REVIEW

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Macrolide Structure, Bioactivity and Interactions: a Review

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Abstract: Since the discovery of macrolide antibiotics, extensive research has been focused on the development of new bioactive compounds in response to an emerging number of multidrug-resistant pathogens. In the last several decades, macrolide antibiotics have made a significant contribution to human healthcare. Nevertheless, interest in the development of broad-spectrum macrolides remains due to increasing bacterial resistance. This review summarizes recent advances in macrolide synthesis and discusses alternative strategies for structural modification of macrolides, highlighting how these modifications affect their bioactivity through structure-activity relationship (SAR) and interaction studies. It provides an insight into the macrolide binding mechanisms to their biomolecular targets that play key roles in the overall biological profile of a drug. Recently reported structures of macrolide complexes with biomolecules have also been summarized, providing a platform for the design of new antibiotics active against resistant bacteria.

Keywords: macrolides, bacterial resistance, structure and bioactivity, biomolecular targets and interactions.

INTRODUCTION

ERYTHROMYCIN and azithromycin belong to a macrolide class of antibiotics (Figure 1) that are widely prescribed to treat respiratory tract infections because of their high efficacy and safety.[1] Macrolides and their derivatives have a wide spectrum of biological activities, such as anti-bacterial, anticancer, anti-inflammatory, and antiparasitic properties.[2]

The emergence of multi-drug resistant (MDR) pathogens has rendered marketed macrolide antibiotics less effective, driving an urgent need for discovery of novel and more potent antibacterials. Antimicrobial resistance (AMR) has become a critical global human health threat, driven by rising resistance to existing antibiotics and a lack of new bioactive compounds to address this urgent medical need. The number of antimicrobial agents in the clinical pipeline declined from 2023 to 2025, and experts predict that AMR might cause up to 40 million deaths by 2050.[1]

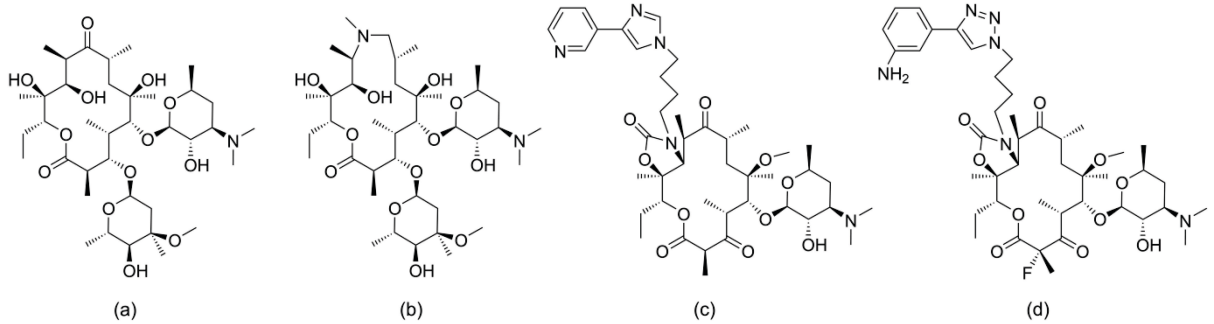

Figure 1. The main classes of macrolide antibiotics: (a) erithromycin, (b) azithromycin, (c) telithromycin and (d) solithromycin.

Ketolides are antibiotics that posses activity against some resistant strains, but they have been withdrawn from the market due to severe side effects. Hence, it is important that new generations of macrolides structurally differ from telithromycin and solithromycin to avoid toxic effects.

Macrolides bind to domain V of the 23S ribosomal RNA (rRNA) at or near the peptidyl transferase center (PTC) and sterically block the nascent peptide synthesis during the early stages of translation. Macrolides have a bacteriostatic effect by inhibiting bacterial growth.[3]

Crystal structures of several ribosome-macrolide complexes have been determined,[4–6] shedding a new light on the binding mechanisms and interactions of these antibiotics to ribosomes and hence providing a good basis for the rational design of new ligands and inhibitors. In this review we describe the discovery of new macrolide compounds, their bioactivity, and biomolecular interactions reported from 2019 onwards.

MACROLIDE SYNTHESIS, STRUCTURE CHARACTERIZATION AND BIOACTIVITY

Janas and Przybylski[2] have previously given an overview of the structure and *in vitro* antimicrobial activity of macrolide derivatives up to 2019, focusing on 14- and 15-membered lactone macrolides as well as their analogues, congeners, and hybrids. Their review described the mechanisms of action and bioactivity and compared the antimicrobial activity of these derivatives with commercially available antibiotics.[2] Furthermore, Undheim detailed in his review[7] the modifications of 14-membered macrolides, represented by erythromycin and clarithromycin.

Over the years, intensive efforts have been focused on the modification of natural and semi-synthetic 14- and 15-membered macrolides. Chemical modifications were performed on the aglycone ring as well as on the sugar moieties, cladinose and desosamine.[2] Studies of macrolide–ribosome complexes have shown that desosamine (an amino sugar) is critically important for macrolide binding, whereas cladinose is not essential for the overall antibacterial activity.[2,3] Based on these insights, Yan et al.[8] designed azithromycin derivatives by modifying the 4″ position of desosamine. The 4″-*O*-aralkylacetylhydrazine-acyl azithromycin derivatives exhibited significantly improved activity against methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, penicillin-resistant *S. aureus* ATCC 31007, and additional methicillin-resistant *S. aureus* strains. The minimum inhibitory concentrations (MICs) of the 4″-substituted azithromycin derivatives ranged from 2 to 4 μg/mL, whereas the MICs of azithromycin against the same strains exceeded 128 μg/mL.[8]

Additionally, Undheim summarized research conducted up to 2019[7] that focused on scaffold modifications of 14-membered macrolides at inactive positions in the aglycone ring, providing insights into the structure-activity relationship (SAR) and potential sites for chemical optimization.

Decades after their initial discovery, natural macrolides still remain central to drug discovery efforts, highlighting the enduring significance of these bioactive secondary metabolites. Macrolides represent a group of natural antibiotics isolated from plants and microorganisms, exhibiting a wide range of bioactivities, including antiviral, antifungal, antibacterial, immunosuppressive, herbicidal, and cytotoxic effects. Evidente[9] focused on macrolides isolated from fungal sources, describing their chemical and biological characterization, stereochemistry, and SAR. Recently, Li et al.[10] reported the isolation of two structurally related 13-membered macrolides, cyclopiumolides A and B, from the deep-sea sediment-derived fungus *Penicillium cyclopium*. Their structures were elucidated using NMR spectroscopy and mass spectrometry, and absolute and relative configurations were determined by quantum mechanical calculations combined with NMR analysis. These macrolides, featuring a verrucosidinol unit condensed with a spiculisporic acid moiety, demonstrated cytotoxic activity against SF-126, FaDu, and TE-1 cell lines, with IC_{50} values ranging from 5.86 to 17.05 μM.[10]

Some recently conducted research has been focused on the design of macrolide derivatives that target novel binding sites in the bacterial ribosome to enhance activity against resistant pathogens. Ma et al.[11] discovered potent derivatives of macrolones by SAR-aided drug design. Macrolones are hybrids of two distinct antibiotic classes - macrolides and quinolones, designed to enhance antimicrobial activity against resistant bacterial strains. Authors focused on the development of fourth-generation macrolide derivatives, 2-fluoro ketolides. Unlike third-generation macrolides (e.g., telithromycin, Figure 1), 2-fluoro ketolides feature a 2-fluorine substituent that inhibits tautomerization. Structural similarity to telithromycin was intentionally avoided to minimize the risk of similar adverse effects, such as cardiovascular toxicity, vision problems, nausea, diarrhea, fainting, liver damage and worsening of myasthenia gravis symptoms. Thirty-six 2-fluoro-9-oxime ketolides were designed, synthesized, and evaluated *in vitro* for antibacterial activity and *in vivo* for pharmacokinetic properties. Heteroaryl and carbamoyl substituents were introduced under the hypothesis that additional hydrogen bonds and π–π interactions would increase binding affinity, thereby restoring antibacterial activity against resistant strains. Hybrids of 2-fluoro-9-oxime ketolides and carbamoyl quinolones exhibited high potency

against Erm-mediated resistant (*S. pneumoniae*, *S. pyogenes*) and efflux-mediated resistant strains. Furthermore, *in vivo* pharmacokinetic studies showed that these derivatives are more metabolically stable than other ketolides such as telithromycin (*i.e*. prolonged half-life and mean residence time *in vivo*).[11]

In a related approach, Zhang and coworkers[12] focused on improving the activity of 3-keto ketolides by designing alkylides (3-*O*-alkyl derivatives) and hybrids of 3-*O*-decladinosyl erythromycin incorporating quinolone motifs. Their objective was to improve efficacy against pathogens harbouring the erythromycin resistance methyltransferase (*erm*) gene. Twenty novel ether-linked alkylides, macrolone derivatives, were synthesized, evaluated for *in vitro* antibacterial activity, and subjected to SAR analysis. The resulting 3-*O*-decladinosyl macrolide–quinolone hybrids represent a promising antibiotic class successfully directed toward resistant strains of C3-modified macrolides, particularly *Streptococcus pneumoniae* and *Streptococcus pyogenes*. The newly synthesized macrolones showed significantly improved activity due to the dual-targeting mechanism, binding both to the bacterial ribosome (primary target) and to topoisomerases (secondary target). The authors claimed that the proposed mechanism could explain simultaneous inhibition of protein synthesis and bacterial DNA replication.[12]

Building on previous studies of macrolone derivatives, Fan et al.[13] also investigated dual synergistic activity of studied compounds, highlighting its significance in addressing clinically resistant pathogens. They synthesized hybrids of azithromycin with ciprofloxacin or gatifloxacin and demonstrated that substitution at the C6 position of azithromycin enhances activity against *Escherichia coli*. SAR studies on 28 azithromycin–quinolone derivatives further improved efficacy against *Streptococcus pneumoniae*, efflux-mediated resistant *S. pneumoniae*, Erm-resistant *Staphylococcus aureus*, and *Haemophilus influenzae*. Substitutions were introduced at positions C3, C6, C11, and C12 of the azithromycin aglycone ring (Figure 1). This dual mechanism of action—simultaneous inhibition of protein synthesis and poisoning of DNA gyrase and/or topoisomerase—proved highly effective against resistant *S. aureus* strains. The newly synthesized derivatives exhibited greater potency, while market-available macrolides (azithromycin, telithromycin, and clarithromycin) showed MICs exceeding 256 μg/mL against these resistant *S. aureus* strains.[13]

Further efforts in macrolone development were directed toward MLS_BK (macrolide–lincosamide–streptogramin B–ketolide) resistant pathogens. In contrast to the classical dual-targeting strategy characteristic of macrolones, an alternative design concept was introduced based on the incorporation of a secondary binding site within the nascent peptide exit tunnel (NPET) of the ribosome. This approach enabled the design of macrolides with additional stabilizing interactions, including sandwich-like π–π stacking and a water–magnesium bridge, which enhanced binding to A2058-methylated ribosomes commonly found in resistant strains such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*. As a result, the newly designed compounds effectively restored antibacterial activity against MLS_BK resistant pathogens.[11]

Nafithromycin is the first macrolide antibiotic developed and approved in India, representing a lactone ketolide that has been under development for over 30 years (Figure 2). Unlike traditional macrolides, it exhibits dual-target binding to domains II and V of the 23S rRNA, with enhanced tissue penetration, resulting in increased potency. Clinical studies have shown it to be well-tolerated with no significant side effects and effective against a wide range of respiratory infections. Paliwal et al.[14] provided detailed stages of its development, mechanism of action, SAR, clinical trial outcomes, and bioactivity. Structural modification at the C3 position, specifically the introduction of a keto group, enhanced ribosomal binding and respiratory tissue penetration, while incorporation of an alkyl-aryl side chain improved bacteria biofilm penetration and further increased binding affinity. Nafithromycin demonstrates significant activity against pathogens including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus pneumoniae*, *Streptococcus pyogenes*, *Moraxella catarrhalis*, and atypical respiratory pathogens such as *Mycoplasma pneumoniae*, *Legionella pneumophila*, and *Chlamydophila pneumoniae*.[14]

In addition to their antibacterial activity, macrolides have also demonstrated antiviral potency. Notably, Zhong et al.[15] identified a novel class of 16-membered macrolides derived from carrimycin, expanding the therapeutic potential of macrolides beyond bacterial infections. They

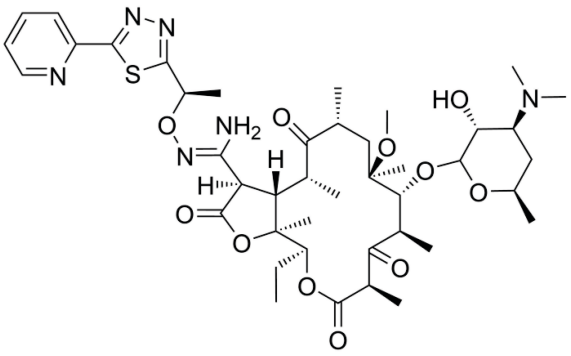

Figure 2. Chemical structure of nafithromycin.

demonstrated that modification of carrimycin at the 4″ position induces a favorable U-shaped conformation, enabling stronger binding to the coronavirus FSE (frameshift stimulation element) RNA pseudoknot, thereby sterically inhibiting protein replication and translation.[15]

Certain macrolide derivatives have also demonstrated anticancer potential by targeting the protein actin. Actin is a key cytoskeletal protein that regulates cell shape, motility, and intracellular transport and plays a critical role in cancer metastasis. Macrolide derivatives that target actin can disrupt its polymerization and cytoskeletal organization, preventing cancer cells from metastasizing. The mechanism of action was elucidated by studying X-ray crystal structures of macrolide–actin complexes. Based on these insights, Trofimova et al. focused on designing Mycalolide B analogues with an acyclic side chain (tail). The long aliphatic chain (tail) inserts into a narrow hydrophobic cleft located between two protein subdomains 1 and 3, while the macrocyclic ring interacts with a hydrophobic surface on actin subdomain 1.[16]

Ohyoshi et al.[17] have an ongoing study investigating the synergistic antitumor activity of Aplyronine A–Swinholide A hybrids. Aplyronine A is a marine natural macrolide, and by linking it with the side chain of Swinholide A, cytotoxic and antitumor agents were successfully designed. Aplyronine A is a 24-membered marine macrolide, and Swinholide A is a 42-membered marine macrolide known for its potent actin-targeting properties. By linking Aplyronine A with the side chain moiety of Swinholide A, cytotoxic and antitumor agents were successfully designed, demonstrating promising antitumor activity.[17]

Another cytotoxic macrolide family is the 22-membered Scytophycins, isolated from the cyanobacterium *Scytonema pseudohofmanni*. The relationship between the structure and cytotoxicity of Scytophycins A–E was studied. It was assumed that highly potent Scytophycins A and B likely interact with multiple protein targets. To explain activity differences and identify the pharmacophore in this class, Ohyoshi et al. performed a fragment-based analysis (marolactone and side chain fragments), concluding that the integrity of the entire molecule is crucial for bioactivity.[18]

Furthermore, Cheng et al.[19] described the broad therapeutic potential of neopeltolides and their analogues. Neopeltolide is a macrolide natural product of marine origin, initially isolated from deep-sea sponges. Its structure consists of a 14-membered macrolactone ring, a pyran moiety, and an oxazole side chain. Neopeltolides have demonstrated anticancer and antifungal activities, making them attractive scaffolds for the development of synthetic analogues with enhanced therapeutic potential. However, their limited natural availability presents challenges for large-scale production.[19]

Three elansolid-type polyketide-spanned macrolides were isolated from a marine bacterium *Bacillus amyloliquefaciens* MTCC 12716 and structurally characterized using NMR spectroscopy, including 1D and 2D techniques (COSY, HMBC, and NOESY). Predicted protein-ligand interactions and binding energies were evaluated through *in silico* molecular docking analyses. *In vitro* studies demonstrated broad-spectrum antibacterial activity, notably against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE), *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.[20]

It is well known that natural macrolides are produced *via* chemoenzymatic synthesis. The polyketide synthase (PKS) is an enzyme that catalyzes the formation of 12- and 14-membered macrolides. By modifying PKS enzymes, it becomes possible to introduce non-natural functional groups and heteroatom substitutions, thereby enabling the chemoenzymatic synthesis of new complex antibiotic scaffolds. This approach enables the generation of novel macrolide analogues with improved biological properties, representing a promising strategy for the development of new antibiotics.[21,22]

Since their discovery, 14- and 15-membered macrolides have been the main focus in the development of new macrolide antibiotics. However, the macrolide family includes structurally diverse antibiotics that differ in macrocyclic ring size, and therefore larger-ring macrolides should also receive greater attention.

Song and coworkers.[23] summarized research conducted from 1959 to 2019 on guanidinium-containing polyhydroxyl macrolides. They described the structural features, bioactivity, acute toxicity, mechanism of action, and SAR of both naturally isolated compounds from actinomycetes and semi-synthetic derivatives. These macrolides include 32, 36 and 40-membered compounds with a polyhydroxylated lactone ring and a guanidinium side chain. They exhibited diverse bioactivities, including broad-spectrum antibacterial, antifungal, antitrichomonas, and antitumor effects, as well as inhibition of H^+/K^+-ATPase, mitochondrial oxidative phosphorylation (NADH dehydrogenase), and phospholipase C. Notably, studies have shown that the guanidinium side chain targets lipoteichoic acid (LTA) in *Staphylococcus aureus*. LTA is a polymer that anchors to the cell membrane and plays a key role in antibiotic resistance and cell division in Gram-positive bacteria, making LTA synthase a proposed drug target for the development of novel antibiotics against Gram-positive pathogens.[23]

Similarly, Al-Fadhli and coworkers[24] highlighted the wide spectrum of antibiotics isolated from actinomycetes and emphasized the medical relevance of this class for future drug discovery. These macrolides, with ring sizes

ranging from 9 to 60 members, often substituted with hydroxy and alkyl groups, display unique antibacterial, antifungal, cytotoxic, antimicrobial, insecticidal, anti-tripanosomal, antimalarial, antiprotozoal, antimycobacterial, and antiherpetic activities, reflecting their remarkable structural diversity and versatile bioactive potential.[24]

Alferova et al.[25] investigated the mechanism of action of a 20-membered macrolide irumamycin. Using NMR techniques (ROESY, NOESY, and HSQMBC), they elucidated its stereochemical configuration, contributing to better understanding of the relationship between structural features and biological properties of 20-membered macrolides. In addition, they successfully isolated an isomer of irumamycin, iso-irumamycin, an 18-membered macrolide.[25]

Two 20-membered polyether macrolide analogues, marginolides A and B, were isolated from the veined octopus *Amphioctopus marginatus*.[26] Their isolation, purification, structural characterization, and biological evaluation were described in detail. Both compounds were identified as inhibitors of angiotensin I-converting enzyme (ACE), a key regulator of blood pressure. SAR analysis correlated biological activity with physicochemical parameters, including steric, electronic, and lipophilic properties. Molecular docking studies provided insight into the mechanism of action and macrolide–ACE interactions by evaluating hydrogen bonding patterns, binding energy, intramolecular energy of the protein–ligand complex, and docking score. Notably, increased polarity together with a balanced hydrophobic–hydrophilic profile (octanol–water partition coefficient, log P_{ow} 2–4) was found to favour an enhanced ACE inhibitory activity, highlighting the importance of fine-tuned physicochemical properties for optimal biological performance.

Continuing the investigation of larger macrolides, An et al.[27] isolated hamuramicin C, a bicyclic 22-membered macrolide, from the gut bacteria of wasps. Its structure was characterized using NMR and mass spectrometry, and the relative configuration was determined by analyzing coupling constants with the NOE effect and heteronuclear long-range coupling NMR experiments. Hamuramicin C exhibited significant cytotoxic activity against human cancer cell lines, including HCT116, A549, SNU-638, SK-HEP-1 and MDA-MB-231.

Koštrun and coworkers[28] focused on macrolide-inspired synthetic and semi-synthetic macrocycles, highlighting a strategy to bridge the gap between proteins and small molecules. They described the design, synthesis, and *in vitro* profiling of various macrocycles aimed at inhibiting interleukin-17 (IL-17), a key mediator in numerous autoimmune diseases and therefore an attractive target for drug discovery. X-ray crystallography of IL-17, NMR conformational analysis (NOESY), and coupling constants were used as the starting point for molecular docking studies. Subsequently, STD NMR was employed to study ligand–protein interactions in solution. Different macrocycle ring sizes were examined, along with stereochemistry and modifications of the linear side chain, providing a broad SAR framework for optimization. This approach highlights the potential of large molecules to access binding sites that are inaccessible to conventional small molecules.

In contrast to larger-ring macrolides, Myers and Clark[29] highlighted the importance of developing macrolides with lower molecular weight (MW = 600 – 700 Da), as this modification results in altered physicochemical properties. Reducing molecular weight and introducing more polar functional groups may facilitate improved penetration into Gram-negative bacteria, thereby advancing the bioactivity (ideal range $cLogD_{7.4}$ between –1 and 3). Removal of lipophilic substituents and the introduction of secondary and tertiary amines improved the permeability of azithromycin-like 15-membered azalides. Furthermore, the replacement of C10–C13 macrocyclic fragment with 1,2-aminoalcohol moieties led to the synthesis of 13-membered azalides. These compounds emerged as promising candidates with potent activity against multidrug-resistant Gram-negative bacteria, including *Enterobacteriaceae* and ESKAPE pathogens.

In contrast to broad-spectrum macrolides, narrow-spectrum macrolides have received relatively limited research attention over the years. However, increasing bacterial resistance to existing antibiotics has renewed interest in narrow-spectrum antimicrobials. One class belonging to this group are the nargenicin-like macrolides, first reported in 1980. The nargenicin family is a group of structurally related antibiotics characterized by an oxa-bridged decalin scaffold, with individual compounds differing in their spectrum of activity. Although their precise mechanism of action has not yet been fully elucidated, these macrolides are considered to have clinical potential. Their ability to target a narrow range of pathogens may preserve the patient's microbiota and potentially limit the development of resistance.[30]

Another class of macrolide derivatives are macrozones - conjugates of azithromycin and thiosemicarbazones (Figure 3).[31] Macrozones have demonstrated improved activity compared to macrolides against resistant strains, such as *Streptococcus pneumoniae* and *Staphylococcus aureus*. They also exhibited strong efficacy against susceptible *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Enterococcus faecalis* strains, with activity comparable to or exceeding that of azithromycin. In the chapter *Macrolide Interaction Studies*,

a detailed mechanism of binding and mode of action of macrozones, responsible for their activity, was described.[31–33]

Furthermore, chemical modifications of azithromycin and clarithromycin were investigated by Janas et al.,[34] who synthesized *N*-alkylammonium and carbonate triazole derivatives of azithromycin and clarithromycin and characterized their bioactivity. In particular, the quaternary *N*-alkylammonium bromide salts derived from clarithromycin exhibited an enhanced antibacterial activity compared to the parent macrolides. A SAR study of clarithromycin salts indicated that less bulky *N*-substituents are important for an optimal fit inside the ribosomal exit tunnel, leading to an increased antibacterial activity. This

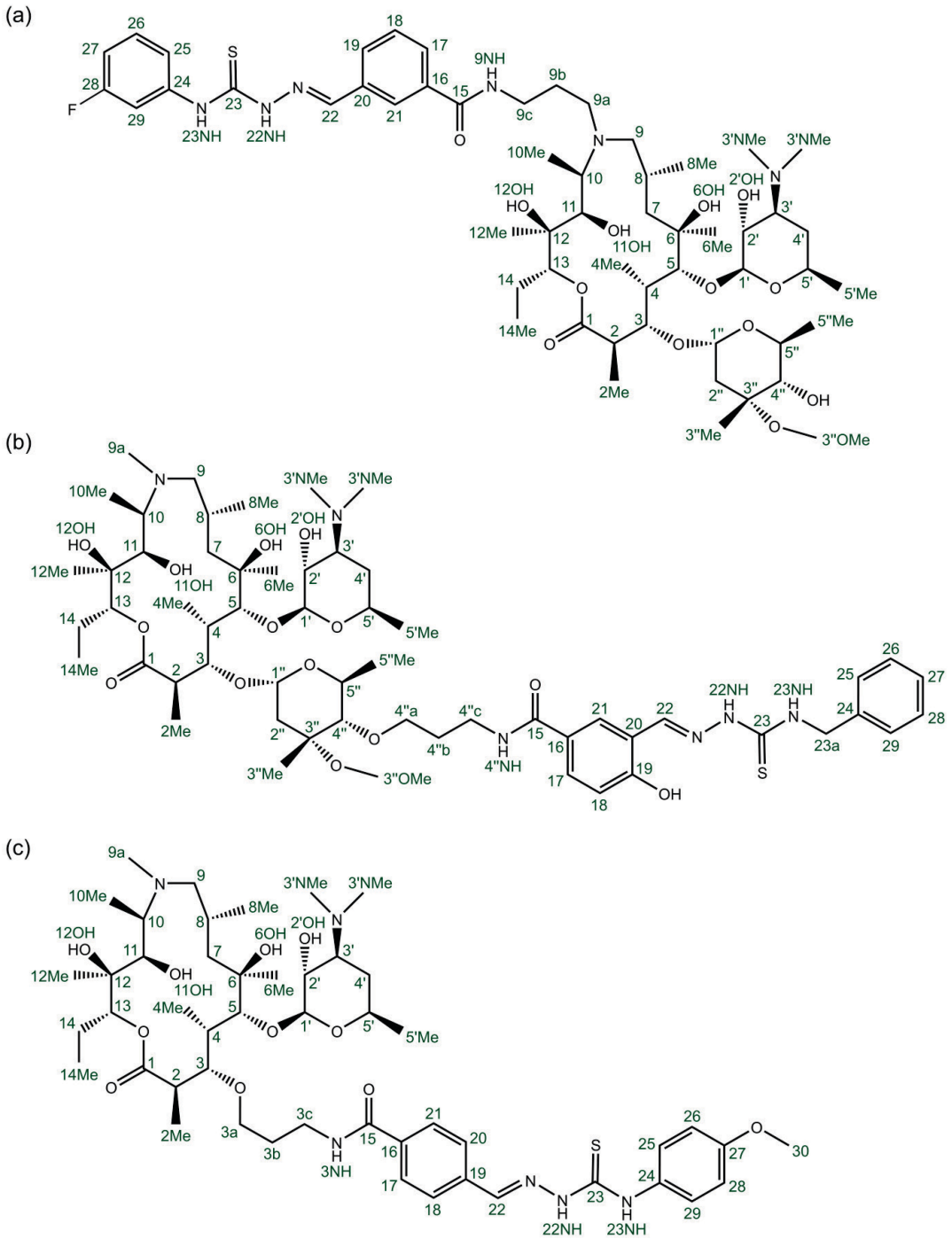

Figure 3. Bioactive macrozones substituted at (a) 9a-, (b) 4''- and (c) 3- positions.

was reflected in improved activity against *S. pneumoniae* and *S. pyogenes* strains, with MIC values of 0.25 and 0.5 μg/mL, respectively. The SAR analysis was supported by conformational studies and molecular docking calculations, providing an additional insight into the binding interactions responsible for the observed activity.[34]

Despite extensive efforts directed toward the development of new antibiotics, it is important to thoroughly investigate the SAR of classical macrolides that once represented the golden era of antibiotic therapy. In this context, Pradhan and coworkers analyzed atomic-scale structural details and nuclear spin dynamics of clinically successful macrolides in order to better understand the basis of their strong antibacterial activity.[35] Using solid-state NMR spectroscopy, they evaluated chemical shift anisotropy (CSA) and site-specific spin-lattice relaxation of carbon nuclei. The observed differences among antibiotics indicated variations in molecular dynamics and local structural features, which can influence binding interactions with the ribosome. These findings highlight the importance of atomic-level analysis for understanding the SAR and guiding novel macrolide design.

Among other factors, the increase in bacterial resistance is strongly influenced by the presence of antibiotics in wastewater and the environment. In recent years, growing awareness of antibiotic contamination in wastewater has raised environmental concerns and potential risks to human health. Chlorination, which is widely used as a disinfection process, affects antibiotic transformation, and the products formed during this process have therefore been extensively investigated. Li et al.[36] examined chlorination-derived products of erythromycin and roxithromycin by combining algal growth bioassays with ECOSAR predictions. Their results showed that chlorination reduces antibiotic ecotoxicity. However, attention should be paid to the accumulation of these chlorination degradation products and potential environmental risks.

BACTERIAL RESISTANCE TO MACROLIDES

Increasing AMR trends to marketed antibiotics, especially those of Gram-negative bacterial pathogens, pose a growing threat to human health globally.[37] Based on a comprehensive data analysis using statistical modeling, Naghavi and coworkers[1] evaluated the global impact of AMR from 1990 to 2021, providing projections up to 2050. Their analysis revealed an annual mortality exceeding one million people due to drug-resistant infections during the 1990–2021 period with projections indicating a potential rise to nearly 2 million deaths per year by 2050. The statistics also showed that for both deaths associated with and deaths attributable to AMR, MRSA increased the most globally. The United Kingdom Health Security Agency reported that there was a 33.0 % rise in resistant *S. aureus* between 2019 and 2024, while the proportion of AMR due to other Gram-positive pathogens remained relatively stable, accounting for 12.9 % of the overall AMR burden in 2024.[38] Due to these facts, the World Health Organization (WHO) included antibiotic-resistant *S. aureus* in the 2024 Bacterial Priority Pathogens List (BPPL) as a high-priority pathogen together with *Salmonella*, *Shigella*, *Pseudomonas aeruginosa*, and *Neisseria gonorrhoeae* due to its multidrug-resistance and limiting treatment options.[39] Gram-negative bacteria that are resistant to last-resort antibiotics, such as *Acinetobacter baumannii,* rifampicin-resistant *Mycobacterium tuberculosis*, and various *Enterobacterales*, are listed as of critical priority in the 2024 BPPL because of their ability to transfer resistance genes. The medium-priority category of the 2024 BPPL, however, includes Group A and Group B *streptococci*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*.[39] A Group A *Streptococcus pyogenes* (GAS) is a Gram-positive human pathogen responsible for serious skin and soft tissue infections.[40] Although GAS had nearly been eradicated by the end of the twentieth century, it re-emerged with outbreaks reported in the United Kingdom and in East Asia.[41,42] Although this pathogen typically responds to conventional antibiotic therapy, growing resistance to macrolides and tetracyclines has been documented. Currently, there is no vaccine candidate for GAS that has passed phase II of clinical trials, but there are several candidates in the development pipeline.[43] Moreover, among Gram-negative organisms, carbapenem resistance exhibited the most significant increase compared to all other antimicrobial classes.[1] Therefore, there is an urgent need for the discovery of new, effective, and safe anti-infectives to combat resistance.

Macrolides are a well-known and significant class of compounds with diverse structures and bioactivities. They are used worldwide primarily as antibiotics. In addition to antibacterial activity, macrolides have shown antiviral, antiparasitic, antifungal, and immunosuppressive actions described systematically in the review by Lenz et al.[44] Among the broad spectrum of macrolides antibacterial activity, they are mostly used for respiratory infections. Their widespread use in the treatment of airway inflammation, especially of azithromycin during the COVID-19 pandemic, has contributed to the growing prevalence of bacterial resistance. Owing to its immunomodulatory effects and its capacity to strengthen the protective barrier of the lung epithelium against pathogens, azithromycin has proven superiority over other macrolides in treating chronic airway diseases such as bronhiectasis,[45] asthma,

and chronic obstructive pulmonary disease (COPD).[46] Previtero and coworkers critically reviewed the use of macrolides for a long-term treatment of chronic respiratory diseases with an emphasis on risks to the community and the environment.[47] Authors highlighted that prolonged macrolide therapy additionally contributes to a faster increase in macrolide resistance and that there is a need for targeted and judicious use of macrolides. Furthermore, there were many cases of delirium as a side effect associated with the use of macrolide antibiotics, namely azithromycin, erythromycin, clarithromycin, and telithromycin.[48]

Mechanisms of Antimicrobial Resistance

A detailed and better understanding of the macrolide mechanism of action, as well as bacterial resistance mechanisms, is crucial for future macrolide structure modifications in order to increase their potency and effectiveness, especially against known resistance mechanisms. The fight against bacterial resistance began in 1953 when resistance was first discovered in *S. aureus* (previously known as *Micrococcus pyogenes*) at Minneapolis General Hospital, a few months after the first clinical use of erythromycin.[49] Since then the bacterial resistance issue has become much more serious and alerting fact.

There are four main mechanisms of the bacterial resistance reported in the literature: (1) modification of target sites at the 23S rRNA or modification of large ribosomal subunit proteins such as L4 and L22; (2) decreasing of macrolide intracelullar concentration by altering the bacterial membrane permeability or by efflux pump expression; (3) macrolide structure modification which includes phosphorylation of a 2'-hydroxyl group of the cladinose catalyzed by phosphotranferases and hydrolysis of the macrolactone ring by esterases and (4) target/ribosome protection by changing the molecular conformation caused by ATP-binding cassette family of proteins.[50–52]

As reported in many SAR studies, macrolides interact with nucleotides A2058 and A2059 of the 23S rRNA.[53] These nucleotides are the essential point of contact for all macrolides, lincosamides, and streptogramin B (MLSB) group of antibiotics. Accordingly, most of the binding site modifications or mutations in macrolide-resistant bacteria were found at these key nucleotides. The modification mechanism involves the methylation or dimethylation of nucleotide A2058 at the N6 position, which mediates the interactions with the saccharide moiety located at the C5 position of the macrolactone ring. Methylation is catalyzed by methyltranferases, which are encoded by the *erm* gene. There are more than thirty *erm* genes specific to a given bacterial strain.[51,54,55] Erm-mediated resistance can be inducible and constitutive. The inducible form of resistance requires macrolide to be transported into the bacterial cells before ribosomal methylation.[56] Hosts that are constitutively resistant to macrolides do not require the presence of the macrolide in cells for the Erm-catalyzed methylation.[57] Mutation of the nucleotide A2058 confers high-level resistance to all macrolides and many ketolides with the exception of *S. pneumoniae*, *S. pyogenes*, and *S. aureus*, where the A2058 mutation confers macrolide resistance but low-level resistance to ketolides such as solithromycin.[50,58,59] Bearing in mind that solithromycin binds to the bacterial ribosome *via* three binding sites, Fernandes and coworkers explained the diversity in pharmacodynamics and toxicity of macrolides and ketolides.[60] Furthermore, the authors highlighted that resistance could be overcome through the synthesis of molecules that target the 23S rRNA at several distinct sites. In that case, pathogens would have to gain mutations at multiple ribosomal sites, which could be unfavorable for them. The resistance of *S. pneumoniae* to macrolides is facilitated by modification of ribosomal target sites *via* methyltranferase enzyme carried by the *ermB* gene.[61] Additionally, one of the resistance mechanisms of *S. pneumoniae* includes efflux pumps expressed by macrolide efflux *mefA*, *mefE*, and *mel* genes. Another group of gram-negative bacteria that developed resistance to macrolides by the modification of 23S rRNA binding sites, as well as modification of ribosomal proteins L4 and L22, are *Enterobacteriaceae*.[62] This group of bacteria also developed transferable mechanisms of macrolide resistance (TMMR), which are encoded within the bacterial chromosome. Dimethylation of nucleotide A2058 in the 23S rRNA of *S. aureus* leads to resistance to the MLSB group of antibiotics. The gene encoding Erm methylase synthetase in *S. aureus* may be expressed in a constitutive or in an inducible manner. In the case of constitutive gene expression, bacterial strains show resistance to all MLSB. If there is an inducible gene expression, resistance occurs only when antibiotics induce the methylase synthesis, like 14-membered macrolides except ketolides (erythromycin, clarithromycin, oleandomycin) and 15-membered macrolides, like azithromycin. *ErmA* and *ermC* genes determinate an inducible resistance in *S. aureus*.[63]

Arsić and coworkers[64] briefly described the advantages of 16-membered macrolides in comparison with 14-membered and 15-erythromycin-based derivatives, focusing primarily on the most explored compounds, tylosin A and josamycin, and compared the resistance mechanisms of the 14- and 16-membered macrolides, respectively. The authors highlighted that, in the case of bacterial resistance against josamycin, a much bigger role played the A2059G mutation than the methylation of the A2058 nucleotide in 23S rRNA, which is the conventional

resistance mechanism for 14-membered macrolides. Furthermore, experiments with *Mycobacterium smegmatis* revealed the importance of A2503U and U2504G substitutions, as well as certain dual mutations that had cumulative effects on *M. smegmatis* resistance against josamycin. Mycoplasmas, distinguished by their lack of a cell wall, are the tiniest self-reproducing prokaryotes, with a diameter of 0.2–0.3 μm and a limited genome of 500–1000 genes. The most frequently used antibiotics for the treatment of Mycoplasma infections are macrolides and tetracyclines. Consequently, the highest resistance was observed for macrolides, as stated in the review by Gautier-Bouchardon.[65] Resistance mechanisms in mycoplasmas include modification of target sites, ribosomal protein mutations, efflux pumps expression and drug modification. In most Mycoplasma bacterial strains, mutations at A2058 and A2059 nucleotides are responsible for high-level macrolide resistance.

Alterations in the large ribosomal subunit proteins L4 and L22, specifically through the insertion or deletion of amino acid residues, also confer bacterial resistance to macrolides.[51] Mutations in the genes encoding L4 and L22 proteins have been found in clinical isolates of *E. coli*, *S. pneumoniae*, *S. pyogenes*, *S. aureus*, *H. influenzae*, and *Mycoplasma genitalium*, respectively.[52] The crystal structure of the large ribosomal subunit, as reported by Wekselman et al.,[66] reveals that an insertion mutation in protein uL22 confers erythromycin resistance. This mutation induces conformational shifts in the uL22 loop and surrounding rRNA nucleotides, thereby increasing the flexibility of the erythromycin binding pocket within the exit tunnel. Furthermore, uL4 and uL22 ribosomal protein mutations were found in clinical isolates of *Pseudomonas aeruginosa*, bacteria which causes cystic fibrosis, a chronic lung infection, and is considered intrinsically resistant to macrolides.[67,68]

Bacteria can reduce intracellular macrolide levels through the action of efflux pumps, which are encoded by chromosomal genes. Based on their structure and energy source, there have been five known main families of efflux pumps in bacteria: (1) the ATP-binding cassette (ABC) family; (2) the multidrug and toxic compound extrusion family; (3) the small multidrug-resistance family; (4) the major facilitator superfamily (MFS) and (5) the resistance modulation cell-division family.[51] Macrolides can be extruded out from the bacterial cell by the Mef (**M**acrolide **ef**flux) and Msr (**M**acrolides-**s**treptogramines **r**esistance) subfamilies of efflux pumps, which belong to the MFS and ABC family, respectively. Mef pumps are proteins that work as antiporters, exchanging the bound macrolide with a proton. There are two major subclasses associated with macrolide resistance: MefA, identified in *S. pyogenes* isolates, and MefE, first identified in *S. pneumoniae*.[61] Mef pumps confer resistance to 14- and 15-membered macrolides, but not to 16-membered macrocyclic lactone rings. On the other hand, Msr subfamily of efflux pumps are proteins that displace macrolide antibiotics by binding themselves to the ribosome. In order to transport macrolides, Msr proteins require ATP as an energy source. There are four classes of Msr proteins, namely types A, C, D and E, which confer resistance to 14- and 15-membered macrolides and low-level resistance to ketolides.[50,51] Furthermore, in clinical *S. pneumoniae* and *S. pyogenes* isolates, M-type resistance was originally attributed to the *mefA* gene. It was subsequently discovered that *msrD*, located adjacent to *mefA*, also contributes significantly to the macrolide efflux mechanism in these streptococci.[69] To elucidate the individual roles of *mefA* and *msrD* in macrolide resistance, Iannelli et al. developed and analyzed isogenic mutant strains of *S. pneumoniae* and *S. pyogenes*, respectively. Their findings suggest that M-type resistance in streptococci is mediated by an ABC superfamily efflux system, where *mefA* encodes the transmembrane channel, while *msrD* encodes the two ATP-binding domains.[70]

ABC-F proteins constitute the predominant group of soluble proteins within the ABC superfamily and they all show some involvement in the translation process. These proteins are involved in the most recently discovered macrolide resistance mechanism called target/ribosome protection by changing the molecular conformation.[51] Protein MsrE is the first of the antibiotic resistance ABC-F proteins characterized in the bound state to the ribosome. It can cause a conformational change of the macrolide binding site in the ribosome by inserting a needle-like domain with two crossed helices connected by an extended loop into the PTC/NPET. This results in the macrolide being displaced from the binding site and interfering with translation inactivation.[71] Ousalem and coworkers gave a detailed description of the structure, function and theories for the mechanism of action of the ABC-F protein family, encompassing those that confer resistance to antibiotics targeting the ribosome. The authors explained four different mechanisms of action proposed on the basis of the cryo-EM structures of ribosome-bound ABC-F proteins. Those include: (1) direct drug displacement; (2) dynamic destabilization of drug binding; (3) drain-snake displacement of drug binding and (4) alternative A/P site tRNA translocation to disrupt drug binding. It has been suggested that each of these mechanisms may play a role in the ABC-F-mediated resistance to antibiotics interacting with the ribosomal PTC/NPET.[72]

Research over the last four decades has established that bacteria naturally occur in two distinct life forms. The first form is a free-floating bacterial cell (plankton) and the second form includes surface attached or non-surface attached aggregates called biofilms. Surface associated

chronic biofilm infections were noticed in patients with prosthetic implants or indwelling medical devices. Non-surface associated biofilm infections include oral and respiratory tract infections, soft tissue and urinary tract infections, wounds, gastrointestinal infections and prosthetic joint infections very often not curable with macrolide antibiotics. The most common microorganisms involved in biofilm-related infections are *P. aeruginosa*, *A. baumanni*, *K. pneumoniae*, *E. coli*, *E. faecium* and *S. aureus*. The processes of biofilm formation are very complex and can be divided into four stages: initial adhesion, formation of microcolonies, biofilm maturation, detachment and dispersion. These formation stages were discovered by studying *P. aeruginosa* biofilm formation.[73,74] The resistance associated with biofilms arises from genetic and phenotypic modifications of the bacteria encapsulated in the extracellular matrix. Biofilm matrix, also called „matrixome", consists of proteins, polysaccharides, water-insoluble compounds and lipids. Complex interactions among „matrixome" components and bacteria surrounded by them are closely related to biofilm-induced AMR as stated by Flemming et al.[75] Previously described AMR mechanisms such as gene mutations, efflux pump expression and antibiotic structure modifications were associated with metabolically active planktonic bacteria targeted by antibiotics. Since bacteria inside biofilms have lower levels of oxygen and nutrients, they are metabolically less active which contributes to their enhanced antibiotic resistance.[76] There are many physical and biological mechanisms of antimicrobial resistance in biofilms described in detail in the review by Pai et al.[74] Ma and coworkers reviewed the strategies for preventing and eliminating biofilm formation based on the characteristics of periods in the biofilm life cycle.[76] Rather than direct treatment of biofilm formation with singular therapy, authors recommended combination therapies that will target biofilm material composition at each stage of biofilm formation. Furthermore, the authors described the application of nanomaterials, many of which possess excellent antibacterial properties and good biocompatibility.

Nowadays, nanotechnological innovations are increasingly applied to combat and overcome antimicrobial resistance. Metallic nanoparticles (NPs), especially gold and silver NPs, polymeric nanomaterials, protein NPs, carbon-based NPs, and lipid-based NPs were designed. Some of them were successfully applied for delivering macrolides into bacterial cells. In the review by Siraj and coworkers, a few nano-based delivery approaches for macrolides were described together with their recent accomplishments, challenges and future perspectives.[77] Although combined macrolide nanomaterials represent a promising tool against resistant pathogens, the authors emphasized the need for further and detailed research of nanomaterials in terms of their safety, biocompatibility, intellectual property, regulations, production time and costs, as well as mechanisms for bacterial cell penetration.

Besides nanomaterial-based drug delivery, another idea in the field of combating antimicrobial resistance was focused on bacterial peptidoglycan (PG) cell wall and on designing new antibiotics that target PG biogenesis factors.[78] Lipid II is a precursor in the synthesis of bacterial cell wall. A Lipid II flippase called MurJ translocates Lipid II from the cytoplasmic leaflet to the periplasmic side of the membrane. Developing new antibiotics that target MurJ protein might help to fight bacterial resistance. In that case the peptidoglycan synthesis would be disturbed, hence bacteria would not be protected from being lysed.[79]

In recent years, artificial intelligence (AI) applications have been increasingly applied for designing novel antimicrobials and for investigating joint effects of combined drug therapies. Furthermore, AI models and algorithms for the prediction of AMR, analysis of resistant infections and rational use of antibiotics, were designed.[80] Recently, an AI-based method named Whole Genome Sequencing for Antimicrobial Susceptibility Testing (WGS-AST) has emerged as a fast and precise diagnostic tool for AMR. Currently, AI-based applications can only process datasets with the same distribution which means that their universality is unsatisfying. Hence, some other AI approaches like few-shot learning and transfer learning will be more applicable for AMR in the future.

MACROLIDE INTERACTION STUDIES

Binding of a macrolide antibiotic **M** to a biomolecular target **T** in its simplest form can be described as a bimolecular association reaction with second-order kinetics (Figure 4).

The total concentrations of a macrolide $[M]_{tot}$ and its target $[T]_{tot}$ can be expressed as:

$$[M]_{tot} = [TM] + [M]_{free} \quad (1)$$

$$[T]_{tot} = [TM] + [T]_{free} \quad (2)$$

where [TM] is the concentration of the macrolide-target complex, while $[M]_{free}$ and $[T]_{free}$ are the concentrations of the free macrolide and its target, respectively. We can then describe the reaction gradient of Gibbs energy ($\Delta_r G$) by the

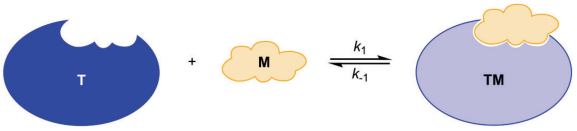

Figure 4. Schematic view of macrolide (**M**) binding to a biomolecular target (**T**) resulting with the formation of a complex **TM**.

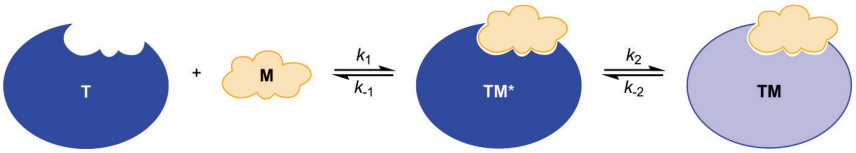

Figure 5. Schematic view of a macrolide (**M**) and its biomolecular target (**T**) forming the steady-state complex **TM*** and the stable complex **TM**.

equation:

$$\Delta_r G = \Delta_r G^\circ + R\,T \ln\left(\frac{[\mathrm{TM}]}{[\mathrm{T}]_{\mathrm{free}}[\mathrm{M}]_{\mathrm{free}}}\right) \quad (3)$$

where $\Delta_r G°$ is the reaction gradient of standard Gibbs energy, R is the ideal gas constant (8.314 J mol K^{-1}) and T is the temperature in Kelvin. At equilibrium, where $\Delta_r G = 0$, the equation (3) becomes:

$$\Delta_r G^\circ = -R\,T \ln\left(\frac{[\mathrm{TM}]}{[\mathrm{T}]_{\mathrm{free}}[\mathrm{M}]_{\mathrm{free}}}\right) \quad (4)$$

or

$$\Delta_r G^\circ = R\,T \ln(K_\mathrm{d}) \quad (5)$$

where K_d is the equilibrium dissociation constant of the complex TM:

$$K_\mathrm{d} = \frac{[\mathrm{T}]_{\mathrm{free}}[\mathrm{M}]_{\mathrm{free}}}{[\mathrm{TM}]} \quad (6)$$

Since the $\Delta_r G°$ can be described by the reaction gradient of standard enthalpy ($\Delta_r H°$) and standard entropy ($\Delta_r S°$), these parameters are related to K_d:

$$\Delta_r G^\circ = \Delta_r H^\circ - T\Delta_r S^\circ = R\,T \ln(K_\mathrm{d}) \quad (7)$$

The dissociation constant can also be expressed in kinetic terms, as the ratio of the complex dissociation (k_{-1}) and formation (k_1) rate constants (Figure 4):

$$K_\mathrm{d} = \frac{k_{-1}}{k_1} \quad (8)$$

If a large excess of macrolide is required to obtain a high degree of binding to the target, the binding interaction results with negligible change in the free macrolide concentration and the reaction can be described with pseudo-first-order kinetics:

$$[\mathrm{TM}]_t = [\mathrm{TM}]_{\mathrm{eq}}\,(1 - \exp(-{}^1k')) \quad (9)$$

where $[\mathrm{TM}]_t$ is the concentration of the macrolide-target complex at a time t, $[\mathrm{TM}]_{\mathrm{eq}}$ is the concentration of the complex at equilibrium and $^1k'$ is the pseudo-first-order rate constant. For reversible binding, the $^1k'$ depends linearly on the free macrolide concentration, $[\mathrm{M}]_{\mathrm{free}}$:

$${}^1k' = k_{-1} + k_1[\mathrm{M}]_{\mathrm{free}} \quad (10)$$

The K_d can be calculated from the slope (k_1) and the intercept (k_{-1}), using the equation (8). However, these values occur on a short timescale and are often difficult to measure, so the K_d is usually measured at equilibrium.

Many binding interactions important for drug development are slower than the diffusion-controlled limit and usually proceed through a two-step mechanism (Figure 5).

In the first step, a macrolide binds to its target and forms the steady-state complex **TM***. Subsequently, an initial interaction tightens and a conformational change occurs, resulting with a more stable complex **TM**. The corresponding equilibria are described by dissociation (inhibition) constants $K_{\mathrm{i},1}$ and $K_{\mathrm{i},2}$:

$$K_{\mathrm{i},1} = \frac{k_{-1}}{k_1} = \frac{[\mathrm{T}][\mathrm{M}]}{[\mathrm{TM}^*]} \quad (11)$$

$$K_{\mathrm{i},2} = \frac{k_{-2}}{k_2} = \frac{[\mathrm{TM}^*]}{[\mathrm{TM}]} \quad (12)$$

The concentration change of the steady-state complex can be expressed as:

$$\frac{\mathrm{d}[\mathrm{TM}^*]}{\mathrm{d}t} = k_1[\mathrm{T}][\mathrm{M}] - k_{-1}[\mathrm{TM}^*] - k_2[\mathrm{TM}^*] + k_{-2}[\mathrm{TM}] \quad (13)$$

If the concentration of **TM*** is constant (steady state) and $k_2 >> k_{-2}$, $[\mathrm{TM}^*]$ corresponds to:

$$0 = k_1[\mathrm{T}][\mathrm{M}] - k_{-1}[\mathrm{TM}^*] - k_2[\mathrm{TM}^*] \Rightarrow [\mathrm{TM}^*] = \frac{k_1}{k_{-1} + k_2}[\mathrm{T}][\mathrm{M}] \quad (14)$$

and the rate of formation of the stable complex **TM** is described by:

$$\frac{\mathrm{d}[\mathrm{TM}]}{\mathrm{d}t} = k_2[\mathrm{TM}^*] = \frac{k_2 k_1}{k_{-1} + k_2}[\mathrm{T}][\mathrm{M}] \quad (15)$$

If the steady-state complex dissociates faster than forming the complex **TM** ($k_{-1} >> k_2$), the rate of **TM** formation is:

$$\frac{\mathrm{d}[\mathrm{TM}]}{\mathrm{d}t} = \frac{k_2 k_1}{k_{-1}}[\mathrm{T}][\mathrm{M}] = \frac{k_2}{K_{\mathrm{i},1}}[\mathrm{T}][\mathrm{M}] \quad (16)$$

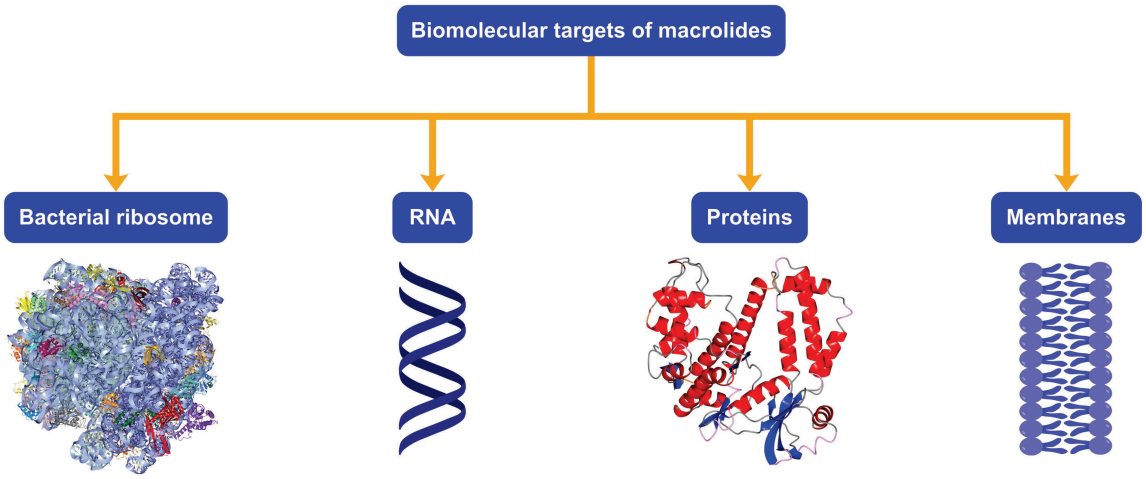

Figure 6. Biomolecular targets of macrolide antibiotics. Ribosome and protein structures are taken from pdb entries 6V39 and 5YJZ, respectively.[81,82]

In the case when a slow reaction step is the formation of **TM*** ($k_2 >> k_{-1}$), the reaction rate follows a simple second-order kinetics:

$$\frac{\mathrm{d[TM]}}{\mathrm{d}t} = \frac{k_2 k_1}{k_2}[\mathrm{T}][\mathrm{M}] = k_1[\mathrm{T}][\mathrm{M}] \qquad (17)$$

Considering different physico-chemical and biological properties of macrolides, the characterisation of their interactions with biomolecular targets is crucial for assessing the overall biological activity and ensuring efficacy. Macrolide antibiotics may target various biomolecules or biomolecular complexes, ranging from bacterial ribosomes, RNA, proteins and membranes (Figure 6).

Since the mechanism of action of macrolide antibiotics is based on inhibition of bacterial protein translation by binding to the NPET, the largest number of studies was focused on explaining the interactions with bacterial ribosome. Recent findings, summarized in excellent reviews of Vazquez-Laslop and Mankin,[53,83] have shown that ribosome-targeted macrolides act as translation modulators rather than simply inhibiting the protein synthesis. Hence, they affect protein synthesis in a context-specific manner by arresting the ribosomes at a limited number of mRNA sites defined by specific sequence motifs, such as Arg/Lys–X–Arg/Lys, where X represents any amino acid. According to biochemical studies, an important role in making this sequence problematic for a macrolide-bound ribosome play both the positive charge of the key amino acids in the +X+ motif and the length of their side chains. It was demonstrated that the interruption of only one essential protein by macrolide binding is sufficient to stop bacterial proliferation and growth. A molecular basis for these findings was revealed by the cryo-electron microscopy (cryo-EM) structure of the ribosomes from *E. coli* arrested by erythromycin or telithromycin during the synthesis of an Arg6-Leu7-Arg8 sequence.[84] The authors explained that drug binding promotes a conformation of the ErmDL nascent chain where the Arg6 side chain extends directly into the A-site and interferes with an incoming Arg-tRNA. It was also suggested that the two drugs direct ribosome stalling by different mechanisms, with telithromycin-induced translation arrest depending more strictly on the +X+ motif. Further insight into context-specific inhibition of bacterial translation was provided by Syroegin et al.[85] They characterized the crystal structures of several ribosome-nascent chain complexes from *T. thermophilus* with various peptidyl- and aminoacyl-tRNAs, alone or in combination with erythromycin or telithromycin. The obtained structural data indicated that the inactive conformation of the A-site Lys-$tRNA_{Lys}$ is largely a consequence of steric hindrance from the penultimate −1 Arg of the nascent chain, rather than allosteric reconfiguring of the PTC induced by macrolides. It was also shown that the dimethylamino group of the desosamine is crucial for macrolide binding and for mediating the rearrangement of Arg/Lys residues in the peptidyl-tRNA. The same group provided a new explanation of how A2058 dimethylation confers resistance to macrolides by solving the high-resolution crystal structures of the Erm-dimethylated and unmethylated ribosomes from *T. thermophilus* in complex with mRNA and aminoacyl-tRNA molecules.[86] They suggested that the high affinity of desosamine-containing macrolides is the result of a strong hydrogen bond mediated by the water molecule, which is tightly coordinated by the phosphate group of G2505 and the exocyclic N6-amino group of A2058 in 23S rRNA. Dimethylation of the N6-amino group of A2058 by Erm methyltransferases prevents coordination of this water

molecule and leave the dimethylamine of desosamine without the mediator of its interaction with G2505. Similar water-mediated networks play an indispensable role in the mechanism of action of other ribosome-targeted antibiotics, pointing towards a high structural conservation of interactions with ribosomes.[87] Another viewpoint on the Erm-mediated bacterial resistance was reported by Rivalta et al., who determined the high-resolution cryo-EM structures of *S. aureus* ribosomes in complex with solithromycin, including those with dimethylated A2058.[88] Their analysis revealed the specific interactions that enable solitromycin binding both in unmodified and dimethylated states. It was suggested that binding occurs directly to the dimethylated ribosome, while the water bridge is partially retained. This finding is in sharp contrast with the results obtained for *T. thermophilus* ribosomes,[86] where dimethylation was shown to completely disrupt the water-mediated interaction and thus prevent drug binding. Hence, it was emphasized that structural and functional observations of *T. thermophilus* may not fully explain complex resistance mechanisms in human pathogens. An additional resistance mechanism identified in the resistant *S. aureus* strains involves mutations in ribosomal protein uL22, which alter the shape of the ribosomal exit tunnel. Halfon and coworkers reported cryo-EM structures of the *S. aureus* ribosome and its erythromycin-bound complex with a two-amino acid deletion mutation in the β-hairpin loop of uL22.[89] Their analysis demonstrated that shortening of the flexible uL22 loop shifts it toward the tunnel wall, thereby widening the passage for nascent proteins and allowing them to bypass the obstruction created by the antibiotic. In addition, macrolide binding further expands the tunnel and induces conformational rearrangements associated with the deletion.

New ways for rational improvement of macrolides were proposed by Khabibullina et al., who determined the crystal structure of dirithromycin bound to the 70S ribosome from *T. thermophilus* carrying mRNA and A-, P- and E- site tRNAs.[90] It was found that the side chain of dirithromycin is engaged in a lone pair π-stacking interaction with the imidazole ring of the residue His69 in ribosomal protein uL4. This additional contact in the macrolide binding pocket may be responsible for more pronounced *in vitro* translation inhibition by dirithromycin compared to its parent compound, erythromycin.[90,91] Another strategy in the search for more potent antibacterials is to explore the clinical potential of 16-membered macrolides, such as mycinamicins. The major advantage of mycinamicins is their ability to overcome Erm-mediated macrolide resistance. Structure elucidation of the 50S ribosomal subunit from *D. radiodurans* with mycinamicins was reported by Breiner-Goldstein et al.[92] They hypothesized that additional interactions of mycinamicins with the upper rim of NPET might delay emergence and attenuate resistance. An interesting research with implications for new antibiotic development was reported by Morgan et al., who applied a combination of cryo-EM and docking analysis to structurally characterize the *A. baumanii* ribosome and predict the drug binding modes.[81] Their analysis showed that erythromycin, azithromycin and clarithromycin target a location on the *S. aureus* 50S ribosomal subunit that serves as the binding site for macrolide antibiotics.

However, when studying interactions of macrolides with their biomolecular targets, it is important to consider that the structural features of the complexes may not be exactly the same in solution and in the solid state. Structures obtained by X-ray diffraction of a single crystal or cryo-EM of a frozen sample cannot always explain the mechanism of interactions at physiological conditions. In order to obtain a more detailed description of drug binding, this approach can be combined with solution-state methods based on NMR spectroscopy and gel electrophoresis. Ligand-detected NMR methods, such as saturation transfer difference (STD), paramagnetic relaxation enhancements (PRE) and transferred nuclear Overhauser effect (trNOE) spectroscopies can provide a wealth of information about the groups responsible for binding, immersion depth, ligand conformation, binding modes and epitopes in solution, resembling physiological conditions.[93,94] Furthermore, toeprinting and inverse toeprinting assays based on primer extension and gel electrophoresis have emerged as valuable tools for measuring the ribosome position on the mRNA where stalling occurred.[94,95] An interesting application of the toeprinting assay was reported by Svetlov et al., who found that distant nucleotides A752 and U2609 of 23S rRNA likely form a base pair and thus facilitate modulation of the ribosomal response to regulatory nascent peptides.[96] On the other hand, the disruption of this base pair accelerates the departure of macrolides with the extended side chain from the ribosome and decreases their ability to kill bacteria. Further mechanistic details about the interactions of antibiotics with rRNA and their effect on translation were summarized in a review by Krawczyk et al.[97] The authors compared the antibiotic binding sites and highlighted the importance of a two-site binding mechanism unique to ketolides. Beyond the usual macrolide binding site, a ketolide telithromycin also targets a secondary site that involves interactions with domain II of the 23S rRNA. This mechanism enhances the inhibitory activity of telithromycin since it is less affected by mutations in rRNA related to bacterial resistance. A similar binding mechanism was reported for macrozones, bioactive thiosemicarbazone-based azithromycin conjugates (Figures 3 and 7).[31,32] Their interactions with the *E. coli* ribosome and bovine serum

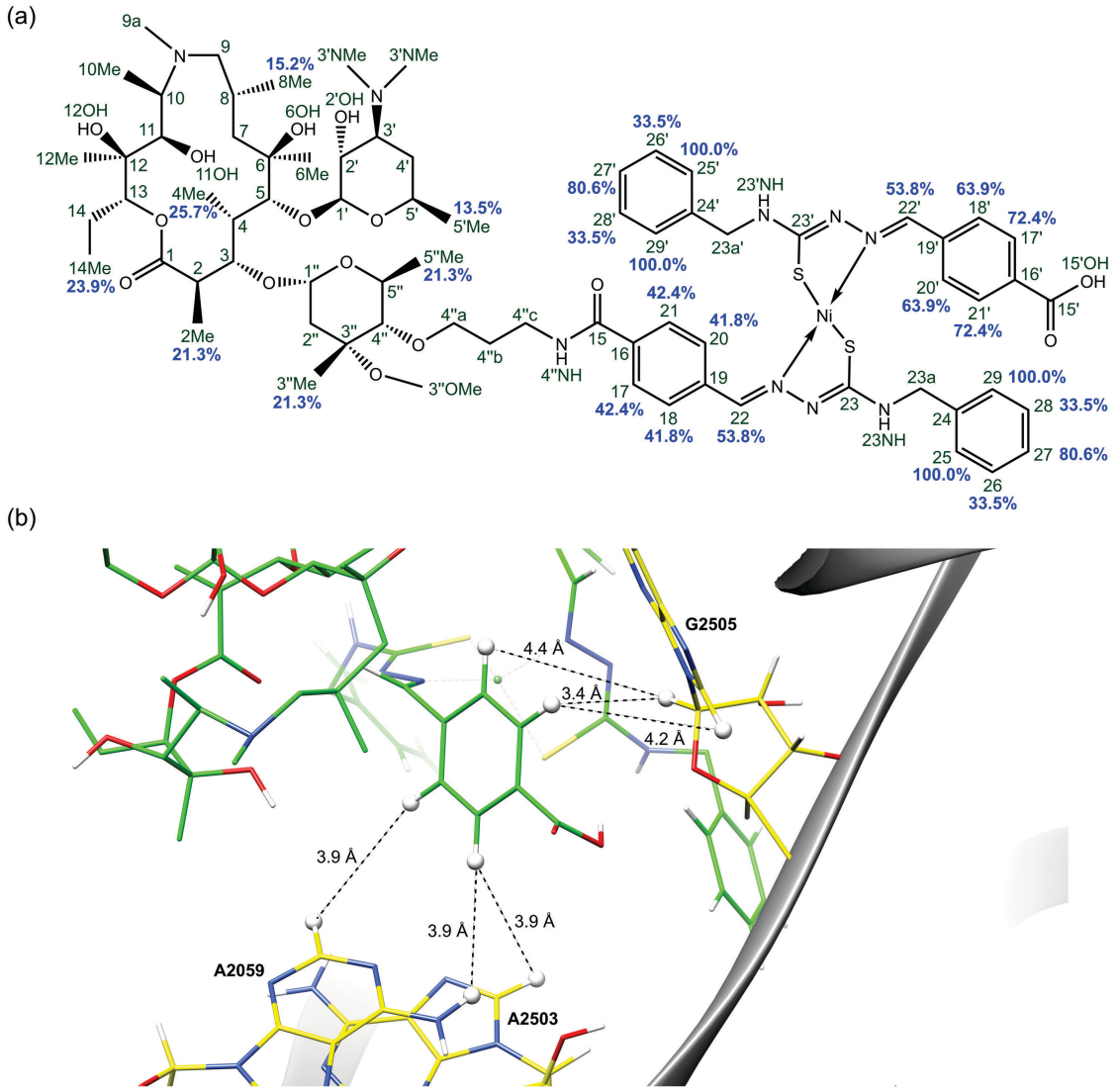

Figure 7. Interactions of the nickel-coordinated macrozone with the *E. coli* ribosome studied by (a) STD NMR and (b) molecular docking. STD enhancements showing the groups in contact with the ribosome were normalized with the highest peak intensity set to 100 %. Nucleotides which form contacts with the macrozone are shown as yellow sticks.[32]

albumin (BSA) were studied by a combined use of NMR, fluorimetric and docking methods. It was shown that macrozones bind to the ribosome *via* the two-site mechanism: the macrolide part occupies the usual macrolide binding site, while the thiosemicarbazone part reaches deeper into the exit tunnel. These additional interactions were found to be responsible for the activity of macrozones against resistant bacterial strains.[32,33] An analogous approach was applied to characterize the structure and interactions of three aminopropyl–azithromycin derivatives, precursors in the macrozone synthesis.[98] It was demonstrated that the studied compounds adopt similar conformations in the free and bound states, while binding epitopes resemble those in erythromycin and azithromycin.

One of the most effective strategies for overcoming bacterial resistance is based on synergistic interactions between macrolides and other bioactive molecules. Linking them covalently can result in conjugates, such as macrolones[11,12] and macrozones,[31–33] with an enhanced bioactivity compared to the parent compounds. Alternatively, macrolides can be administered together with other antibiotics to act against resistant pathogens. Chen et al. reported that an aminocyclitol antibiotic hygromycin-A cooperatively binds to the ribosome with erythromycin, azithromycin or telithromycin and slows down macrolide dissociation.[99] Structural basis for cooperative binding was explained by the X-ray structure determination of wild-type and dimethylated ribosomes

from *T. thermophilus* in complex with macrolide-hygromycin-A pairs. The authors concluded that the potentiating effect of hygromycin-A might be due to direct interactions with ribosome-bound macrolides and induced rotation of A2062 which is required for efficient macrolide binding. On the other hand, concomitant administration of macrolide antibiotics with antibody-drug conjugates (ADC) may affect the activity of ADC. Kiyomiya et al. discovered that both macrolides and ketolides inhibit the cytotoxic effect of an ADC trastuzumab emtansine in human KPL-4 breast cancer cells.[100] Their results pointed towards potential interactions that may reduce the therapeutic effect of trastuzumab emtansine in patients undergoing long-term macrolide treatment.

Classes and general characteristics of RNA-based therapies and RNA-targeted drugs, including macrolide and ketolide antibiotics, were summarized in a comprehensive review of Yu et al.[101] Furthermore, different strategies for the development of RNA-targeted drugs and recent advances in structural understanding of antibiotic interactions with rRNA were discussed. The importance of this research lays in the fact that the interactions of drugs with unique RNA structures or motifs could modulate RNA functions or expression of genes important for control of the disease. An interesting example of a genome-wide analysis of macrolide targets in mammalian cells was reported by Gupta et al.[102] In this research, the authors applied a genome-wide screening of short hairpin RNAs in human erythroleukemia K562 cells to pinpoint the genes responsible for sensitivity to josamycin. It was shown that the macrolide affects mitochondrial translation and cellular energy production at early time points in a relatively nontoxic manner both in cancer and primary cells.

Macrolide antibiotics can also modulate the function of proteins that play important roles in bacterial resistance, molecular transport, proliferation, immunological pathways and chemoenzymatic syntheses. Zhang et al. discovered and structurally characterized the first macrolide antibiotic binding protein (MABP-1) in *Mycobacterium tuberculosis*.[82] The protein was found to confer inducible resistance to erythromycin, clarithromycin and azithromycin, with a preference for 14-membered over 15-membered macrolides. Further functional and structural studies revealed the accessory domain of MABP-1 responsible for its dimerization and macrolide binding. Transport proteins represent another important group of macrolide targets. Albumins are the transport proteins with the highest concentration in blood plasma that are crucial for macrolide metabolism and can serve as drug delivery systems. Rotzinger et al. applied NMR spectroscopy to study interactions of azithromycin aglycon, erythromycin and telithromycin with human serum albumin (HSA).[103] Diffusion coefficients calculated from diffusion-ordered spectroscopy (DOSY) NMR spectra in the absence and presence of the protein provided information on the relative binding strengths to HSA. It was observed that the interaction strength increases with the decreasing polarity of the macrolide side chain. The conformations of telithromycin were determined from trNOE distance restraints, which were used in force field (FF) and density functional theory (DFT) calculations. Previous studies showed that 14- and 15- membered macrolide antibiotics may exist in the two main conformations, folded-in and folded-out.[94,104] However, the characteristic trNOE cross peaks supported by the results of FF and DFT calculations indicated that HSA-bound telithromycin adopts a 3-endo-folded-out conformation with structural features of both conformational families.

Several research groups focused on the use of macrolides and macrolide-inspired macrocycles as antiproliferative agents,[10] enzyme substrates,[22] and potential disruptors of protein-protein interactions,[28] pointing towards the diversity of their protein targets. On the other hand, some macrolide derivatives simultaneously target proteins and ribosome. Dual-targeting macrolones that inhibit either the bacterial ribosome, DNA gyrase, or both are found to be less prone to resistant bacterial strains.[105] In the case of concurrent inhibition of both targets, a spontaneous single mutation in either of them is not sufficient to make cells resistant to the macrolone.

Before reaching bacterial ribosomes, RNA or proteins, macrolides have to penetrate the cell membrane. Understanding how macrolides interact with bacterial membranes is a crucial step in the discovery of novel compounds with increased efficacy. The interactions of macrolides, aminogylcosides and fluoroquinolones with bacterial membrane mimetics were studied by Khondker et al.[106] High-resolution X-ray diffraction analysis was applied to determine the exact drug location in the bilayers and to assess their effect on membrane fluidity and thickness. It was demonstrated that antibiotics increase membrane disorder in a dose-related manner, without changing membrane thickness either at low (1:100 drug/lipid) or high (1:10 drug/lipid) concentrations. Furthermore, all studied classes of antibiotics were found to partition in the lipid heads and tails, while additional partitioning in the membrane core was observed for macrolides and fluoroquinolones.

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