TB episodes in the more recent years, opened new opportunities and urged the need for the development of new and more effective medicines for TB treatment. Currently, some drugs are undergoing mid-stage clinical development, which means that there may still be a market for new classes of medicines that prove their efficiency against MDR-TB and less susceptible to the acquisition of resistance over time. Indeed, there are currently about 10 new AMPs in clinical trials for the treatment of bacterial and fungi infections.

AMPs are key players of the human innate immune system. Thus, from a medical and regulatory approval perspective, such molecules may have a more straightforward and cheaper regulatory pathway. Also, regulatory entities have realized not only the urgency in developing new drugs, but also the potential of antimicrobial peptides as therapeutic molecules.

A promising strategy for TB treatment may comprise the administration of AMPs in combination with conventional anti-TB drugs. This synergistic approach would result in enhanced killing of bacteria and prevention of drug resistance, as suggested by Yeaman and Yount (2003). On a different approach, Bauer and Shafer (2015) have suggested that finding a strategy to cripple AMP resistance systems may help ameliorate host defense. Also, the use of AMPs in vaccination has gained recent interest. Indeed, Cervantes-Villagrana et al. (2013) reported a significant improvement in immunization against *M. tuberculosis* strains after DNA vaccines containing β-defensin-2 sequences were used as an adjuvant in BCG vaccination.

In conclusion, AMPs hold a great therapeutic potential against tuberculosis, with several advantages over commonly used antibiotics. Recent changes to the regulations of new drug approval and a rising peptide market, pave the way for efficient new molecules, namely AMPs, provided their pharmacological properties are enhanced and issues like manufacturing costs, stability, toxicity and delivery are addressed.

Disclosures

The authors declare no conflicts of interest.

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