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Review

STRATEGIES TO COMBAT PERSISTENT INFECTIOUS DISEASES

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Abstract: Antibiotic failure is one of the most worrying health problems worldwide. Nowadays we are facing an international crisis where several issues are involved: new antibiotics are not being discovered any longer, resistance mechanisms become spread in nearly every clinical isolate of bacteria and the appearance of recurrent infections caused by persistent bacteria complicates the overcoming of infections. In this context, it has been explored new anti-infectious strategies against MDR and persistent bacteria as well as the rescue of FDA-approved compounds (drug repurposing). Among the highlighted new anti-infectious strategies we find anti-microbial peptides, anti-virulence compounds, phage therapy and new molecules. On the other hand, as drugs of repurposing that have been described, we have anti-inflammatory compounds, anti-psychotics, anti-helmintic drugs, anti-cancerous and statins.

Keywords: Anti-MDR strategies, anti-persistent treatments, drug repurposing

1. Introduction

Antimicrobial resistance is currently considered one of the principal threats to global public health by the WHO (World Health Organization) [1], especially because of the global spread of MDR (multidrug resistant) bacterial pathogens. MDR are pathogens that can develop resistance to different antimicrobials by gene horizontal transfer and by gene mutations as a consequence of the exposition to these agents. Although the resistance acquisition is a natural process, it has been exacerbated by the misuse of antibiotics, the inadequate surveillance and the poorly controlled regulations of antibiotics in clinical medicine and in the livestock industry, which has led to the appearance of MDR and their expansion all over the world [1,2][3].

Mortality caused by resistance to antibiotics is a major health problem, causing more than 23000 deaths per year just in the U.S. alone (CDC's *Antibiotic Resistance Threats in the*

United States, 2019 (2019 AR Threats Report)) and more than 33000 in Europe [4]. Infections account for 13 to 15 million deaths annually worldwide [5]. These alarming numbers are predicted to increase to ten million deaths worldwide by 2050, even if predictions in this field are difficult to make [6].

Rice *et al*., in 2008 [7], grouped for the first time the six species that contribute to the more frequent MDR bacterial pathogens and named this group "ESKAPE", standing for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp.* It is noteworthy that there are some other opportunistic pathogens, such as *Escherichia coli, Enterococcus faecalis* or *Burkholderia cepacia,* which can also become MDR strains able to cause severe infections.

Bacteria can evade the antimicrobial activity of antibiotics by three different but related mechanisms: resistance, tolerance and persistence [8]. Resistance is the ability of bacteria to grow under antibiotic pressure and is due to the inherited mutations which affect the efflux pumps or to the drug target [8,9]. These **resistant populations** can be found in every kind of environment: water, animals, inanimate surfaces, humans, plants or food [10-14]. Moreover, resistant bacteria are able to grow under antibiotic pressure, their resistant phenotype is inheritable and a significant increase in the minimal inhibitory concentration (MIC) of antibiotics is required for an effective killing [15].

However, a resistance phenotype is not always due to the acquisition of resistance genes or mutations in the bacterial genome, but it is often explained by the appearance of a persistent phenotype which includes the **tolerant populations** of bacteria and/or the presence of **persistent sub-populations** (known as "persister cells" or "persisters") within a population of susceptible cells (i.e. killed by an antibiotic at a concentration equal or inferior to the MIC). Bacteria exhibiting this persistent phenotype are able to overcome an antibiotic treatment but none of them affect the MIC of the drugs. Just as happened with resistance, tolerance and persistence were first observed shortly after the introduction of penicillin, as reviewed very recently by Windels *et al.* [16]. Antibiotics become then ineffective due to the lack of cellular metabolism: protein synthesis is stopped, as well as replication of DNA, transcription or cell wall synthesis. "Persisters" are non-growing, metabolically inactive, dormant bacteria that exhibit high levels of tolerance to antibiotics in a transient way and play a non-negligible role in chronic or recurrent infections [15], as they can survive not only antibiotic-therapy but also host immune responses. These dormant bacteria can indeed rapidly restore their wild-type phenotype (and become susceptible again) when drug pressure is removed, reactivating their metabolism. The signaling pathways involved in this "awaking" process are being further investigated [17,18]. Persister cells can survive in immune-compromised patients but also in patients where antibiotics do not effectively kill pathogenic bacteria by immune-evasion strategies [17]. Currently, there is strong evidence suggesting that the ability of bacteria to live inside some cells (as macrophages) and the formation of biofilms are two strategies associated with persistent infections [19]. Furthermore, clinical isolates from chronic infections by *Candida albicans* [20], *P. aeruginosa* [21] or uropathogenic *E. coli* [22] that have been under antibiotic pressure during a long period of time were also associated with persistent infections, exhibiting increased persister levels when compared to isolates from acute or early-stage infections.

The presence of persisters in common bacterial infections has been reported in patients and linked to relapses of infection: Mulcahy [21] and coworkers isolated clinical strains of *P. aeruginosa* from lung infections of cystic fibrosis patients and observed increasing persister levels during antibiotic treatment. Schumacher *et al.* [23] showed that in *E. coli*, high-persister *hipA* mutations, including *hipA7*, were selected over time in recurrent urinary infections; consistently, they also observed the importance of the *hipA7* mutation in persister formation *in vitro.*

On the other hand, tolerant bacteria also exhibit a resistance phenotype against antibiotic treatment. Tolerance is defined as the ability to survive transient exposure to high concentrations of antibiotics and it can be inherited or not [9]. Differently from persistent subpopulations, tolerant bacteria are metabolically active even if it has been shown that their vital processes are slow down [24].

Both tolerant and persistent cells are nowadays being underestimated by the scientific community even if there is evidence that they help the evolution of resistance in bacteria. Back in 1988, Moreillon and Tomasz [25] demonstrated that cyclic exposure of pneumococcus (*Streptococcus pneumoniae)* to high concentrations of penicillin selects for tolerant mutants, while resistant mutants evolve during exposure to low (but sustained) levels of penicillin. A decade after, Novak *et al.* established that the clinical isolates of pneumococcus that were vancomycin-tolerant had mutations in the *vncS* gene. They realized that streptomycin- or penicillin-tolerant *S. pneumoniae* mutant showed greater efficiency in transformation with DNA compared to a WT *S. pneumoniae* strain [26]. This fact consolidated the idea of tolerant bacteria facilitating the resistant-genes acquisition by horizontal transfer, such as transformation.

In 2017, two important experiments added some light in the driving of resistance evolution by persisters and tolerant bacteria: first, studies of long exposition of *Mycobacterium tuberculosis* to rifampin concluded that persisters are a source of *de novo* resistant mutants [27]; secondly, the group of Nathalie Balaban found that the evolution of increased tolerance or persistence confer mutations in *E. coli* populations under intermittent ampicillin exposure [28]. Windels and coworkers, in 2019 [29], discovered a strong, positive correlation between persister levels and the likelihood to evolve resistance in natural isolates and lab strains of *E. coli*. Even if these three findings used different types of antibiotics and different experimental conditions, they still provided consistent results, strongly suggesting a widespread link between persistence/tolerance to antibiotics and the evolution of resistance against these drugs. From now on, considerable efforts should be devoted to the development of strategies able to eliminate tolerant and persistent cells, as these have the potential to favor the evolution of resistance [29].

Given the immense area of possibilities to treat MDR and persistent bacteria from different points of view, it is time to take a look into what has been achieved so far, therapy by therapy. Thus, the present review aims at gathering the results from the most relevant studies, organized by type of administered drug or therapeutic strategy, in order to provide some help to researchers and doctors in their fight against infectious diseases caused by MDR and persistent bacteria.

Along this review, the most studied strategies used against MDR bacteria and/or persisters were presented as follows: one block of new therapies, including AMPs,

antivirulence compounds (such as QS inhibitors), phages and some new molecules that have been chemically synthesized, as vitamin-A derivatives (retinoids). On the other block, we have summarized the most important drug repurposing strategies (use of anti-inflammatories, anti-psychotics, anti-parasitics, anti-cancerous or statins) with a potential antibacterial effect. The next diagram summarizes these approaches (Figure 1).

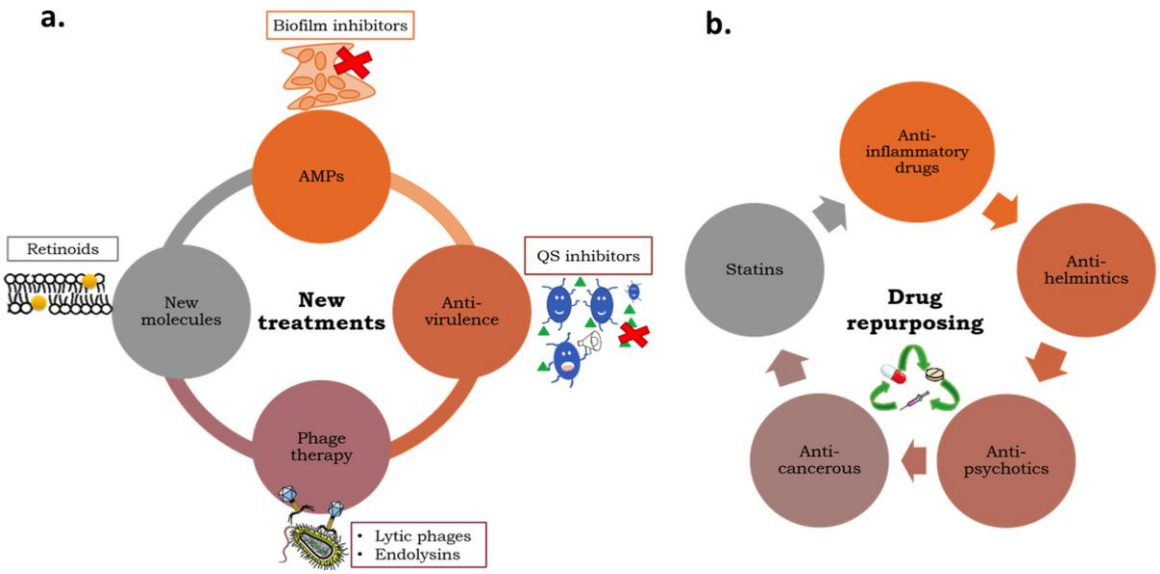

Figure 1. Anti-infectious strategies that are being nowadays investigated to fight resistant and persistent bacteria. (**a**) From the upper part and clockwise: AMPs: antimicrobial peptides, among which inhibitors of biofilms are found; QS: quorum sensing. The green triangles illustrate the acyl-homoserin lactones that bacteria secrete as QS modulators. Phage therapy includes all the clinical trials where a combination of lytic phages or parts of these (endolysins) are being used against bacterial infections with a therapeutic goal. Finally, new molecules harbor all the synthetic compounds showing any antibacterial activity, as retinoids (symbolized as yellow circles inserted in the membrane); (**b**) Repurposing encompass all the FDA- (Food and Drug Administration) approved drugs with potential antibacterial effects.

All these fields are gaining currency in the scientific community from some decades ago. In the era of multidrug and persistent pathogens causing severe infections against which we have no longer an effective cure, interest in these therapies or strategies is increasing, and a proof of that are two new research topics in which we have recently participated, focusing on the quorum network of MDR pathogens [30] and the drug-repurposing for bacterial and viral infections [31]. To provide some more light into the tendencies that have been worrying scientists around the globe, the next plot summarizes the number of annual publications available on PubMed search engine by decade, from the 40s of the nineteenth century until nowadays (Figure 2):

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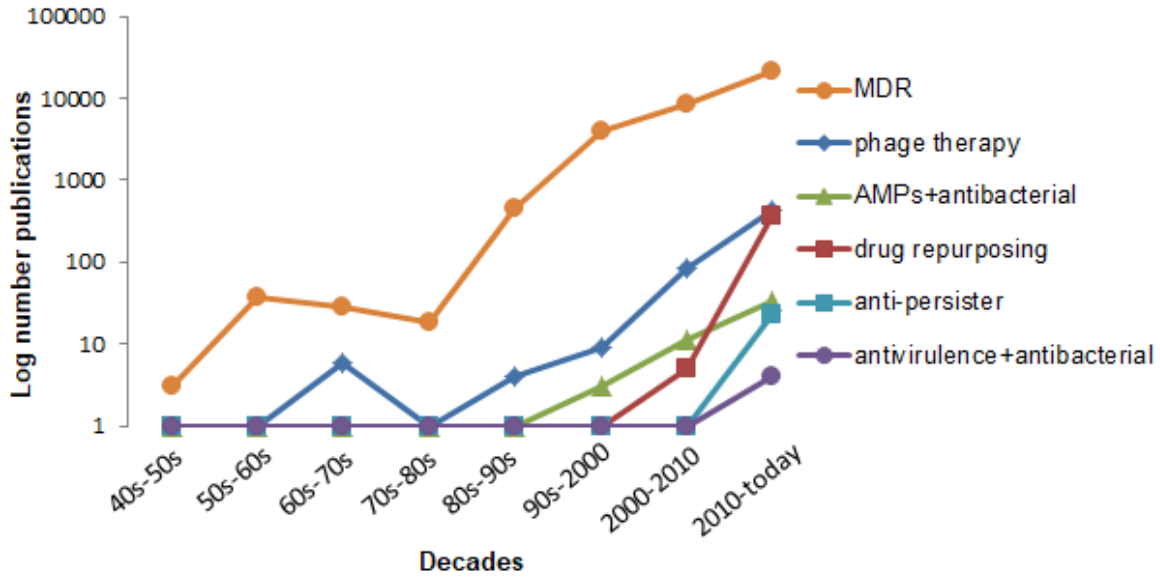

Figure 2. Number of annual publications available on PubMed search engine with the keywords "MDR OR multidrug resistance", "phage therapy", "antimicrobial peptides AND antibacterial", "drug repurposing", "anti-persister OR anti-persistence OR resuscitating drugs", "antivirulence AND antibacterial". The publications are expressed in logarithmic scale.

2. New anti-infectious treatments against MDR and persistent bacteria.

Among the new anti-infectious treatments against MDR and persistent bacteria we highlight in this review:

2. 1. Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are currently being studied and developed for the treatment of recalcitrant bacterial infections, in many circumstances due to persistent bacteria. Regarding the issues involved in this relatively new therapy, the synergy between AMPs and antibiotics together with the anti-biofilm activity of AMPs are of great interest. Many AMPs are naturally produced by a broad range of organisms while others are being newly designed and chemically synthesized in the laboratory.

Recently, Yang *et al*. [32] have characterized nine fungal defensin-like peptides (peptides synthesized by neutrophils with antimicrobial and cytotoxic properties) as potent antibacterial compounds. This team expressed them in *Pichia pastoris* and tested their antibacterial and anti-biofilm abilities against MDR and persistent *S. aureus*. Results showed that, among them, a defensin-like peptide called P2 exhibited the highest activity and expression level with low toxicity, no resistance, high stability and a MIC of < 2 μg/ml. P2 bound to bacterial DNA, wrinkled the outer membrane, permeabilized the cytoplasmic membrane and inhibited *S. aureus* biofilm formation. Importantly, P2 killed 99% persistent bacteria, which were resistant to 100×MIC of vancomycin. These data suggest that P2 may be a candidate for novel antimicrobial agents against MDR and persistent staphylococcal infections.

Li *et al.* [33] have characterized two novel peptides, P5

(YIRKIRRFFKKLKKILKK-NH2) and P9 (SYERKINRHFKTLKKNLKKK-NH2), that exhibited potent antimicrobial activities against both methicillin-sensitive *S. aureus* clinical isolates and methicillin-resistant *S. aureus* (MRSA) strains. Just as previously described for P2, these peptides did not show any significant hemolysis or cytotoxicity to renal epithelial cells. P5 and P9 significantly inhibited the biofilm of *S. aureus* and disrupted the cell membrane, in addition to the down-regulation of several virulence genes. P5 and P9 could be promising antibacterial agents for the treatment of MRSA infections.

Also this year, Liu *et al.* [34] investigated the potential applications of cationic peptides in the fight against vancomycin-resistant *Enterococcus* (VRE). They found some peptides displaying moderate bactericidal activity against VRE (with MIC values of 2-8 µg/ml) and a significant synergistic activity between these peptides and vancomycin. The mechanism of action of these peptides is the inhibition of *vanRS* transcription, a two-component system (TCS) key in the resistance against vancomycin. Researchers showed that the inhibition of *vanRS* transcription restored vancomycin activity. Consistent with *in vitro* results, scientists observed better survival rates of *Galleria mellonella* larvae when treated with these cationic peptides plus vancomycin compared with vancomycin alone. Taken together, these results suggested that cationic peptides did not only show antibacterial activity against VRE but also reversed vancomycin resistance in *Enterococcus*, providing good candidates to combat vancomycin-resistant pathogens.

2. 2. Antivirulence compounds

Antivirulence treatment targets bacterial virulence without interfering with their growth. In 2013, Pan *et al.* [35] characterized a chemical compound called BF8 ((*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one) able to reduce persistence during *E. coli* growth and revert the antibiotic tolerance of persisters. This BF8 is a QS inhibitor of *E. coli* which disrupted *E. coli* biofilms and rendered associated cells more sensitive to ofloxacin. BF8 appeared to be safe to mammalian cells *in vitro* and showed no long-term cytotoxicity in a healthy mouse model *in vivo.*

In November 2013, Conlon *et al.* [36] developed the acyldepsipeptide antibiotic, called ADEP4, able to activate the ClpP protease and therefore resulting in the death of bacterial persistent cells by degrading around 400 proteins. Furthermore, this ADEP4 was active against persisters both in planktonic and biofilm states. A combination of ADEP4 with rifampicin completely killed *S. aureus* biofilms *in vitro* and in a chronic infection murine model *in vivo.*

The first compounds able to reduce the formation of antibiotic-tolerant persister cells of *P. aeruginosa* were identified by Starkey *et al.* [37]. Some of these compounds, such as M64, blocked the synthesis of both pro-persistence and pro-acute MvfR-dependent signaling molecules. MvfR, also referred to as PqsR, is the global virulence QS transcriptional regulator of *P. aeruginosa*. M64 was active against MDR strains of *P. aeruginosa* that cause acute and persistent murine infections and did not perturb bacterial growth, which limited selective resistance.

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Concerning *E. coli,* it was the team of Frimodt-Møller [38] who was able to re-sensitize *E. coli* cells to antibiotics by inhibiting AcrAB-TolC pump. The AcrAB-TolC apparatus in *E. coli* increases the DNA mismatch repair system, which induces spontaneous mutations that can lead to high-level resistance to antibiotics. Frimodt-Møller and collaborators used inhibitors of the AcrAB-TolC efflux pump that not only sensitized *E. coli* cells to antibiotics but also were able to restore their mutation rates, potentially leading to improved treatment.

2. 3. Phage-therapy alone or in combination with antibiotics to treat MDR and persistent bacteria

It seems clear that some alternative antibacterial therapies need to emerge in order to stop the spread of MDR organisms. In this context, **phage therapy** is an antibacterial approach that involves introducing natural bacterial viruses (known as bacteriophages or phages) that infect and lyse bacteria to cure or prevent infectious disease [39,40]. These viruses are the natural predators of bacteria and, as it has been recently reviewed by Divya and colleagues [41], phages were a primary cure against bacterial diseases since their discovery in the 1900s, for 25 years, until they were absolutely eclipsed by antibiotics.

One of the advantages of phage therapy over broad-spectrum antibiotics is the high specificity toward target bacterial pathogens, without adversely affecting the host itself or the host commensal microbiota [42], thus minimizing secondary effects. An interesting issue in the phage-therapy is the synergy between phages and the host immune system [43]. Indeed, it is not in phages' best interest to kill every host bacteria in the infection site (otherwise they could not continue to replicate themselves), but they can be expected to bio-control the bacterial pathogens and significantly reduce their population, thus giving the patient's immune system the chance to eliminate the remaining pathogens. Let's give a few examples of phage-based treatments over MDR and persistent infections:

In 2017, one of the successful trials consisted of treating a 68-year-old diabetic patient with necrotizing pancreatitis complicated by an MDR *A. baumannii* infection with a personalized phage-based therapy consisted of 9 lytic phages that saved the patient's life [44]. Five days after the bacteriophage-based therapy was started (and therefore the infection was controlled), minocycline was administered and infection was finally overcome.

In 2011, Khawaldeh *et al.* [45] described the successful use of six lytic *P. aeruginosa* phages as adjunctive therapy to cure a recurrent bladder infection in a 67-year-old woman, most likely caused by a persistent subpopulation of *P. aeruginosa* cells. Antibiotics alone fail to cure the infection, as it is normally the case of recalcitrant, persistent bacteria. However, the combination of meropenem and colistin with this phage cocktail led to the symptomatic enhancement and a significant reduction of the bacterial load, while it was well-tolerated by the patient. The cocktail was applied every 12 h for 10 days and from the eighth day, the bacterial count decreased until no viable bacteria could be detected anymore.

Experiments using *P. aeruginosa* carried by Torres-Barceló's team [46] and Chaudhry's team [47] showed an improved therapeutic effect when the antibiotic was introduced once the phages had already started to fight bacteria. These two studies highlight the importance that must be given to the sequential application of both treatments in order to achieve a

better result, as it appears that bacterial pathogens become more vulnerable to some antibiotics after confrontation to the phages [48] and even that the phages can restore their sensitivity to antibiotics [49]. Importantly, phages can use bacterial efflux pumps as receptors, which would affect the clearance of antibiotics, one of the most spread resistance mechanisms of bacteria [48].

Blasco et al., in 2019, published a sophisticated assay describing an engineered lysogenic phage (Ab105-2phiΔCI) in which repressor CI was deleted, thus becoming lytic. This was the first study using a mutant lytic phage in combination with imipenem and meropenem antibiotics, and this combination resulted in a significant decrease of *A. baumannii* MDR cells both *in vitro* and *in vivo*, with lower MICs needed for these carbapenems to be effective against the resistant pathogen.

For the first time in 2007 it was described the anti-biofilm activity of phage phi11 endolysin, able to kill staphylococcal biofilms via its two endopeptidase domains [50]. Fenton and Shen's teams described lytic endolysins (CHAP(K) and PlyC) which successfully removed staphylococcal and streptococcal biofilms, respectively [51,52]. As biofilms are mainly constituted by persistent cells, the use of phage-derived endolysins may represent an effective "anti-persister" strategy. It is also noteworthy the endolysin PlyE146 [53], which displays lytic activity against *E. coli*, *P. aeruginosa* and *A. baumannii*, as these three bacteria are important nosocomial pathogens that represent a major danger for health. Finally, LysAB2 endolysin, described for the first time in 2011, showed activity against different bacterial species, such as MRSA, *A. baumannii* or *E. coli* [54]. Interestingly, peptide-induced modification of endolysin LysAB2 broadened the range of lytic activity [54].

2. 4. New molecules

One year ago, Kim *et al.* [55] described the antibacterial activity of two synthetic retinoids (vitamin A analogues), named CD437 and CD1530. These compounds exhibited high killing rates *in vivo* of both growing and persister *S. aureus* cells, by disrupting lipid bilayers. More in detail, their mechanism of action is the following: the carboxylic acid and the phenolic groups anchor these retinoids to the surface of the bacterial membrane bilayer, by strongly binding to hydrophilic lipid heads. As a result, the retinoids penetrate the bilayers and become embedded orthogonally to the lipid molecules in the outer membrane leaflet, inducing substantial perturbations and causing permeabilization of MRSA and persister membranes. Besides, both compounds showed synergism with gentamicin, a low probability of resistance selection and potent activity against a panel of clinical *S. aureus* and *E. faecium* strains. The major obstacle for developing retinoids as therapeutics is their potential cytotoxicity, which is a matter of considerable debate. However, none of the tested molecules exhibited cytotoxicity *in vitro* [55].

This discovery brings a broad range of possibilities to chemically change these molecules and synthesize more antimicrobial retinoid-based compounds, by studying and minimizing their cytotoxicity. Indeed, approximately 4,000 retinoid analogues have been synthesized so far [56]. It will be crucial to decipher their chemical properties and

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interactions between bacterial compounds and the candidate molecules in order to achieve a good antibacterial agent.

3. Repurposing treatments against MDR and persistent bacteria

In the era of MDR pathogens, where the discovery and development of new antibiotics are limited and unsatisfying, we need to employ new strategies to ensure the fight of infectious diseases. In this optic, the utilization of non-antibiotic compounds (drug repurposing strategy, also known as "repositioning") is of great interest. Focusing on drug repurposing strategies that have been tested on MDR bacteria, it has been reported that resistance is rarely crossed, normally because the active molecule affects a different target, independent of the antibiotic one. Moreover, time- and economic-related advantages of FDA-approved drugs have to be considered.

Among the repurposing treatments against MDR and persistent bacteria we describe in this review:

3. 1. Anti-inflammatories as antibacterial agents

By using *in silico* tools, Vijayashree *et al.* [57] have recently started to study the antibacterial effects of acetaminophen and ibuprofen (normally anti-inflammatory, anti-pyretic and analgesic drugs) against red-complex pathogens (*Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*), since these bacteria are associated with inflammatory conditions related to periodontal disease. Acetaminophen and ibuprofen were found to interact by *in silico* approximations with bacterial cytoplasmic proteins involved in cellular process, metabolism and virulence. The authors claimed that bioinformatic prediction tools revealed multiple epitopes in the virulent proteins that should be focused on by *in vitro* assays.

A well-known class of anti-inflammatory drugs is the group of glucocorticoids but its use in patients with sepsis is highly controversial because of their immunosuppressive effects [58]. Betamethasone is an anti-inflammatory steroid belonging to this class. Indeed, once in the nucleus, betamethasone stimulates the transcription and translation of lipocortin and vasocortin, two proteins that inhibit the release of inflammatory mediators such as prostaglandins, leukotrienes or histamine [59]. It might seem then contra-productive to use them in sepsis patients, as they mitigate the immune response, but actually, Emgard and co-workers showed that the topical steroid betamethasone was effective, on its own, for external otitis caused by infection with *P. aeruginosa* and *C. albicans* [60]. These findings could be explained considering that inflammation is a major mechanism in the development of external otitis, similarly to what happens in periodontal disease, described above.

Finally, we would like to include in this section a cathelicidin-like antimicrobial peptide, Cbf-K_{16}, characterized by Jiang *et al.* [61] and proposed as an anti-inflammatory and antibacterial compound able to effectively kill clarithromycin- and amoxicillin-resistant *Helicobacter pylori* SS1, both *in vitro* and in a gastritis mouse model. Cbf-K_{16} showed time-dependent killing kinetics, protection of *H. pylori*-infected gastric

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epithelial cells and inhibition of IL-8 secretion. Indeed, Cbf-K_{16} binds to genomic DNA, down-regulating the expression of adhesion genes (*alpA* and *alpB*) and the virulence gene (*cagA*), which makes this peptide a potential candidate for anti-infective therapy.

3. 2. Anti-psychotics

One decade ago, Lieberman and Higgins [62] screened the antibacterial activity of some compounds that affected the neurological function and identified 68 that disrupted the infection of macrophages by *Listeria monocytogenes.* After further examination of the compounds, the authors indicated that thioridazine (an antipsychotic drug that has been used to treat schizophrenia for 40 years) and bepridil (a calcium channel blocker) decreased intracellular infection by *L. monocytogenes* in a dose-dependent manner by significantly inhibiting vacuole escaping *in vitro*. Treatment of host cells with thioridazine or bepridil significantly decreased the ability of *L. monocytogenes* to escape the phagocytic vacuole, a step required to initiate the intracellular replication. Even if experiments were performed using the WT *L. monocytogenes* strain 10403S, it is an elegant example of drug repurposing strategy, as a calcium channel blocker can be as useful in the brain tissue as to combat bacterial pathogens. Thus, bepridil and thioridazine would be interesting compounds to be tested on persistent and resistant bacteria. Besides, antimicrobial activity of thioridazine against other bacterial species, such as *S. aureus* [63] and *M. tuberculosis* [64] has also been reported in the past. For the first case, scientists observed that just 0.1 mg/l of thioridazine completely inhibited the growth of *S. aureus* that has been phagocytosed by macrophages, suggesting an intracellular bactericidal activity. Nevertheless, thioridazine was withdrawn worldwide in 2005 because it caused severe cardiac arrhythmias in some patients (it binds to histamine receptors). However, there are still some generic forms of this drug available in the US.

It was Andersson's team [65] who described in 2016 the antibacterial effects of another antipsychotic drug, trifluoperazine. Although trifluoperazine MIC values were too high to be achieved in plasma and it did not affect the growth of *Yersinia pestis* cells nor the expression/production of their type 3 secretion system (T3SS) (an important virulent factor for this bacteria), this antipsychotic did increase the survival of *Y. pestis*-infected macrophages *in vitro* and the survival of infected mice *in vivo*. Trifluoperazine was then tested for both *Salmonella enterica* serovar Typhimurium and *Clostridium difficile* infection murine models, where once again it significantly increased the survival of infected mice. Interestingly, its antibacterial activity has not been described yet, but a significantly higher survival rate of infected mice has been reported for *Y. pestis, S. enterica* and *C. difficile.* It could be possible that its bactericidal activity is intracellular, as it turned to be the case for thioridazine, another antipsychotic drug with a similar structure.

3. 3. Anti-helmintic drugs

Salicylanilides are a well-studied group of antiseptics used worldwide. These compounds are thought to act as uncouplers of oxidative phosphorylation, thereby impairing the motility of parasites. Rajamuthiah *et al.* [66] described the efficacy of **niclosamide** (a bacteriostatic agent included in the salicylanilides family) against

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methicillin-, vancomycin-, linezolid- or daptomycin-resistant *S. aureus* isolates, probably damaging the bacterial membrane. Niclosamide inhibited QS and virulence genes in *P. aeruginosa* [67], such as phospholipase C, LasA protease, pyocyanin, chitinase, rhamnolipids… It also increased the negative charges of the cell walls from *A. baumannii* and *K. pneumoniae*, which results in synergy with cationic colistin, resensitizing both pathogens against this antibiotic [68]. These summarized experiments show consistent results while they were performed using four different bacterial species and conditions.

In 2016, Gooyit and Janda [69] reported that other members of the salicylanilides family, such as rafoxanide and closantel, presented greater bactericidal activity against the logarithmic and stationary phases of *C. difficile* than vancomycin. Continuing with anti-parasitic drugs, avermectins, a broad-spectrum class of anti-helmintics, demonstrated *in vitro* efficacy against *M. tuberculosis* and *Mycobacterium ulcerans* with MIC values ranging from 1 to 8 mg/l and 4 to 8 mg/l, respectively [70]. Ashraf *et al.* observed the bacteriostatic effect of ivermectin (one type of avermectins) against clinical isolates of *S. aureus in vitro* [71]. Ten years before, Zhang *et al.* [72] had described that ivermectin improved the survival of mice challenged by lethal doses of LPS, significantly reducing the levels of TNF-α, IL-1b and IL-6. Consistently, the same findings were obtained *in vitro.* Ivermectin also blocked the NF-$\kappa\beta$ pathway and reduced the endotoxemia (presence of endotoxins in the blood) and its associated inflammation.

3. 4. Anti-cancerous drugs as antibacterials

The use of anti-cancer agents to treat bacterial pathogens may seem surprising, but even if cancer and infectious diseases are seemingly different, drug-tolerant persisters are present in cancer cell populations as well, where they are implicated in tumor recurrence.

One year ago, Cheng *et al.* [73] used an *A. baumannii* strain resistant to most antibiotics (AB5075) and reported that 3 antineoplastics (5-fluorouracil, 6-thioguanine and pifithrin-μ), an anti-rheumatic (auranofin), an antipsychotic (fluspirilene), an anti-inflammatory (Bay 11-7082) and an alcohol detergent (disulfiram) inhibited the growth of a MDR *A. baumannii*. 5-fluorouracil and 6-thioguanine seemed to be the best candidates among all the repurposed drugs in the treatment of MDR clinical *A. baumannii*: their IC_{90} values and MIC were lower than standard plasma drug concentration levels in human, suggesting a possible use without major adverse events. The authors hypothesized that 5-fluorouracil may have the same inhibitory mechanism against bacterial pathogens as it has against tumor cells: inhibition of the thymidylate synthase. Similarly, 6-thioguanine may also share its mechanism of action over tumoral cells and bacteria, as it works as a guanine analog, disrupting DNA and RNA synthesis.

Antibacterial activity of metal gallium has been known for more than 80 years. Due to its chemical similarity to iron, gallium inhibits ferric redox reactions or pathways affecting bacterial growth. Gallium has a broad spectrum of activity, in particular against MDR ESKAPE pathogens [74,75]. In fact, a phase 2 trial in cystic fibrosis *P. aeruginosa*-infected patients [76] assessed the activity of gallium and provides evidence of its safety and efficacy for human infections, improving their pulmonary capacity without affecting the activity of essential human enzymes, as the superoxide dismutase or the aconitase.

Mitomycin C is also known to induce DNA cross-linking in a growth-independent manner, killing persisters and actively-growing cells of several pathogens such as *E. coli*, *S. aureus* and *P. aeruginosa* [77]. However, three years ago Chowdhury *et al*. [78] claimed that the anti-cancer drug cisplatin forms intra-strand DNA crosslinks and therefore eradicates *E. coli* K-12, *S. aureus* and *P. aeruginosa* persister cells through a growth-independent mechanism and more efficiently than mitomycin C, which forms inter-strand DNA crosslinks.

Finally, hormonal modulators used as anti-cancerous might also have a role in the anti-infectious fight. Selective estrogen receptor modulators (SERM) are a group of drugs widely used against breast cancer. Among this, we find clomiphene, nowadays in preclinical development for the treatment of fertility [79]. Clomiphene has shown efficacy against *S. aureus in vitro*, with a MIC value of 8 mg/l. Its mode of action is the inhibition of undecaprenyl diphosphate synthase (UPPS), an enzyme involved in the synthesis of the teichoic acid wall and peptidoglycan of *S. aureus.* It has also been described that due to its mode of action, clomiphene exhibits synergy with β-lactams in restoring MRSA susceptibility.

3. 5. Statins

Back in 2008, Jerwood and Cohen [80] observed that certain statins directly inhibited the growth of species belonging to genera *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Moraxella*, thus suggesting a possible antimicrobial activity of statins. In humans, statins inhibit the enzyme class 3-hydroxy-3-methyl-glutaryl-CoenzymeA reductase (HMG-CoA) leading to decreased synthesis of cholesterol and increased removal of low-density lipoprotein (LDL) circulating in the body [81]. As statins are lipid-lowering molecules which display pleiotropic effects, their potential antibacterial ability has been analysed:

Graziano and co-workers tested one type of statin, simvastatin, and observed no synergy between this latter and vancomycin [82]. However, simvastatin was able to reduce the formation and viability of mature biofilms, decreasing cell viability and extra-polysaccharide production. Simvastatin also exhibited a significant decrease in *M. tuberculosis* bacterial load, presumably by reducing cholesterol synthesis due to the inhibition of HMG-CoA reductase within the phagosomal membrane (reviewed in [83]).

Using a murine MRSA skin infection model, Thangamani *et al.* [84] confirmed that simvastatin significantly reduced the bacterial burden in the infected wounds, displaying an excellent anti-biofilm activity against established staphylococcal biofilms *in vivo*. Taken together, these studies suggest a potential bactericidal activity of simvastatin alone or in combination with topical antimicrobials currently used to treat MRSA skin infections.
One year ago, the antibacterial effects of statins *in vitro* were verified against skin pathogens as *S. aureus*, *E. coli*, *P. aeruginosa* and *Serratia marcescens* [85].

The following tables aim at recapitulating the anti-MDR bacteria (Table 1) and anti-persisters (Table 2) treatments that have been already described in the text above.

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Table 1. Anti-MDR molecules that have shown results against multidrug resistant bacteria.

Name of the drug	Type of drug	Active against	Mechanism of action	Reference
P5 and P9	AMPs	MRSA	Inhibition of biofilm, disruption of membrane integrity, down-regulation of virulence genes	[33]
Cationic peptides	Inhibition of TCS *vanRS*	VRE	Restoration of vancomycin activity	[34]
Inhibitors of AcrAB-TolC	Inhibition of AcrAB-TolC	MDR *E. coli*	Restoration of antibiotic activity and reduction of mutation rates	[38]
LysAB2	Endolysin from a phage	MRSA	Bactericidal activity against MRSA, *A. baumannii* and *E. coli*	[54]
Cbf-K_{16}	AMP anti-inflammatory	Clarithromycin and amoxicillin-resistant H. pylori SS1	Inhibition of IL-8, down-regulation of virulence and adhesion genes	[61]
5-fluorouracil and 6-thioguanine	Anti-cancerous	*A. baumannii*	Pirimidin and purin analogues	[73]

Table 2. Anti-persister molecules that have shown results against persistent bacteria.

Name of the drug	Type of drug	Active against	Mechanism of action	Reference (PMID)

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BF8*	QS inhibitor	*E. coli*	Disruption of *E. coli* biofilm, restoration of ofloxacin activity	[35]
M64	QS inhibitor	*P. aeruginosa*	Inhibition of PqsR, down-regulation of virulence genes	[37]
P2* (Defensin-like peptide)	Permeabilizer	*S. aureus*	Binding to DNA, inhibition of biofilm	[32]
phi11 endolysin	Endolysin from a phage	*S. aureus*	Disruption of S. aureus biofilms and bactericidal activity	[50]
CHAP(K)	Endolysin from a phage	*S. aureus*	Removal of staphylococcal biofilms	[51]
PlyC	Endolysin from a phage	*Streptococcus spp.*	Removal of streptococcal biofilms	[52]
PlyE146	Endolysin from a phage	*E. coli, P. aeruginosa and A. baumannii*	Disruption of *E. coli, P. aeruginosa* and *A. baumannii* biofilms	[53]
Cisplatin	Anti-cancerous	*E. coli* K-12, *S. aureus*, *P. aeruginosa*	Forms intra-strand DNA crosslinks	[78]
CD437* and CD1530*	Retinoids (analogues vit. A)	*S. aureus*	Disruption of membrane	[55]
ADEP4	ClpP activator	*S. aureus*	Degradation of hundreds of proteins	[36]

* Also efficient against MDR cells.

4. Discussion

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The first cause of deaths worldwide are microbial infections [86], and, among them, persistent infections have a big clinical impact. One of the main causes of these recurring infections is persistent cells [87]. As it has been said above, persistence to antibiotics might not be due to genetic change; instead, it is caused by metabolic inactivity and dormancy, strictly regulated by complex molecular processes such as ppGpp network, QS or toxin-antitoxin (TA) systems, all of them recently reviewed by Trastoy *et al.* [24]. Normally, strategies to avoid antibiotic-therapy failure focus on genetic resistance, while other bacterial survival strategies are increasing, like persistence or tolerance to antibiotics. Giving them a closer look would be necessary in order to combat persistent and re-incident bacterial infections caused by resistant pathogens. Theuretzbacher *et al.* [88] wrote a interesting review where they analyzed some of the new treatments that have been summarized all along the present review, together with vaccines and immune-modulators-based therapies.

Here we have put the focus first on new treatments (antimicrobial peptides, anti-virulence strategies, phage-based therapies and new molecules) then in drug repurposing assays (anti-inflammatory and anti-psychotics compounds, anti-helmintic drugs, anti-cancerous and statins). In what concerns antimicrobial peptides, they open a huge spectrum of possibilities as they are less prone to generate resistance than antibiotics and they can be chemically modified to enhance their antibacterial activity. Here, we have mentioned a couple of them (Table 1) but the mechanisms of action of several AMPs that are being currently investigated have been already reviewed by Kang *et al.* [89] and can be resumed as interference of the membrane or disruption of cellular processes. Another exhaustive review about the advances concerning AMPs is the one from Sierra *et al.* [90], where they mention that there are 20 AMPs currently being tested in clinical trials, going from preclinical stage to phase III, and mostly (but not exclusively) for topical indications. The vast majority of them are cyclic polycationic peptides.

Focusing on anti-virulence strategies, we have included here inhibitors of QS, inhibitors of global virulence regulators and inhibitors of pump-efflux, as well as an activator of ClpP protease. Much information about these anti-virulent compounds can be found in this exhaustive review made by Dickey *et al.* [91]. In the same optic, López and collaborators [92] took a deep look into the 26 patents that have been published from 1994 until 2012, including some inhibitors of adhesion and colonization, secretion systems or cellular signalling systems, among other virulence factors. This high number of patents in barely 2 decades reflects the increasing interest in AMPs and their antibacterial properties.

Concerning now the bacteriophages or parts of them (as endolysins reviewed in the text), it is well known that in western countries there are regulatory hurdles and legal problems in the use and administration of viruses as clinical tools. Among the factors that could explain the controversy of using phages as an antimicrobial tool in clinics, it is to note the complicated regulatory issues, the safety concerns and the scepticism about their therapeutic efficacy, for example, because of phage resistance evolution. Till these days, only 9 current trials using phage therapy are ongoing [93]. As Harper suggested in 2018 [94], an important factor worthy to be considered would be the choice of appropriate targets: the high species-specificity of phages can be desirable in monomicrobial infections but can limit polymicrobial infections. The phage-antibiotic synergy (PAS) has been proved

both *in vitro* and *in vivo* [95] and, even when benefits were not obtained, a minimizing in the emerging of antibiotic or phage resistant phenotype was remarkable. As reviewed by Torres-Barceló and Hochberg [96] and by Tagliaferri *et al.* [97], the combination of phage-therapy and antibiotics would have an increased benefit because of the improved bacterial clearance together with the reduced bacterial capacity of developing resistance to one or both therapies. Moreover, and just as happens with synthetic molecules with potential antibacterial effect, some biochemical modifications of phages or phage-derive endolysins can extend the range of susceptible organisms. The potential activity of bacteriophage endolysins to supplement or replace antibiotics is an exciting issue that has been already reviewed by Love *et al.* [98] and by Gondil *et al.* [99], where they also mentioned the safety of these endolysins in humans.

Given the time and economic problems associated with the development of a brand new drug, "the rescue" of drugs already approved by the FDA that exhibit antibacterial activity might be a suitable option to fight persistent infections. Non-antibiotics molecules can be effective in combination with other drugs or antibiotics, but further studies need to be pursued to determine effective concentrations that are clinically tolerated and safe. In terms of economic issues, we agree with the review published this year by Leyclit *et al.* [100] where they mention that pharmaceutical industries have little interest in re-profiling existing drugs due to the lack of profits. However, drug repurposing can present real economic advantages, as all studies about the structure and pharmacological properties (as bioavailability or safety profiles) had been already conducted [83]. Because toxicity and pharmacokinetics would be already known, it is possible to skip preclinical trials and start directly by clinical phase 2 to test their effectiveness [101], which also represents an advantage in terms of time.

Following with the drug repurposing strategy, Gupta *et al.* recently published a review [102] about some of the properties of farnesol, a QS molecule described in *C. albicans* that can be used as an anti-inflammatory but also as an anti-biofilm, anti-cancer and anti-tumor agent. Similarly, Liu and coworkers also reviewed the topic of triazines [103], nitrogen-containing heterocyclic molecules displaying a wide range of pharmacological activities, such as anti-bacterial, anti-malarial, anti-HIV, anti-cancer or anti-oxidants. Their anti-bacterial activity has been tested both *in vitro* and *in vivo.*

About antipsychotic drugs that have displayed an anti-infective activity, thioridazine and bepridil are the most promising compounds. Some antipsychotics can function as calcium channel inhibitors. As bepridil is also a calcium channel blocker, it is clear that calcium fluxes within host cells following infection by *L. monocytogenes* are implicated in bacterial entry and vacuole escaping. As it was the case for trifluoperazine, effective against *Y. pestis, S.* Typhimurium and *C. difficile*, these drugs may have broad-spectrum as many pathogens rely on similar mechanisms to modulate virulence or host pathways.

In what concerns anti-helmintic drugs, niclosamide seems to show the best results *in vitro* and it is efficient against a broad spectrum of pathogens (*P. aeruginosa, S. aureus, K. pneumoniae, A. baumannii, H. pylori*…). Niclosamide also showed therapeutic efficacy in an experimental infection model of *Galleria mellonella* larvae infected with *P. aeruginosa* and *H. pylori* [67,104], but further experiments in vertebrates *in vivo* need to be conducted to assess a proper dose without causing major adverse effects. The formulation of niclosamide under

nanosuspension showed lower toxicity in a rat lung infection model involving *P. aeruginosa*; the results of this study are potentially favorable for the further study of this formulation [105].

Finally, statins have also been tested as a possible anti-bacterial treatment, with good results due to their capacity of attenuating virulence factors, interfering with teichoic and lipoteichoic acids and disrupting cellular structures. In this review by Ko *et al.* [106] they compare several statins with each other and concluded, just as said above, that simvastatin shows the best results and seems to be an appropriate adjuvant antibiotic.

Thus, repurposing approved drugs may be highly effective against multiple antibiotic-resistant pathogens, taking into account the current (and increasing) problem of antimicrobial ineffectiveness and resistance [83,100].

5. Conclusions

In short, we believe that a possible via of study to fight persistent infectious bacteria would reside in analysing the coordination of several networks associated with molecular mechanisms of bacterial tolerance or persistence. The combination of new anti-infectious treatments, as well as drug repurposing alone or in association with antimicrobials, could be an efficient way to counter persistent infectious bacteria.

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