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Reactive Oxygen Species contribute to antibiotic efficacy

Towards a common mechanism for cellular death to combat multi-resistance

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19-11-2016

Literature Essay
Molecular Biology and Biotechnology

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As multi drug resistant bacterial strains are becoming a significant problem within our current society, there is a continuous need for developments in the field of antibiotics. Unfortunately, over the last two decades, the advancements in this field did not lead towards a usable solution for the current problems. Around one decade ago, a possible link between Reactive Oxygen Species and the lethality of antibiotics has been suggested. By acquiring knowledge on this possible linkage, a controversy on the contribution of Reactive Oxygen Species towards antibiotic efficacy was initiated. This resulted in experiments suggesting that Reactive Oxygen Species do contribute to this process, mainly when bactericidal antibiotics are combined with the presence of molecular oxygen. This contribution could give new options in utilizing current antibiotics and elucidating unknown relations between stress impulses like antibiotics and the protective systems developing within bacteria.

Contents

Introduction

In the present day, to protect us from infectious diseases, many useful methods have been developed. Over the last century, our general hygiene has increased dramatically and the current scientific community has more knowledge than ever before. But in time when we are diagnosed with an infectious disease, many variations and combinations of antibiotics are used to combat the bacteria that cause them. Apart from the natural anti-bacterial compounds, that have been around for thousands to millions of years, the first modern chemotherapeutic drug was discovered by Paul Ehrlich, Alfred Bertheim and Sahachiro Hata in 1909. By systematic screening on syphilis-infected rabbits, they found compound 606, arsphenamine, which is a derivative of Atoxyl. This showed to be a great improvement over the commonly used inorganic mercury compounds[1]. Another better-known event in this field was the discovery of penicillin by Alexander Fleming in 1923[2]. Even though penicillin's antibacterial properties were already known before this moment, it made a large impact during the last century. This was mainly thanks to the ability of mass production, which was made possible by a purification protocol from Howard Florey and Ernest Chain[3]. These discoveries are examples of the times antibiotics were truly on a rise, but this would come at a prize as usage of these compounds would start an arms race between man and bacteria.

Over the course of time, three major classes of antibacterial compounds have been proposed. These can be divided into two large groups: bactericidal drugs and bacteriostatic drugs. The first group induces cell death by inhibiting the synthesis of either deoxyribonucleic acid (DNA), ribonucleic acid (RNA), cell walls or proteins. The second group only inhibit bacterial growth. The first group of compounds is dividable into three different classes based upon the systems they target; the first class targets complexes involved in DNA replication and DNA repair systems. These systems are exploited by the compounds within the quinolone class. These compounds interfere with the bacterial enzyme gyrase, which makes transient double-strand breaks in the DNA to resolve DNA supercoiling. The quinolone antibiotics will covalently bind the cleaved DNA and the gyrase enzyme, which will arrest the DNA replication process and will induce cellular death[4]. The second class of compounds targets cell-wall synthesis. This is mostly done by β-lactams and glycopeptides. Each of these compounds interferes with the synthesis process, yielding failure to peptidoglycan cross-link production and therefore killing the bacterium, in its own way. β-lactams block this process as they can act as a substrate for the enzyme which catalyzes peptide bond formations. By binding to the active site of the penicillin binding protein (PBP) enzyme, it prevents crosslinking of the peptide chains[5]. Next to β-lactams, glycopeptides, like vancomycin, can also interfere with the cell wall synthesis. In contrast to the β-lactams, glycopeptides bind to the substrate of this synthesis reaction. These compounds have a high affinity towards the D-alanyl-D-alanine dipeptide of peptidoglycan. If they bind to the peptidoglycan, they form a steric hindrance for the PBP enzyme[6]. The last class of compounds targets protein synthesis. Due to a large number of reactions of which the protein synthesis system is comprised, there are also many compounds that can infer with this system. However, only one compound is broadly bactericidal, namely aminoglycosides. The binding of this compound promotes protein mistranslation. This is done by incorporating improper amino acids during elongation of the peptide chain. However, this compound does rely on the presence of oxygen to enable absorption through electrostatic interactions by fluctuations in the membrane potential[7]. The efficiency of absorption is also increased by the drug itself, through incorporating faulty amino acids into membrane proteins, which results in a more permeable membrane[8]. Furthermore, bacteriostatic drugs inhibit bacterial growth. These compounds are divided into two groups: 50S- and 30S inhibitors. 50S inhibitors can block the initiation of the translational process[9]. By binding to the 16S ribosomal RNA (rRNA), 30S inhibitors can interfere with the elongation factor-catalysed translocation and with complex formation between the messenger

RNA (mRNA) and the transfer RNA (tRNA), which can result in the mistranslation of the amino acid chain[10]. Both inhibitors can block the access of the tRNA molecule into the ribosome.

The suggestion that resistance to antibiotics will eventually lower the efficiency of antibiotics, in general, was already suggested as humans first started to use these new compounds. However, in the present day, there is a multitude of issues that made way for bacteria to develop resistance to one or multiple antibiotics. Over-prescription is the first of these issues. Often this is done due to uncertainty in diagnostics to ensure successful treatment of the condition. This issue becomes even larger in areas where there is no professional health care system present. In these areas, patients purchase and administer drugs without proper consolation. Another large issue lies within the hospitals, where the events of transferring resistant microbes or resistance marker are frequent[11]. The last issue is the current frequency in which new compounds are currently successfully made accessible for public use. The main reason for this is a non-functional development pipeline for new antibiotics. This is due to the exceptionally high costs that are accompanied by the third phase of clinical trials, where new compounds are tested in large groups of patients. This holds back pharmaceutical companies to a return on their investments. This way they will not get involved in the development of many new compounds. Economical solutions have been proposed and even been legislated already to lower the cost required to enter the market by involving government budget or prolonging patents on drugs for an additional 5 years. This way the risk that the involved companies carry becomes lower. However, these (proposed) changes did not significantly influence the process of antibiotic development of the past years[12].

As we failed to discover new classes of antibacterial agents, there might be chances to improve the currently known and used antibiotics by learning more about their complex interaction with and influences on bacterial cells. Since the beginning of the millennium, it has been suggested that anti-bacterial agents could rely on the formation of reactive oxygen species (ROS), which put the cells under oxidative stress to help to kill them[13-15]. From 2007 onwards, more research has been done to this connection between antibiotics and ROS, which started by linking gyrase blocking to the promotion of ROS formation[16]. Around this point in time, it became clear more and more that antibacterial compounds can interfere with many cellular systems to either induce cellular death or inhibit bacterial growth. But this is a complex process, which can be influenced on many levels as this whole route from antibacterial compound interaction to cellular death contains a huge amount of reaction and an even bigger amount of combinations of reactions. In the end, many inhibitions or interactions by/with these antibacterial compounds result in perturbations within the tricarboxylic acid (TCA) cycle. These perturbations could affect the electron transport chain (ETC), which in turn may produce superoxide molecules. These superoxide molecules are highly reactive and can be converted to hydroxyl radicals by the Fenton reaction. These hydroxyl radicals will then cause excessive damage which could induce cellular death. The involvement of ROS could result in a drastic, change of our current view towards antibiotics as they could contribute, be the main component but also could be the downfall to the efficacy of antibiotics. This essay will discuss the current knowledge on antibiotic-induced ROS and how it might adapt the way we look at antibiotics.

Reactive Oxygen Species

Particles known as ROS are free radicals derived from molecular oxygen. As free radicals, they contain unpaired electrons. This makes such particles highly reactive, by their urge to acquire more electrons to meet the octet rule[17]. Therefore radicals react with the nearest possible particle from which they can take an electron. In turn, these particles will turn into radicals, as they now have an unpaired electron. This way, a chain reaction is created, which could potentially disrupt

the entire cell. If cellular death is evaded, these cells could now contain oxidized lipids, carbohydrates or DNA mutations[18]. This damage is mainly caused by the hydroxyl radical (OH•). This radical is converted from hydrogen peroxide (H_2O_2) and ferrous iron (Fe^{2+}) together with hydroxyl (OH^-) and ferric iron (Fe^{3+}) in the Fenton reaction. In turn, the H_2O_2 is converted from the superoxide anion radical (O_2^-) by reactions in the respiratory chain, which in *Escherichia coli* is mainly responsible for the production of H_2O_2[19,20]. Molecular oxygen is a weak univalent electron acceptor, so it cannot efficiently oxidize biological macromolecules such as nucleic- and amino acids. Molecular oxygen often interacts with transition metals or accessible catalytic redox cofactors of flavoenzymes. These interactions enable the production of O_2^- and H_2O_2. This happens when oxygen collides onto the reduced cofactor before the electrons can be transferred to secondary redox partners like in the ETC. Even though these particles are better oxidants, due to the anionic charge in O_2^- and the oxygen-oxygen bond in H_2O_2, the reactivity of these particles is relatively low. Moreover, both these particles can be eradicated through interaction with suitable enzymes. Superoxide dismutases can convert two O_2^- together with two protons into H_2O_2 and molecular oxygen and peroxidases can convert two H_2O_2 into two water and molecular oxygen. OH• has a highly reactive character due to the fact that both of these features do not apply to this particle[21].

Even though some of these particles are better counteracted then others, all of them can cause damage within bacteria in their own degree. For instance, O_2^- can be destructive towards iron-sulfur clusters. These clusters are widely used as a redox cofactor, for instance by the enzyme dihydroxy-acid dehydratase, which catalyzes a reaction in the biosynthesis of valine, leucine, isoleucine and coenzyme A[22]. O_2^- can bind to the cluster, together with two protons, to produce H_2O_2, but mainly oxidize the cluster. The oxidized cluster is not stable enough to keep its associated, so it lost by degradation. This shuts down the synthesis pathways, which results in a loss of catalytic iron group function that leaves the bacterium highly vulnerable[23].

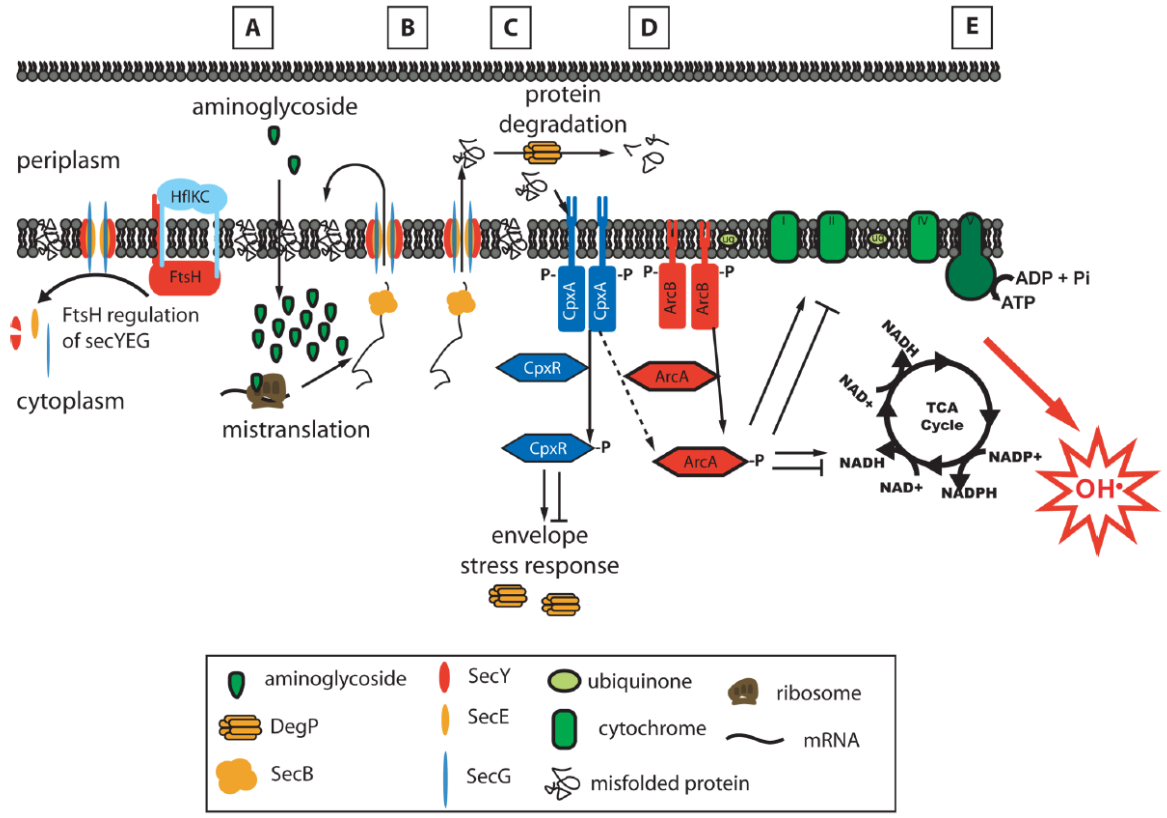
Antibiotic-induced ROS

As mentioned above, antibiotics play a role in inducing cellular death of bacteria. Bactericidal drugs do this by truncating processes like DNA replication, cell wall-, or protein synthesis. Fluctuations in these processes will lead to drastic changes in the cell's metabolism, and mainly in the TCA cycle, as the fluxes in this cycle can be completely rewired to cope with any stress that is applied to the cell. After treatment with antibiotics, nucleotides and lipids are downregulated and the relative concentrations of carbohydrates, adenosine triphosphate (ATP) and antioxidants are increased. Different antibiotic classes induce a similar metabolic response after a short incubation with the antibacterial compound (± 30 minutes). Metabolites that were uniformly upregulated during this short incubation were citrate, succinate, nicotinamide adenine dinucleotide (NAD^+), NAD precursors and coenzyme A. This suggests large changes in the TCA cycle and the oxidative phosphorylation pathway. Unique responses to each class of antibiotics are better visible over a longer period of time (one or more hours). For instance, quinolones tend to decrease metabolite concentrations, while β-lactams generally increase them. This suggests that the target-specific effects are influencing the metabolic pathways prior to cell death. Additional changes that suggest lethal antibiotic stress are a diminished nucleotide pool and an increased GSH biosynthesis as they are linked to higher amounts of DNA damage and antioxidant activity[24].

Over the course of time, the intricate interactions occurring within downstream metabolism, are appreciated for their self-regulation, which is terribly interconnected and often occurs before genetic regulation processes. Even though it is difficult to explain why different antibiotics show their own expression pattern of TCA genes, it is thought that it does give rise to ROS formation in

the oxidative phosphorylation pathway for aerobe growing bacteria under the influence of mainly bactericidal antibiotics[25]. Studies have shown that quinolone treatment activates multiple responses increasing the OH• production. This production is mainly powered by two compounds: *atpC* and *iscS*. *AtpC* is an ATP synthase which translocates protons and *iscS* is a component of the iron-sulfur cluster synthesis complex. This increase in production of OH• goes for both Gram-positive and Gram-negative bacteria. The general mechanism of lethal OH• production involves tricarboxylic acid (TCA) cycle metabolism and a transient depletion of NADH, in addition to iron–sulfur cluster destabilization and iron misregulation[21]. Based on systems-level analysis it was confirmed that certain antibiotics (aminoglycosides) trigger specific event which leads to the increased production of OH•. Based on gene expression microarray analysis, 6 networks from the system were selected, based on similar shifts in expression under influence of aminoglycosides. Multiple elements showed enrichment during these experiments: First were the ArcA-regulated elements. These elements are part of the Arc

two-component system, which are important components in the bacterial cell cycle[26]. Activation of this system is regulating genes that are dedicated to processes like metabolism and respiration, which links the response to the aminoglycosides to the processes which produce ***Figure 1**: **the***



proposed mechanism by which aminoglycosides trigger hydroxyl radical formation and cell death. *(A) The primary interaction between the antibiotic (aminoglycosides or β-lactams) and the cell causes protein mistranslation. (B) Mistranslated, immature membrane proteins are brought to membrane translocation complexes and are translocated across the inner membrane into the periplasmic space or inserted into the membrane. (C) Due to mistranslation, many of these proteins are misfolded, leading to phosphorylation of CpxA. (D) Activated CpxA phosphorylates CpxR, which upregulates expression of envelope stress response proteins, such as the periplasmic protease DegP. CpxA may also activate ArcA, which regulates a large number of metabolic and respiratory genes. These changes shift the cell into a state that (E) provokes free radical formation, ultimately culminating in hydroxyl radical formation and cell death. It was found that β-lactams and quinolones also trigger hydroxyl radical formation and cell death through the Cpx and Arc two-component systems (D, E). Adopted from [29].*

OH•[27]. Furthermore, this compound is involved with protein mistranslation. Enriched elements included proteases which are regulated by the heat shock factor σ^H. Others consist of elements

related to electrochemical transport across the cell membrane. By constructing multiple single-gene knockouts on genes that are associated with these elements, their involvement in the OH• production was determined. Through experiments, where the OH• production was directly measured, using hydroxyphenyl fluorescein (HPF), which only emits fluorescence once the hydroxyphenyl is cleaved off by interaction with ROS[28]. The results of these experiments suggest the following; First, aminoglycosides induce the expression of the CpxR-regulated stress response system. This is done through the activation of the genes *degP* and *cxpP*. Through knockouts of these genes, they were confirmed to have a positive effect on the OH• production. Second, cross-talk between *degP* and *arcA* was confirmed as the *arcA* knockout had a significantly slower membrane depolarization. Thirdly, the trafficking of mistranslated proteins through or into the membrane by translocation complexes is an essential component for OH• production. Lastly, over the course of these experiments the cells of all knockout strains eventually were killed, so aminoglycosides have more than one way to kill a cell. Based on these results the system visualized in figure 1 was proposed. Interestingly, this increased OH• production is initiated by the same system that provides protection against misfolded proteins by the activation of the CpxR-regulated stress response system. This was determined by the removal of *degP* and the CpxR-regulated stress response system components, which led towards a decreased OH• production. This could be possible when the increased DegP decreases the repression on CpxA which eventually leads to an increased OH• production[29].

Controversy

Since the publication of this model, other work has been presented, which undermines the plausibility of this model. These studies stated that, even though this model was widely accepted, it did not explain some of the made observations. The first study "decided to re-examine the role of ROS in cell death and consequently found that killing by antibiotics is unrelated to ROS production"[30]. This was based upon the following results from their experiments: first, the protective compound thiourea inhibited killing of the cells at clinically applied concentration. Second, there was no correlation between kill-count due to norfloxacin and the amount of fluorescence detected from the potentially oxidized HPF, concluding that there was no link between cellular survival and ROS formation. Lastly, antibiotics were equally or more effective under anaerobic conditions, which are situations where ROS could not have been formed. According to the study, the possible reasons for these different results could be the use of low concentrations of antibiotics, a different amount of surviving cells and the inability of detecting ROS accurately using HPF. The other study "devised experiments to directly test the molecular events that underpin" the proposed model[31]. These experiments concluded that the cells were more likely killed by the traditional targets of the different antibiotics, DNA replication, protein synthesis and cell-wall assembly, then by the formation of ROS. This conclusion is based on the fact that antibiotic treatment did not sufficiently increase respiration to produce a toxic concentration of ROS and therefore do not promote ROS formation, the present ROS formation did not activate genes for the H_2O_2 sensor of the cell, HPF dye can be oxidized by high-valence iron, which is formed in the early stages of Fenton chemistry and, again, cellular death was not dependent on the presence of oxygen.

As a reaction to these criticizing studies towards this common lethality system, a study was done to confirm that ROS does contribute to the lethality of bactericidal antibiotics to some extent. The original hypothesis was extended by specifying this partial contribution, next to the traditional target modifications of each class of antibiotics. First, it was proven that ROS were indeed generated when antibiotics were administered. For these experiments, a broad selection of fluorescent reporters was used besides the criticized HPF. Most of these dyes showed significantly increased fluorescent signal in all classes of antibiotics[32]. This was not the case when quinolone-

resistant cells were exposed to norfloxacin, which confirms that ROS is generated as a result of the traditional antibiotic reactions to the DNA gyrase. Also, a new assay was developed, using an engineered ascorbate peroxidase (APX) and the diffusible fluorophore Amplex Red to ensure that antibiotics promote the intracellular production of H_2O_2. In comparison to the old method, the peroxidase is active in the cytoplasm of the bacteria where it can directly measure the H_2O_2 production, instead of the traditional external measurement of H_2O_2, where it was assumed that the external concentration of H_2O_2 would be comparable to the internal concentration. After antibiotic treatment, all classes of antibiotics resulted in a signification two- to threefold increase of Amplex fluorescence. Bacteriostatic antibiotics did not generate this increase. Based on this results, it was assumed that the produced H_2O_2 would cause the activation of oxidative stress regulators. By applying a GFP-fused promoter for the most important regulators, it was shown that bactericidal drugs activated OxyR and SoxR. These quantitative experiments, using 10 μM H_2O_2, also showed that the necessary concentration of this activation exceeded the amount or DNA damage. Next, the issue of the linkage of ROS towards the presence of oxygen/respiration was tackled. It was hypothesized that the ROS was produced by either auto-oxidation reactions or the ECT. Both these possibilities would rely upon an increased respiratory activity. This was confirmed by addition of bactericidal antibiotics and there was no increase after treatment with bacteriostatic antibiotics. Based on the results described above the study claims that "bactericidal drugs antibiotics induce redox-related alterations to cell physiology, and that these alterations are sensitive to respective primary target effects". In this study, they continue to fortify their assumptions around the involvement of ROS in the lethal properties of antibiotics by determining the actual contribution of ROS to this process by damaging nucleic acids in double-stranded DNA or single nucleotides through Fenton chemistry. Together with these assumptions the question of mutagenesis at sub-lethal conditions also arises. To investigate this territory, multiple complexes were analyzed in combination with antibiotics. First, it was determined that the mismatch repair enzyme MutS protects the cell against all classes of bactericidal antibiotics. This was the same for KatG and AhpCF, the H_2O_2 scavengers at high and low concentrations, respectively. These results have implied that there is a common component which damages cells through corrupting DNA and protection against antibiotics is established by activation of oxidative stress regulons by the presence of oxidative compounds. This protection can also be achieved through antioxidants like glutathione or ascorbic acid, which do not impair bacterial growth by directly interacting with oxidative compounds, although the effectiveness of this varies based on the used antibiotic, based on their primary target. Finally, the link to oxygen presence was fortified by determining the effectiveness of the antibiotics under aerobe and anaerobe conditions. Strictly anaerobe grown bacteria showed a 1- to 4-log increased survival, but not all bacteria survived the antibiotics. This supports that ROS does contribute to the killing of bacteria in aerobe conditions besides other contributors. After collecting all this data, their initial hypostasis was confirmed by observing that priming the anaerobe grown cells with antibiotics after which they were exposed to environmental oxygen resulted in a better killing efficiency[33].

Disadvantages of ROS

However, there could be a significant downside to the involvement of ROS as a result of treatment with antibiotics. As described above, ROS formation can result in OH•, who interact with the closest molecule around. This can lead to DNA damage and therefore mutation. These mutations can result in an increased resistance against the used antibiotic. Studies on this subject revealed that, even though bacteria contain protective systems like the error-prone SOS response and error-correcting repair systems, sub-lethal levels of antibiotics can give rise to antibiotic resistance. This was observed by conducting the following experiments; first, antibiotics from all

classes were administered to *E. coli* cultures in sub-lethal concentrations, which resulted in an increased mutation rate comparable to an increase resulting from the addition of 1 mM H_2O_2. These increases correlated with a peak signal of HPF fluorescence to confirm this was as a result of ROS formation. To enforce this statement, it was shown that adding a hydroxyl scavenger complex of growing the bacteria anaerobically, did not result in a significantly increased mutation rate. Also, it was shown that sub-lethal levels of antibiotics could give rise towards resistance of other and multiple antibiotics. Treatment with norfloxacin increased resistance to norfloxacin and kanamycin and ampicillin towards resistance against ampicillin, chloramphenicol, kanamycin, norfloxacin and tetracycline. Moreover, these changes were not observed under anaerobic conditions. This suggests that the mutation causes by the antibiotic-mediated ROS can lead to resistance to a wide range of antibiotics. Therefore, experiments were performed to determine the possible consequences of these mutations. These experiments elucidated that these mutations can influence drug efflux systems, cross-resistance and some traditional drug targets like gyrase, *arcA,* and *rpsL*, which can contribute to multidrug resistance. These results hold up for both Gram-positive and Gram-negative, as well as clinically isolated bacteria[34]. The influence of ROS on antibiotic resistance was also shown in *S. aureus*[35].

Discussion

The described studies over the past decade have brought fascinating insight into the much-desired development around known antibiotics as creating new efficient compounds were not introduced on the market for many years. As we keep discovering more details about the mechanisms behind antibiotics and the protective system developed by bacteria, a possible link to ROS has been established. A model has been proposed to elucidate the mechanism behind aminoglycosides inducing ROS formation. This is the first step toward a common mechanism behind cellular death, which could show previously unknown relations between the reaction induced by different classes of antibiotics and the protective alterations within bacteria. The development of such a common mechanism is a great step towards a solution for the current problem of multi-resistant bacterial strains. On the other hand, it has been shown that the involvement of ROS also can contribute in favor of the birth of these multi-resistant strains. It could be that efficiency of such reactions makes the difference between successfully killing bacteria and truncating them so they possibly can develop into resistant strains. This risk is the exact reason why a further understanding of the systems behind antibiotics is essential for effective future use of these compounds without creating an environment where these very compounds can no longer be used.

The scientific community has shown significant progress by performing the cited studies and questioning them when needed. The latest research shows the physical alterations within the bacterial cell after the administration of both bactericidal and bacteriostatic drugs. Bacteriostatic drugs seem to slow down mRNA and protein synthesis which leads to an accumulation amino acid and nucleotide precursors. This is thought to block the usage of compounds generated in the central metabolism, which in turn slows down the ETC. It also has been shown that a stimulated respiratory track increases the efficiency of bactericidal drugs, but there is no clear evidence if bactericidal drugs initiate a stimulation on the respiratory track. Even though anaerobic knockout studies have shown that the absence of respiration in a bacterial cell significantly decreased the efficiency of bactericidal drugs[36]. Support for the crucial role of respiration in the efficiency of bactericidal drugs was also presented by another recent study showing that the decay of peptidoglycan by β-lactams could initiate increase cellular respiration as the cell attempts to synthesize new peptidoglycan[37]. This is strong evidence that central metabolism and possible

downstream ROS formation are a vital component in the contribution towards efficient cellular killing by antibiotics.

This assumption generates a monumental task for the scientific community. We are yet to discover most details about the involvement of the central metabolism in many cellular processes. As mentioned before, a bacterial cell has an endless number of minute changes it can apply to its central metabolism in order to combat stress-introducing factors from their internal and external environment. This makes isolating and understanding the required changes, in times of possible antibiotic-induced oxidative stress, an intricate task. To tackle these issues, methods from the field of systems biology could be used to elucidate the important details from the network that is the central metabolism[38]. As these studies are based on *in silico* analysis using algorithms that outplay the human brain in terms of solving complex equations to a large list of pre-set constraints, they could reveal essential details about the adaptions made in the central metabolism as antibiotics are administered and the bacterial cell is attempting to combat them. As these sorts of studies will unveil more knowledge about the internal regulation as the reaction on the administration of antibiotics for bacteria, it will provide us with better chances to create strategies to combat the current problems our modern society faces regarding multi-resistant bacterial strains. This, to makes sure that we will be able to use antibiotics, as a viable tool in once was, for many years to come.

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