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Aminoglycoside uptake, stress, and potentiation in Gram-negative bacteria: new therapies with old molecules

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SUMMARY Aminoglycosides (AGs) are long-known molecules successfully used against Gram-negative pathogens. While their use declined with the discovery of new antibiotics, they are now classified as critically important molecules because of their effectiveness against multidrug-resistant bacteria. While they can efficiently cross the Gram-negative envelope, the mechanism of AG entry is still incompletely understood, although this comprehension is essential for the development of new therapies in the face of the alarming increase in antibiotic resistance. Increasing antibiotic uptake in bacteria is one strategy to enhance effective treatments. This review aims, first, to consolidate old and recent knowledge about AG uptake; second, to explore the connection between AG-dependent bacterial stress and drug uptake; and finally, to present new strategies of potentiation of AG uptake for more efficient antibiotic therapies. In particular, we emphasize on the connection between sugar transport and AG potentiation.

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INTRODUCTION

Since their introduction in the 1940s, antibiotics have played a major role in the development of modern medicine and have saved many lives. However, bacterial resistance to treatment has been observed since the first administrations during the Second World War (1), and it has only increased since then. After decades of use, the World Health Organization (WHO) warns that if nothing is done by 2050, millions of lives could be lost in the battle against pathogenic bacteria. A study published in 2022 (2) reports on the impact of multi-antibiotic resistant [multidrug-resistant (MDR)] bacteria on mortality: in 2019, 1.27 million deaths were directly due to MDR bacterial infections. Of these deaths, nearly 75% were related to six pathogens: *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. Additionally, studies show the significant proportion of hospital infections related to Gram-negative bacteria (3–6).

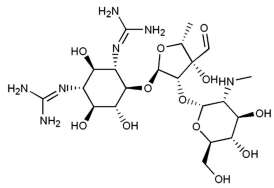
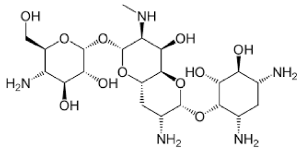
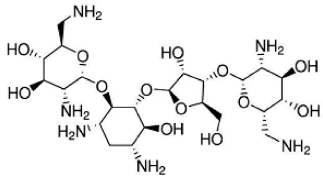
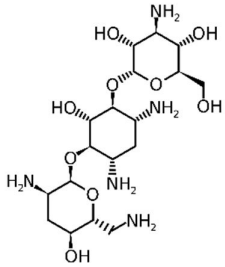
This review focuses on the use of a specific class of antibiotics, the aminoglycosides (AGs), against Gram-negative bacteria. AGs are "old" molecules. The first member of AGs, streptomycin, was discovered in 1944 by Albert Schatz and isolated from *Streptomyces griseus* (7). The discovery of this major antimicrobial earned Professor Selman Waksman the Nobel Prize in Medicine in 1952. Despite their efficacy, one limitation of the use of aminoglycosides is their side effects on patients. Nephrotoxicity and ototoxicity are observed in variable proportions depending on the study, averaging between 3% and 15% of cases, although these figures remain difficult to determine as they depend on the patient and the type of infections (8, 9). AG use decreased with the approval of new treatments such as fluoroquinolones or cephalosporins, but they are now experiencing renewed interest because of their broad-spectrum efficiency and the development of semi-synthetic derivatives (10). AGs are now classified as a critically important class of antimicrobials by the WHO (11), particularly with the arrival of plazomicin, the latest AG approved in the United States (2018) since amikacin in 1981. Plazomicin is active against MDR *Enterobacteriaceae*, including those resistant to carbapenemases (12).

AGs can be classified into four groups based on the core structure as illustrated in Table 1 (13). They are composed of an amino-sugar core structure (14). The amino-moieties are most often protonated under biological conditions, which confers them a highly positive charge at neutral pH, making these molecules basic and hydrophobic. These properties influence their transport across the bacterial membranes.

AGs act primarily by disrupting protein synthesis, because they bind the ribosome on the 16S ribosomal RNA, near the decoding site (15, 16). The 4,5- and 4,6-substituted AGs (Table 1) additionally bind the 23S ribosomal RNA (17), impeding ribosome assembly (18) and recycling (17, 19, 20). Consequently, these AGs have been associated with an overall slowing of translation (20, 21). Importantly, AGs are bactericidal, which makes them an exception among antibiotics targeting translation. Other antibiotics targeting the ribosome, such as chloramphenicol or tetracycline, prevent translation initiation and show a bacteriostatic action (22). On the other hand, AGs do not stop translation, but they cause mistranslation (23–27). AG-dependent translation errors have recently been shown to cluster together (27). As soon as a translation error occurs, others follow, leading to a protein synthesis catastrophe, which has implications in terms of lethality but also AG uptake, as we will detail below [energy-dependent phase II (EDPII)].

The primary target of the AGs is thus the ribosome. However, AGs' mode of action and how they exert their bactericidal effect are still incompletely understood and sometimes under debate. As synthetically illustrated in reference (28), first understanding of AG lethality was that mistranslation would lead to insertion of misfolded proteins to the membrane and eventually membrane disruption (29, 30). Reactive oxygen species (ROS) have later been proposed as key players in AG-induced cell death (30), while iron-sulfur cluster biosynthesis was shown to be involved in AG lethality through increased uptake

TABLE 1 Classification of aminoglycosides according to their core structure and examples[a]

Group	Examples	Molecule
No core (streptidine core)	Streptomycin	
Mono-substituted deoxystreptamine	Apramycin	
4,5-disubstituted deoxystreptamine	Neomycin Ribostamycin Paromomycin	
4,6-disubstituted deoxystreptamine	Kanamycin Gentamicin Amikacin Tobramycin Plazomicin	

[a]The molecule that is represented is underlined. Adapted from Krause et al. (13).

in *E. coli,* in a ROS-independent way (31). Moreover, these mechanisms are not necessarily mutually exclusive and may even depend on each other. For example, membrane disruption was shown to lead to ROS production (30, 32). Additional complexity stems from the fact that not all bacterial species susceptible to AGs bear the same pathways for response to oxidative, envelope, and Fe-S stresses. AG mode of action is thus complex and available data indicate that there are many factors at play.

Several resistance mechanisms to AGs have been described, primarily involving enzymes that modify and inactivate AG molecules, through acetylation, adenylylation, or phosphorylation (14). Another resistance mechanism involves alteration of the target site, the ribosome, through mutations in the 30S ribosomal subunit or methylation of the AG binding site. Although this second mechanism also confers high levels of resistance, it may be associated with a fitness cost in the absence of AGs, with a general alteration of the ribosome functional structure. The third resistance mechanism involves reducing intracellular AG concentrations, which can be achieved by decreasing uptake or increasing efflux (33, 34). In a study on 1,349 clinical isolates including ESKAPE pathogens, *E. coli*, *Proteus* spp., *Providencia stuartii*, and *Serratia marcescens*, it was found that when resistance was not due to AG inactivation enzymes, it was attributed to a decrease in their ability to transport AGs inside the cell. Although the molecular mechanisms have not been addressed in the corresponding study, it was observed that such altered transport was responsible for 90% of resistance to amikacin (35). It would be thus

important to determine the exact mechanisms by which AGs enter the bacterial cells, in order to understand resistance mechanisms and potentially the means to counteract them.

Despite their long history of use, the complete understanding of AG uptake and action remains incomplete. Ongoing research continues to uncover new insights into their transport into bacterial cells and the mechanisms underlying their bactericidal effects. This review aims to consolidate both the long-standing and recent discoveries pertaining to AG entry.

We will first review the methods available for quantifying AG uptake in bacteria, as these have been crucial in understanding the mechanisms involved in their entry through the bacterial envelope. We then focus on how AGs cross the Gram-negative double membrane barrier to enter bacterial cells, including recent discoveries on active transport mediated by carbohydrate transporters. Subsequently, we will explore the relationship between AG-induced cellular stress and its impact on further AG uptake. Finally, based on our understanding of AG uptake, we will discuss possible potentiation mechanisms for enhancing the efficacy of AG therapies.

METHODS FOR MONITORING AG UPTAKE

Several methods have been developed to quantify the entry of AG into bacterial cells, and to construct and consolidate the current model of AG uptake.

Indirect methods

Initially, indirect methods based on evaluating antimicrobial action were employed to assess intracellular AG uptake. The supernatant from bacterial cultures treated with AGs was collected and used to treat naive bacteria. The uptake by the initial batch of AG-treated culture was determined by measuring the concentration of AG remaining in the supernatant. This evaluation was performed by comparing the inhibition rate after treatment with the supernatant to the inhibition rate observed with the initial drug concentration (36). A similar approach was used to assess intracellular drug concentrations by measuring the inhibition rate after treatment with a lysate of bacteria previously exposed to the antibiotics (37). Quantification of AG in bacterial lysate can also be performed with high-performance liquid chromatography (38, 39).

More recently, the remaining amount of tobramycin in the supernatant of treated *P. aeruginosa* cell was evaluated with electroanalytic quantification through tobramycin binding on electrodes, which characterizes its electrochemical behavior through voltammetry, redox potentials, and pH dependence. In this assay, the decrease in the electrochemical current corresponds to consumption of tobramycin (40). This has the advantage to discriminate sensitive and various degrees of resistant bacteria in the biological sample and not only in laboratory media, and appeared as an interesting alternative for clinical antibiotic susceptibility testing.

Although these indirect approaches can provide valuable information, they cannot be employed to directly assess the penetration of the drug into the bacterial cell, and especially at the single cell level.

Labeled drugs

The first labeling of AG molecules was accomplished using radioactivity. Various radiolabeled AG drugs have been developed, with tritium-3H (3H-gentamicin, 3H-tobramycin, or 3H-amikacin, for example) which has the advantage of being a very low-penetration β emitter (41), with Iode 125 (42), Carbon 14 (43, 44), or technetium 99m (45). Radioactive molecules offer the advantage of sensitive detection, but their use is not always practical as it involves a considerable investment by the laboratory, and they cannot be used in microscopy for localizing the antibiotics. Moreover, radioactive isotopes could be subject to passive adsorption on bacteria (42).

Subsequently, fluorescent labeling techniques were developed to measure AG concentrations in bacteria. One such method is the automated fluorescence

immunopolarization assay, which involves competition between gentamicin in the test sample and fluorescein-labeled gentamicin on sheep anti-gentamicin serum (42). Compared to radiolabeled AG entry measurement, this method is less sensitive (threshold of 300 µg/mL) and requires a higher inoculum, although the transport characteristics are similar. As a result, this method is more suitable for monitoring AG concentration in patient fluids rather than bacterial cells (46).

The use of Texas red-AG conjugate molecules offers advantages as they exhibit a similar range of minimum inhibitory concentration (MIC) compared to unconjugated gentamicin (47). They have been tested in *E. coli* (48, 49), *A. baumannii* (50, 51), and *P. aeruginosa* (52, 53). However, the Texas red dye itself can penetrate the cell, making it difficult to differentiate between the uptake of Texas red alone and the Texas red-AG conjugate.

Kanamycin labeled with fluorescent CFDA-SE [5,(6)-carboxyfluorescein diacetate, succinimidyl ester] has been developed for microscopy studies (54). However, the dyes from the aromatic carboxylics acids family, including CFDA-SE, can also permeate cells and exhibit pH-dependent properties (55).

An alternative approach involves labeling neomycin with cyanine fluorophores Cy5 and Cy3, which retains the properties of aminoglycosides for uptake and activity (56). Neomycin-Cy5 has been employed for uptake study in *Vibrio cholerae* (57–59), *E. coli*, and *P. aeruginosa* (60). By coupling neomycin with Cy5, it becomes possible to visualize the precise localization of the antibiotic, which can be found in the periplasm or cytoplasm depending on the duration of treatment, antibiotic concentration, and the species (56).

MECHANISM OF AMINOGLYCOSIDE ENTRY FROM THE EXTERNAL ENVIRONMENT INTO THE CYTOPLASM

In this part, we describe how AGs can bind to and cross the outer membrane, to enter the periplasm, before passing the inner membrane to reach the cytoplasm. We include long-known and recent discoveries on non-specific and specific transport of these molecules through the Gram-negative double membrane. Note that the majority, if not all of the studies of AG entry into the bacterial cell, has been in the context of exponentially growing cells.

AGs can pass through the Gram-negative bacterial envelope

The outer membrane of Gram-negative bacteria consists of an asymmetric bilayer of phospholipids associated with a layer of lipopolysaccharides (LPS). The LPS displays an outwardly directed O-antigen, an oligosaccharide (sugar) core, and lipid A. The composition of O-antigen varies according to the bacterial species. Lipid A is composed of a disaccharide linked to fatty acids (61). This arrangement of the outer membrane forms a highly hydrophobic lipid bilayer which contains embedded pores that act as a selective filter for exogenous compounds (62).

The periplasmic space between the outer and the inner membranes contains a layer of peptidoglycan, which provides mechanical and osmotic protection to the cell. The inner membrane is composed of a phospholipid bilayer embedded with proteins, including the respiratory chain responsible for generating the proton motive force and enabling ATP synthesis.

The outer membrane's hydrophobic nature serves as a protective barrier against the external envirnoment. This constitutes a challenge for antibiotics that need to reach the periplasm or the cytoplasm to exert their action. Several antibiotics can hence be only used against Gram-positive bacteria. One such example is vancomycin, which targets peptidoglycan synthesis. Both Gram-negative and Gram-positive bacteria synthesize peptidoglycan. However, despite its high efficiency against Gram-positive bacteria, vancomycin is generally ineffective against Gram-negative bacteria because it cannot cross the Gram-negative outer membrane (63).

AGs are one class of antibiotics which can cross both membranes and are efficiently used against Gram-negative bacteria. In the 1960s, the entry of AGs into the

Gram-negative bacterial cell was proposed to occur as follows. First, a small amount of the antibiotic enters by passive diffusion through both membranes. Since AGs target translation, their entry affects protein synthesis, among which membrane proteins, and eventually causes a deterioration of membrane integrity, and further AG entry. Then, after a lag, increased entry leads to death (64). This model was further developed in the 1980s, where most of the discoveries were made, that led to the currently accepted uptake model [see reference (65) for a comprehensive review]. The entry of AGs into the cytoplasm is now described as primarily dependent on the proton motive force, and divided into three phases: (i) a slow linear concentration-dependent entry up to a plateau; (ii) a second phase of linear entry, referred to as energy-dependent phase I (EDPI); and (iii) a rapid entry which saturates after several minutes, just before cell death, referred to as energy-dependent phase II (66–69).

Adding to this model, new discoveries have been made regarding AG uptake through outer membrane porins, and a specific entry mechanism at the inner membrane through sugar transporters, as detailed later in this review.

Reaching the target: initial binding to the outer membrane

AGs are small hydrophobic molecules that can bind to the LPS of the outer membrane. Similar to the polymyxin family of antibiotics, AGs bind to the cell through electrostatic interactions between the positively charged groups of the antibiotic and negatively charged components of the outer membrane (70). The binding sites for polymyxin on the outer membrane can be antagonized by the presence of cations, indicating that polymyxin B and cations compete for binding sites on the cells (71). Interestingly, a polymyxin-resistant *E. coli* also exhibits lower outer membrane binding of the AG gentamicin (72). This competition between polymyxin and an AG suggests that both antibiotics may share the same binding sites on the outer membrane (73). It was observed in *P. aeruginosa* that the addition of cations, such as magnesium and polyamines, raises the MIC of AGs by preventing their adsorption to the outer membrane (74). However, addition of cations after AG exposure cannot not rescue viability, demonstrating the irreversible binding of AGs (75). Also note that addition of magnesium (as well as changing other parameters of the external environment) may have pleiotropic effects including binding to the outer membrane, changing metabolism and gene expression, and impacting ribosome stability. Such experiments should thus be interpreted with caution.

The initial entry phase through the outer membrane occurs rapidly upon the addition of AGs, and the rate of uptake is influenced by AG concentration (67, 76, 77). Remarkably, initial observations in *P. aeruginosa* showed that 30 seconds of incubation with an AG was sufficient to result in a 312-fold increase in the intracellular concentration of the AG compared to the external medium (74). This binding and transport across the outer membrane do not appear to require energy, as indicated by the fact that following a cold treatment that inactivates energy-dependent transport, AG binding to the outer membrane remained possible (78).

It is possible to decrease the binding of AGs to the outer membrane. AGs form strong hydrogen bonds with the lipids of the LPS (79, 80). Altering the LPS can thus, in some cases, decrease the binding and the susceptibility to AGs as observed in *P. aeruginosa* (81, 82), *E. coli* (83), and others (84). External agents can also alter the binding of AGs to the outer membrane. One illustrative example is observed in *P. aeruginosa,* with the outer membrane protein OprH. OprH binds and occupies the magnesium cross-bridging sites of the LPS (85), thus masking these cationic sites on the outer membrane where AGs bind (73, 86–88). Magnesium starvation induces the expression of OprH (89), highlighting how environmental factors can significantly impact the effectiveness of AGs.

Crossing the outer membrane

Outer membrane disruption

Once bound to the outer membrane of bacteria, AGs are able to permeabilize it before reaching the periplasmic space. The hydrophobic fluorescent dye 1-N-phenylnaphthylamine was used to observe disruption of the outer membrane by AGs. This dye can only penetrate the Gram-negative cell upon outer membrane permeabilization. Upon treatment with AGs, the dye could enter inside *P. aeruginosa* cells, demonstrating the permeabilization effect of AGs (90). Such outer membrane permeabilization could even be observed in a setup where AGs were prevented from reaching the cytoplasm, indicating that the action of AGs on the outer membrane is independent of their action in the cytoplasm (87). In fact, AGs insert themselves into the bacterial lipid bilayer and initiate a threshold response, ultimately leading to surface disruption (91).

Importantly, this disruption occurs without altering the width or fluidity of the membrane, but it does induce membrane disorder and increase lipid dynamics (92). This results in rapid leakage of cytoplasmic materials (75). For example, lethal exposure to AGs in *P. aeruginosa* has been found to cause a decrease of 34% in total proteins. Moreover, even a short gentamicin treatment was observed to lead to removal of outer membrane constituents (67). Formation of small transient holes were also observed in the same study conducted by Martin and Beveridge.

Implication of porins

In addition to a general membrane disruption, non-specific transport through porins has been proposed as an uptake mechanism for AGs at the outer membrane, similar to what has been observed for other antibiotics, such as β-lactams (93, 94). Porin function does not require an energy supply such as ATP. While porins are often non-specific for the substrates they transport in *Enterobacteriaceae*, some porins may exhibit selectivity, such as LamB for maltose and maltodextrins (95) or ScrY for sucrose (96). The exclusion size of porins varies among bacteria, and in *E. coli*, it is estimated to range between 600 and 800 Daltons. Thus, porins are capable of transporting small, hydrophilic solutes and proteins, and they also serve as signal transduction relays. In some cases, AGs were even proposed to induce their own passage through porin opening as exemplified with the MscL mechanosensitive channel which bears an attachment site for streptomycin (97, 98).

AGs can diffuse through pores formed by porins, at a rate as rapid as that of hexoses and disaccharides, except for pores formed by LamB (93). Another indirect evidence of the interaction between AGs and porins comes from the observation that kanamycin affects the intrinsic tryptophan fluorescence of the OmpF porin, indicating direct binding (99, 100). In *E. coli*, it has been estimated that approximately 10–20 kanamycin molecules per second can pass through OmpF and OmpC. Permeation to AGs, including kanamycin, gentamicin, and amikacin, is also possible through ChiP (chitoporin) at a rate of approximately three molecules per second. However, LamB maltoporin does not show significant permeation, consistent with previous observations (101, 102).

On the other hand, there is limited direct *in vivo* evidence regarding the transport of AGs by porins, as few porin-deficient mutants that are resistant to AGs have been isolated. Most porin-deficient mutants showed no change in susceptibility to AGs (93, 99, 103). A Δ*ompF* (also called *tolF*) strain was identified as resistant to AGs (103); however, this effect could also be indirect, as the loss of OmpF alters the protein composition of the outer membrane (104). Later, it was shown that a single deletion of either *ompF* or *ompC* did not affect susceptibility to AGs, but the double mutant did (101). This suggests that these two porins may act in a redundant way and compensate for each other's function in AG entry. Additionally, the probability of occurrence and selection of a double mutation is low, which could explain why the involvement of porins is mainly identified using specific experimental setups. An *E. coli* strain resistant to both polymyxin and AGs was isolated, and exhibited reduced porin levels, suggesting that decreased

permeability to both compounds may be a result of reduced porin abundance (105). However, a more recent study on a knockout strain lacking all 40 porins in *P. aeruginosa* did not demonstrate increased resistance to AGs (106), indicating that even if AGs are transported by porins, this may not be the primary route of entry into bacterial cells.

In addition to studying AG uptake in porin deletion mutants, overexpression studies have also been conducted. This is relevant because the expression of porins is usually inducible and they may not be expressed at high levels under normal growth conditions. For example, PhoE is induced under phosphate limitation, and the expression of LamB, OmpC, and OmpF is regulated by variations in the physicochemical environment (osmolarity, temperature) (107). It has been shown that only 5% of the population produces open channels for the OprF porin (108). Therefore, under non-induced conditions, the expression of porins may not be sufficient to facilitate significant uptake of AGs, resulting in negligible impact on the MIC. However, there have been instances of AG transport by porins, such as OprB in *P. aeruginosa*, which increases susceptibility to AGs when overexpressed (60). It is important to mention that, to the best of our knowledge, no studies have been conducted on AG uptake under conditions where OmpC and OmpF porins are artificially overexpressed *in vivo*. Moreover, the overexpression of OmpC in *E. coli* has been shown to inhibit the translation of other porins, namely LamB and OmpA (109). This indicates that the expression of different porins can be intertwined, and could potentially minimize any effect on AG susceptibility phenotypes upon overexpression of a given porin. This factor should be considered when investigating the impact of multiple porins on antibiotic uptake using overexpression techniques.

Importantly, a species-specific effect on AG recognition by porins should not be overlooked. For example, while overexpression of LamB in *E. coli* did not enhance sensitivity to AGs (60), AG passage through LamB appears to be possible in *V. cholerae* (59). This may further complicate the identification of specific porins responsible for AG transport.

In conclusion, AGs can cross the outer membrane through electrostatic binding and membrane disruption, and probably also through non-specific transport by porins.

Energy-dependent phase for aminoglycoside transport across the inner membrane

Once AGs have crossed the outer membrane, they accumulate in the periplasmic space and need to traverse the inner membrane to enter the cytoplasm, where they can target the ribosome. This uptake process occurs in two distinct energy-dependent phases, which have been a topic of debate, leading to different conclusions among different research groups (68, 76, 110). However, the role of the membrane potential in facilitating AGs' crossing of the inner membrane is widely acknowledged (65, 68, 111–113).

We describe below the details of this process.

The EDPI: slow uptake in the cytoplasm

The proton motive force (PMF)

The PMF relies on both membrane potential (Δψ) and ΔpH. The membrane potential (ΔΨ) is generated by the respiratory complexes. The respiratory chain consists of a set of protein complexes located at the inner membrane, responsible for the oxidative phosphorylation of ADP to produce ATP, i.e., energy. Unlike eukaryotes, the bacterial respiratory chain exhibits diversity depending on the species, allowing for the utilization of various electron donors and acceptors. Despite their diversity, respiratory chains share a common organization: a dehydrogenase receives electrons from a donor and gets oxidized. The electrons are then transferred to a co-factor, which becomes reduced, and pass through several co-factors (which are subsequently re-oxidized) until they reach a final acceptor, which is oxygen in the case of aerobic respiration. ΔpH depends on the proton concentration on either side of the membrane (114). Respiratory chain complexes are capable of coupling their redox reactions with the transfer of protons across

the membrane to balance charges, thereby establishing an electrochemical gradient generated by proton pumps. This potential is referred to as the proton motive force. Thus, the PMF is tightly linked with energy production, and the uptake of AGs depends on the PMF, (111–113), as described below.

The EDPI

A threshold membrane potential must be reached in order to initiate AG transport. In *E. coli* cells, AG uptake occurs when Δψ is between −107 and −125 mV (115). As an indication, in *E. coli*, the ΔΨ value is −105 mV during fermentation, −130 mV in anaerobic conditions (116), and ranges from −220 mV at the beginning of the exponential phase to −140 mV at the end under aerobic conditions (117, 118). On the other hand, ΔpH is measured as −117 mV during fermentation, −144 mV in anaerobic conditions, and −160 mV in aerobic conditions (116).

This implies that the amount of AGs that can enter the bacterial cells varies with growth conditions: AGs are less effective in an environment with low pH (119, 120) and also less effective against anaerobic or facultative anaerobic bacteria that have a reduced PMF. For example, bacteria with anaerobic nitrate respiration are resistant to higher AG doses (121). Mutants of *E. coli* with defective respiratory chains exhibit reduced AG entry and susceptibility, indicating that a deficiency in cellular respiration hinders AG accumulation within the cell. Therefore, reduced AG uptake and susceptibility may be observed when the respiratory chain is altered, as it happens in mutants for NADH-dehydrogenase (31, 122), cytochrome oxidase (123), succinate dehydrogenase (124), or heme biosynthesis (which is a co-factor of the respiratory chain) (111, 125–127). Recent literature also indicates that the PMF-dependent uptake of AGs is hindered in mutants deficient for iron-sulfur (Fe-S) cluster machineries of the respiratory chain (31). Thus, mutations altering iron-sulfur cluster biogenesis can also have an impact on AG susceptibility.

Inhibition of the EDPI

The inhibition of the EDPI uptake phase is possible and can be achieved through the inhibition of membrane potential, by uncoupler, also called protonophores. These are compounds that dissipate the gradient of protons and decouple it from oxidative phosphorylation of ADP to ATP. Uncouplers include dinitrophenol, carbonyl cyanide m-chlorophenylhydrazone (CCCP), potassium cyanide (KCN), oxamic acid, sodium arsenate, and sodium azide (31, 128, 129). Moreover, nitric oxide and nitrite, by binding to terminal oxidases, can halt respiration (130–132). Other inhibitors such as N-ethylmaleimide (76, 128), p-chloromercuribenzoate (sulfhydryl reagents) (129), and low temperature (128, 133) have also been shown to be effective.

The direct role of ATP in AG transport was addressed in an *unc* mutant (F-ATPase), in which ATP production is uncoupled from the electron transport and the PMF. Treatment with the protonophore CCCP dissipates Δψ, and leads to a decrease in ATP levels in wild-type conditions, but the ATP content of the cell remains unchanged in the *unc* mutant. CCCP treatment could still abolish AG uptake in the *unc* mutant despite normal ATP levels, suggesting that ATP alone does not have a direct role in the transport of AG (134, 135). AG uptake does not depend on the levels of intracellular ATP, as a 10-fold reduction in ATP content does not modify AG uptake (136). In the same line, the use of the ATPase inhibitor N,N′-dicyclohexylcarbodiimide (DCCD) does not change the rate of AG uptake in the Gram-positive *S. aureus* (77), but no similar experiment was performed in Gram-negative bacteria.

In an interesting unpublished observation, Miller and Dougherty observed that AG uptake could occur in CCCP-treated cells lacking ΔΨ [as also observed in Muir et al. (137) on anaerobic cells or or Nielsen (138)], but only after a lag phase [their personal communication to Taber in Taber et al. (65)], which probably corresponds to the resumption of bacterial growth and restoration of intracellular ATP levels (134). They hypothesized that this could be attributable to the induction of an alternative

uptake pathway, such as an active transport mechanism that relies on ATP rather than membrane potential ΔΨ. This may be one of the first instances where the idea of active AG transport, e.g., through ATP-binding cassette (ABC) transporters, was mentioned (see "Active transport through the inner membrane" below).

In summary, during EDPI, a small amount of AGs cross the inner membrane through PMF-dependent passive diffusion and this amount is concentration dependent.

The EDPII: fast cytoplasmic uptake prior to cell death

The second phase of energy-dependent entry, known as EDPII, relies on the action of AGs on protein synthesis. As mentioned previously, AGs induce translation errors (23–25, 27), leading to the production of non-functional, misfolded, or truncated proteins. Recent research has demonstrated the presence of error clusters following AG treatment, with an error rate within these clusters that is 60,000 times higher than the predicted random error rate. AGs can remain bound to the ribosome over multiple protein elongation cycles, inducing consecutive errors and causing proteotoxic stress and protein aggregation (27). Furthermore, AGs stimulate the accumulation of ROS (139, 140), which are also associated with the misfolding of mistranslated proteins (141), further exacerbating proteotoxic stress (see Aminoglycoside uptake and stress section below).

How mistranslation primes AG entry during EDPII is still unclear, although it is widely accepted that poorly translated membrane proteins can create pores and facilitate the entry of AGs into the cell (29, 65, 142, 143), in a so-called leakage pathway. This, in turn, disrupts various cellular processes and ultimately leads to cell death due to the irreversible nature of absorption (29). It has been proposed that after protein mistranslation, rapid proteolysis occurs, trapping the AGs inside the cell and contributing to its irreversible action (144).

Inhibition of the EDPII

Protein synthesis and interaction with the ribosome are necessary to initiate EDPII (145). Inhibiting AG binding to the ribosome prevents EDPII. A specific example can be found in an *rpsL* (ribosomal protein S12) mutant, which lacks the high-affinity ribosomal binding site for streptomycin (146, 147). In this mutant, the second phase of uptake for the AG streptomycin cannot occur, demonstrating the essential role of ribosomal binding of AGs in EDPII.

Reduced ribosomal activity has also been associated with a decrease in EDPII, likely due to a reduced amount of mistranslated proteins: for example, chloramphenicol supplementation, which halts protein synthesis, during or before treatment with AGs, hinders AG uptake (128) by blocking EDPII, not EDPI (148–150). Therefore, protein production is required for both the initiation and continuation of EDPII (145).

Although this EDPII is not directly linked to respiration or alteration of the PMF, this step is still referred to as energy-dependent because it is prevented by uncouplers (as described above), and requires the presence of the PMF (76, 115). This energy dependency is inconsistent with a simple leakage pathway resulting from membrane damage but rather suggests the involvement of energized channels in the passage of AGs. Furthermore, a possible link between the protein translation machinery (essential for EDPII) and the PMF (essential for EDPI/EDPII) cannot be excluded. For example, PMF drives the translocation and resolution of protein folding problems in the inner membrane (151–153). However, no studies have provided substantial evidence to support such a hypothesis.

During EDPII, a large amount of AGs enter the bacterial cytoplasm following mistranslation of membrane proteins. This phase necessitates the presence of a membrane potential.

Active transport through the inner membrane

In addition to unspecific uptake during EDPII due to membrane damage, the possibility of active transport of AGs through the inner membrane has been proposed and

debated for half a century. In 1978, Holtje observed that streptomycin induces the active transport of polyamines and competes with them for entry into the cell. He proposed that streptomycin could be actively recognized and carried by the polyamine transporters (129). Since then, new insights invalidated this hypothesis. Firstly, it was observed that polyamine uptake is inhibited during amino acid depletion in a stringent strain of *E. coli* but not in a relaxed strain (154). A stringent strain constitutively activates the stringent response. We now know that AGs also induce the stringent response (155), which could explain the observed "competitive" effect with polyamine uptake. Additionally, polyamines have the ability to displace gentamicin from the ribosome *in vitro* (156), suggesting that the competition between AGs and polyamines may be due to their opposed effects on the ribosome. In *P. aeruginosa*, polyamines have been shown to increase the MIC of several antibiotics, including AGs, but this effect was found to be linked to LPS perturbation and not to an active transporter (157).

Another observation supporting the hypothesis of active transport of AGs arose from the finding that AGs accumulates in *E. coli* when chloramphenicol is present, provided that the AG is added prior to chloramphenicol treatment. Initially, this observation was interpreted as evidence of the AG inducing an active transport system (66). Subsequent studies showed that active protein synthesis is necessary for AG accumulation (EDPII), only during the early stages of treatment. Current theory suggests that the requirement for protein synthesis during early AG accumulation can be explained by the fact that, at later stages, some AGs have already entered the cell (EDPI) and induced a certain amount of misfolded proteins, thereby initiating EDPII (147). However, this does not rule out the potential involvement of active transport in AG entry (128).

Active transporters of nutrients in bacteria usually exhibit both compound selectivity and size selectivity. For example, in contrast to the AG dihydrostreptomycin, its adenylated derivative does not enter *E. coli* cells. This disparity could be attributed to charge modifications or to the size of the modified molecule being too large to pass through an active transporter. These findings support the notion of transport occurring via specific carriers (158). Another supportive fact came from a study using N-ethylmaleimide, which is a compound known to inactivate several transporters with thiol groups in *E. coli*, without affecting the growth rate or the PMF. Similar to lactose, melibiose, and proline, the uptake of the AG tobramycin is hindered when N-ethylmaleimide is added. This suggests that AGs may be transported by proteins with accessible thiol groups on the external face of the cytoplasmic membrane (76).

A recent study suggests that AGs are transported by amino acid carriers in *E. coli*. This hypothesis is based on the fact that deletion of the *gcvB* sRNA, which represses amino acid transporters, increases sensitivity to several AGs (159). Moreover, AG import was reduced in the *btuD* ATP-binding subunit mutant of the vitamin B12 transporter, which was attributed to an active transport of the AG in *Lysobacter* (54), but this has not yet been further studied.

Finally, recent studies have suggested that AG transport occurs through carbohydrate transporters, regulated by carbon catabolite repression (CCR). CCR is a regulatory mechanism in bacteria that controls the transport and utilization of carbon sources. It ensures efficient utilization of the most favorable carbon source, e.g. glucose for *E. coli,* before utilizing secondary carbon sources (160). Thus, in the presence of the preferred carbon source, bacteria repress the transport and utilization of alternative carbon sources. The repression is mediated by catabolite repression protein, such as the cAMP receptor protein (CRP), in complex with cyclic AMP (cAMP). The specific mechanisms vary between bacterial species, as different organisms may have their own set of regulatory proteins and signaling pathways involved in CCR.

Although sugar utilization-related genes have sometimes been detected (161, 162), the vast majority of high-throughput studies of resistant/persistent populations usually identify and study PMF and membrane-related mutants (e.g., electron transport and oxidative respiration) (163–165). Initial data leading to the hypothesis of a link between sugar transporters and AG uptake came from the observation in *E. coli*, that glucose

supplementation reduces the rate of uptake of the AGs (129). These findings suggested a connection between AG uptake and CCR. Recent research in *V. cholerae* (59) and other Gram-negative bacteria (*E. coli*, *P. aeruginosa*, and *A. baumannii*) (60) has demonstrated that AGs are actually transported by a diverse array of sugar transporters. In the case of *V. cholerae,* high-throughput studies have identified the involvement of a small non-coding RNA called *ctrR* in AG susceptibility. Inactivation of *ctrR* leads to increased tolerance to AGs (59). A homolog of *ctrR* has in parallel been identified in *Vibrio tasmaniensis* (166), and it has been shown to interact with mRNAs of carbohydrate utilization and transport genes, resulting in the stabilization of these transcripts. In both species, upregulation of this small RNA leads to an increased number of the corresponding transporters.

This discovery highlighted the connection between carbon source utilization and AG uptake. Even though no *ctrR* homolog was identified in other genera, this mechanism of AG uptake by sugar transporters is shared among other Gram-negative bacteria. Indeed, at least 11 transporters have been confirmed as involved in AG-specific susceptibility and uptake when overexpressed in *E. coli*. They are primarily located at the inner membrane (PTS [phosphotransferase system] and ABC transporters) and are non-preferential sugar transporters, induced by the CRP-cAMP complex, upon glucose limitation. Thus, AG uptake seems to be regulated by CCR and *de facto*, under the control of environmental factors. Moreover, other small regulatory RNAs can regulate sugar transport, such as Spot 42 (*spf*) in ɣ-proteobacteria (167, 168), but their involvement in AG susceptibility has not been tested.

The recognition of AGs by sugar transporters could be attributed to the sugar moiety of AGs (60). However, there are still unresolved questions regarding the relationship between this active transport mechanism and the PMF, as the PMF may play a direct

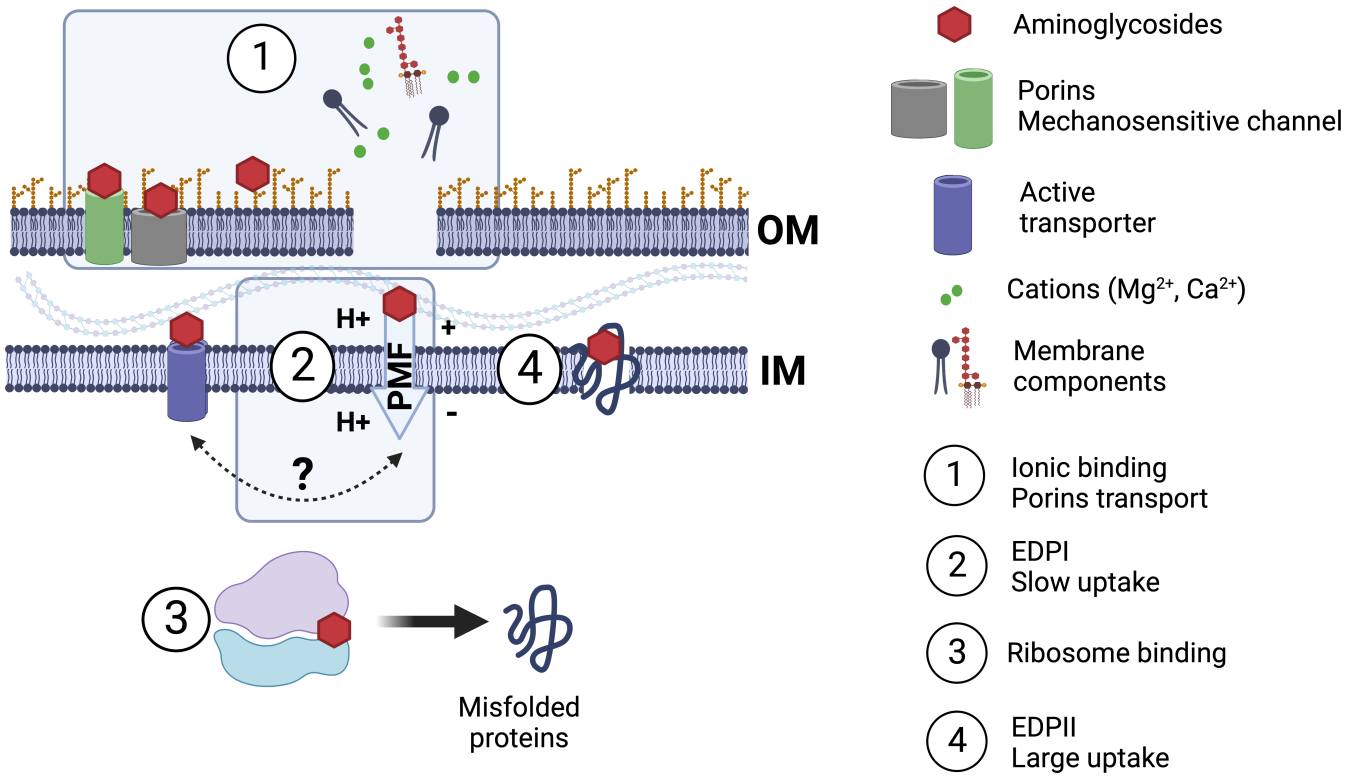


FIG 1 Model of AG entry in Gram-negative bacteria. (1) Electrostatic binding. The interaction between AG and the outer membrane induces its destabilization. Entry through porins is possible. (2) Energy-dependent phase I. Slow uptake, proton motive force dependent. Active transporters (notably carbohydrates transporters) are also able to carry AG. The link between active transporters and the role of the PMF in AG entry remains to be fully elucidated. (3) Ribosome binding. AGs induce protein mistranslations. (4) Energy-dependent phase II. Misfolded protein induces membrane permeability and large uptake, preceding cells death. "Active transporter" corresponds to carbohydrate transporters and others.

or indirect role in powering or regulating active sugar transporters (169–171). Moreover, several active transport systems, such as lactose permease, are proton-driven transporters (172), and their functionality can be inhibited by the uncoupler CCCP, which is also an inhibitor of AG entry (173). These recent findings raise the question of the extent to which the dependence on membrane potential is connected to active transport systems that are coupled to proton transport.

In summary, once AGs have reached the periplasm, there is an initial slight increase in entry known as the energy-dependent phase I, followed by a plateau, and then a rapid increase in entry referred to as energy-dependent phase II. The latter is dependent on the production of proton motive force by cellular respiration, which drives the uptake process (68). In addition, specific transport of AGs through carbohydrate transporters has also been demonstrated. Fig. 1 summarizes the current AG uptake model in Gram-negative bacteria.

Once AGs have entered the cytoplasm, the uptake process becomes irreversible, as the molecules cannot exit the cell even when the proton gradient is artificially removed using a protonophore to dissipate the membrane potential (68).

AMINOGLYCOSIDE UPTAKE AND STRESS

Once AGs have entered inside the bacterial cell, they can exert their action primarily by targeting the ribosome, but also, and consequently, by triggering various stresses, some of which can also influence their further uptake.

Bacterial stress responses are important molecular mechanisms that bacteria deploy to survive and adapt to a variety of stressful conditions and challenges, including exposure to antibiotics. Indeed, as part of the everlasting arms race among microorganisms, antibiotics have been well-known stressors for bacteria with several studies showing how the presence of these compounds, sometimes even at very low concentrations, induces different bacterial stress responses (174–180). Activation of these mechanisms can enhance bacterial survival in the presence of antibiotics by either modulating the accumulation of antibiotics inside the cell or by interacting with the factors required for their efficacy. In this section, we will briefly review how AGs trigger different bacterial stress responses and how these affect AG entry.

Aminoglycosides and heat shock response

As previously mentioned, after their entry in the cell, the primary target of aminoglycosides is the 16S rRNA at the 30S small subunit of the ribosome (143, 181). The binding of AGs to the ribosome's 16S rRNA, specifically at the tRNA acceptor A site (aminoacyl site), disrupts the proofreading mechanism responsible for accurately discriminating between cognate and non-cognate tRNAs during protein synthesis (182–186). Consequently, ribosomes bound by AGs result in a greater occurrence of erroneous amino acid incorporation during protein synthesis (27) which in turn leads to the synthesis of proteins with a higher propensity to misfold. The presence of misfolded proteins in the cell is the main stress signal triggering the heat shock response (187), characterized by the induction of a set of heat shock proteins that help protect cells from the damaging effects of misfolded proteins. Establishing the link between AGs and misfolded proteins, several studies have shown the induction of heat shock genes and the importance of several chaperones and proteases in both Gram-positive and Gram-negative bacteria exposed to these antibiotics (30, 179, 188–193). For example, microarray analysis of *Pseudomonas aeruginosa* exposed to lethal concentrations of the AG tobramycin revealed the induction of several heat shock genes including a Lon-type protease important for this activation (179). Similarly, a study on the proteomic response of *P. aeruginosa* to different classes of antibiotics also showed a higher abundance of the chaperones DnaK/GrpE and GroESL, in cells treated with tobramycin or gentamicin (194). Moreover, the induction of these chaperones and other heat shock genes have equally been observed in streptomycin-treated *Acinetobacter baumannii* (195) and *E. coli* (177), and tobramycin-treated *V. cholerae* (196, 197).

Misfolded proteins have long been suggested to be key factors in AG lethality because several of these misfolded proteins would be inserted in the membrane, forming pores and disrupting membrane stability (29, 198, 199). This would in turn lead to the PMF-dependent second phase of AG uptake (EDPII) as described above. How adaptative can heat shock response activation be in this process?

It is almost intuitive that stressors and conditions that could induce heat shock genes in bacterial cells, independently of aminoglycoside treatment, would lead to expression of chaperones and proteases which in turn would limit the amount of misfolded proteins inserted in the membrane and thus temporarily increase AG tolerance once in presence of these drugs. This was in fact shown to be case, with numerous studies reporting that overexpression of products of the heat shock machinery, such as chaperones and/or proteases, increased tolerance and led to short-term adaptation to AGs in different species (179, 188, 189, 192, 195). Moreover, some of these studies provide evidence of reduced membrane depolarization during the first hours of aminoglycoside treatment upon heterologous expression of different heat shock proteins in *E. coli*, which highlights the link between heat shock response and EDPII phase of AG uptake (188, 189).

However, even though it is clear that AGs induce the heat shock response in bacteria, the consequences of such response on further aminoglycoside uptake and lethality are not yet totally understood and may vary in different contexts. Indeed, a recent study challenges some of these previous observations, showing that a pre-heat shock induction of *E*. coli cells could also potentiate AG lethality through enhancing of the PMF (200).

Aminoglycosides and envelope stress

Misfolded proteins generated during AG treatment may have as target both the inner and outer membranes of the cell. As previously said, insertion and accumulation of these aberrant proteins at bacterial membranes are thought to generate envelope stress leading to entry of more AGs. This is sensed by bacteria which trigger several envelope stress responses (ESRs) to mitigate such stress (201, 202). Thus, it is perhaps not surprising that aminoglycoside susceptibility has been often linked to the activation of some of these ESRs in many *Enterobacteriaceae*. One example of a component of the ESR tightly linked to aminoglycoside susceptibility is the Cpx.

The Cpx is a well-known two-component system (TCS) composed of a sensor protein CpxA that senses misfold protein insertion in the inner membrane and the periplasm, and the regulator CpxR which regulates many genes including proteases responsible for cleavage of the aberrant proteins (203). The first observations on the link between the Cpx system and aminoglycoside susceptibility are not new, with a 1970s study showing that mutations in *cpx* genes are associated with changes in AG resistance levels (204). More recently, in *E. coli*, it was shown that a *cpxR* mutant leads to increased sensitivity to aminoglycosides (205) and, in *S. enterica cpxAR* system activation has also been shown to increase AG resistance (206). Moreover, *cpxP*, a member of the *cpx* regulon, was found to be highly induced by the aminoglycoside gentamicin in *E. coli*, in a process dependent on *cpx* system (207). The Cpx system is also important for the AG-tolerant phenotype of a *V. cholerae ΔravA-viaA* mutant (124). The RavA-ViaA complex of *E. coli* is known to modulate AG sensitivity (208–210) but mechanisms are still elusive.

Because the cpx regulon is complex and involves several genes, the role of the cpx system on AG susceptibility may be highly pleiotropic. For example, the activation of the Cpx response in *E. coli* leads to a reduction in the transcription of genes involved in the electron transport chain (205), and more recently, it was also observed in *E. coli* that the Cpx stress response is involved in the turnover of respiratory chain proteins (211). These observations thus suggest an indirect role of *cpx* system on the metabolic state of the cell, which in turn may affect membrane polarization and consequently aminoglycoside uptake.

Similar to the *cpxAR* system in *E. coli*, aminoglycoside resistance in *Pseudomonas aeruginosa* has been linked to the AmgRS TCS (190, 212). One of the primary effects of

the AmgRS system activation is the upregulation of specific genes involved in aminoglycoside resistance, such as several membrane proteases like FtsH and HtpX (190). The mechanism of intrinsic aminoglycoside resistance through the AmgRS are proposed to be similar to that of Cpx in *E. coli,* i.e., efficient degradation of aberrant misfolded membrane proteins and maintenance of envelope homeostasis.

Another TCS of high importance in AG resistance in *E. coli* is the EnvZ/OmpR system. This system, which can be activated by several environmental cues, is known to control the relative levels of the outer membrane proteins OmpC and OmpF to adjust membrane permeability and maintain cell envelope stability. As AGs can passively diffuse through these OMP (as previously discussed in this review) (101), it is likely that EnvZ/OmpR system ends up by affecting AG uptake.

Finally, TCS systems linked with uptake or response to AGs are still being discovered, such as the Zra system which was proposed to be a functional homolog of Cpx in *E. coli* (213) and a Zra/Cpx hybrid system newly discovered in *V. cholerae* (124).

Aminoglycosides and oxidative stress response

Oxidative stress in bacteria occurs due to excessive production of ROS (214). ROS are highly reactive molecules derived from oxygen and include superoxide anions (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH), which can react with nearly all biomolecules in the cell including proteins and ribonucleic acids. ROS are naturally byproducts of aerobic metabolism because leakage of electrons from the electron transport chain can react with oxygen. To cope with the presence of ROS, bacteria encode several proteins capable of detoxifying the cell from these molecules (215). However, external factors such as antibiotics can also induce ROS production in bacteria and generate excessive ROS, overwhelming the antioxidant machinery of the cell. For example, because of AG-induced mistranslation, misfolded membrane proteins are thought to generate ROS through disruption of membrane integrity and alterations in the PMF (198). Indeed, some studies have reported a link between AGs and ROS formation in different bacterial species (139, 198) and others have shown that overproduction of ROS scavengers increase AG resistance (191). It is also important to note that not only misfolded proteins induce ROS production, but also that misfolded proteins may be themselves more prone to oxidation by ROS (191, 216). Indeed, excessive levels of ROS cause post-translational modification of specific amino acids and side chains which in turn affects folding chemistry and can result in protein aggregation (217, 218).

Thus, from the moment of the initial uptake until the collapse of proteostasis, AGs are involved in the activation of several bacterial stress responses that interplay and dictate the fate of AGs uptake and their efficiency.

POTENTIATION OF AMINOGLYCOSIDES

Potentiation as a strategy to enhance antibiotic efficacy

In response to the lack of new antibiotic molecules, researchers have been exploring alternative approaches for the treatment of bacterial infections (219–221). One promising strategy lays in methods improving the efficacy of existing antibiotics (222). One such approach is antibiotic potentiation, which involves combining an antibacterial agent with a non-active agent (molecule, physical treatment, or chemical treatment) to enhance the effectiveness of the antibiotic. This concept has been classified into two types by reference (223): class I adjuvants/potentiators that act directly on the pathogen by inhibiting active resistance mechanisms (e.g., modifying enzymes or efflux pumps) or passive resistance mechanisms (e.g., targeting the membrane barrier or biofilm formation); and class II adjuvants that enhance the host's ability to eliminate the pathogen. It is important to note that this review does not discuss potentiation of the lethal effect of aminoglycosides that are independent of uptake. We will focus below on class I adjuvants that have been proposed as potentiators of AGs.

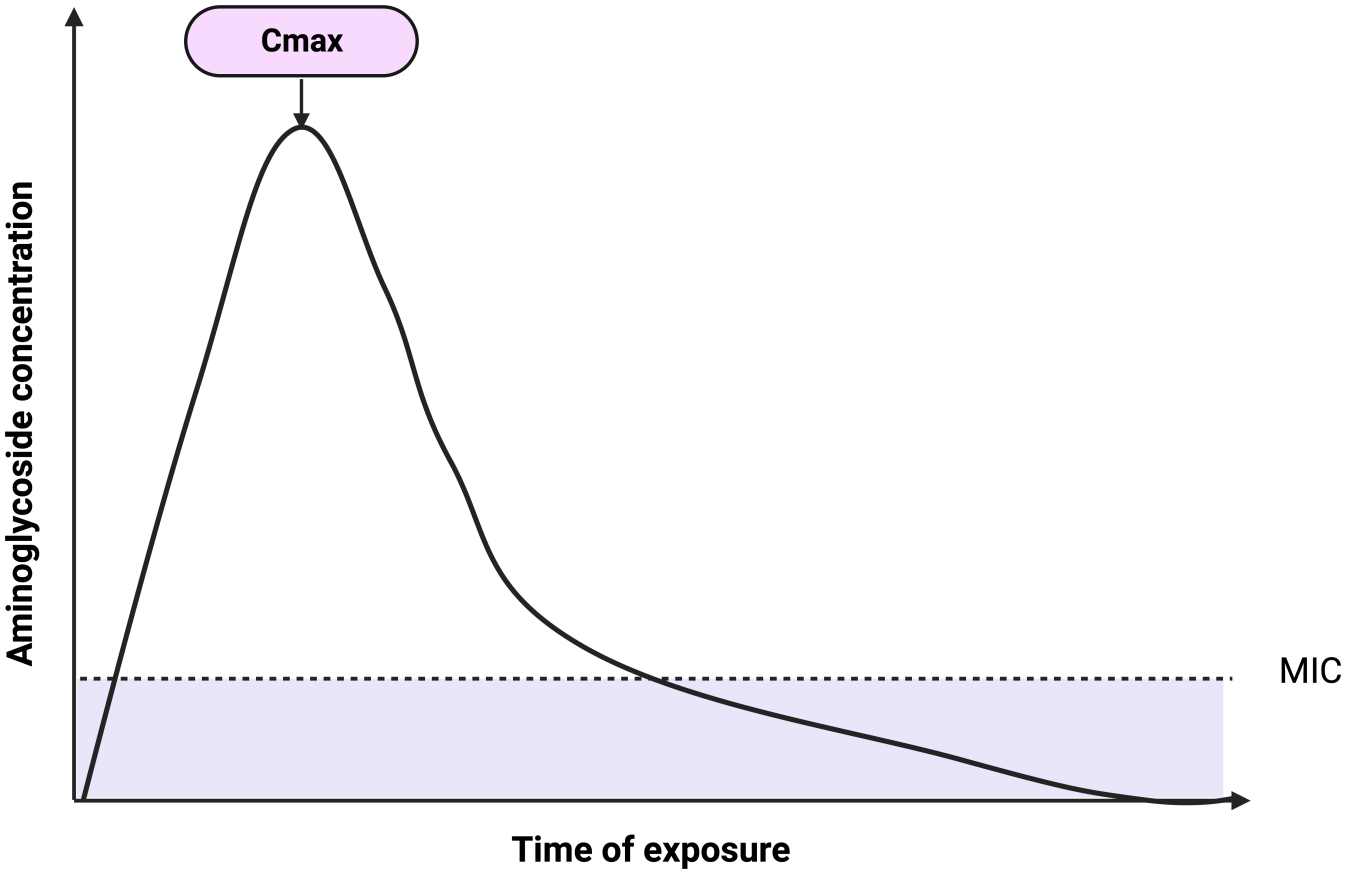


FIG 2 Pharmacokinetic and pharmacodynamic parameters of aminoglycosides concentration. The probability of clinical response is correlated with the dose administered (peak Cmax/MIC), rather than the time of exposure.

The use of aminoglycosides in modern medicine

AGs are crucial molecules in the fight against infectious diseases due to their broad spectrum of action, targeting aerobic Gram-negative bacteria, *Staphylococci*, and Gram-positive bacilli. However, they are ineffective against *Streptococci*, *Pneumococci*, and anaerobic bacteria. AGs are prescribed in humans for prophylaxis during surgery or as part of the treatment for various infections, including urinary tract infections, pyelonephritis, abscesses, septicemia, pneumonia, and endocarditis (224). The primary AGs used in hospital settings are gentamicin, amikacin, and tobramycin (225). Initially, these antibiotics were administered in multiple doses until the effectiveness of single doses was demonstrated, attributed to their prolonged post-antibiotic effect resulting from irreversible binding to ribosomes (226). This is conditioned by a normal renal function (227, 228). The administration of multiple doses is however still necessary for specific infections such as endocarditis, pediatric treatment, or in synergistic combinations with other antibiotics such as β-lactams, colistin, fosfomycin, or glycopeptides (225).

Enhancing uptake for aminoglycoside potentiation

Once administered in the human body, the antibiotic reaches a peak concentration that gradually decreases over time. Thus, the choice of the dosage protocol for a given antibiotic is determined by its pharmacokinetic and pharmacodynamic parameters. AGs exert their effects in a concentration-dependent manner, and the dosing strategy for these molecules aims to achieve a higher peak concentration (Cmax) in relation to the MIC (Fig. 2). The probability of a clinical response, indicating treatment efficacy,

has been shown to be strongly correlated with the dose administered and the peak concentration (Cmax/MIC) (229). However, the occurrence of adverse effects (230), limits the flexibility in therapeutic options. Generally, a dose at least 10 times higher than the MIC is recommended to ensure effective treatment and to minimize the risk of selecting antibiotic-resistant mutations (231, 232). This means that, in the case of AGs, increasing the intracellular drug uptake in bacteria increases the peak at Cmax and enhances the likelihood of treatment efficacy. Recently, Webster and Shepherd (28) provided a comprehensive review of the environmental and metabolic factors that can influence the efficacy of AGs. Some of these factors act as booster of AG entry into cells and can be exploited to potentiate AGs and improve their effectiveness in killing bacterial pathogens. This becomes particularly relevant in the context of rising antimicrobial resistance, where each effective molecule plays a crucial role.

PMF-dependent potentiation of aminoglycoside uptake

In the case of AGs, the literature suggests that the modes of potentiation primarily involve increasing drug entry by stimulating the PMF. Various approaches have been tested and published to enhance aminoglycoside uptake by increasing PMF.

As mentioned earlier, PMF is generated by respiration, and also depends on pH. Thus, modifying respiration, energy metabolism, or pH can be a strategy to increase PMF and, consequently, drug uptake. The use of bacterially metabolizable compounds, such as amino acids, nucleosides, and sugars, provides the advantage of reduced toxicity in humans. Below are examples of the proposed use of such compounds to potentiate AGs.

Regarding pH, the use of basic molecules such as L-arginine or L-lysine has been shown to potentiate AGs against persistent cells of *A. baumannii* (233), *S. aureus*, *E. coli*, and *P. aeruginosa* (234). Interestingly, lung epithelial cells have been found to secrete host metabolites such as succinate and glutamate, which also increase intracellular pH (235), and contact between *P. aeruginosa* and mouse epithelial cells sensitizes the bacteria to AGs (236).

Activating respiration by fumarate inhibits the formation of persistent *P. aeruginosa* cells (53, 237). On the other hand, the biosynthetic pathways of specific amino acids, such as serine, glycine, glutamine, tryptophan, threonine, and alanine, were found to potentiate AG uptake by *E. coli* persisters by influencing the cellular energy state (49). In the same line, a study on lab-evolved *E. coli* revealed that AG-resistant clones had low levels of metabolites involved in amino acid metabolism which fuels the energy metabolism (238). However, this effect was not specific to AGs (239). Adenosine has been shown to enhance the effectiveness of various antibiotic families, including AGs, against persister bacteria, through increase of PMF, and suppression of stress responses (240). NADH plays a central role in energy metabolism and respiration. Promoting NADH production directly leads to increased PMF (241). Metabolites such as glutamate, L-tryptophan, or alanine have been shown to increase NADH production, thus AG uptake, re-sensitizing resistant bacteria or reducing persister cell formation (49, 242–245). Alanine's role in AG sensitivity has also been associated to ROS production (246).

In a similar way to the above-mentioned amino acids, some sugars have also been shown to increase AG uptake through increased NADH. These include D-ribose in *Salmonella* (247), fructose in *Edwardsiella tarda* biofilms and persister cells (248), glucose in cefoperazone/sulbactam-resistant *Pseudomonas* (249), and fructose in resistant *Salmonella enteritidis* (250).

One promising carbohydrate potentiator of AGs is mannitol. Potentiation of AGs using mannitol stimulates PMF and leads to eradication of persistent *E. coli* cells and biofilms (48), and of persistent *Pseudomonas* cells (251). Importantly, such a potentiating effect of mannitol can lead to the use of effective concentrations below the threshold of renal toxicity (252).

But other carbohydrates can also potentiate AGs without stimulating the PMF, because they can regulate the expression of carbohydrate transporters, which internalize AGs. One promising strategy of potentiation comes from the search for molecules

capable of increasing the number of sugar transporters at the membrane (60) identifying uridine and other nucleosides as metabolites that increase the expression of sugar transporters. Supplementation with uridine increases AG uptake into *E. coli* and improves the efficiency of AGs, without changing the PMF. However, it is important to maintain the PMF for this potentiating effect to be observed (60), likely because its presence is necessary to power the sugar transporters.

Alternative approaches to metabolites have also been proposed as potentiators of AGs, such as heats shock which, similarly to AGs, can induce proteotoxic stress, protein misfolding, and ROS formation. A synergistic effect is thus almost expected between high temperature and AGs. However, high temperature appeared also to boost the PMF in *E. coli* stationary phase cells, and facilitate AG uptake (200).

The use of n-butanol has demonstrated effectiveness in rapidly eradicating persistent cells of *S. aureus*, as well as various Gram-negative and Gram-positive species within a short period (1 minute). This potentiation effect of n-butanol is PMF-dependent since it is abolished by CCCP, although PMF is not modified, and still relies on the action of AGs on the ribosome, as it is abolished by the presence of a ribosomal mutation (253). However, implementing this type of potentiation strategy would be challenging due to concerns regarding toxicity of such an alcoholic compound.

Synergy between AG and other drugs for the uptake of AGs

In addition to potentiators devoid of any bactericidal action when administered alone, AG uptake can also be increased due to synergistic effect with other drugs (254). The majority of these examples involve boosting of PMF by the co-administered drug. This is the case for tigecycline, an inhibitor of protein synthesis (255), the silver-containing antimicrobial "AGXX" (256), or even anticancer drugs such as vincristine or vinblastine (257), which are indole derivatives. Another indole derivative, 4-fluoroindole, not only acts on PMF, but also possibly inhibits the efflux pump MexXY-OprM (258). One PMF boosting adjuvant was recently identified through an approach based on the idea that antibiotic-producing bacteria could also produce adjuvants: the actinomycete product VentA (for venturicidin A) potentiates gentamicin against multidrug-resistant clinical isolates (259). Agents that target the bacterial cell wall, such as β-lactam antibiotics, have also been shown to facilitate AG entry (260–262). The combination of AGs with penicillin, for example, has been proven to be effective and is currently used in clinical therapies. The synergistic effect between penicillin and AGs can be suppressed by electron transport inhibitors (263), indicating that penicillin also enhances AG entry through PMF-dependent uptake into the cytoplasm.

PMF-independent potentiation of aminoglycoside uptake

PMF-independent potentiation of uptake primarily involves strategies that enhance AGs uptake by destabilizing the outer membrane and modulating porins, or through EDPII.

One mechanism is believed to occur through physical perturbations applied to the cell membrane, and the inhibition of cellular responses to such stress. For instance, hypotonic shock has been proposed to potentiate AGs entry through the mechanosensitive MscL porin, which is activated in response to membrane destabilization (264). Cold shock also significantly increases the entry of AGs in *P. aeruginosa* in a skin infection model in mice (265). Rifampicin was found to interact with the *Pseudomonas* AmgRS two-component system (mentioned earlier), counteracting the stress response against AG-induced membrane damage (266).

Potentiators that act on AG export through efflux pumps have also been described (267). For example, meropenem, which inhibits the MexXY-OprM efflux system in *P. aeruginosa*, increases sensitivity to AGs (268).

Other potentiators act on protein synthesis and potentiate EDPII, like silver (269). While the precise mechanism is not yet fully understood, silver is believed to destabilize the membrane, bypassing the EDPI process and increasing the entry of AGs into the

cytoplasm. This requires EDPII, i.e., the production of non-functional proteins. Alternatively, the action may occur directly at the level of the translating ribosome, leading to the generation of more misfolded/non-functional proteins and consequently increasing entry (269). Silver has actually been observed to interact with various bacterial cellular processes, ultimately leading to membrane permeability (270). In a *Caenorhabditis elegans* model, silver nanoparticles reduced the MIC of amikacin by 22-fold without toxicity against the host (271).

Certain potentiation pathways are specific to Gram-positive bacteria and cannot be exploited in Gram-negative bacteria. For instance, rhamnolipids increase PMF-independent entry of AGs in *S. aureus* and other Gram-positive bacteria by altering membrane charges, fluidity, and permeability. However, it is ineffective against *E. coli* (150).

The process of developing a new molecule and bringing it to market can span over 20 years, involving extensive research, clinical trials, and production setup, incurring significant costs. Potentiating antibiotics by enhancing the entry of existing antibiotics represents a rapid, effective, and cost-efficient alternative to the development of new molecules.

CONCLUSION AND PERSPECTIVES

In the current scenario, the rise in antibiotic resistance is anticipated to result in up to 50 million deaths per year within the next 30 years, leading to significant economic implications such as increased treatment costs and work absences, in addition to the obvious public health concerns (272). Gram-negative bacteria are associated with high lethality: for example, in the case of MDR *P. aeruginosa* septicemia, the rate of associated death is 67%. For a soft tissue infection with *K. pneumoniae*-necrotizing hyper-virulent strain, it is from 25% to 47% (273). Concurrently, pharmaceutical companies are progressively disengaging from antibiotic research due to the high development costs and limited financial returns associated with them (274).

Therefore, the need for new treatments has become imperative. A comprehensive review by Walesch and colleagues provides insights into the present and future of antimicrobial development (275). Discovering new active compounds faces numerous challenges, including the isolation of molecules from non-cultivable organisms, the necessity for novel mechanisms of action to overcome pathogen resistance, concerns regarding toxicity and lower efficacy in humans compared to animal models, as well as the difficulty of crossing the bacterial outer membrane. Alternatively, improving existing antibiotics through structural modifications, targeted delivery, and potentiation or combination therapies is another avenue (276).

Understanding the different mechanisms by which AGs enter bacterial cells can contribute to the development of new treatments, as exemplified by several promising studies on AG potentiation. Among ongoing antimicrobial projects, 8% involve potentiation approaches (277). Most of the potentiators currently being developed are β-lactamase inhibitors, such as those from the diazabicyclooctane family, which are currently undergoing clinical trials (278). In terms of improving antibiotic uptake, SPR741, a novel polymyxin derivative, has shown the ability to enhance the penetration of macrolides or glycopeptides in Gram-negative bacteria and is currently in clinical trials for systemic use (279, 280).

AGs uptake now appears to be tightly linked to carbon sources utilization, as demonstrated by numerous studies that show the relevance of using sugars to stimulate PMF, and the role of active sugar transporters in AG transport. This can pave the way for future research on AG uptake through specific transporters, and have implications in terms of potentiation.

From a fundamental perspective, further research is needed to establish the profiles of carbon source-related regulation (CCR) in different Gram-negative pathogens and the identification, for each species of interest, of sugar transporters that also transport AGs. This would enhance our understanding of the conditions that lead to increased uptake of AGs in a variety of bacterial species.

The regulation of AG transporters can be species-specific, particularly regarding the CRP-cAMP-dependent CCR. CCR is well-characterized *E. coli*, and the regulator CRP is highly conserved, but the regulatory mechanisms can vary among different species (281), as different bacterial species are specialized in their own natural environment. This means that, although it would be ideal to identify a universal molecule to increase the number of AG transporters, the use of a particular sugar would not necessarily produce the same outcome in every species. For instance, bacteria belonging to the order *Pseudomonadales*, including the genera *Pseudomonas* and *Acinetobacter*, exhibit a diverse range of metabolic capabilities. Unlike for Enterobacteria, glucose does not play a central role for them. Instead, they preferentially utilize amino acids or succinate as carbon sources, which repress the enzymes involved in glucose utilization. CCR in *Pseudomonadales* is thus referred to as "inverted" (282). Unlike for *E. coli* (283, 284), the levels of cAMP in *P. aeruginosa* and *Pseudomonas putida* remain relatively constant regardless of the growth conditions (285), and does not alter CCR (286).

Transcriptional regulators of CCR can also be unexpectedly different among different species. In *Pseudomonas*, while the transcriptional regulator Vfr shares 67% identity with CRP (287), the functional homolog of CRP in *P. aeruginosa* is, in fact, not Vfr (288), but Crc (289–292). Consequently, understanding the impact of sugar transporters and different carbon sources on AG uptake can be more challenging in *P. aeruginosa*.

Additional regulatory layers even complicate the complete understanding of regulation of sugar transporters, as observed with the example of *Vibrio* spp., where the *ctrR*, a non-coding RNA is involved in regulation of the abundance of sugar transporters, resulting in increased AG uptake (59). Although no sequence homolog of this RNA has been found in other species, one cannot exclude the existence of such RNA-related regulation. One indication is that in *Pseudomonadales,* CCR involves the RNA chaperone Hfq and the small RNAs CrcZ and CrcY (293–296).

While the regulatory mechanisms can be complex, sugar transporters may be able to transport AGs in numerous, if not all, bacterial organisms, and they can easily be identified through overexpression strategies (60).

From a clinical perspective, the use of sugars as potentiators of AGs could be a promising strategy due to the expected innocuity of these metabolizable compounds and the wealth of previous clinical studies on the (non-)toxicity of these molecules in the human body. In fact, mannitol and uridine, described above as potentiators of AGs, are already used in clinics for other indications. Mannitol is currently used for managing cerebral edema or for kidney protection (297). In addition, numerous studies have been conducted to compare the pharmacokinetic and dynamic properties, as well as side effects, considering different modes of administration, for compounds such as uridine, used in cancer therapies and rheumatology, and ribose, used as dietary supplement [e.g., (298–300)]. This offers the possibility of potentiating AGs using existing treatments, with new applications.

In several countries, including France, there is the provision for the use of drugs for purposes other than those specified in their marketing authorization, particularly when no therapeutic alternatives are available. This approach, known as drug repositioning, often applies to older drugs. With drug repositioning, the initial steps of assessing toxicity and tolerance in humans have already been conducted, eliminating the need for repetition. Similarly, "off-label" use of drugs is not prohibited in the United States. This rationale has also been applied to the development of treatments for coronavirus disease 2019 (COVID-19). For instance, the combination of the drugs remdesivir (originally developed for ebola) and diltiazem (an anti-hypertensive) could provide significant benefits to COVID-19 patients (301). Given the concerning rise of antibiotic resistance, this approach is gaining increasing interest among scientists. For AG, potentiators have been identified among various molecules that are already approved and available on the market for treating other diseases, as described above.

These investigations help to reduce costs and time-to-market by leveraging existing knowledge and data, thereby minimizing the need for extensive clinical trials.

In addition to the advantage conferred by the use of potentiators together with known antibiotics in the context of drug repurposing, potentiation also offers the possibility of a novel treatment, while avoiding the necessity of identifying new bacterial targets for new antibiotics. Potentiation can also allow for bypassing bacterial resistance mechanisms, through efflux compensation or saturation of antibiotic-inactivating enzymes, as is the case for a widely used antibiotic *Augmentin*, which combines the β-lactam amoxicillin with a β-lactamase inhibitor. AGs are particularly suitable for potentiation through enhanced uptake, as their efficacy is closely tied to their concentration within bacteria. While the use of AGs is limited due to their side effects (302, 303), increasing the intracellular concentration of AGs in bacteria could enable the administration of lower doses and mitigate side effects in humans. The study of potentiation largely focuses on stimulating the PMF or CCR, both of which are highly conserved mechanisms. This suggests broad-spectrum efficacy, thus simplifying patient recruitment compared to pathogen-specific drugs.

Considering the escalating threat of antibiotic-resistant infections, AGs could play a crucial role in the effective treatment, and potentiation appears to be a fast and efficient approach of unlocking the full potential of these molecules.

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