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Review

Zinc: Multidimensional Effects on Living Organisms

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Abstract: Zinc is a redox-inert trace element that is second only to iron in abundance in biological systems. In cells, zinc is typically buffered and bound to metalloproteins, but it may also exist in a labile or chelatable (free ion) form. Zinc plays a critical role in prokaryotes and eukaryotes, ranging from structural to catalytic to replication to demise. This review discusses the influential properties of zinc on various mechanisms of bacterial proliferation and synergistic action as an antimicrobial element. We also touch upon the significance of zinc among eukaryotic cells and how it may modulate their survival and death through its inhibitory or modulatory effect on certain receptors, enzymes, and signaling proteins. A brief discussion on zinc chelators is also presented, and chelating agents may be used with or against zinc to affect therapeutics against human diseases. Overall, the multidimensional effects of zinc in cells attest to the growing number of scientific research that reveal the consequential prominence of this remarkable transition metal in human health and disease.

Keywords: metalloproteins; zinc transporters; metal chelators; antibiotic resistance; antimicrobials

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1. Introduction

Zinc, an essential component of life in the three domains, follows iron as the second most abundant transition metal ion in living organisms [1,2]. About 5–6% and 9–10% of proteins from prokaryotes and eukaryotes, respectively, depend on this metal to fulfill their biological functions [1]. A bioinformatics study found that over 50% of zinc-bound proteins are enzymes, and in the vast majority of them, the metal plays a catalytic role [3]. About 20% of them use zinc as a structural component, and in a small percentage, it is a regulator or substrate of enzymatic activity [3–5]. The requirement of zinc in such a high number of proteins illustrates its fundamental role in numerous biological processes [6–11]. The essential nature of zinc for cellular viability, together with the toxic nature of the element in higher concentrations, led prokaryotes [12] and eukaryotes [13] to evolve export and import systems to keep ionic homeostasis.

This review summarizes representative examples of zinc's action on bacterial virulence and antibiotic resistance gene dissemination, different bacterial mechanisms to maintain zinc homeostasis, and possible uses of the metal ion in the development of novel therapeutics. It also discusses multiple roles that zinc ions play in mammalian cells, including its modulatory effect on cell signaling and cell death, in which a wide variety of therapeutic applications is continuously being researched and developed to produce an immense volume of information. Therefore, this review offers the reader a representative rather than an exhaustive description of mechanisms of action, uptake, and export as well as pharmacological uses of zinc and its chelators.

2. Prokaryotes

2.1. Zinc as Antimicrobial

Zinc is a member of the group of metals that participate in the nonspecific mechanisms of defense against infection [14,15]. The host defenses reduce trace elements' availability to starve the infecting bacterial cells in a response known as "nutritional immunity", a term

coined in the mid-70s [16]. The first hint to this defense strategy's existence occurred in the mid-40s, when the high-affinity iron-binding transferrin was discovered [17,18]. Immediately after the invasion of infecting bacteria, the body responds, reducing free iron levels in the blood and tissue (hypoferremic response) [19,20]. Posterior studies have shown that nutritional immunity is a strategy that is not limited to iron sequestration and also includes restricting the availability of other essential elements, including zinc [21–25]. In the face of these nutritional limitations, microorganisms evolved stratagems to scavenge sufficient quantities of trace elements necessary to support their metabolism and growth. Zinc is a component of nutritional immunity; in human serum, which contains 0.1% of the total body zinc, about 98% is bound to proteins, mainly albumin (80–85%) and alpha-2-macroglobulin (5–15%), and in marginal quantities to other proteins [26–28]. Additionally, zinc is further restricted to pathogens in ongoing infections by releasing calprotectin, a protein that sequesters this metal and creates zinc-limited microenvironments [29–32]. Calprotectin, a heterodimer formed by the S100A8 and S100A9 proteins, also binds manganese and iron [31,33,34], and it has a proven effect against infection [23,33–38]. Other proteins, like the S100 family calgranulin C (S100A12) and psoriasin (S100A7), have also been shown to be able to bind zinc and could contribute to nutritional immunity response [39,40]. As zinc is a component of nutritional immunity, bacteria need to sense the intracellular concentrations and put in motion the different mechanisms involved in this element's homeostasis. Interestingly, while essential to support growth, zinc is also known to inhibit the progress of infectious processes caused by bacteria [41,42] and viruses, including SARS-CoV-2, the causative agent of COVID-19 [43–46]. Zinc also inhibits SOS-induced antibiotic resistance and horizontal transfer of antibiotic resistance genes in enteric bacteria [47,48]. Another utilization of zinc as defense by the human host is through macrophages, which use it within the phagolysosome to intoxicate invading bacterial cells [49–53]. A common mechanism by which zinc in excess is toxic to bacterial cells is by binding to noncognate proteins [52,54–56].

The effect of zinc in the progress of infection was also investigated utilizing zinc-deficient murine models [45,57,58]. Furthermore, zinc-deficient mice were found to be suitable models for more general studies of infections caused by enterotoxigenic *Escherichia coli*, *Shigella flexneri*, and *Campylobacter jejuni* and potential treatments and immunization [45,57,58].

While zinc uptake is an essential process for bacterial pathogens to cause disease, this element can also be detrimental to some infections. Since long ago, zinc has been used as treatment and prophylaxis of diarrheal diseases [59–61]. It was originally thought that the beneficial effect of zinc in treating enteropathogenic *E. coli* (EPEC)-produced diarrhea was entirely caused by enhancement of the immune response and inhibition of ecto-5′-nucleotidase, an enzyme that catalyzes the conversion of the 5′-AMP to the secretagogue adenosine [57,58]. However, this initial idea proved to be insufficient to explain the therapeutic effects observed [57,58,62]. Addition of zinc acetate at sublethal concentrations caused a decrease in the expression of various virulence factors [62]. The mRNA species corresponding to the *bfp* gene (bindle forming protein) and various *esp* genes (EPEC-secreted proteins) were expressed at reduced levels. Furthermore, zinc acetate lowered the bacterial cells' adherence, inhibited secretion of infection-induced fluids into ileal loops, and reduced histopathological damage in an animal model of infection [62]. Zinc acetate also had effects on the virulence of Shiga-toxigenic *E. coli* (STEC) and enteroaggregative *E. coli* (EAEC) [44,45,63]. It inhibited STEC adherence to cultured cells, expression of enterohemorrhagic *E. coli* (EHEC)-secreted protein A (EspA) and Shiga toxin. In vivo, it reduced fluid secretion and toxin levels in the loops and reduced STEC-induced histological damage [64]. In several forms like oxide, sulfate, and acetate, zinc was also used to test its effects on EAEC [42]. A decrease was observed in biofilm formation, cell adhesion, and expression of other potential or confirmed virulence factors [42]. The observation that zinc reduced the expression of *recA* suggests that inhibition of the SOS response may be one mechanism by which zinc acts on *E. coli* virulence [41]. This finding also prompted other studies to test if

zinc could also reduce SOS-induced hypermutation response to antibiotics or horizontal transfer of resistance traits [48,65–67]. Zinc blocked the SOS-induced (hypermutation response) development of resistance in *E. coli, Klebsiella pneumoniae*, and *Enterobacter cloacae,* probably by inhibiting RecA binding to single-stranded DNA [47]. Zinc also interfered with horizontal transfer of a β-lactamase gene from *Enterobacter* to *E. coli* strain [47]. As observed with other zinc effects [63,68], the complex zinc ionophore showed significantly higher activity than zinc salts in inhibition antibiotic-induced hypermutation [47].

Environmental enteropathy, a small intestinal disorder caused by subclinical intestinal infections, produces chronic low-grade intestinal inflammation and dysregulation of tight junctions [69,70]. A study on adults with environmental enteropathy showed disruptions that cause leakage in the patients' epithelial barrier [69]. The authors hypothesized that the sites with epithelial defects could be responsible for bacterial translocation. In vitro experiments utilizing enteropathogenic *E. coli* and *Citrobacter rodentium* showed that these bacteria induced barrier dysfunction, and treatment with numerous compounds like zinc, epidermal growth factor, colostrum, trefoil factor 3, resistin-like molecule-β, hydrocortisone, and ML7 (an inhibitor of the myosin light chain kinase) increased transepithelial resistance while reducing bacterial translocation [71]. This effect was nutrient-independent, and, of all the tested compounds, only zinc exhibited an antimicrobial activity [71].

2.2. Zinc Oxide Nanoparticles as Antibacterial

Zinc ions at concentrations higher than those needed for the cells' normal physiology are detrimental [12]. Multiple effects of excess zinc concentrations lead to bacterial cell death [12,52]. Interestingly, zinc ions at sublethal concentrations inhibit biofilm formation but do not disrupt preformed biofilms in *Actinobacillus pleuropneumoniae*, *Salmonella typhimurium*, *Haemophilus parasuis*, and at a lesser level, *E. coli*, *Staphylococcus aureus,* and *Streptococcus suis* [72]. Zinc oxide is the most common, but not the only, zinc compound used as an antibacterial [73,74], and it has attracted great interest in nanoparticle form [75,76]. Zinc oxide nanoparticles are being researched for applications not only against infections but also as drug delivery tools and as therapies for a variety of conditions [75]. The enthusiasm for nanoparticles' uses as antibacterial agents is partly fueled by their particular mechanisms of action that differ from those utilized by currently used antibiotics and have targets reducing the frequency of appearance of resistant strains [77,78]. Various research teams have tested the activity of zinc oxide nanoparticles as antibacterials against numerous species [75,76,79–84]. The mechanism by which zinc is toxic to bacterial cells is ultimately that of other forms of the ion; however, zinc oxide seems to be more effective when it is administered in nanoparticle form [80,81,85]. Zinc oxide nanoparticles release zinc in an aqueous medium, which then penetrates the cells and produces toxic effects [75]. Zinc oxide nanoparticles are robust candidates to be developed as standalone antimicrobials or as components in combination therapies against multiresistant bacterial infections.

2.3. A Brief Overview of Zinc Homeostasis in Bacterial Pathogens

The intracellular concentration of zinc must be tightly controlled to ensure that it is high enough to sustain life but low enough not to cause toxic effects that can lead to cell death [6,12,85–88]. Through the molecular interactions with biomolecules, zinc participates in a wide variety of metabolic processes as well as repair and maintenance of cell structures and biomolecules that are essential for bacterial cell growth [7,13,81,89,90]. However, higher than needed concentrations of zinc are highly detrimental to the life of the bacterial cell. Infecting bacteria may need to overcome zinc deprivation or toxicity depending on the location of the infection. While nutritional immunity produces deprivation of the metal ions, intracellular and other niches have the opposite effect [50,56]. As a consequence of the strict requirements for intracellular zinc concentrations, bacteria evolved several tightly regulated uptake, efflux, binding, and sensing mechanisms critical for pathogenicity [6,12,23–25,33–37,76,86,89–94]. This section will discuss the major and better-known

mechanisms of zinc import and export. Major regulation mechanisms will be described in the following section.

Import systems can be specific, usually coupled to an energy source, and nonspecific, mostly exerting their function through diffusion gradients across the cytoplasmic membrane [12]. A major import mechanism is ZnuABC, an ATP-binding cassette (ABC) transporter [95,96]. The ZnuA and ZnuB are the periplasmic and inner membrane components, respectively, and ZnuC is the cytosolic ATPase. ZnuA, the periplasmic component, belongs to the cluster A-I of substrate binding proteins [97]. These proteins are associated with ABC transporters in prokaryotes. They are periplasmic in Gram-negatives or lipid-anchored in Gram-positives. They can also exist as domains of periplasmic or inner membrane components [97,98]. The *znuA* gene is transcribed in opposite orientation to *znuC–znuB* [96,99]. The transport complex consists of one molecule of the periplasmic ZnuA and two ZnuC and ZnuB monomers [96,99]. Another component of this zinc uptake mechanism is ZinT (also known as YodA) [100–102]. This periplasmic metal-binding protein recruits zinc and delivers the ion to the ZnuABC complex through specific interaction with ZnuA [103–105]. It is worth mentioning that ZinT is not exclusively a zinc-binding protein. It shows affinity for other divalent metal ions and also participates in detoxification [106]. In Gram-negatives, there is a need for one or more outer membrane receptors. The protein ZnuD found in bacteria belonging to several families like *Neisseriaceae*, *Moraxellaceae*, *Acinetobacteriaceae*, *Pseudomonadaceae*, and *Bordetellaceae* was proven to be a zinc transporter and a requirement for a high virulence phenotype [107,108]. The 82 kDa ZnuD is a member of the TonB-dependent siderophore receptor family of proteins, and it may be able to recognize free or metallophore-complexed zinc ions [107]. ZnuD has been identified as a potential candidate for a vaccine against at least two bacteria: *Acinetobacter baumannii* and *Neisseria meningitidis* [103,109]. Interestingly, while the *znuD* gene is included in the *A. baumannii* chromosome, another homolog, *znuD2*, is present in about half of the isolates, in at least one case within a plasmid [93]. The role of ZnuD in *A. baumannii* virulence was proven but that of ZnuD2 remains to be clarified because of difficulties in generating a mutant that inactivates the gene [93]. Although most studies about the ABC transporter mechanism of uptake of zinc were carried out on Gram-negative models, research on Gram-positives showed that this mechanism also exists in these bacteria. The proteins AdcB and AdcC are the integral membrane and ATPase components, respectively. In addition, there are substrate-binding lipoproteins, AdcA and AdcAII, that are attached to the membrane's surface and are the functional equivalents to ZnuA and ZnuT [110]. Structural and functional studies showed that AdcA and AdcAII are substrate-binding proteins that bind zinc, but they do it via different structural features [97,110]. In particular, the structure of AdcA resembles a fusion between the zinc-binding domains of ZnuA and ZnuT [110]. Although AdcA and AdcAII have redundant functionalities in vitro, they are both required for full virulence in *Streptococcus pneumoniae* [110].

An outer membrane protein found in *N. meningitidis* is CbpA, which binds calprotectin in complex with zinc ions and, in a process that resembles the uptake of iron from transferrin or lactoferrin, mediates acquisition of zinc in a TonB-dependent manner [104].

Until recently, the mechanism to scavenge an essential metal ion by means of small molecular weight, high-affinity compounds known as siderophores was mostly known for the uptake of ferric iron. Siderophores compete with the host organism's high-affinity binding proteins, and the ferric–siderophore complexes are internalized into bacterial cells through specific receptors [105,111]. Recent research showed that bacteria possess a zinc uptake mechanism that mimics siderophore-mediated iron-uptake systems. Metal chelators, which are chemically unrelated to siderophores but play a similar role transporting several metals, were found in many bacteria [112]. They are known as metallophores, and when they transport zinc, are sometimes called zincophores. A broad-spectrum metallophore, staphylopine, produced by *S. aureus*, is one of the earliest metallophores found in a human pathogen [112]. Staphylopine participates in the transport of nickel, cobalt, zinc, copper, and iron [112]. Furthermore, the staphylopine biosynthetic pathway is conserved in

numerous bacterial pathogens, and in some cases, it is associated with virulence [112,113]. Although other zincophores were subsequently discovered, research on these elements is in its infancy. Many more will most probably be detected and characterized in the future [90,114–116].

ZIP (zinc–iron permeases or ZRT/IRT-like proteins) mediate zinc uptake into the cell's cytosol. They were thought not to exist only in bacteria, but it is now known that they can be found in all kingdoms [117]. Expression of some of them is regulated by zinc concentration like the Zip11 and Zip63 from *Nostoc punctiforme* [118] or the *Salmonella enterica* ZupT, which was shown to be a virulence factor [119]. Conversely, expression of the *E. coli* ZupT seems to be constitutive [120].

Transcriptomic analyses using mutants that lack a functional Zur (zinc uptake regulator, a regulator discussed below) led to the identification of elements involved in zinc homeostasis [99,121,122]. One of them, the *A. baumannii* ZrlA, is a zinc-binding peptidase induced in zinc-limiting conditions. A lipoprotein anchored to the inner membrane, ZrlA is necessary for appropriate uptake levels under zinc starvation and may promote uptake through modifications of the peptidoglycan [94]. Interestingly, mutants lacking a functional ZrlA are also more susceptible to antibiotics. Both effects may be related to the protein's contribution to cell wall integrity [94]. Another protein involved in *A. baumannii* zinc homeostasis is ZigA, a zinc-binding GTPase necessary for full growth in zinc-limiting conditions and full virulence [123].

Since zinc is highly toxic at higher concentrations, bacteria are also equipped with efficient efflux systems that contribute to the homeostasis. Gram-negative as well as Gram-positive bacteria can export zinc through P-type ATPase transporters, a ubiquitous superfamily of membrane ion transporters that couple ATP hydrolysis to ion passage from inside to outside of the cell [97,124,125]. The presence of P-type ATPase transporters in cells from all kingdoms of life and the characterization of many of them led to their classification in at least five subfamilies, P1–P5, that are further subdivided (P1B–P1B, P2A–P2D, P3A–P3B, and P5A–P5B) [126]. Zinc is mostly exported through P1B-type ATPase transporters, which can also transport other metal ions [91,92]. The ATPase's general structure includes a transmembrane domain that has a central core of six α-helices and a cytoplasmic portion that contains three domains: nucleotide-binding, phosphorylation, and actuator. The protein adopts two possible conformations, E1 and E2, and each one can be phosphorylated (E1P and E2P) or not. In conformation E1, the metal ion can reach an intramembrane site, and ATP–Mg can bind the nucleotide-binding site. The protein adopts the E1P state after phosphorylation by the transfer of a phosphate from ATP. A conformational change to E2P closes the possibility that the metal ion flows back to the cytosol. A further conformational modification releases the metal ion to the outside of the cell, followed by hydrolysis of the phosphate group, which leads to the adoption of the E2 state [92,126]. Several P1B-type ATPase transporters from Gram-negative and Gram-positive bacteria have been mechanistically and structurally studied, and many of them were confirmed as virulence factors [127–132].

Another family of transporters that export zinc outside the cytosol is the cation diffusion facilitators (CDF). Proteins in this family are subdivided into groups according to the metal they transport [121,122,124]. In prokaryotes, these transporters play a role in metal ions homeostasis, tolerance, and resistance [121,122,125,133,134]. ZitB, an *E. coli* member of the CDF family, was first shown to be an antiporter that effluxes the divalent metal cation in exchange for protons [135]. Studies on CDFs in several bacteria later confirmed that they export the cations through an antiport mechanism driven by the proton motive force [136–138]. In at least one case, the exchange could also occur with potassium [139].

The structure of the 32 kDa CDF YiiP protein from *E. coli* has been resolved. It exists as an integral membrane Y-shaped homodimer with a C-terminal 89-amino acids cytoplasmic domain [140,141]. The two upper arms of the Y are the transmembrane domains, which include six α-helices, and the lower stalk of the Y is the C-terminus cytoplasmic region.

Studies on YiiP and other structures that were resolved indicate that the C-terminal region is structurally related to metallochaperones. Therefore, one of the functions of this sector of the protein could be to sense zinc and deliver it to the transmembrane region [137,140–144]. At least two mechanisms have been proposed for the transfer of zinc through the cytoplasmic membrane. Both consider conformational changes as the ion contacts the protein, and it is subsequently exported [121]. Interestingly, CDF proteins that lack the C-terminus have been recently identified. Although the existing evidence points to a role in the export of zinc and other metal ions, research of these elements is still in the early stages [121,145].

The CDF and ATPase-mediated export mechanisms of Gram-negative bacteria deliver zinc to the periplasmic space, and then the ions must be exported outside of the cell wall. Conversely, heavy metal efflux (HME) systems, which belong to the resistance–nodulation–cell division efflux system superfamily, transport zinc from the cytosol to the milieu [13,95,97,146]. Three proteins assemble the HME export machinery. Components known as A and C are integral inner and outer membrane proteins, respectively. They usually exist as trimers and are connected through a periplasmic protein component (B) that forms a hexameric or trimeric ring around the inner and outer membrane components [147]. A HME system from *A. baumannii*, *czcCBA*, was recently studied, and it was found that all three genes are highly regulated, together with the CDF proteins coded for by *czcE* and *czcF*, when the cells were treated with 2.5 mM zinc sulfate for 1 h [148]. This work found that cytoplasmic zinc can be transported from the cytoplasm to the periplasmic space by the CDF member CzcD; a metallochaperone CzcI may bind the metal ion and deliver it to the CzcCBA system that completes the efflux process [148].

More detailed descriptions of bacterial systems and elements involved in zinc homeostasis can be found in numerous excellent reviews and publications [12,36,56,85,86,91,92,119,121–124,126,131,132,147,149,150].

2.4. Zinc-Driven Regulation of Gene Expression

Cells keep a delicate balance to avoid zinc deficiency or toxicity. For this, they are equipped with numerous systems, some of them discussed in the previous section, to take up or export zinc. Cells possess regulators, mainly transcription factors, that control the levels of expression of the different uptake and efflux systems to ensure that ions are imported or exported according to their needs [6].

The Fur family is an essential group of metal sensors that regulate gene expression. The first known member of this group of proteins was Fur (ferric uptake regulator), which functions as a classical repressor, interfering with transcription when complexed to iron [151,152]. Further research showed that most bacteria possess Fur homologs [15,146,153–158]. Fur is a dimer that binds iron, which acts as corepressor and activates it to favor binding to the Fur box (an operator) to turn off the expression of genes mainly involved in iron-uptake systems [159,160]. While this is the only identified factor regulating gene expression in many cases, Fur can serve as part of regulatory circuits that inhibit expression of genes when the concentration of cellular iron is high [161,162]. Fur also acts as a positive regulator of certain genes by repressing expression of a protein required for expression of another gene, facilitating binding of RNA polymerase when binding to the Fur box located upstream of a responsive gene, or binding to a Fur box located near an operator and interfering with repressor binding to such an operator [163]. Fur functions as a dimer and binds one iron per monomer. It binds iron at the C-terminus region and the DNA Fur box through a winged-helix DNA-binding domain located at the N-terminus [164]. A signature, histidine-rich motif ($HHHXHX_2CX_2C$), is located close to the dimerization domain between the DNA-binding domain and the C-terminal region [153,164]. Research on Fur through the years have shown that related regulator proteins have different metal-binding sites with affinity for other metal ions or compounds, such as hydrogen peroxide. Of particular interest to this article are the Zur regulators, which, mirroring the Fur proteins, act mainly, but not always, as negative regulators of gene transcription and repressors of uptake of zinc [6,165]. A search at the Research Collabora-

tory for Structural Bioinformatics Protein Data Bank revealed that three crystal structures of bacterial Zur proteins have been resolved [166–168]. The *E. coli* Zur structure was solved in association with zinc and bound to the promoter region of the *znuABC* operon [166]. The authors showed repression of gene expression mediated by highly cooperative binding of two adjacent dimers [166]. Studies using other Zur-regulated promoters showed significant differences in binding affinity between Zur–zinc complexes and the cognate DNA protein binding sites (Zur boxes) and the strength of inhibition of gene expression [166]. Zur proteins, as it is the case of other Fur family members, have two conformations. When bound to zinc (activated, closed conformation), it has high affinity for the Zur binding DNA regions. When not complexed to the metal (open conformation), it has negligible affinity for DNA [6]. Numerous experiments designed to elucidate aspects of regulation of gene expression by Zur and its requirement for virulence have shown that most of the time, albeit not always, it is essential for virulence [169,170]. An extensive listing of bacterial operons regulated by Zur can be found in a recent review [6].

A zinc-dependent gene regulator with functions similar to Zur, but with unrelated structural characteristics, has been identified and characterized in different species of *Streptococcus* [56,171–173]. This regulator, AdcR (adhesion competence resistance), is part of the MarR family of transcription factors. Proteins in this family participate in the regulation of expression of genes involved in virulence, antimicrobial resistance, and other physiological functions [174,175]. Similarly to Zur and other metalloregulators, AdcR is a homodimer that includes a DNA-binding and a zinc-binding domain. After associating with zinc, a conformational change increases the affinity for DNA operator sequences (known as *adc* motif) and leads to binding and inhibition of gene expression [176]. A recent study on *Streptococcus pyogenes* showed that by sensing the cytosol's zinc concentration, AdcR regulates about 70 genes. As is the case with other metalloregulators, AdcR acts as a negative and positive regulator, and its effects extend to functions other than zinc homeostasis, including virulence and metabolism [171].

Mechanisms to export zinc outside the bacterial cell, discussed in a previous section, are necessary to counter the host immune system based on zinc intoxication. They were first discovered when studying interactions between macrophages and *Mycobacterium tuberculosis* [49,53] and later described as a host defense against some enterobacteria and *Streptococcus* [50,51]. As is the case for systems that mediate uptake, expression of export systems is tightly regulated. The expression of the exporter CzcD proteins of *S. pyogenes*, *Streptococcus agalactiae*, and *S. pneumoniae* are activated by GczA (*S. pyogenes* and *S. agalactiae*) or SczA [50,177]. These are related transcriptional regulators that belong to the TetR family [50]. As is the case for the regulators Zur and AdcR, these regulators can also have the opposite function to which they were discovered. Therefore, GczA and SczA can also act as a negative regulator [177,178]. A recent study on SczA showed that it is a homodimer, with each monomer containing two zinc-binding sites necessary for transcriptional activation [177]. Studies on different species of *Mycobacterium* showed that as opposed to Zur's action, the SmtB binds DNA when it is bound to zinc, which happens when the metal ion concentration is too high. In consequence, an increase in the concentration of zinc results in detachment of SmtB from the operator region, inducing expression of zinc exporters like ZitA [179–181]. It is of interest that SmtB and Zur are part of an operon in *Mycobacterium* species, a characteristic that seems to be specific to mycobacteria and a small group of actinobacteria and *Corynebacterium diphtheriae* [1,182–184]. Goethe et al. proposed an elegant model of regulation of gene expression in *Mycobacterium smegmatis* at high and low zinc concentrations mediated by Zur and SmtB. Both regulators are expressed from a unique operon containing binding sites for both proteins (Figure 1) [185]. At low zinc concentrations, apo-SmtB binds the DNA and induces expression of the regulators (apo-SmtB and apo-Zur), then apo-SmtB binds the operator of the gene coding for an exporter protein [185]. Meanwhile, the expression of importers is allowed to proceed because apo-Zur does not bind DNA efficiently. At high zinc concentrations, the holo-Zur binds the Zur binding site upstream of the *smtB–zur* operon and represses the expression

of SmtB. It also binds the operator regions of genes coding for zinc importers. SmtB bound to zinc does not bind DNA, and zinc exporters are therefore expressed (see Figure 1) [185].

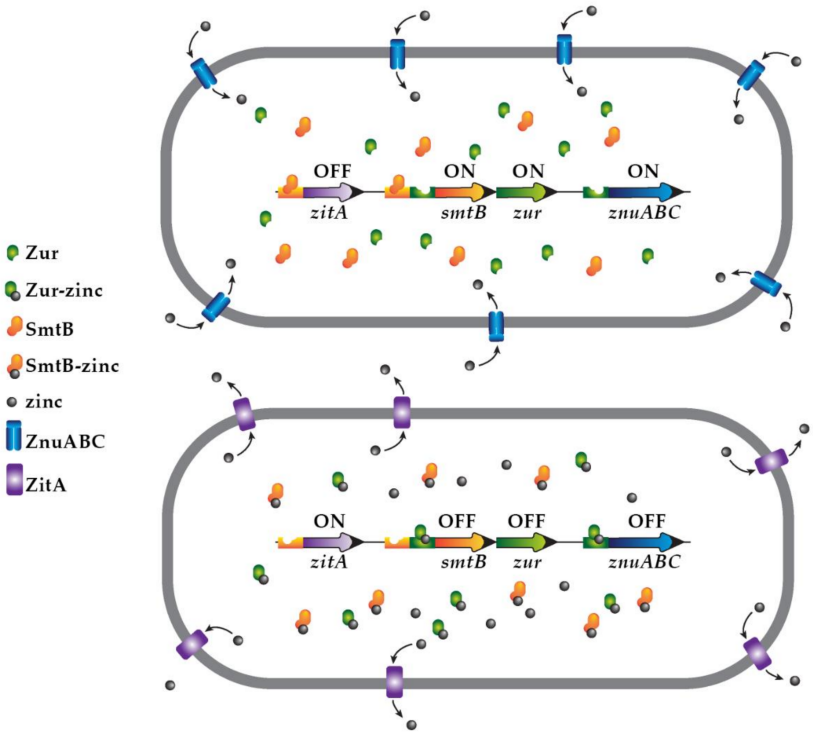

Figure 1. *Mycobacterium smegmatis* SmtB and Zur regulation of gene expression in response to the concentration of zinc. Model proposed by Goethe et al. [185]. The upper diagram shows a cell in an environment with low zinc concentration. In these conditions, SmtB is a homodimer that acts as a repressor of the expression of ZitA (zinc exporter). Binding of ZitA also facilitates expression of the *smtB* and *zur* genes. Zur depleted from zinc is unable to efficiently bind the Zur boxes upstream of *znuABC* (zinc importer) and *smtB*/*zur*. As a consequence, expression of ZnuABC and the regulators set up the cell to internalize zinc ions. The bottom diagram shows a condition where zinc ions are abundant; Zur binds the metal ions as a homodimer and binds the operators, turning off expression of *smtB*/*zur* and *znuABC*. SmtB loses affinity to the DNA, permitting high expression of ZitA, which facilitates export of zinc ions.

2.5. Zinc as Adjuvant to Antibiotic Treatment

Aminoglycoside antibiotics have a general chemical structure consisting of an aminocyclitol nucleus (streptamine, 2-deoxystreptamine, or streptidine) linked to amino sugars [186–188]. They are used to treat a broad spectrum of bacterial infections. Aminoglycoside modifying enzymes are responsible for the vast majority of aminoglycoside treatment failure in clinics [182,189]. Because therapies combining an antibiotic and an inhibitor of resistance proved to be potent weapons against β-lactam-resistant infections [190,191], there have been numerous attempts to find equivalent compounds to overcome resistance to aminoglycosides caused by modifying enzymes [183,184,190,192–195].

Recent studies to identify inhibitors of this resistance mechanism showed that zinc interferes with enzymatic inactivation by acetylation catalyzed by the aminoglycoside 6′-*N*-acetyltransferase type Ib (AAC(6′)-Ib) and others [68,193,196–198]. The initial experiments showed that zinc ions very efficiently inhibited AAC(6′)-Ib-mediated acetylation of aminoglycosides in vitro [68]. However, attempts to interfere with resistance in cellulo by treating bacteria with amikacin and zinc chloride ($ZnCl_2$) required enormous concentrations of the salt. The differences between the concentrations necessary for efficient inhibition in vitro and in cellulo could be due to permeability issues or the action of zinc export systems (see previous sections). Therefore, the combination treatment was mod-

ified to include ionophores that could increase the number of zinc ions that reach the cytosol. The addition of zinc complexed to pyrithione reduced the concentration required to add to the growth medium to reverse resistance to amikacin by 1000-fold [68,196]. Similar results were obtained utilizing 8-hydroxyquinoline analogs, such as clioquinol or 5,7-diiodo-8-hydroxyquinoline [63,197]. An aminoglycoside-resistant *A. baumannii* strain that resists these antibiotics' effects through a mechanism other than enzymatic modification grew equally in the presence of amikacin or amikacin plus clioquinol or 5,7-diiodo-8-hydroxyquinoline ionophores [63]. These experiments expanded the scope of zinc's therapeutic uses to encompass being an inhibitor of the clinically most relevant resistance mechanism to an important group of antibiotics as it is of aminoglycosides.

3. Eukaryotes

3.1. Importance of Zinc in Eukaryotic Cells

The effectiveness of antibiotics against bacteria exemplifies the importance of these drugs against many human infections. Unfortunately, the same drugs that kill bacteria have similar effects on the mitochondria present within eukaryotic cells [199]. The synergistic effect of zinc with antibiotics may also be the reason why zinc itself is toxic to eukaryotic cells at high intracellular levels because zinc enters the mitochondria and enhances production of reactive oxygen species (ROS) [200,201].

In eukaryotic cells, zinc exists in labile or free ion form (chelatable) [202], while zinc bound to proteins serve in a structural or catalytic capacity [202–204]. Zinc is a small ion (~0.65 angstrom) that binds nitrogen- and sulfur-containing molecules and readily exchanges ligands due to its low ligand field stabilization energy. Intracellular zinc levels range from 10^{-12} to 10^{-9} M in most cells, but zinc-enriched cells such as neurons, hepatocytes, splenocytes, and thymocytes may contain an estimated 10^{-6} to 10^{-5} M amount [205–208]. Zinc concentrations range from 10^{-9} M within the cytoplasm in most cells to 10^{-3} M in some vesicles [209]. In transgenic baby hamster kidney cells that express zinc influx transporter proteins, Palmiter and colleagues (1996) estimated that vesicular zinc concentrations reach 14 μM when these cells are exposed to high levels of exogenous zinc [210]. Cell survival in vitro is compromised when cells are exposed to extracellular zinc concentrations ($[Zn^{2+}]e$) between 225–1000 μM in neuronal cells and 7.5–200 μM in non-neuronal cells [211–214]. It is therefore essential that cells regulate their intracellular zinc concentrations through protein influxers and effluxers as well as physiological chelation by apo-thionein or other zinc-sequestering apoproteins [210,215–218].

3.2. Zinc-Rich Cells

Zinc-rich cells in mammals are found in various tissue and organs, particularly in the brain, mammary gland, intestine, pancreas, thymus, prostate gland, testes, and ovaries [202,219–221]. In the brain, high levels of chelatable or labile zinc is synaptically co-released with glutamate during normal neuronal communication [202], with ionic levels reaching 100–300 μM, particularly during a seizure activity [222,223]. Intracellular zinc is typically buffered but is exocytosed from neurons or secretory cells that release vesicles or granules, respectively. Zinc release in the hippocampal mossy fiber terminals is calcium-dependent whether it was evoked via potassium or kainic acid administration and spontaneous activity [222,224–226]. Intracellular zinc elevation may occur due to high exogenous zinc levels [227–231] or caused by cytoplasmic zinc release from compartments or proteins due to oxidation by ROS or nitrosylation by reactive nitrogen species [232–234]. Zinc overload kills neurons, and thus it is imperative that cells tightly regulate intracellular zinc concentration via zinc transporters and buffering of zinc-binding amino acids or proteins.

3.3. Zinc Transporters

High- and low-affinity uptake mechanisms for zinc have been identified with dissociation constants (Kd) of 15 and 361 μM, respectively [222]. An even higher binding

affinity constant (Ka) of 0.25 μM has been reported for zinc, which is saturable, ATP-independent, and unaffected by Na^+ concentration gradient [235]. Indeed, zinc levels are strictly maintained by tissue-specific and highly conserved low molecular weight transport protein families known as the ZnTs (also known as SLC30 for solute carrier 30) and ZIPs (also known as SLC39 for solute carrier 39) [236–238]. Early studies in the field led to the discovery of mammalian ZnTs involved in the extrusion of zinc out of cells named ZnT1 [215] and sequestration of zinc into compartments called ZnT2 [210]. ZnT1 is mainly localized in the plasma membrane [215]. Meanwhile, ZnT2 is localized within vesicular (acidic) compartments, such as lysosomes, but has a low affinity for zinc [210]. Another effluxer termed ZnT3 was cloned and identified to localize within synaptic vesicles of zinc-rich neurons [217]. ZnT3 is expressed in the mammalian brain, such as in the cerebral cortex and the hippocampus, and strongly detected in the dentate granule cells. Over the course of time, other members of the ZnT effluxers (ZnT4–ZnT10) and ZIP influxers (ZIP1–ZIP14) were identified through sequence similarity analyses, cloning, and functional experimentations [223,238]. More recently, transmembrane 163 protein (TMEM163; also known as SV31) [239,240] was functionally characterized as a dimeric protein that effluxes zinc [241], and one of us proposed that TMEM163 be now classified as ZnT11 as a new member of the ZnT efflux family of proteins [241]. One commonality among ZIPs and ZnTs is that histidine (H) and/or aspartic acid (D) residues, such as the HXXXD motif (where X is a nonpolar amino acid) typically located within transmembrane domain (TMD)-4 and TMD5 helices of ZIPs, as well as HXXXH motif found in TMD2 and TM5 helices of ZnTs have been shown to be responsible for tetrahedral zinc coordination [223,242]. For a relevant review on certain zinc transporters, we refer the reader to the paper by Styrpejko and Cuajungco (2021) as part of this Special Issue.

In addition to transporters, intracellular buffers offer a secondary defense mechanism to prevent intracellular zinc overload, such as the metallothioneins (MTs)—a group of low molecular weight (~6–7 kDa), single polypeptide chains with four functional mammalian isoforms (MT1−MT4) [219,243]. MTs, however, are not a long-term storage for zinc due to its short biological half-life [244]. Thus, vesicular or compartmental storage mediated by ZnTs and ZIPs provide important contributions to zinc homeostasis.

3.4. Zinc Interactions with Various Proteins

Zinc is crucial to over 200 proteins and enzymes [204]. Most of the endogenous and exogenous zinc in biological systems is bound to proteins with different degrees of affinity (see Table 1). The ultrastructural localization of several neurotransmitters present in the hippocampal mossy fiber terminals, especially glutamate, coincides with the location of zinc-containing neurons [245–248]. Zinc is known to bind and permeate ionotropic glutamate receptors (e.g., amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA)) and metabotropic glutamate receptors [249–253]. Zinc also binds to the gamma-amino-n-butyric acid (GABA) receptors and noncompetitively inhibits GABA-mediated responses [254,255]. Zinc antagonizes both NMDA [256] and GABA [257] with approximate Kd of 13 and 11 μM, respectively. These are well within the concentrations released during synaptic activity, which implies an association of zinc in the regulation of neurotransmission. Likewise, zinc can modulate the responses of ligand-gated or voltage-gated ion channels, such as the ATP-gated P2X-purinergic ion channels, glycine, and sigma receptor [258–260].

Table 1. Tabulated list of selected proteins and enzymes with known binding affinity and inhibitory constant for zinc.

Protein Name	Binding Affinity * [Reference]
Superoxide dismutase (Cu/Zn)	Kd = 5×10^{-5} M [261]
Na^+/K^+ ATPase	Kd = 3×10^{-6} M [262]
Ca^{2+} ATPase	Ki = 8×10^{-12} M [263]
Mitochondrial complex III	Kd = 1×10^{-7} M [264]
Protein tyrosine phosphatase	Ki = 2×10^{-13} M [265]
Protein kinase C	Kd = 1×10^{-13} M [266]
Caspase-3	Ki = 1×10^{-13} M [267]
Caspase-6	Ki = 1×10^{-13} M [268]
Sp1	Kd = 1×10^{-10} M [269]
Glutathione	Kd = 5×10^{-7} M [270]
Metallothionein	Kd = 1×10^{-12} M [271]
Calmodulin	Kd = 8×10^{-5} M [272]
NMDA Receptor	Kd = 1×10^{-7} M [256]
GABA receptor	Kd = 1×10^{-7} M [257]
Tubulin	Kd = 2×10^{-7} M [273]
Heme	Kd = 2×10^{-8} M [206]

* Kd = dissociation constant; Ki = inhibitory constant. Adapted and modified from [274].

3.4.1. Enzymes

Zinc impedes the activity of several ionic transporters, such as the sodium–potassium (Na^+/K^+)-ATPase with IC50 = 20 µM [262,275] and Ca^{2+}-ATPase in vitro IC50 = 100 µM (Table 1) [276]. The Na^+/K^+-ATPase enzyme activity has been found to be highly concentrated in the hippocampus and hypothalamus [275], and the inhibition of Na^+/K^+-ATPase has been shown to cause neuronal death [277,278].

Zinc can inhibit the cell respiratory chain (Ki = 10^{-7} M; see Table 1) by blocking the initial step of respiration of electron transfer between ubiquinone (coenzyme Q) and cytochrome b of the bc1 complex (complex III) [264,279]. At higher concentrations (10^{-3} M), zinc may further inhibit at the levels of flavoprotein 1–2 (complex I–II) and cytochrome c oxidase (complex IV) activities [280]. The inhibitory effects of zinc may explain why mitochondrial zinc overload is cytotoxic to eukaryotes.

In addition to inhibitory effects on energy metabolism, zinc also negatively impacts a number of enzymes critical for neurotransmitter metabolism [202]. For example, it was reported that zinc inhibits glutamate and GABA reuptake transporters (Km ~50 µM) in mouse synaptosomal fractions [281]. Although zinc inhibition of glutamate transporters results in extracellular glutamate accumulation and death of cortical neurons in vitro [282], it was reported that physiological levels of zinc released within the synapse facilitate glutamate homeostasis through its effect on glutamate transporter activity [283]. For further information regarding the inhibitory effects of zinc on additional enzymes, see the review by Maret (2013) [284].

3.4.2. Signaling Proteins

It has been observed that zinc influx may depolarize the cell and consequently trigger a rise in intracellular calcium concentration ($[Ca^{2+}]i$). Calcium is then bound by a number of EF-hand proteins that contain distinct zinc binding sites, such as S-100A6 (calcyclin), S-100A7, S-100A8, S-100A9, S-100A12, calmodulin (CaM), and calgranulin C (CAGC) [272,285,286]. Zinc binds CaM (Kd ~8×10^{-5} M) and induces a slight conformational change; however, calcium has a greater affinity for CaM (Kd = 10^{-8} M) [272]. The binding of zinc to these calcium-binding proteins under physiological conditions remains a point of contention. Notwithstanding, recent reports indicate that zinc and calcium play important roles in the function of these S100 proteins [287].

3.4.3. Cytoskeletal Proteins

Zinc has been shown to influence both assembly and disassembly of tubulin (Table 1) and microtubule-associated proteins in vitro [273,288]. An excess of zinc levels in nervous tissues could interfere with the microtubule structure by disrupting the normal functions of the cytoskeleton [289]. The metal chelator, *N,N,N,N*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), prevented protein kinase C (PKC)-mediated actin cytoskeletal disruption induced by phorbol ester in cultured C6 rat glioma cells, while addition of zinc reversed the protective effects of TPEN [290].

3.5. Zinc-Induced Cell Death

Abnormal zinc metabolism results in an excess or deficiency of intracellular zinc concentrations ($[Zn^{2+}]i$), which could be detrimental to cells. The mechanism of zinc-induced cell death has been extensively researched. Zinc may augment or directly trigger cell death through its effects on various cellular pathways. The following discussion summarizes the connection between zinc and programmed cell death (apoptosis).

Zinc Mobilization in Apoptosis

Apoptosis is characterized by distinct morphological changes in cells that include membrane blebbing, chromatin condensation, DNA fragmentation, organellar packaging, and cell shrinkage [291]. Cells undergoing apoptosis induce zinc-dependent transcription factors [292]. Recently, cell death involving iron has been observed and coined ferroptosis (for reviews on this topic, see the following references [293,294]).

Cultured lymphoblasts undergoing early events of apoptosis exhibit an increase of $[Zn^{2+}]i$ [208,295]. Mobilization of $[Zn^{2+}]i$ is possibly due to compartmental release and zinc dissociation from metalloproteins mediated by free radicals and ROS such as HOCl, H_2O_2, and O^{2-} [296–298]; amino acids such as L-glutamate, L-aspartate, and L-cysteic acid [299]; and disulfides [271]. It remains to be shown, however, if intracellular zinc mobilization is a primary or secondary cause of apoptosis. As a case in point, apoptosis is observed in animal models of a neurological disorder called mucolipidosis type IV (MLIV), which is caused by the functional loss of the TRPML1 ion channel [300,301]. Coincidentally, lysosomal permeabilization and the release of cathepsin B have been shown in a cell culture model of MLIV [300], which correlates with lysosomal zinc accumulation in MLIV cells, as well as cell culture and mouse models of MLIV [237,248,302]. It is worth noting that in the MLIV mouse model, the downregulation of the ZnT3 vesicular zinc transporters may explain why cortical zinc levels are abnormal [303], but further research is necessary to determine if this is a cause or consequence. Notwithstanding, targeting $[Zn^{2+}]i$ to reduce pathological conditions observed in certain human diseases could be a therapeutic approach. Indeed, in the case of cerebral ischemia [304] or seizure activity [305], degenerating neurons show increased zinc staining, and these neurons undergo an apoptotic process [306]. In the case of ischemia, the pathological process results in $[Zn^{2+}]i$ elevation but precedes other biomarkers of cellular damage. Interestingly, neuronal death is rescued by the chelator ethylenediamine tetraacetic acid (EDTA), which suggests that zinc mobilization influences cell death, at least in cerebral ischemia [304].

Intracellular zinc mobilization has biphasic effects on non-neuronal cells by either initiating or preventing apoptosis [221,222,307]. There is, however, a variable range of concentrations where zinc may induce or preclude apoptotic death. For example, cultured mouse thymocytes incubated with zinc at concentrations of 15 μM underwent apoptosis [212], while other studies have found zinc-induced apoptotic death between 80 and 200 μM [213]. These results contrast markedly with induction of *c-myc*-dependent apoptosis by zinc (37.5 μM) in the absence of DNA fragmentation [292]. Based on these findings, intracellular zinc elevation in non-neuronal cells undergoing apoptosis may be a protective mechanism, while zinc mobilization in post-ischemic neuronal cells would appear to be neurotoxic because a metal chelator rescues cell death.

3.6. Zinc Chelators

Metal chelators have unique and specific binding properties on various metal ions [308,309]. Certain chelators are able to permeate cell membranes or become lipid soluble, while others become membrane permeable after esterification or by acquiring a nonpolar state following metal complexation. Further, some chelating agents demonstrate particular attributes known as ionophores. Ionophores selectively enhance the permeability of metal ions in lipid membranes of cells [308]. For example, pyrithione and clioquinol are zinc chelators that also act as ionophores [302,310–312], in which both compounds increase intracellular zinc by virtue of their membrane permeable property. Meanwhile, a number of chelators avidly associate and sequester metals from metal–protein complexes, while others are incapable of such interaction. The former are classified as high-affinity metal chelators, while the latter are low-affinity chelators. Moreover, metal complexing agents have different denticity or means to bind metal ions. Whereas some chelators form multidentate complexes, others can only bind a mono- or bidentate complex. Note that multidentate ligands do not necessarily result in the formation of high-affinity metal–ligand complex [309]. Thus, properties of chelating agents may depend on the affinity or stability constant they form with the target ion(s), the metal-to-ligand ratio, and the accessibility of the ion within the metal–ligand complex that the chelator is in competition with. Overall, researchers must fully consider the characteristics of the chelator before they use them for experiments.

Chelators That Bind Zinc and Their Effects on Cells

Certain chelating agents could exhibit high binding affinity for a particular metal ion; however, these chelators may also bind other metals, albeit less avidly than the target metal. Such a problem of metal selectivity is typically encountered in biological studies. For example, diethylenetriamine pentaacetic acid (DTPA) avidly binds zinc but forms more stable complexes with copper and iron [313], while diethyldithiocarbamate (DEDTC) and zincon bind copper even though both can also complex zinc. Note that DTPA and zincon do not affect histochemical staining for brain zinc, suggesting that both are membrane impermeable, but DEDTC can penetrate cell membrane and quench histochemical stain for brain zinc [314]. One commonly used and highly specific zinc chelator is TPEN. However, although it is highly selective for zinc than calcium or magnesium, it also has a higher affinity for copper and iron [307,315]. One advantage of TPEN is its cell membrane permeability [307,315]. Because TPEN avidly binds zinc, its use to chelate intracellular zinc results in induction of apoptosis in thymocytes [217], lymphocytes [295,316], isolated hepatoma cells [317], and splenocytes [208]. These results are comparable to other reports in which apoptosis was induced by exposure to low levels of zinc [212,213]. Note that zinc chelation-mediated death is not only independent of, but also additive to apoptosis induced by exogenous addition of Ca^{2+} [318]. These observations show that zinc is important for apoptotic cell death pathways.

The use of metal complexing agents against certain disease states is quite common. Indeed, membrane permeable chelators, such as TPEN and pyrithione, attenuate zinc neurotoxicity in vivo [314,319], while the membrane impermeant chelator EDTA reduces zinc toxicity in vitro [211,219,304,314,319–321]. It is interesting to note that intrahippocampal co-administration of zinc with several metal chelators have been reported to produce differential effects on neuronal damage in vivo. Specifically, several zinc–chelator complexes showed behavioral side effects, such as seizures, which also correlated with increased neuronal loss [314]. In contrast, however, the same chelators intra-hippocampally injected alone were not significantly toxic compared with the zinc chelate treatment.

In summary, the problem of specificity may be overcome by using metal chelators with varying degrees of affinity for zinc and determining different chemical structures that inform denticity for zinc. Finally, recent advances have now allowed researchers to develop chelating agents with more specificity and affinity for zinc than other metal ions.

3.7. Concluding Remarks

Zinc is critical for the growth and survival of cells. However, an abnormal metabolism of zinc in cells can have deleterious effects. Zinc is required by a number of transcription factors, proteins, and enzymes. Studies have shown that zinc can induce cytotoxicity in prokaryotic and eukaryotic cells once a threshold is reached. Zinc inhibits many critical enzymes and regulates receptors or ion channels. Finally, zinc plays a role in apoptotic death and is thus a potential target of specific chelating agents. The importance of zinc in cells cannot be overstated, but further research is necessary to determine when and how zinc may be used for therapeutic intervention in the case of antibiotic resistance as antimicrobial adjuvant and when zinc can be tackled to prevent cell death in various human diseases.

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