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The Future Challenges Facing Antimicrobial Therapy: Resistance and Persistence

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Abstract The emergence of resistance to antimicrobial agents is a pressing concern for human health that increases the need for the development of novel antimicrobial drugs. Antimicrobial resistance means that microorganism keep on growing even in the presence of a drug due to specific defense mechanisms [e.g. efflux-pumps]. Many of infectious diseases are difficult to be treated with antimicrobials not due to resistance but persisters [non-multiplying cells]. Distinction is important as persistent cells need an entirely design of new antimicrobial agents. Non-multiplying cells, do not cause overt disease but prolong the duration of therapy, increasing the chance of the emergence of resistance [i.e. bacterial or fungal biofilms and latent tuberculosis] resulting in therapy failure. Persisters are phenotypic variants of the wild type that present in all microorganisms which are able to survive antimicrobial treatment without acquiring resistance-conferring genetic changes and upon re-growth they produce a population of sensitive cells and new persisters. Persistence may arise spontaneously regardless to the presence of drug or environmentally induced due to starvation, DNA damage, oxidative stress and quorum sensing. Many approaches targeting non-multipliers would shorten the duration of therapy and decrease the emergence of resistance. Some depends on studying the effectiveness of the existing therapeutics against non-multipliers [i.e. pyrazinamide and gatifloxacin] and others depend on the discovery of new compounds targeting microbial genes that might be essential to non-multipliers viability or specific enzymatic or metabolic pathway [i.e. TG44 targets outer membrane of *Helicobacter pylori* and TMC207 targets proton pump of the ATP synthetase in *Mycobacterium tuberculosis*]. Clinical trials and studies are needed to produce a marketed antimicrobial agent active against both multiplying and non multiplying organisms and to know whether the approach of targeting non-multiplying bacteria is clinically relevant and will produce compounds that reduce the rate of emergence of bacterial resistance.

Keywords: *antimicrobial resistance, persisters, non-multiplying microorganisms*

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1. Introduction

Antimicrobials can be obtained from three sources: moulds or fungi, bacteria, or synthetic or semi-synthetic compounds. Antimicrobials may be for internal or topical use, and their function is either bacteriostatic [to inhibit the growth and the multiplication of pathogens] or bactericidal [kill them]. However, the distinction between Bacteriostatic and bactericidal drugs is difficult. As it depends on the concentration of the antimicrobial, the targeted bacteria, and the growth phase. Also, Antimicrobials may be divided into broad-spectrum and narrow-spectrum antibiotics [1].

Antimicrobials can affect bacteria by targeting certain vital processes in the bacterial cells or metabolism [2]. So, antibiotics can be divided into five major classes which are: **inhibition of cell wall synthesis** as β-lactams and glycopeptides, **inhibition of cell membrane function** as polymixins**, Inhibition of protein synthesis** as aminoglycosides, chloramphenicol, tetracyclines, macrolides, lincosamides and streptogramins, **inhibition of nucleic acid synthesis as** Fluoroquinolones and Rifamycins and **inhibition of other metabolic processes as** Sulfonamides and trimethoprim

From the previously mentioned antimicrobial mechanisms of action, we notice that they need metabolically active bacteria to exert their reactions. So, antimicrobials are more active against actively growing bacteria, than against non-growing cells [persisters] or spores. As persisters or metabolic resting bacteria show down-regulation for major biosynthetic pathways which results in the failure of acute infections chemotherapy and extending the duration of treatment.

The emergence of multi-drug resistant pathogens is considered as a great problem challenging treating infectious diseases that increases the need for the development of new antimicrobial agents. Few decades ago, bacterial resistance to antibiotics began to increase rapidly, demanding urgent action to avoid returning back to times before antibiotic discovery [3,4,5]. Despite of the medical need for new antimicrobials, clinical development of new antimicrobials is limited and most of the antibiotics used recently in the market are derivatives or modifications of existing antibiotic classes [6].

The aims of this review are to describe antibacterial resistance, mechanisms of resistance, difference between resistance and persistence, how are bacteria switch from the metabolically active state to the metabolically resting state, importance of persistent cells in clinical diseases, Antibiotics showing activity against non-multiplying bacteria and new strategies to target non-multiplying bacteria.

2. Antimicrobial Resistance:

Antimicrobial resistance is defined as the ability of a microorganism to withstand the effects of biocides or the agents that are intended to kill or control them. A microorganism is considered to be resistant in 3 situations when it is not killed or inhibited by a concentration attained *in vivo*, or it is not killed or inhibited by a concentration to which the majority of strains of that organism are susceptible or it is not killed or inhibited by a concentration acting upon the majority of cells in that culture.

Increasing drug resistance implies that people with different infections can no longer be effectively treated, epidemics due to resistant strains will spread, prolonged and a lot of people become at a high risk of getting infected and die. Also, it results in a great expensive problem. Resistance may be emerged due to some factors as antibiotic overuse, abuse, misuse, incorrect diagnosis, the use of counterfeit drugs [7,8].

No single mechanism of resistance is considered to be responsible for the observed resistance in a bacterial organism. In fact, several different mechanisms may work together to confer resistance to a single antimicrobial agent.

Bacterial resistance may occur due to one or more of these strategies:

[1] Prevention of the antimicrobial agent from reaching its target by reducing its ability to penetrate into the cell.

[2] Expulsion of the antimicrobial agents from the cell via general or specific efflux pumps.

[3] Inactivation of antimicrobial agents via modification or degradation.

[4] Modification of the antimicrobial target within the bacteria by mutation, by specialized enzymatic changes [9,10].

Resistance and Persistence:

Microbes were found to be capable of entering a dormant state where all growth and reproduction processes ceases in which bacteria can tolerate extremely high doses of antimicrobials without becoming resistant or undergoing genetic changes. On the other hand, antibiotic resistance means that bacteria have certain defense mechanisms that keep them growing even in the presence of an antibiotic [11].

Distinction between persistence and resistance is very important. As persisters need antibiotics of different designs and new targets. In addition, persistent cells may be cultivable or non-cultivable. So, Persisters are difficult to be studied and isolated from normal growing cells [12]. Administration of antibiotics results in killing of multiplying bacteria but not non-multipliers, resulting in an incomplete eradication of the total population of microorganisms which leads to the administration of repeated-dose regimens associated with poor patient compliance. Repeated dose regimens of antibiotics for long time leads to the emergence of drug resistance. Also, non-multipliers can switch on multiplying bacteria, causing recurrent diseases as that seen in tuberculosis, bacterial endocarditis, sore throat and infected eczema [13,14,15].

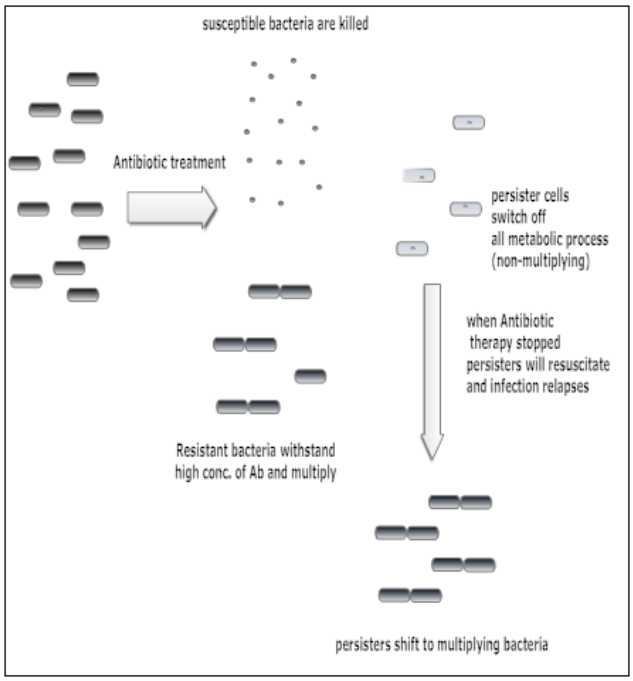

Figure 1. The figure shows that susceptible bacteria were killed by antibiotics while resistant cell can withstand the effect of an antibiotic through specific defense mechanism allowing the bacteria to replicate. Persisters cells [non-multiplying] can tolerate antibiotics. When Antibiotic therapy stopped, bacteria resuscitate and multiply

How are bacteria switch from the metabolically active state to the metabolically resting state:

Persistance was first discovered by Hobby *et al.* [16], who found that 1% of *Staphylococcus aureus* cells were not killed by penicillin and became persister cells. In 1944 Joseph Bigger reported that 99% of the bacteria can be killed by penicillin but the remaining 1% of the bacteria were found to be persisters. when the remaining cells were cultured in fresh media, they regained susceptibility to antibiotics. Also, Bigger was the first to term antibiotic-tolerant cells as persisters [17].

Bacteria enter the metabolic resting states to overcome the undesirable environmental conditions. In these metabolic resting state, most of metabolic pathways as cell wall and protein synthesis are down-regulated resulting in the decrease of their susceptibility to antibiotics and the increase in their ability to persist during chemotherapy [18,19].

Many researches discuss the mechanisms of persister cells formation, some reported that the rate at which persisters form is a function of inoculum age in that older inocula have more persister cells. Some showed that the degree of persistence depends on the type of antibiotic used [20]. The most discussed persister formation in terms of genetic basis, in which toxin-antitoxin [TA] pairs are primarily responsible for the induction of dormancy [21,22] that enables cells to escape the effects of antibiotics.

The Toxin-Antitoxin [TA] Model

TA systems [23] consist of a stable toxin [always a protein] that disrupts an essential cellular process [e.g., translation via mRNA degradation] and a labile antitoxin [either RNA or a protein] that prevents toxicity [24]. There are 5 types of RNA antitoxins. RNA antitoxins known as type I if they inhibit toxin translation [antisense RNA]. Type II antitoxins proteins if they inhibit toxin activity by

direct protein-to-protein binding [25]. type III if they inhibit toxin activity by binding the toxin protein. Type IV protein antitoxins if they prevent the toxin from binding its target instead of inhibiting the toxin directly [26], and type V antitoxins if they cleave the toxin mRNA specifically [27].

Moyed and Bertrand 28 isolated a strain of *Escherichia coli* showing a thousand fold increase in persistence. A mutation, named hipA7, was mapped to a gene encoding the HipA toxin [29] of the hipBA toxin–antitoxin module. Toxin–antitoxin [TA] modules are formed of pairs of genes, usually located in the same operon. One of these genes acts as a toxin that down-regulates transcription and translation and the other gene reverses its effect. Firstly, TA modules were found on plasmids [30] but later, new TA modules, were found in the chromosomes of bacteria. Fifty TA modules were found on the chromosome of *Mycobacterium tuberculosis* alone [31]. The action of hipBA module is to control the expression of HipA toxin and the HipB antitoxin genes. Overexpression of the toxin gene leads to growth arrest that reversed by the expression of the antitoxin gene [32,33,34,35] which confirm that the hipBA TA module plays a key role in bacterial persistence.

Moreover, there are a lot of ways to form persister cells. For example, TisB/IstR-1 system, in which over expression of the toxin TisB is effective for inducing persistence in the exponential phase but is not effective in the stationary phase. TisB toxin causes the cells to enter the dormant state by decreasing the proton motive force and ATP levels, [36]. TisB toxin producing cells showed persistence to different types of antibiotics such as ampicillin, ciprofloxacin, and streptomycin.

Another TA module is MqsR/MqsA [37,38,39], in which MqsR toxin cleaves most of the transcripts in the cell stopping translation. By using DNA microarrays, it was observed that MqsR causes persistence by decreasing the ability of the cell to respond to stress [40].

Dörr and Lewis [36] also suggested that cells try to do at least two things when stressed, the first is to activate genes to respond to the stress and the second is to convert part of the population to a dormant state, which is an alternative strategy that allows a fraction of the population to survive the stress through inactivity. These two different responses are important for pathogens to resist host-related stresses [oxidants, high temperature, low pH, and membrane-acting agents].

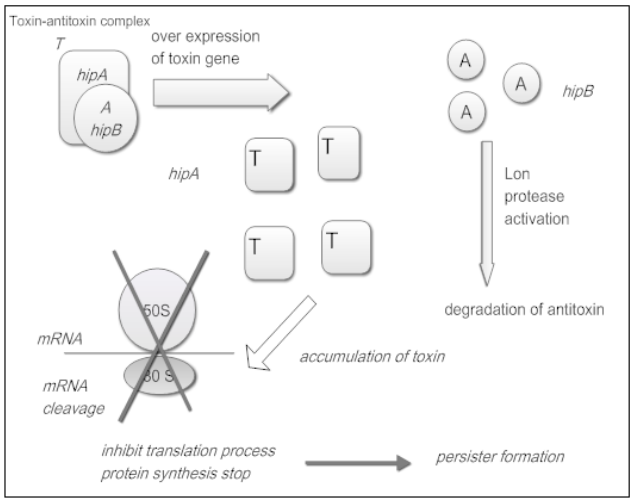

Figure 2. Toxin-antitoxin module

Factors that can affect TA system:

To activate TA systems, First, the cell should respond to stress and this stress response is most likely transferred to TA systems by certain molecule know as guanosine tetraphosphate [ppGpp]. ppGpp is produced via RelA and SpoT [which can also degrade ppGpp] during nutrient limitation [i.e., the stringent response] and other stresses e.g., acid stress. ppGpp can change transcription due to direct interactions with RNA polymerase and by its activation of RpoS, the stress response sigma factor for the stationary phase, and RpoE, the stress response sigma factor for misfolded proteins in the periplasm. ppGpp also directly reduces DNA replication and protein synthesis [41].

ppGpp was first definitively linked to persistence in 2003 via the HipA toxin. Using an *E. coli* strain with the gain-of-function mutation *hipA7* in which persistence is increased 1,000-fold, it was shown that persistence conferred by the HipA7 allele was both diminished by *relA* knockout and eliminated by *relA spoT* mutation. Therefore, ppGpp is required for HipA7 to increase persistence [42].

Kwan *et al.* [43] also demonstrate that persistence can be induced by extracellular stress factors like antibiotics. By testing the effect of some antibiotics on persister cell formation, it was found that a major route to persistence is via activation of toxins [a group mimicked a type II endonuclease toxin [e.g., MqsR]. By treating an initial population of 0.01% with rifampin [affect transcription], a 10.000 fold increase in persister cells was achieved [100%]. Hence, cells that are not producing protein are persisters. The cells also pretreated with tetracycline [which alters translation], nearly 100% of the cells were converted into persister cells. Similarly, carbonyl cyanide *m*-chlorophenyl hydrazine [affect ATP synthesis] converted nearly 100% of the cells into persister cells. The same results were observed also with ciprofloxacin [5µg/ml] and ampicillin [100 µg/ml]. So, we can say that pretreatment of cells by antibiotics only reduced the viable cell population by about one half, so the dramatic increase in persister cells was achieved by converting nearly all of the initial exponential culture into persisters which revealed that persister cells lack protein synthesis [43].

PhoU

PhoU, a new persister gene, was identified through a transposon-based screen in *E. coli*. PhoU is a negative regulator of phosphate metabolism in *E. coli*. Deletion of *phoU* results in a metabolically hyperactive state with increased expression of numerous genes involved in energy production. While deletion of *phoU* does not affect the initial percentage of persister cells, the *phoU* mutant persisters die more rapidly in the presence of ampicillin, with 100- fold-reduced CFU/ml after 3 h in comparison to their occurrence in the wild type. PhoU mutant shows high susceptibility to different antibiotics as ampicillin, streptomycin, sulfa drugs, and quinolone drugs. Also, mutants are more sensitive to conditions, such as heat, starvation, acidic pH, and weak acids. PhoU was found to be a suppressor for cellular metabolic activity. Its expression results in shutting down the cellular metabolism but its mechanism is not clear [44].

Are persister cells considered as dormant state or a state of active response to a stress?

It has been argued that the study of Wakamoto *et al.* [45] with *Mycobacterium smegmatis* implies that persister cells

are not dormant. This study showed that cells surviving lethal treatment with the prodrug isoniazid were metabolically active. However, isoniazid requires activation by the catalase KatG, so the cells tolerant to isoniazid were simply cells with low levels of KatG activity. In effect, these tolerant cells were never exposed to a lethal antibiotic treatment because the isoniazid remained inactive. Hence, the metabolic activity observed for cells surviving isoniazid is not indicative of metabolic activity in persister cells and this report is a special case of a prodrug requiring activation. Therefore, there is little evidence indicating that persister cells are not dormant but a wealth of evidence indicating that persister cells are dormant. Although Balaban *et al.*, [46] reported that spontaneous *E. coli* persisters have been shown to be growth arrested, a microfluidics-based study following the fate of individual Mycobacteria during antibiotic treatment found that the apparent stability of persister numbers was in fact due to a dynamic state of balanced death and division, rather than generally arrested growth [45].

Persisters are dormant or an active response to stress, has become a fashionable question in most of studies dealing with persistence [47,48,49]. It is better to understand the genetic mechanism by which these cells are converted to the dormant state. As cells respond to stress in an active manner (via genetic circuits) only as a means to achieve dormancy. It is important to know what these circuits are.

Persistence by self-produced and interspecies signaling [indole production]:

In a recent study done by Vega *et al*., [50], he showed that the signaling molecule indole controls persistence in the intestinal bacterium *E. coli* by activating stress conditions. Also, he reported that Indole can act as an interspecies signal to control antibiotic tolerance in mixed infections with non-indole producing strains as *Salmonella typhimurium.*

Bacteria under stress conditions (environmentally induced persistence):

The SOS response is a stress response mechanism which is induced by Oxidative stress and DNA damage, such as that caused by the treatment with paraquat, hydrogen peroxide, or sub-lethal doses of antibiotics promote persistence to fluoroquinolone antibiotics in both *E. coli* and *P. aeruginosa* [50,51,52,53]. This response was found to be involved in persistence. So, interference with this process may be another drug target [36,51,54]. Similarly, bacterial envelope stress has been associated with enhanced persister formation [55,56]. Heat shock, for example, increases the survival of both *Acinetobacter baumannii* and *P. aeruginosa* in the presence of aminoglycosides or β-lactams, respectively. As Genes regulating the heat-shock response have been reported to play a role in tolerance to aminoglycosides [55,57]. Also, signaling mediated by soluble quorum sensing [QS] molecules including phenazine pyocyanin in *P. aeruginosa*, peptide alarmones in *Streptococcus mutans*, and indole [a stationary-phase QS molecule secreted and sensed by a wide range of bacteria] were found to play important role in persister cells development [50,52,58,59]. Nutrient starvation and diauxic carbon-source transitions can also induce persistence [46,60,61,62].

Under stress conditions, translation of proteins by ribosomes were stopped and incomplete polypeptides are formed which are toxic to the bacterial cells. Bacteria resolve this problem by the addition of tmRNA to the stopped mRNA [63,64]. As tmRNA binds to SmpB and EF-Tu forming a complex. This complex [alanyl tmRNA/SmpB/EF-Tu complex] can recognize the stopped ribosomes at the 3′end of an mRNA without stop codon. Translation restarts again using tmRNA as a message in which the tmRNA-encoded peptide tag started to be added to the C-terminus of the incomplete polypeptide. The tagged protein and mRNA are then degraded by proteases and RNases, leading to save of the stopped ribosomes in a process known as trans-translation process.

Importantly, data suggest that in some cases, bacterial persistence that appears spontaneous may in fact have been environmentally induced. Specifically, maintenance of *E. coli* cultures in log phase through repeated reinoculation leads to the loss of a detectable persister population. These data suggest that persisters found in actively growing, low-stress populations may in fact represent leftover cells from high-stress stationary-phase inoculums rather than actively growing bacteria that have switched phenotypes spontaneously [33]. Together, these data underscore the role of environmental parameters such as nutrient availability, population density, and oxidative stress in the modulation of the persistence levels.

From the above we notice that, persistence increases by the response of cells via genetic circuits, as a result of extracellular factors such as that shown by the effect of antibiotics like ciprofloxacin [a bactericidal antibiotic shown to induce the toxin TisB at sub-inhibitory concentrations] [36], indole [50] an interspecies [64,65,66] and interkingdom signaling molecules [67].

3. Mechanisms of Survival

Most stress responses lead directly or indirectly to a slowing or stalling of bacterial growth and division. This slowed or arrested cellular growth has been suggested as the major factor underlying drug persistence, given that the ability of antibiotics to kill bacteria is generally proportional to their growth rate [68]. Reduced growth rates have indeed been correlated with increased drug persistence both in vitro [33,69,70] and in vivo, when bacterial growth tapers over the course of an infection due to immune pressure or lack of nutrients [71]. The reduced rates of DNA replication, translation, cell-wall synthesis, and metabolism directly targeted by antibiotics have been assumed to account for the relative drug tolerance of dormant bacteria. Although stalled biosynthesis probably promotes persistence, its effects are difficult to untangle from those of accompanying stress-response processes, and it is becoming clear that many active cellular processes occurring in parallel with growth-rate reduction are central for cellular survival in a toxic environment. For instance, a number of active intracellular detoxification mechanisms that can be triggered by stress play an important role in persistence. Multidrug efflux pumps are up-regulated in response to various cues, including oxidative stress and [72], and can contribute to both *in-vitro* and *in-vivo* persistence. For example, paraquat-induced persistence in *E. coli in-vitro* is dependent on the AcrAB-TolC multidrug efflux system [53]. Similarly, drug tolerance in a Mycobacterial infection model was reported to depend on drug efflux pumps induced by

macrophage-mediated oxidative stress [73]. In some cases, persisters exhibit a reversible defect in drug uptake [74]. Beyond active efflux, detoxification can take other forms. For example, the exposure of bacteria to antibiotics can enhance their synthesis of nitric oxide, which chemically modifies certain drugs and inactivates them [75,76]. In Mycobacteria, stress can also promote alternative metabolic pathways that produce lower levels of reactive oxygen species [ROS], thus elevating the threshold for antibiotic-mediated death [77]. Finally, generation of ROS following antibiotic exposure has been proposed to contribute to the lethal effects of antimicrobials [78,79,80,81]. Although the role of ROS in antibiotic-mediated bacterial killing has recently become a matter of debate [82,83], active mechanisms of oxidative stress relief have been found to promote drug tolerance. For example, activation of Catalase and superoxide dismutase, antioxidant enzymes, promotes stress induced drug persistence and biofilm-associated persistence in bacteria and fungi [46,76,84,85]. In addition to detoxification, stress can promote active microbial mechanisms of physical sequestration. For instance, bacterial surface adhesiveness and the subsequent formation of protective biofilms can be enhanced in response to sub-lethal antibiotic treatment and other stresses [86,87]. Although tolerance mechanisms in biofilms are still a subject of intense investigation, it is clear that mechanisms other than dormancy are at play [88].

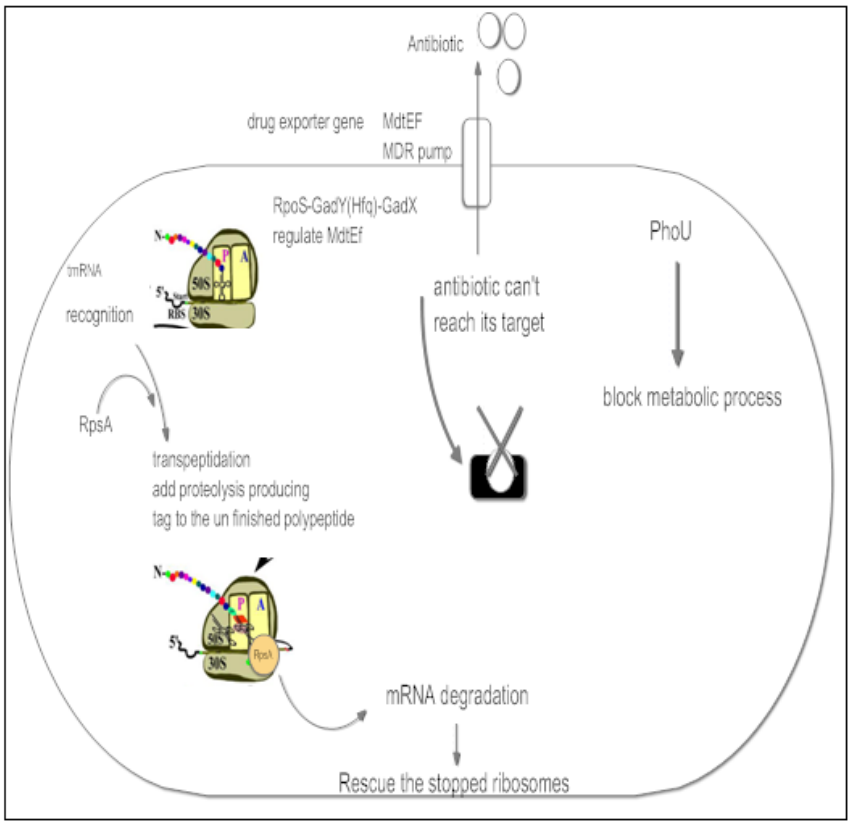

Figure 3. A view on the nature of persisters and their formation

Uncultivable bacteria and dormancy:

Over 99% of all species that are present in the environment fail to grow on laboratory media [89]. Many studies observed that uncultivable organisms can grow on nutrient medium *in-vitro* in the presence of its natural environment components and other species from the same environment [90]. These findings might indicate that growth depends on substances [perhaps signalling molecules] that give an indication of the presence in its familiar environment. Most bacterial species were found to grow in a specific environment and can not grow when placed in a synthetic medium. So, it can be said that uncultivable cells enter into a dormant state when transferred to an unfamiliar environment. Dormancy might be the default form of most bacterial life in the absence of its natural habitat and a protective tool.

Uncultivable cells were observed in populations of *Mycobacterium tuberculosis* cells. These cells are small ovoid or coccoid in shape with intact cell walls and unobservable respiratory activity, it can be regarded as dormant cells. These cells shows low ability to grow on solid media. Also, very small population of these cells can be cultivated in liquid medium, which is characterized by transition from ovoid to rod-like cell shape bacteria. It was found that the resuscitation of dormant cells in liquid media is due to the effect of substance[s] secreted by actively growing cells as bacterial growth factor Rpf [91].

On the other hand, Uncultured organisms have recently been reported to produce interesting compounds with new structures/modes of action—lassomycin, an inhibitor of the essential mycobacterial protease ClpP1P2C1 [92], and diverse secondary metabolites present in amarine sponge *Theonella swinhoei* which are actually made by an uncultured symbiotic Entotheonella spp. [93]. teixobactin, discovered in a screen of uncultured bacteria. Teixobactin [a new antibiotic] inhibits cell wall synthesis by binding to a highly conserved motif of lipid II [precursor of peptidoglycan] and lipid III [precursor of cell wall teichoic acid]. It is not active against bacteria with an outer membrane such as gram negative pathogens, particularly

carbapenem resistant enterobacteriaceae, or those with New Delhi metallo- beta-lactamase 1 (NDM1). Teixobacin is a product of new species of b-proteobacteria provisionally named *Eleftheria terrae* [uncultivable bacteria] showed good activity. The genome of *E. terrae* was sequenced. Based on 16S rDNA and in silico DNA/DNA hybridization, this organism belongs to a new genus related to Aquabacteria. The researchers did not obtain any mutants of *Staphylococcus aureus* or *Mycobacterium tuberculosis* resistant to teixobactin. The properties of this compound suggest a path towards developing antibiotics that are likely to avoid development of resistance [94].

Biofilm and persister cells formation:

Biofilm is defined as structured communities of microbial species embedded in a biopolymer matrix on either biotic [living tissues] or a biotic [inert non living material] substrata [95]**.** It was found that exopolymer matrix act as a penetration barrier to nutrients and oxygen for deeper cells in the biofilm mass. In addition, the accumulation of metabolic products and wastes resulting in the conversion of bacteria to the dormant state in which all metabolic processes are down-regulated [96]. Microbial cells embedded in biofilms or those in the stationary phase developed in media which are deprived of certain nutrients and subjected to QS signaling, are found to show persistence at levels higher than those observed by logarithmically growing organisms [33,97].

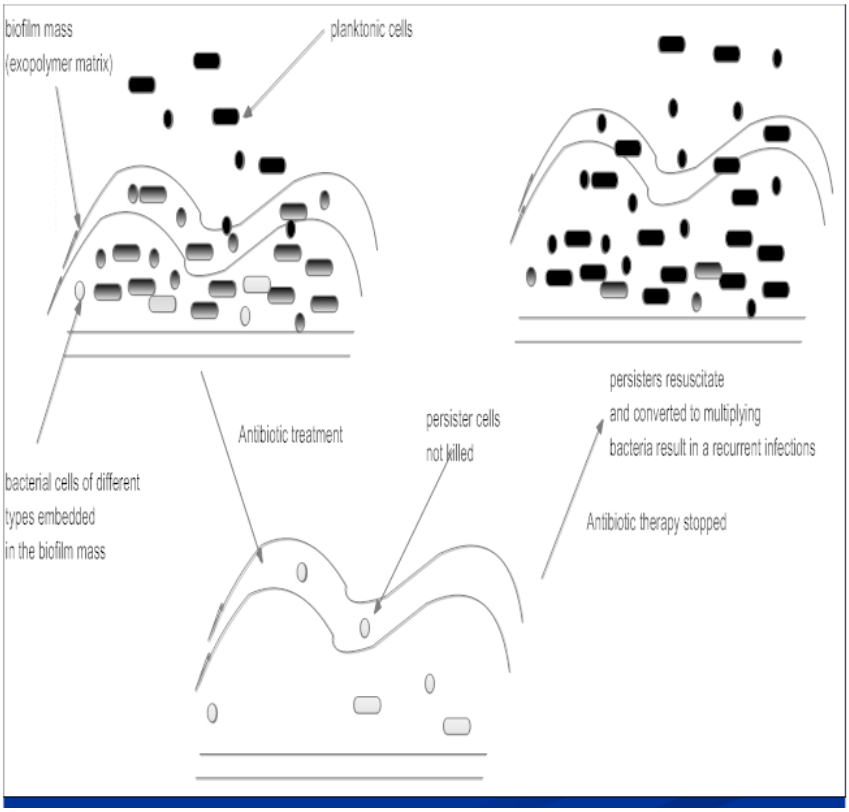

Figure 4. Resistance of bacterial biofilms to drug therapy due to the presence of persisters

Importance of persistence in clinical diseases:

Most bacterial diseases are not completely cured due to the presence of non-multipliers [98]. Targets for non-multiplying bacteria in tuberculosis are well recognized and the treatment of tuberculosis with rifampicin, isoniazid, ethambutol and pyrazinamide achieves cure in approximately six months [13]. Infections with these organisms require treatment with unusually protracted courses of combinations of antibiotics. Mtb therapy can last up to 12 months, and NTM, such as Mycobacterium avium complex (MAC), can require up to 2 years of treatment. Furthermore, relapse rates of mycobacterial infections are found to be high, indicating the importance of persistent sub-populations that can't be detected using current diagnostic technology [99]. In addition, detailed studies of infection in mice revealed that treatment of Mtb infections reduces organism titers, but fails to sterilize the animal [100]. Treatment of mice Mtb infections reach a plateau during which numbers of viable bacteria are stabilized. In addition to the mouse infection model, the inability to sterilize the infected subject has been observed in the zebra fish [*Mycobacterium marinum*], guinea pig [*M. tuberculosis*], and macrophage [*M. tuberculosis*] infection models [101]. The ability of *M. tuberculosis* to enter in the dormant state, where intact cells lie dormant and survive despite exposure to bactericidal concentrations of antibiotics, may contribute to the need for long and complex treatment regimens to eradicate TB infection. Also, some studies demonstrate that bactericidal antibiotics with a variety of different mechanisms of action increase ROS production within cells via the Fenton reaction (Fenton's reaction term means that several metals have a special oxygen transfer properties which improve the use of hydrogen peroxide) [79,80,81,82]. Numerous ROS, and in particular hydroxyl radicals, are toxic to cells and can result in cell death.

Tolerance to antibiotics may therefore depend on the ability of the cell to defend itself against ROS, as suggested by several recent studies [46,75,76]. For example, the coordinated stringent response to nutrient limitation in *P. aeruginosa* and *E. coli* was shown to increase antioxidant enzyme expression and decrease

production of prooxidant molecules, resulting in antibiotic tolerance [54]. Bacteria also produce nitric oxide [NO] as well as hydrogen sulfide (H2S), both of which result in antibiotic tolerance via suppression of the Fenton reaction as well as increased antioxidant enzyme expression in both Gram-positive and Gram negative bacteria [36,50].

Non-multipliers exist in most of bacterial diseases [99], especially in biofilms such as urinary tract infections in catheterized patients which is difficult to be treated by antimicrobials alone and needs the removal of the catheter. Biofilm resistance to killing by antibiotics is mainly based on persisters included in the biofilm matrix. As, initial treatment with antibiotic kills the metabolic active bacteria in both planktonic and biofilm population, immune cells can kill planktonic persisters but persiters embedded in biofilm matrix are protected by exopolymer matrix. By discontinuing chemotherapy, persisters can resuscitate and infection relapse occurs [63,103]. e.g. Recurrent infections with methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA skin carriage is suppressed by intranasal mupirocin but recurrence occur after short period in most cases [104].

Also, Recurrent infection is common in patients with cystic fibrosis (CF). As CF hundreds of bacterial species colonized their lungs, *Pseudomonas aeruginosa* is the most common cause of recurrent pneumonia in these patients [105]. Antibiotic treatment of pneumonia in CF patients is the mainstay of therapy. But the presence of persister cells of non-bacterial infections can complicate therapy. Biofilm-associated infections with fungi, for example, are highly recalcitrant to routine antifungal therapy. Even in the absence of genetic resistance, management of *C. albicans* infection can frequently require prolonged courses of drugs or the removal of an infected implanted device.

Another example of persistence, referred to as dormancy, has been described for the blood stage of Plasmodium species, the protozoal parasites that cause malaria. In dormancy, It was found that a small subpopulation of parasites survives drug treatment and can cause recurrent infection after completing therapy. This phenomenon was found in many antimalarial drugs, including mefloquine, atovaquone, and most recently with artemisinins, the most commonly used therapy for malaria worldwide [106,107].

Persistence may be a cause of the emergence of resistance:

It was discovered that microorganisms can undergo cellular activity and division during persistence which raises the possibility that persisters may be an intermediate state in the development of drug resistance. Also, Stress-response mechanisms which are essential for the survival of persisters can accelerate mutagenesis and horizontal gene transfer. Thus, persisters may play a role in the emergence of resistant mutants [107].

Treatment of bacteria with sublethal doses of bactericidal antibiotics results in genome-wide mutations through stimulation of ROS production and through RpoS-mediated activation of the error-prone polymerase PolIV [108,109,110]. Selection of such resistance mutations by antibiotics can occur at drug concentrations orders of magnitude below the inhibitory level of susceptible bacteria [111]. Interestingly, a link between ROS-specific mutagenesis by sublethal antibiotics and microbial stress-response pathways has recently been demonstrated [46]. In *E. coli* lacking the SR [both relA and spoT], treatment of cultures with sublethal antibiotics failed to generate adaptive resistance, a phenomenon that was linked to reduced rates of oxidative stress in the SR null organism [46]. This suggests that an intact SR pathway is required for an antibiotic-stress-caused adaptive mutation to develop.

The structure of bacterial communities may also play an important role in the occurrence of adaptive mutations [112]. Higher mutation rates have been found in biofilm associated *P. aeruginosa* [113] and *S. aureus* [114] than in planktonic cultures, and these increased rates appear to be linked to higher levels of oxidative stress in the biofilm community.

Another major mechanism by which antibiotic resistance is elaborated is by the sharing of resistance determinants on mobile genetic elements. Stress-response pathways, particularly the SOS, have also been found to promote horizontal gene transfer in bacteria. Induction of the SOS response by the quinolone class of antibiotics increases horizontal gene transfer frequency in *E. coli* and *Vibrio cholera* [115], specifically promoting the sharing of integrative conjugative elements that result in resistance to aminoglycosides, lincosamides, and antifolate antibiotics.

Higher rates of phage transduction, which can disseminate antibiotic resistance determinants, have also been reported with treatment of some antibiotics [116] Interestingly, environmental stress from sources other than antibiotics has similarly been shown to increase rates of horizontal gene transfer in bacterial communities [117]. Biofilm formation, in addition to increasing rates of adaptive mutation, can also promote horizontal gene transfer in *S. aureus* [118]. Thus, stress-response pathways and microbial community structures that favor the development of persisters can also potentiate horizontal gene transfer.

Persistence and immunocomprimised patients

Although drug persistence can interfere with treatment, antimicrobial treatment succeeds in most cases to eradicate microorganisms. As It is known that if drugs successfully kill the majority of an infecting population, intrinsic host of healthy individuals can defense the remainder. In immunocompromised, In addition to their increasing susceptibility to infection, persisters that would normally be cleared may cause recurrent infections. Infection with HIV and its concomitant T cell immunodeficiency state is associated with increased severity and recrudescence of tuberculosis in co-infected individuals [119]. Patients with cancer exhibit impaired mucosal barriers leading to the development of oral candidiasis which can not be eradicated by local antifungals. Evaluation of isolates of *C. albicans* and *C. glabrata* from such patients demonstrated the presence of drug tolerant populations, and found that patients with prolonged fungal carriage had significantly higher levels of persisters [98]. In addition, compromised host that undergo implantation of cardiac devices, prosthetic joints, or other devices can develop biofilms. Treatment of microbial biofilms that form and infect these devices is ineffective and unfortunately requires dangerous and costly removal of these implants due to the presence of persisters cells in the biofilm community.

Marketed antibiotics effective against non-multiplying bacteria:

Targeting non-multipliers will lead to complete cure for many bacterial diseases, shorten antibiotic regimens which

will improve the patient compliance, decrease drug side-effects, lower costs and delay the emergence of resistance [120,121].

Some marketed antimicrobials were found to have the ability to kill non-multiplying bacteria. Pyrazinamide is found to be more active against non-multiplying *M. tuberculosis* than against multiplying bacteria [122,123]. The pro-antibiotic pyrazinamide, once uptaken by the bacterial cell, it is converted to an antibiotic that can bind to many targets or might even have nonspecific bactericidal action [124]. Rifampicin is found to be partially active against non-multiplying *S. aureus* [125,126]. Also, the addition of moxifloxacin and gatifloxacin to the regimen of rifampicin, pyrazinamide and isoniazid accelerates the rate death of a culture of non-multiplying *M. tuberculosis* [122,127].

Also there are derivatives of existing antibiotics that showed activity against non-multiplying bacteria. A new rifamycin derivative, Known as rifalazil, showed bactericidal activity for multiplying and non-multiplying *S. aureus* when used in combination with vancomycin [128]. TG44, is another recent antibiotic, was found to target the outer membrane of *Helicobacter pylori* [129]. Also, TG44 showed bactericidal action against non-multiplying *H. pylori* at pH 3–7.

A quinolone derivative, termed HT61, was found to have a stronger potency on methicillin-resistant strains in comparison to vancomycin and daptomycin [130].

Screening for drugs targeting non-multipliers:

Screening for new drugs acting on non-multipliers may be done by [i] whole-cell high-throughput screening testing for: [a] bacterial viability *in-vivo* [using models as zebra fish and *Drosophila* and *in-vitro* [under conditions inducing metabolic resting state]. [b] critical metabolite of a pathway [ii] screening for new molecular targets. [iii] pathway-directed screening [iv] Re-evaluation of the existing antibiotics mechanisms of action. [v] Phage therapy.

[i] whole-cell high-throughput screening testing:

High-throughput screening assays are procedures used for the early-phase drug discovery. These assays can provide more relevant *in vivo* biological information when compared to biochemical assays. As many drugs showed good activity during the *in-vitro* testing procedures but lack of *in-vivo* activity due to modifying the metabolic activity during infection, rendering the targeted pathway non-essential for virulence [131]. So, these procedures are resulted in the reduction of animal tests and facilitating drug discovery process. High-throughput screening assays are 1,000 times faster than the classical screening techniques [132]. On the other hand, there are two technical challenges facing this approach which are how to make conditions suitable for generating metabolically resting bacteria *in-vitro*, or for maintaining the *in-vivo* model, into standard high-throughput screening procedures.

High-throughput screening testing is done for bacterial viability in the presence of the tested compound *in-vitro*, Testing compound activity with simple *in-vivo* system, critical metabolite after the identification of critical pathway *in-vitro* and The essentiality of certain protein or component for pathway function *in-vitro*.

There are no drug in clinical trials or in the market that deal with certain structures that are essential to the non-multipliers. As targeting certain bacterial enzyme on metabolic pathway is too difficult to be applied to non-multipliers which may be due to: [a] low number of drug targets due to the downregulation of all metabolic process. [b] Drug penetration may be low in non-multipliers.

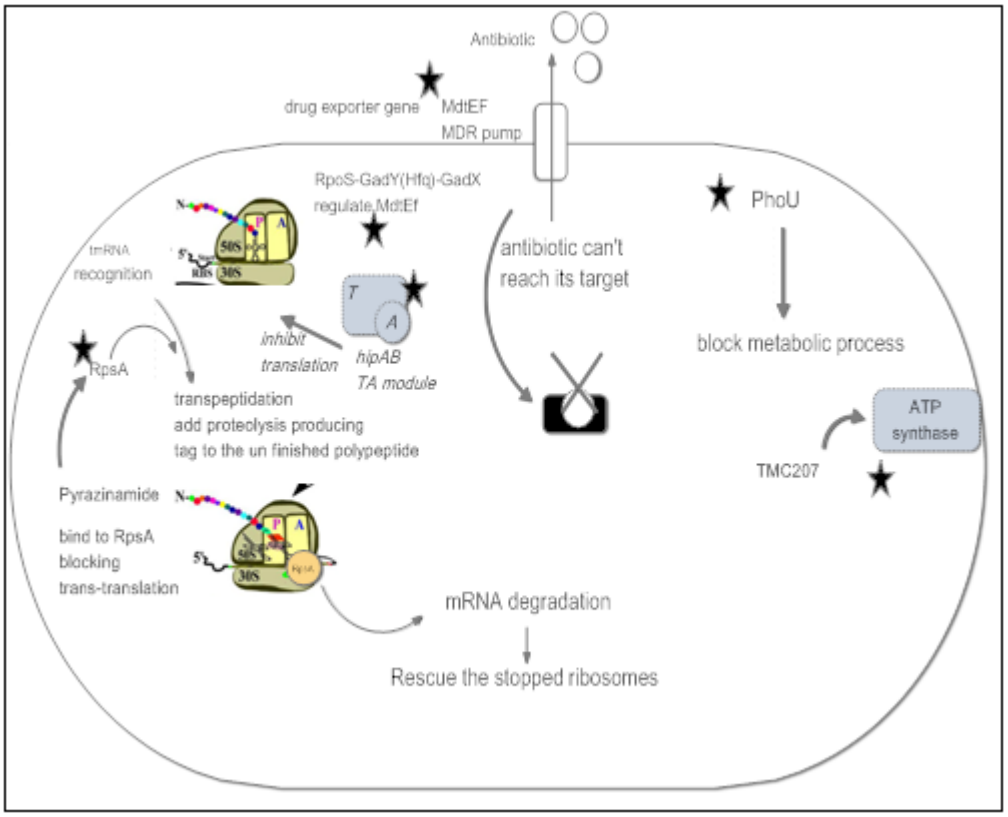

Figure 5. Possible drug targets in persister cells

[ii] Screening for new molecular targets:

It is important to identify a gene that can play a role in antibiotic tolerance shown by non-multiplying bacteria, then search for a compound to interfere with its gene product. By inhibiting or interrupting this product, non-multipliers will be susceptible to antibiotics. Many researches showed that overexpression of genes encoding toxins of TA modules such as HipA [*hipBA modules*], YafQ [*dinJ-yafQ* module], RelE [*relBE* module] and TisB [tisB-istR1 module] may contribute to the non-multipliers phenotype in *E. coli* strains [133].

There are another examples such as stationary-phase alternative sigma factor [RpoS]. It was found that RpoS mutant *P. aeruginosa* showed higher susceptibility to antibiotics than the wild-type bacteria. In *E. coli* rpoS and the RpoS-dependent signaling pathway involving Hfq, GadY and GadX regulate the drug exporter gene mdtEF, whose expression is increased in the stationary phase. So, mdtEF [drug exporter genes] may be an effective target. As if its action is blocked, a reduction in the drug efflux will occur and the drug concentration will be increased in the cell resulting in a high bactericidal effect [134].

PhoU negatively regulates many functions that are linked to the antibiotic tolerance. These functions are phosphates and energy metabolism, cell motility and nutrient transport. So, finding new compounds acting on it will lead to increasing the susceptibility of non-multipliers [44].

Recently, it was found that bacteria may be β-lactam resistant through non-PBP resistance mode. CiaRH was the first to be identified as non-PBP resistance mode. Upon activation of this system in *Streptococcus pneumoniae*, a high tolerance to antibiotics such as vancomycin observed [135].

[iii] Pathway-directed screening

In case of the presence of specific pathway that is confirmed to be essential for replicating as well as resting bacteria, pathway-directed screening can be carried out.

a. Inhibitors for respiratory ATP synthesis.

It was proven that inhibitors of respiratory ATP synthesis are powerful and effective for shortening the duration of tuberculosis treatment. TMC 207 [ATP synthase inhibitor] has a strong activity on mycobacterial strains. As mycobacterial strains cannot grow only depending on fermentation but they are mainly depends on the respiratory ATP synthase [136,137]. Despite of the importance of ATP synthesis to the growth of all bacteria, there are some strains can compensate that by increasing the activity of fermentation using the glycolytic pathway. Thioridine is another compound that can inhibit the NADH dehydrogenase type II in *M. tuberculosis*. Another example is clofazimine which interferes with NADH dehydrogenase producing oxygen reactive species. Also, these compounds showed good activity in clinical trials in humans and mouse models [138,139,140].

b. Membrane active compounds:

For compounds acting on bioenergitic functions, it is important to undergo counter-screening using human cell lines or sub cellular structures as mitochondria to select compounds that act on the bacterial metabolism [128]. These compounds are exemplified by daptomycin, which can block the bioenergitic function indirectly in *S. aureus* and porphirin class agent XF-73 which can eradicate biofilms formed by *S. aureus* [141].

c. Targeting a key metabolite of a pathway:

- ATP as a target:

Benzimidazoles, thiophens and imidazopyridines were found to decrease the cellular ATP levels accelerating the bacterial killing *in-vitro*. Studies showed that these compounds displayed good safety profile when tested by counter-screening tests using human cell lines [142].

- Type II fatty acid synthase complex as a target [FASII]:

Many researches showed that FAS II was proved as a target in many gram positive bacteria as Staphylococci, Enterococci and Streptococci but non-essential for *Streptococcus agalacticae*. As *Streptococcus agalacticae* mutant with a deletion in FASII was found viable *in-vitro* in the presence of exogenous lipids [142-148]. They showed that S. *agalacticae* can compensate the defect in the intrinsic fatty acid synthesis by the uptake of fatty acids from the surrounding media or environment. So, FAS II complex is not suitable for *in-vivo* studies.

d. Modulation of the bacterial metabolic state:

Switching bacteria from resting state to the active replicating state, increase its sensitivity to antimicrobials. This can be achieved by:

- Addition of carbohydrate metabolite:

Addition of mannitol and fructose to *E. coli* and *S. aureus* persisters, significantly increased the sensitivity of persisters to aminoglycosides. As it was found that addition of aminoglycosides increased killing of Gram positive and Gram negative *in-vitro* and *in-vivo* in mouse models. This is because addition of sugars increases drug uptake facilitated by a membrane potential setup formed by metabolization of carbohydrates [143].

- Inclusion of small metabolites:

It is exemplified by 3-[4-[4-methoxyphenyl] piperazin-1-yl] piperidin-4-yl biphenyl-4-carboxylate polycyclic compound known as C10. By using C10 with quinolones, it was found that C10 accelerates killing of bacterial cells in the presence of norfloxacin. Also, Kim et al., [143] explain the ability of C10 to accelerate the reversion of persisters to antibiotic sensitive bacteria with no effect on the normal antibiotic sensitive cells.

e. Blocking of Trans-translation process.

Pyrazinoic acid are found to bind to RpsA and interferes with the interaction of RpsA with tmRNA required for trans-translation. Blocking of trans-translation pathway leads to a defect in saving of stalled ribosome and depletion of available ribosomes and perhaps increased accumulation of toxic or deleterious proteins, ultimately affecting persister survival under stress conditions [62,63].

[iv]. Re-evaluation of the mechanisms of action of the existing antibiotics:

Re-evaluation of the existing antibiotics for mechanisms of action rather than that their known mechanisms of action, may lead to the identification of new targets for the existing antibiotics. Pyrazinamide is considered as the key component of the short course therapy regimens for > 50 years without knowing its cellular targets. Many studies showed that there are many targets have been proposed for pyrazinoic acid as fatty

acid synthase II [149], RpsA required for trans-translation process and keeping proton motive force and ATP levels in the cell.

[V] Phage therapy

Phages are specific and are proposed to be important tools that can help to fight an infection. There are many studies suggesting that phage therapy is effective against bacteria embedded in biofilm mass [150,151]. Also, lytic phages able to infect cells of persisters, wait for their switching to the actively multiplied cells, and eradicate them by lysis [152].

Bacteriophage engineered to interfere with the oxidative stress response by overexpressing SoxR also potentiates antibiotic therapy [153]. Other findings suggest that drugs targeting the SR [154] or interfering with envelope-repair pathways [67] may also help prevent persister formation or enhance bactericidal antibiotic activity

Disadvantages of therapeutic phages are [i] rapid emergence of resistance, increasing the need for a cocktail of phages that is difficult in the manufacture of standard lots, [ii] a phage may result in some undesirable immunologic reactions [155].

For these reasons, a topical phage therapy for chronic otitis— Biophage-PA — is in clinical trials in the United Kingdom against antibiotic-resistant *Pseudomonas aeruginosa* [156]. Also, phage gene products is another potential route for new antibacterials. Phage lysins, produced late in the viral infection cycle, are cell wall hydrolases, bind to peptidoglycan and disrupt the cell wall of Gram-positive bacteria that followed by hypotonic lysis [157]. Lysins can be applied for human use to clear mucous membranes from pathogens. lysins are thought to be active against persisters and biofilms [158,159] which may help in the treatment of medical device-associated infections.

The advantage of finding compounds or new antibiotics that targeting non-multiplying bacteria is shortening the duration of therapy resulting in increasing of patient compliance, particularly in diseases such as tuberculosis [160,161] and decreasing the rate of emergence of bacterial resistance for many infectious diseases [162], particularly in patients with infectious diseases, such as meningitis, endocarditis, osteomyelitis and infections in the neutropenic host [163].

There are many difficulties facing the development of new compounds targeting non-multiplying bacteria. First, non-multiplying bacteria may have a limited molecular targets in comparison to multiplying ones due to the downregulation of their genes [164,165,166,167]. Another problem may be that there are many different types of non-multiplying bacteria, each of which may be susceptible to a different compound. This situation was exemplified by tuberculosis, in which there are at least three forms of non-multiplying bacteria [168]. Also, targeting persisters in biofilm may not give the expected results in curing biofilm associated infections because of the presence of exopolymers that act as a barrier for antibiotic penetration and the presence large amounts of bacteria which may be also of different types. In addition, some mechanisms of persisters development that have been suggested to be targets for new drugs in some species are not present in another species. As HipA and TisB which are known to have important role in persister development are not found in the genomes of *S. aureus*, *Pseudomonas aeruginosa* or *M. tuberculosis* [169].

[vi] Biofilms:

Compounds that can prevent the biogenesis of pili and curli, extracellular bacterial fibers mediate the attachment of microbes to surfaces, may inhibit the initial phases of biofilm development [170]. Although treatment susceptibility was not assessed, these compounds effectively reduced the virulence of uropathogenic *E. coli* in a murine infection model [171]. Therapeutic strategies to disaggregate existing biofilms are also being considered [172,173].

Detoxification mechanisms such as efflux pumps are being investigated as new antibiotic targets or as adjuvants to existing therapies [174]. Persisters can also be antagonized by potentiating antibiotic toxicity though the enhancement of drug uptake. For example, aminoglycoside uptake by persisters and the efficacy of aminoglycoside treatment of catheter-associated *E. coli* infection in mice can be increased through coadministration of specific metabolites that help power the transmembrane proton motive force [75,155]. Finally, the use of adjuvants that maximize antibiotic-induced intracellular ROS production is also being explored [175,176]. Drug screens have traditionally focused on identifying compounds lethal to microbes in stress-free in vitro culture environments. Although this has been a successful way to identify drug targets, it is becoming clear that many microbial processes not required for idealized life in vitro are essential for survival over the course of infection and treatment in humans [177]. Such processes, which include both persistence and virulence, are intimately linked to dynamic and complex networks of stress responses that orchestrate phenotypic adaptation. Learning more about these processes and their role in infection is likely to yield a rich trove of potential new drug targets and hopefully will lead to more effective therapies against persistent, relapsing, and resistant organisms.

Finally, we should take in consideration some aspects:

1. There are not 2 pure categories of antimicrobial agents (one that exclusively kills bacteria and another that only inhibits growth). As according to the Guidelines for performing bactericidal tests published in 1999 by the NCCLS [178], Method for the determination of Minimum inhibitory cincentration (MBC) include an inoculum size of $\geq$ 5X 10^5cfu/mL and a subculture volume of 0.1 mL to accurately predict whether $\geq$99.9%of the bacteria were killed which means that the results may be influenced by growth conditions, bacterial density, test duration, and extent of reduction in bacterial numbers. It is also unclear why a 18–24 h was chosen as a cutoff incubation time in this test. Perhaps by extending the incubation time from 18–24 h to 36 h or even 48 h classification of many antibacterial agents may change from bacteriostatic to bactericidal, or vice versa. So, MBC values represent the result of an in vitro test in which the fixed, static concentration of an antibacterial agent is being tested against an initially fixed concentration of bacteria in an aqueous medium. Also, these in-vitro results may not correspond with the in vivo situation, in which antibacterial and bacterial concentration in various body fluids and tissues may fluctuate widely [179,180].

2. Tolerance of pathogens to antibiotics is due to the presence of persisters which are not mutants, non-growing, dormant cells. Presence of dormant cells explains their

tolerance to bactericidal antibiotics that depend on the presence of active targets for killing the cell [133].

3. Heteroresistance is a phenotypic heterogeneity of microbial clonal populations under antibiotic stress which was observed by gradual decreased colony-forming capability in the presence of antibiotic resulting in a decreased growth rate rather than selection of resistant cells.

4. Conclusion

- Increasing our knowledge about bacterial metabolism process will be conclusive for detecting effective antimicrobials and their application in chemotherapy.
- Identifying essential proteins and pathways in bacterial metabolism is very important to predict the essentiality of certain target and drug efficacy.
- Shifting replicating virulent bacteria to the metabolic resting state may be helpful in the management of sever acute infections and to prevent the resuscitation of metabolic resting bacteria suppressing the recurrence of infections.
- Combining new medicinal chemistry with chemical biology may allow to increase the spectrum of narrow spectrum drug classes to be active on both replicating and resting microorganisms.
- Although the majority of cells respond actively to stress, it is only the dormant cells which demonstrate persistence.
- Re-evaluation of the existing antibiotic known mechanisms of action, even after decades may result in identifying new targets in the bacterial metabolism.
- The difficulty in screening and discovering metabolic resting states observed for many species of pathogenic bacteria is considered a major challenge in the emergence and the development of new antimicrobials.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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