British Journal of Pharmacology

British Journal of Pharmacology (2017) **174** 2967–2983 2967

REVIEW ARTICLE

The macrolide antibiotic renaissance

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Received 21 February 2017; **Revised** 29 May 2017; **Accepted** 20 June 2017

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Macrolides represent a large family of protein synthesis inhibitors of great clinical interest due to their applicability to human medicine. Macrolides are composed of a macrocyclic lactone of different ring sizes, to which one or more deoxy-sugar or amino sugar residues are attached. Macrolides act as antibiotics by binding to bacterial 50S ribosomal subunit and interfering with protein synthesis. The high affinity of macrolides for bacterial ribosomes, together with the highly conserved structure of ribosomes across virtually all of the bacterial species, is consistent with their broad-spectrum activity. Since the discovery of the progenitor macrolide, erythromycin, in 1950, many derivatives have been synthesised, leading to compounds with better bioavailability and acid stability and improved pharmacokinetics. These efforts led to the second generation of macrolides, including well-known members such as azithromycin and clarithromycin. Subsequently, in order to address increasing antibiotic resistance, a third generation of macrolides displaying improved activity against many macrolide resistant strains was developed. However, these improvements were accompanied with serious side effects, leading to disappointment and causing many researchers to stop working on macrolide derivatives, assuming that this procedure had reached the end. In contrast, a recent published breakthrough introduced a new chemical platform for synthesis and discovery of a wide range of diverse macrolide antibiotics. This chemical synthesis revolution, in combination with reduction in the side effects, namely, 'Ketek effects', has led to a macrolide renaissance, increasing the hope for novel and safe therapeutic agents to combat serious human infectious diseases.

Abbreviations

CAP, community acquired pneumonia; MIC, minimum inhibitory concentration; MLS_B, macrolide–lincosamide–streptogramin B; NPET, nascent peptide exit tunnel; PTC, peptidyl transferase centre

DOI:10.1111/bph.13936

Isolation of natural macrolides and their chemical structure

The first macrolide antibiotic was isolated from a *Streptomyces* strain in 1950 and was named pikromycin due to its bitter taste (from the ancient Greek word pikro meaning bitter) (Brockmann and Hekel, 1951). The main chemical characteristic of pikromycin which is common to all later isolated macrolides is the presence of a macrocyclic lactone ring from which the macrolide name derives, as proposed by Woodward in 1950 (see Omura, 2002). Macrolide antibiotics are classified according to the size of the macrocyclic lactone ring as being either 12-, 14-, 15- or 16-membered ring macrolides (Figure 1). The majority of macrolides contain amino sugar and/or neutral sugar moieties connected to the lactone ring via a glycosylic bond.

Methymycin produced by *Streptomyces* sp. is the main representative of the 12-membered macrolides, with only a few other compounds in this class (Figure 1) (Donin *et al.*, 1953). **Erythromycin** (Figure 1) is the best known member of the 14-membered group and was isolated from the *Streptomyces erythraeus* or *Arthrobacter* sp. (McGuire *et al.*, 1952). Oleandomycin (Sobin *et al.*, 1955), lankamycin (Gäumann *et al.*, 1960) and pikromycin (Brockmann and Hekel, 1951) (Figure 1) are also important members of this group. The last group comprises the 16-membered macrolides, with the most important members being tylosin (Hamill *et al.*, 1961), carbomycin (Wagner *et al.*, 1953) and niddamycin (Huber *et al.*, 1962) (Figure 1). In addition to the size of the lactone ring, macrolides can also differ from one another by containing either a disaccharide or a monosaccharide attached to the lactone ring.

Almost all macrolides are produced by strains of *Streptomyces*. However, several species of the genus *Micromonospora* were found to produce either 14- or 16-membered macrolides (Weinstein *et al.*, 1969; Wagman *et al.*, 1972). Because the antibiotic productivity of *Actinomyces* isolated from a soil sample is very low, higher yields were obtained by

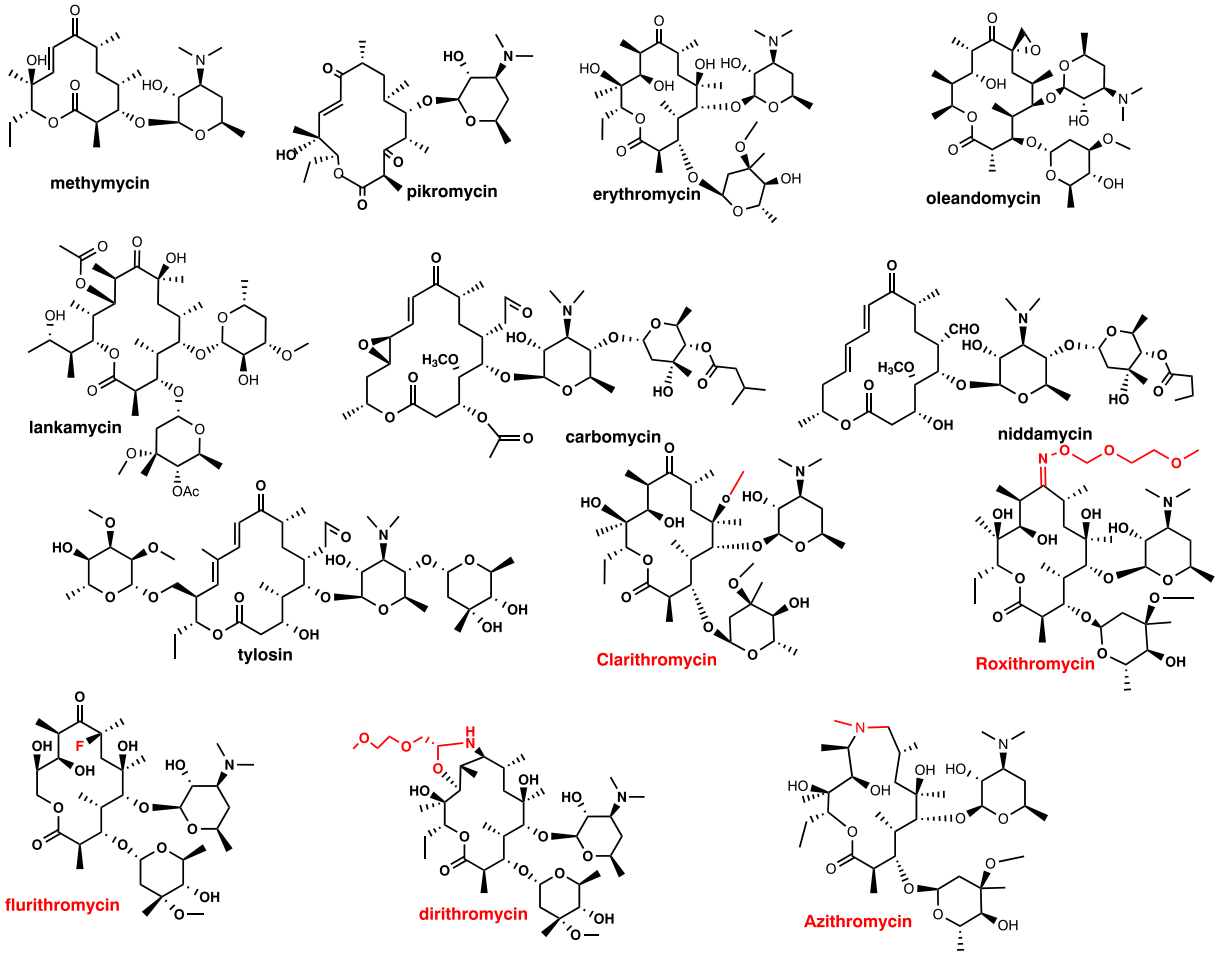

Figure 1

Macrolide structures. First generation: 12-membered (methymycin), 14-membered (pikromycin, erythromycin, oleandomycin and lankamycin) and 16-membered (carbomycin, niddamycin and tylosin), all natural products. Second generation: 14-membered (clarithromycin, roxithromycin, flurithromycin dirithromycin) and 15-membered (azithromycin). Red colour indicates modifications inserted in the erythromycin molecule to generate the second generation of 14- and 15-membered macrolides.

examination of various cultural conditions and by improvement of the producing strain using mutational approaches. Industrial yields of macrolide antibiotics are presumed to reach 10 $mg \cdot mL^{-1}$, although the exact details are not known due to company secrecy. Today, although the total synthesis of erythromycin has been reported (Woodward *et al.*, 1981), the fermentation production is preferred due to higher yields.

In this short review, we describe the historical development of macrolides and their mode of action, which has been completely revised during the past few years. Moreover, all resistance mechanisms that render pathogens resistant to macrolides and are responsible for their decreased usage are presented. Finally, the latest developments that have returned this antibiotic family to the forefront of science are discussed, leading to the conclusion that the next-generation macrolide family members will be highly active with reduced toxicity and will therefore re-enter the market in the near future.

Antimicrobial activity and chemical derivatization

In general, macrolide antibiotics are active mainly against Gram-positive bacteria and have only limited activity against Gram-negative bacteria (Nakayama, 1984). Macrolides are very active against *Staphylococcus*, *Streptococcus* and *Diplococcus* Gram-positive bacteria, and among Gram-negative cocci, *Neisseria gonorrhoea*, *Haemophilus influenzae*, *Bordetella pertussis* and *Neisseria meningitis*. Additionally, they are also extremely active against various *Mycoplasmas*, although there are some susceptibility differences between 14- and 16-membered macrolides (Bébéar *et al.*, 1997; Doucet-Populaire *et al.*, 1998; Morozumi *et al.*, 2008). They have very low activity against eukaryotes due to their low affinity for binding to eukaryotic ribosomes (Corcoran, 1984; Böttger *et al.*, 2001). Additionally, eukaryote rRNAs carry a guanosine in the equivalent position A2058 of prokaryotes (Böttger *et al.*, 2001), although this difference is not the only determinant responsible for the difference in macrolide susceptibility between yeast and prokaryotes (Bommakanti *et al.*, 2008).

Although macrolides display excellent antibacterial activity, their generally poor bioavailability, unpredictable pharmacokinetics and low stability in the acidic pH of the stomach prompted early searches for new derivatives with improved properties. This resulted in the second generation of macrolides, which were semisynthetic derivatives of the first, natural product, generation. Five derivatives of erythromycin were developed and marketed, namely, clarithromycin (Omura *et al.*, 1992), dirithromycin (Counter *et al.*, 1991), **roxithromycin** (Chantot *et al.*, 1986), flurithromycin (Toscano *et al.*, 1983; Gialdroni-Grassi *et al.*, 1986) and **azithromycin** (Girard *et al.*, 1987; Retsema *et al.*, 1987) (Figure 1). Miokamycin (Omoto *et al.*, 1976; Borzani *et al.*, 1989) and rokitamycin (Sakakibara *et al.*, 1981) were the only 16-membered second-generation compounds developed for human use (Figure 2). Tilmicosin (Debono *et al.*, 1989), a semisynthetic derivative of tylosin, was developed solely for veterinary use (Figure 2). Clarithromycin and azithromycin are highly marketed worldwide, whereas dirithromycin (Brogden and Peters, 1994; Kirst, 1995), flurithromycin (Benazzo *et al.*, 1998) and roxithromycin (Jain and Danziger, 2004) have had a much more limited distribution.

Clarithromycin and azithromycin were prepared from erythromycin A in a short, four- to six–step, sequence of chemical transformations (Morimoto *et al.*, 1984; Bright *et al.*, 1988). The second-generation erythromycin derivatives contain all modifications at the C6 or C9 positions of the lactone ring, thereby preventing the formation of the 9,12- and/or the 6,9-hemiketal forms, which degrade to spiroketal inactive derivatives and therefore exhibited immunity to acid-catalysed inactivation. Clarithromycin is still degraded under acidic conditions to form such derivatives, albeit at reduced rates relative to erythromycin A (Nakagawa *et al.*, 1992; Mordi *et al.*, 2000). The above mentioned second-generation derivatives (Figures 1 and 2) have each improved oral bioavailability and increased half-life in plasma, enabling the oral dosage to be reduced to once or twice a day (Foulds *et al.*, 1990; Bahal and Nahata, 1992; Piscitelli *et al.*, 1992; Rodvold, 1999). These compounds also exhibited enhanced tissue penetration because of their higher lipophilicities, relative to that of the parent compound erythromycin A and hence were more effective for treatment of intracellular pathogens such as *H. influenzae* (Alvarez-Elcoro and Yao, 2002). Although the search for the second generation of macrolides was undertaken with the desire to discover compounds with expanded spectra and improved activity, the compounds selected did not exhibit improved activity against Gram-positive bacteria, and some, in fact, such as azithromycin had reduced potency compared with the mother compound erythromycin (Barry *et al.*, 1988, 2001; Fernandes and Hardy, 1988). Nevertheless, they were selected for development mainly because of their enhanced pharmacokinetic profiles, in particular, the ability to accumulate to high levels within lung tissue (Wise, 1989; Foulds *et al.*, 1990; Retsema *et al.*, 1990; Hardy *et al.*, 1992). Clarithromycin is also used generally in combination with other antibiotics, for the treatment of gastric ulcers caused by *Helicobacter pylori* and for AIDS-related respiratory infections caused by the *Mycobacterium avium* complex (Haefner *et al.*, 1999).

While the second generation of macrolides provided solutions with respect to improved pharmacokinetics and acidic inactivation, they provided few answers with respect to antibiotic resistance. As macrolide resistance was becoming increasing dangerous, as was happening with all other antibiotic classes, this prompted research into the development of the next generation of macrolides to combat macrolide resistant strains. This effort yielded the third generation of macrolides, termed ketolides, where the 3-keto group in the lactone ring replaces the L-cladinose present in erythromycin (reviewed by Katz and Ashley, 2005). In addition, nearly all ketolides contained the addition of a fused 11,12-cyclic carbamate as well as an alkyl–aryl side chain tethered to different positions of the lactone ring (Figure 2). Initially, all chemical efforts were focused on the structure and length of the alkyl–aryl side chains and the tethering position on the lactone ring. The first position tethered was the C11 carbon of the lactone ring (Denis *et al.*, 1999; Putnam *et al.*, 2011),

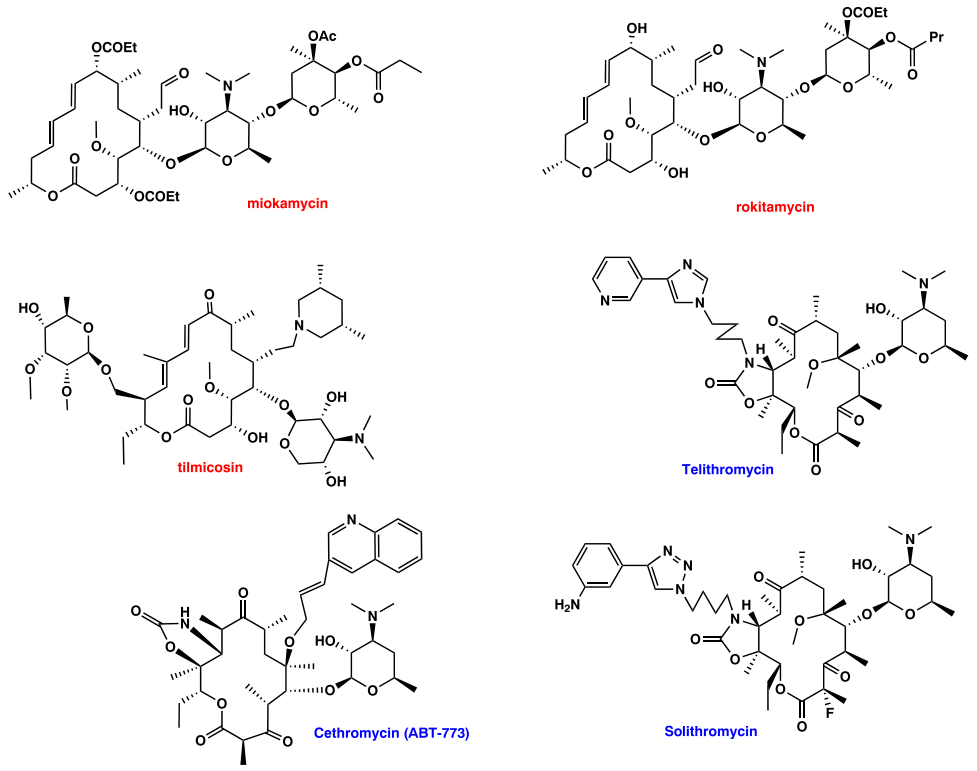

Figure 2

Macrolides structure. Second generation: 16-membered (miokamycin, rokitamycin and tilmicosin). Third generation: ketolides: Telithromycin, cethromycin and solithromycin. Red and blue colour indicates second- and third-generation macrolides respectively.

although in parallel, C6-tethered ketolides were also prepared (Or *et al.*, 2000; Ma *et al.*, 2001; Wu and Su, 2001; Plata *et al.*, 2004). Later, a series of C9-oximes ether ketolides bearing N-aryl–alkyl acetamides were also synthesized, where the length of the alkyl group differed by up to five atoms (Iwaki *et al.*, 2005; Nomura *et al.*, 2005; Nomura *et al.*, 2006). Other structure modifications included the following: modified 5-*O*-desosamine ketolides (Chen *et al.*, 2012), fluorination at the C2- and/or C12 positions (Denis and Bonnefoy, 2001; Krokidis *et al.*, 2014), variation of the cyclic carbonate and hydrazono-carbamate at 11,12 positions (Hunziker *et al.*, 2004; Andreotti *et al.*, 2007; Zhu *et al.*, 2007) or variation of the aglycon ring (Shaw *et al.*, 2005; Ashley *et al.*, 2006; Sugimoto and Tanikawa, 2010). Lastly, modifications also included replacement of desosamine with different sugars (Liang *et al.*, 2005; Romero *et al.*, 2005; Chen *et al.*, 2012). A detailed review covering all the chemical efforts to improve the ketolide activity is presented in the review of Liang and Han (2013). The only third-generation macrolide in the market today is telithromycin (Figure 2), commercialized as Ketek by Aventis, a 14-membered 'ketolide' that was derived from clarithromycin using eight chemical steps (Denis *et al.*, 1999). Another ketolide cethromycin (Figure 2) (Ma *et al.*, 2001) with similar activity to telithromycin was denied FDA approval in 2009. Lastly, solithromycin (Pereira and Fernandes, 2011) (Figure 2) is currently undergoing phase III clinical trials (Farrell *et al.*, 2016) and seems to be the most promising ketolide.

These ketolides (telithromycin, cethromycin and solithromycin) (Figure 2) have outstanding activity against Gram-positive aerobic pathogens, including macrolide-resistant strains of *Streptococcus pneumoniae* (Shortridge *et al.*, 2002; Farrell *et al.*, 2015). In addition, they display good activity against some Gram-negative aerobes, such as *Moraxella catarrhalis* and *H. influenzae*, and gratifying activity against atypical/intracellular CAP pathogens such as *C. pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella pneumophila* (Bébéar *et al.*, 1997; Hammerschlag *et al.*, 2001; Fernandes *et al.*, 2016). Unlike azithromycin and clarithromycin, telithromycin is not a substrate for the efflux pumps found in *S. pneumoniae* and *Streptococcus pyogenes* and does not induce ribosomal methylation associated with inducible macrolide–lincosamine–streptogramin B (MLS_B) resistance in *Streptococci* and *Staphylococci* (Bryskier, 2000). However, *Staphylococcal* and *S. pyogenes* strains that carry constitutively methylated ribosomes are not susceptible to telithromycin. Although rare, ketolide (telithromycin)-resistant strains have been isolated worldwide (Doern, 2006; Felmingham *et al.*, 2007). Unlike macrolides, which are considered as time-dependent bacteriocides, ketolides show concentration-dependent killing (Zhanel and Hoban, 2002; Woosley *et al.*, 2010). In addition to the three ketolides mentioned above, other ketolide compounds have been developed, although

none of them have so far attracted much attention (Fu *et al.*, 2006; Karahalios *et al.*, 2006; Kouvela *et al.*, 2009; Liang and Han, 2013; Ruan *et al.*, 2013; Krokidis *et al.*, 2014).

Side effects

Although first and second generation of macrolides were safe and well tolerated, the third generation of macrolides and more specifically, the ketolide telithromycin exhibited rare but serious irreversible hepatotoxicity named 'Ketek effects' (Young, 2007; Georgopapadakou, 2014; *Telithromycin* 2014; Fernandes *et al.*, 2016). This is why telithromycin use was restricted and led to pharmaceutical companies focusing on improving the safety of macrolides. According to Fernandes and colleagues, it is the pyridine ring included in telithomycin's alkyl–aryl-side chain (Figure 2) that blocks **nicotinic acetylcholine receptors**, resulting under specific conditions in serious hepatotoxicity (Fernandes *et al.*, 2016). Therefore, the first requirement to avoid hepatotoxicity is the absence of a pyridine ring from the alkyl–aryl side chain of a new macrolide.

Procedures for development of the next generation macrolides

A breakthrough in macrolide development and synthesis occurred last year when Seiple and colleagues presented a new approach to total synthesis, capable of generating virtually any kind of macrolide (Seiple *et al.*, 2016). Specifically, they developed a platform of unprecedented versatility for the development and synthesis of novel macrolide antibiotics, employing a design strategy that involved a multi-convergent assembly of macrolides from simple chemical building blocks. The assembly utilizes eight initial building blocks into which they can introduce huge diversities, and follows a sequence of convergent coupling reactions to form two fundamental intermediates, which participate in the next key reaction, the macrocyclization reaction step (Boeckman and Pruitt, 1989). The success of macrocyclization is part of the main contribution of this new procedure because many previous pioneer attempts had all failed (Seiple *et al.*, 2016). In addition to the modifications inserted in the initial building blocks, further modifications are possible either at a later step or after lactone ring complemention. As a result, a library of macrolide antibiotics was constructed containing more than three hundred different compounds. Some of the novel macrolides exhibited high antimicrobial activity against many macrolide-resistant pathogenic bacteria and are undergoing further evaluation. Following this new procedure, many compounds of clinical importance were also obtained, such as azithromycin, telithromycin and solithromycin, bypassing the established semisynthetic method of chemical modification, which starts from the erythromycin molecule as a fermentation product. Furthermore, this procedure enables success in designed macrolide compounds that could not have been synthesized earlier using the derivatization method.

Other tools in the macrolide renaissance include the structure-based drug design procedure (Lounnas *et al.*, 2013) as well as molecular simulations (Pavlova and Gumbart, 2015; Pavlova *et al.*, 2017) for the optimization of a chemical structure, both with the same goal of identifying a compound suitable for clinical testing as a drug candidate. These procedures are based on the knowledge of the three-dimensional structure of the macrolides and how its shape and charge cause it to interact with the 50S ribosomal subunit. Lastly, genetic engineering procedures need to be mentioned, especially with respect to polyketide synthases (Khosla and Zawada, 1996; Khosla, 2009; Walsh, 2017), the multi-enzyme systems responsible for macrolide biosynthesis, which have substantially increased the potential to develop new macrolides.

Mode of action

Regardless of whether first, second or third generation, all macrolide antibiotics bind to the large ribosomal subunit of the prokaryotic ribosome, occupying a site within the nascent peptide exit tunnel (NPET) adjacent to the peptidyl transferase centre (PTC). The binding site of a diverse range of macrolide antibiotics on different bacterial (and archeal) ribosomes has been revealed using X-ray crystallography (Schlünzen *et al.*, 2001; Berisio *et al.*, 2003; Tu *et al.*, 2005; Bulkley *et al.*, 2010; Dunkle *et al.*, 2010) (Figure 3). There was initially a controversy concerning the conformation of the macrolide-bound lactone ring and the contacts they make with the ribosome (Schlünzen *et al.*, 2001; Hansen *et al.*, 2002; Berisio *et al.*, 2003; Tu *et al.*, 2005). However, subsequent studies using *Escherichia coli* (Dunkle *et al.*, 2010) and *Thermus thermofilus* ribosomes (Bulkley *et al.*, 2010) resolved the situation and confirmed that the macrolactone ring of all macrolides is similarly oriented in the ribosomal tunnel, regardless of the type of macrolide or the size of the lactone ring. Macrolides interact with the nucleobase of A2058 of the 23S rRNA, which involves a hydrogen bond between the desosamine hydroxyl and the N1 atom of A2058. In addition, the binding of macrolides is stabilized by tight packing of the hydrophobic face of the lactone ring against rRNA nucleotides 2611 and 2057 (Figure 3C). However, species-specific differences do appear to exist with respect to macrolides or ketolides bearing the alkyl–aryl side chain. In the case of telithromycin, this heterocyclic side chain was observed in different positions when comparing the drug bound to the archaeal *Haloarcula marismortui* 50S subunit (Tu *et al.*, 2005), *Deinococcus radiodurans* 50S subunit (Berisio *et al.*, 2003; Schlünzen *et al.*, 2003), *T. thermophilus* (Bulkley *et al.*, 2010) or *E. coli* 70S ribosomes (Dunkle *et al.*, 2010), as previously described (Wilson *et al.*, 2005) (Figure 3D).

For a long time, macrolides were considered as general inhibitors of translation by simply obstructing the ribosomal exit tunnel and thereby preventing the progress of the synthesis of the nascent polypeptide chain (Menninger and Otto, 1982; Tenson *et al.*, 2003; Mankin, 2008). In contrast to this view, Mankin and coworkers have demonstrated that the mode of action of these drugs is more complicated

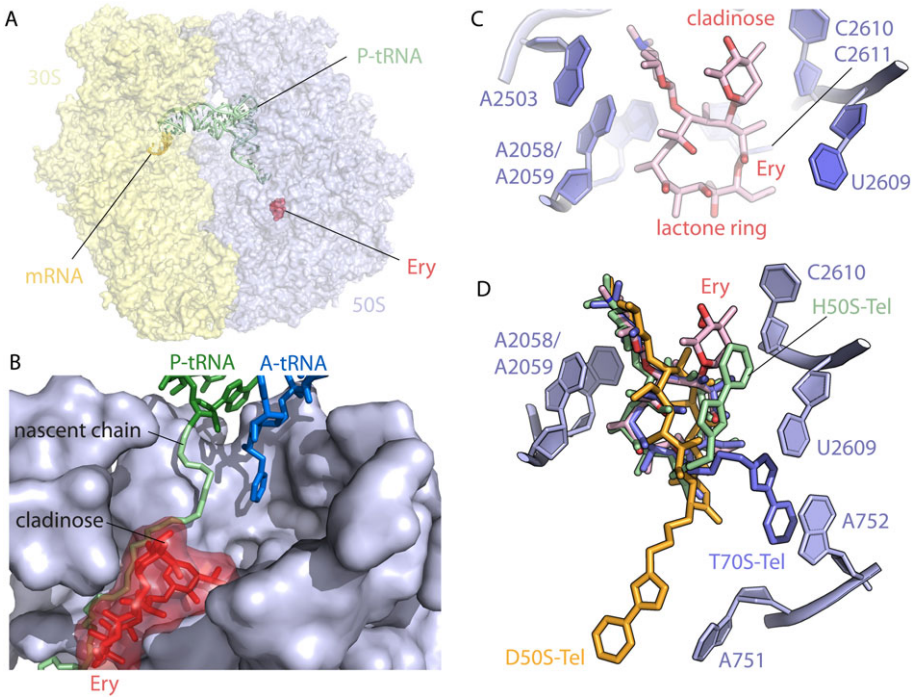

Figure 3

Interaction of macrolides with the ribosome: (A) Overview of the antibiotic erythromycin (Ery, red) bound to the 70S *E. coli* ribosome (PDB entry 4V7U; Dunkle *et al.*, 2010). (B) Close-up view of the erythromycin binding site in the ribosomal exit tunnel in the presence of the P-site peptidyl-tRNA (green) and the A-site tRNA (blue) (prepared with modifications from Wilson 2014). (C) Erythromycin binding pocket within the *E. coli* 70S ribosome is located adjacent to bases A2058, A2059, A2503 and U2609. The desosamine amino sugar of Ery at position 5 of the lactone ring contains a dimethyl amine that makes pivotal contact with the base A2058 (PDB entry 4V7U; Dunkle *et al.*, 2010). (D) Superposition of telithromycin (Tel) bound to the ribosomes from different species. All structures of ribosome-bound Tel were aligned based on domain V of the 23S rRNA. Note that although the lactone rings almost perfectly match in all cases, the position of the alkyl–aryl groups varies significantly depending on the species. Shown are *Haloarcula marismortui* (green, PDB entry 1YIJ; Tu *et al.*, 2005), *D. radiodurans* (orange, PDB entry 1P9X; Berisio *et al.*, 2003) and *T. thermophilus* (blue, PDB entry 4V7Z; Bulkley *et al.*, 2010). All figures were prepared using PyMol software.

(Kannan *et al.*, 2012; Kannan *et al.*, 2014; Sothiselvam *et al.*, 2016). For the majority of proteins, the binding of the drug within the tunnel does cause synthesis to be aborted when the nascent peptide chain reaches a nominal length of 5–11 amino acids where prolongation is prevented, leading to dissociation of the peptidyl-tRNA (drop-off) from the ribosome (Menninger and Otto, 1982; Menninger, 1995; Tenson *et al.*, 2003). A small number of short specific nascent peptides, such as those encoded in the regulatory cistrons of genes rendering macrolides ineffective, can induce ribosome stalling by interacting with the NPET. In this case, the peptidyl-tRNA remains bound, but peptide bond formation with the arriving A-site bound aminoacyl-tRNA is prevented (Horinouchi and Weisblum, 1980; Vazquez-Laslop *et al.*, 2008; Ramu *et al.*, 2011). Moreover, Mankin and coworkers recently demonstrated that some peptide sequences have the ability to bypass the antibiotic within the NPET which leads either to the synthesis of long polypeptides on drug-bound ribosomes or to the interruption at a later state when the length of the nascent chain has already passed the antibiotic binding site (Kannan *et al.*, 2012). Macrolides, such as erythromycin, appear to allow fewer proteins to bypass compared with ketolides, such as telithromycin (Kannan *et al.*, 2012; Davis *et al.*, 2014; Kannan *et al.*, 2014), presumably because erythromycin contains a C3-bound cladinose sugar (Figure 1) that projects into the lumen of the tunnel (Schlünzen *et al.*, 2001; Bulkley *et al.*, 2010; Dunkle *et al.*, 2010).

Ribosome profiling studies have also been used to dissect macrolide action (Davis *et al.*, 2014; Kannan *et al.*, 2014). Ribosome profiling or Ribo-seq is a recently developed high-throughput sequencing technique that allows the identification of RNA fragments resistant to RNAse digestion by translating ribosomes. Therefore, it provides a snapshot of ribosomal movement on the template mRNA (Ingolia *et al.*, 2009; Li *et al.*, 2012). A high number of ribosome-protected fragments mapping to the transcriptome is indicative of prolonged ribosome occupancy at a given position, also known as ribosome stalling/pausing. Ribo-seq carried out recently in Gram-positive and Gram-negative bacteria treated with different macrolides identified the major sites of late translation arrest and allowed classification of problematic sequences for the first time (Davis *et al.*, 2014; Kannan *et al.*, 2014). Several amino acid motifs favourable to macrolide-induced arrest were revealed by these studies. The most dominant motif contained the tripeptide sequence motif R/K-X-R/K, where R and K denote arginine and lysine amino acids, respectively, and X represents any amino acid. *In vitro* biochemical experiments supported the conclusion drawn from the mRNA profiling analysis that the ribosome stalls

when the codon representing the middle amino acid (X) of the motif enters the P site. Accordingly, the first residue of the consensus (R or K) represents the position before the last amino acid of the nascent peptide chain, whereas the last consensus sequence residue (also R or K) corresponds to the amino acid attached to the A site-bound aminoacyl-tRNA (Davis *et al.*, 2014; Kannan *et al.*, 2014; Sothiselvam *et al.*, 2014, 2016). These findings suggest that rather than inducing a universal arrest of protein synthesis, macrolides and ketolides actually allow translation of only a small subset of proteins. Taking into account that telithromycin, which is bactericidal, allows more nascent peptides bypass than erythromycin (which is bacteriostatic), it has been suggested that an inequity in protein inhibition is more harmful for the cell compared with uniform inhibition of protein synthesis (Kannan *et al.*, 2012).

Kinetic analysis of many different macrolides revealed that most of them act as slow-binding inhibitors (Morrison and Walsh, 1988). Moreover, while their association rate constants (k_{on}) are nearly identical, there are large differences in their dissociation rate constants (k_{off} values). The k_{off} values for some macrolides are extremely low, in the range of 10^{-5} s^{-1}, indicating that these macrolides are almost irreversibly bound to the ribosome (Di Giambattista *et al.*, 1987; Dinos and Kalpaxis, 2000; Dinos *et al.*, 2003; Krokidis *et al.*, 2016). Mankin and colleagues have suggested that such very low macrolide dissociation constants from the ribosome may also contribute to their bactericidal effect (Svetlov and Mankin, personal communication).

Champney and co-workers have shown that macrolides inhibit ribosome assembly (Chittum and Champney, 1995; Champney and Miller, 2002; Champney and Pelt, 2002), but this results from a secondary effect due to the inhibition of synthesis of ribosomal proteins (Siibak *et al.*, 2009). Lastly, a novel action of macrolides previously unknown is the promotion of ribosome frameshifting (Gupta *et al.*, 2013).

Macrolide resistance

The two most common resistance mechanisms in the bacterial pathogens are the reduced binding affinity of the drug, firstly, due to modification of either the bacterial ribosome or the antibiotic molecule and, secondly, due to efflux of macrolides from the bacterial cell, arising via altering either the membrane permeability or efflux pump expression (Wilson, 2014; Fyfe *et al.*, 2016). Efflux proteins belong mainly to Mef and Msr families, and ribosome modification mechanisms include either ribosomal 23S rRNA or large ribosomal subunit proteins, while drug-inactivating mechanisms include phosphorylation of the 2′-hydroxyl of the sugar by phosphotransferases and hydrolysis of the macrocyclic lactone by esterases.

Mef family

Mef pumps are proteins that are members of the major facilitator superfamily, consisting of 12 transmembrane domains linked by hydrophilic loops (Pao *et al.*, 1998). Mef pumps work as antiporters, exchanging the bound macrolide with a proton (Law *et al.*, 2008). *mef* genes are found in Gram-positive bacteria but have also been reported in some Gram-negative species (Ojo *et al.*, 2004). There are two major subclasses, *mef*(A) and *mef*(E). Although there is higher than 80% homology, they are carried on distinct genetic elements. Both genes confer resistance to 14- and 15-membered, but not to 16-membered, macrolides, lincosamides and streptogramins B, affording the so-called 'M phenotype' but not the 'MLS_B phenotype'. *mef*(E), as well as the *Staphylococcus aureus msr*(A) family of genes, carries an adjacent ATP-binding cassette-type transporter gene known as the *msr*(D) gene. Msr (D) and Mef(E) co-expression is required for high-level macrolide resistance in *S. pneumoniae*, and both proteins interact synergistically to increase macrolide resistance in *E. coli* (Nunez-Samudio and Chesneau, 2013). Recently, additional *mef* genes were described, namely, *mef*(B) and *mef* (E), with medium homology to *mef*(A) and *mef*(I), as well as *mef*(O) with high homology to *mef*(A) (see Fyfe *et al.*, 2016). *mef* genes are regulated by transcription attenuation, with the induction of the *mef*(E)/*msr*(D) operon occurring by anti-attenuation of transcription in the presence of inducing macrolides. There is also evidence, however, that an additional regulation mechanism is existing with a leader peptide encoded upstream of *mef*(E) (Subramaniam *et al.*, 2011; Chancey *et al.*, 2015).

Msr family

These proteins displace macrolide antibiotics from the ribosome, offering ribosome protection by binding and chasing the bound drug from the ribosome (Sharkey *et al.*, 2016; Wilson, 2016). There are four classes of Msr proteins, namely, types A, C, D and E, with each class having an ATP-binding motif and sequence homology with the ATP-binding superfamily (Ross *et al.*, 1990). The Msr family confer resistance to 14- and 15-membered macrolides and low level to ketolides (Ross *et al.*, 1995; Canton *et al.*, 2005; Reynolds and Cove, 2005; Vimberg *et al.*, 2015). The *msr* genes related to macrolide resistance have been isolated from *Staphylococcus epidermidis* (Ross *et al.*, 1990), *Staphylococcus xylosus* (Milton *et al.*, 1992), *St. aureus* (Matsuoka *et al.*, 1999, 2003), *Enterococcus* (Portillo *et al.*, 2000), *Streptococcus* (Varaldo *et al.*, 2009), *Pseudomonas* and *Corynebacterium* (Ojo *et al.*, 2006). For a long time, the Msr family was thought to confer macrolide resistance by acting as efflux pumps (Fyfe *et al.*, 2016). Now, it seems that these proteins act in a similar way to TetM/TetO proteins, chasing the bound macrolide from the ribosome (Sharkey *et al.*, 2016; Wilson, 2016). Tet(M) and Tet(O) are paralogs of the translational GTPase EF-G and remove tetracycline from the ribosome in a GTP hydrolysis-dependent manner causing tetracycline (not macrolides) resistance (Burdett, 1996; Connell *et al.*, 2003).

23S rRNA modification

Gram-positive bacteria, as well as *E. coli*, can acquire genes that modify the 23S rRNA and confer high-level MLS_B resistance to macrolides, lincosamides and some members of the streptogramin B family (Weisblum, 1995a, 1995b; Roberts *et al.*, 1999). The genes encode methyltransferase enzymes that either mono- or di-methylate the N6 position of nucleotide A2058 (*E. coli* numbering) of the 23S rRNA (Katz *et al.*, 1987). The enzyme class was named Erm for erythromycin resistance methylase, and individual genes as *ermA*, *ermB*, etc. (Roberts *et al.*, 1999). The *erm* genes have been found on

high- and low-copy plasmids and within transposons, usually in association with other genes responsible for resistance expression to other antibiotics (Alekshun and Levy, 2007). The *ermE* gene from the erythromycin-producer *Saccharopolyspora erythraea* has been found in commercial preparations of the drug, causing one to wonder whether resistance in clinical isolates originated from the producing strain and whether it was spread directly because of drug usage (Webb and Davies, 1993, 1994). Some of the enzymes, such as ErmN, catalyse only monomethylation (Liu and Douthwaite, 2002), whereas others, such as ErmE (Katz *et al.*, 1987) and ErmC (Denoya and Dubnau, 1989), catalyse dimethylation, but it is not known whether dimethylation takes place through a concerted two-step process since these latter enzymes can use monomethylated RNA as a substrate. Moreover, induction is dependent upon the presence of the antibiotic with the correct structures of a 14- or 15-membered macrolides that contain a neutral sugar at C3. In contrast, 16-membered macrolides and 14-membered-ketolides are not inducers. Erm-mediated resistance exists in two forms: inducible and constitutive. In the inducible form of resistance, the ribosomal methylation is established only after the macrolide is transported into the cells (Ramu *et al.*, 2009). In hosts that are constitutively resistant to macrolides, Erm-catalysed methylation of the ribosomes does not require the presence of the macrolides (Poehlsgaard and Douthwaite, 2003). Both inducible and constitutive MLS_B resistance require the complete sequence encoding the *erm* gene (Weisblum, 1995a, 1995b).

Recently, it was shown that the tunnel acts as a regulatory compartment where the sequence of the nascent peptide acts together with the drug to slow the rate of translation elongation or even stop translation completely (Otaka and Kaji, 1975; Tenson *et al.*, 2003; Davis *et al.*, 2014; Kannan *et al.*, 2014). A number of genes regulated by recognition of the nascent peptide have been identified in bacteria and eukaryotes (see Wilson *et al.*, 2016). The inducible macrolide resistance genes remain silent in the absence of the antibiotic but are activated in its presence. Activation of the inducible gene is regulated by ribosome stalling at a precise position on programmed, evolutionarily defined site of the regulatory upstream open reading frame (uORF), which precedes the resistance gene (Weisblum, 1995a, 1995b; Ramu *et al.*, 2009; Subramaniam *et al.*, 2011). The ribosomes, which are arrested at the uORF of the regulatory gene, alter the folding of the mRNA, activating the expression of the downstream resistance cistron (Figure 4). The chemical structure of the antibiotic and the sequence and/or the structure of the leader peptide are the two main factors that regulate the position of translation arrest on the mRNA of the leader peptide (Gryczan *et al.*, 1980; Horinouchi and Weisblum, 1980; Vazquez-Laslop *et al.*, 2008, 2010; Ramu *et al.*, 2011; Arenz *et al.*, 2014a, 2014b). As mentioned above, the amino acid sequence motif R/K-X-R/K was recognized as one of the major motifs that induce translation arrest in the presence of macrolides (Davis *et al.*, 2014; Kannan *et al.*, 2014). The R/K-X-R/K motif has been detected in many regulatory uORFs of macrolide resistance genes, including also the ermDL ORF that controls expression of the *ermD* gene that has been studied extensively (Kwak *et al.*, 1991; Kwon *et al.*, 2006; Sothiselvam *et al.*, 2014). Many regulatory peptides like ermCL and ErmBL do not feature this motif and direct translation arrest via distinct stalling sequences (Wilson, 2016). Ketolides which do not induce ribosome stalling at the uORF of the ermC resistance gene trigger its translation through frameshifting, allowing so the translating ribosome to invade the intergenic spacer (Gupta *et al.*, 2013). In all tested regulatory uORFs with the R/K-X-R/K motif, the ribosome stalls when the second codon of the consensus enters the ribosomal P site (Sothiselvam *et al.*, 2014; Almutairi *et al.*, 2015). RNA chemical probing has shown that binding of macrolides to the NPET of the vacant ribosome was sufficient to allosterically induce structural changes in the PTC (Sothiselvam *et al.*, 2014, 2016). This is confirmed by Mankin's experiments where translation of the 5′terminally truncated ermDL ORF, which encodes the peptide starting with the MRLR (methionine–arginine–leucine–arginine) sequence, was efficiently arrested by macrolides at the third codon responsible for leucine, when the length of the nascent peptide chain was only three amino acids and is predicted to establish only limited contacts with the drug (Sothiselvam *et al.*, 2014). These results suggested that the inhibition of translation elongation by macrolides does not block the progress of the nascent chain in the drug-engaged exit tunnel but rather that binding of the antibiotic allosterically modifies the PTC which is unable to catalyse peptide bond formation when certain combinations of donor and acceptor substrates have occupied the A- and P-sites (Kannan *et al.*, 2014; Sothiselvam *et al.*, 2014, 2016). Additionally, the efficiency of stalling by a minimal MRLR sequence allowed Mankin and colleagues to conclude that the amino acids preceding the stalling motif in the ErmDL peptide do not make a significant contribution to the translation arrest. Rather, the length of the side chains and the

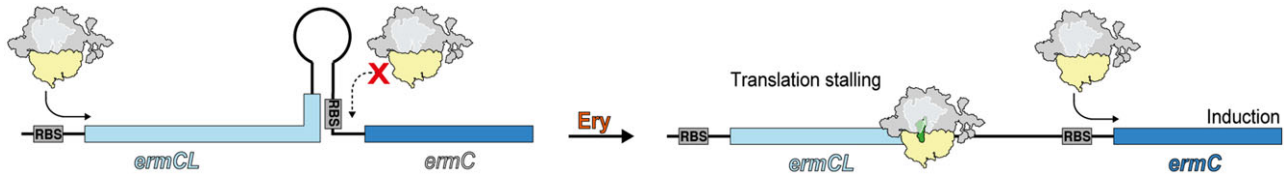

Figure 4

Regulation of gene expression by ribosomal stalling. In the absence of erythromycin, there is no stalling and no translation of ermC. In the presence of erythromycin, there is stalling during translation of the leader peptide ermCL, which causes the ribosome to block stem-loop formation and exposes the ribosome binding site (RBS) of the downstream cistrons, allowing its expression. (Figure was prepared with modifications from Wilson *et al.*, 2016).

positive charge of the amino acids occupying the A- and P-sites appear to be more important for the efficiency of stalling (Sothiselvam, 2016). Such an allosteric modification of PTC after macrolide binding on the ribosome was presented many years before, when addition of erythromycin in a mixture of ribosomes with bound radioactive chloramphenicol caused complete release of the latter despite the apparent lack of overlap in the binding sites of the two antibiotics (Pestka and LeMahieu, 1974; Dunkle *et al*., 2010).

Mutations in ribosomal RNA

All published data indicate that A2058 is the key nucleotide of the 23S rRNA for macrolide resistance (Vester and Douthwaite, 2001; Canu *et al*., 2002; Berisio *et al*., 2006). The mutation A2058G confers high-level resistance to all macrolides including many ketolides (Vester and Douthwaite, 2001; Canu *et al*., 2002; Misyurina *et al*., 2004; Berisio *et al*., 2006). The exception is *S. pneumonia* where the A2058G mutation confers resistance to macrolides but low level to ketolides (Canu *et al*., 2002; Farrell *et al*., 2003). This unusual behaviour is explained by the 2057–2611 base pair, which is part of the cradle housing the lactone ring of the macrolide (Pfister *et al*., 2005; Kannan and Mankin, 2011). The 2057–2611 base pair is conserved across bacteria (Akshay *et al*., 2011), and the polymorphism of this base pair (i.e. the presence of G-C vs. A-U) determines the ketolide susceptibility of A2058G mutants (Pfister *et al*., 2005). Another resistance mutation occurs at the A2059 position and has been found *in vivo* in *Mycobacteria*, *Propionibacteria*, *H. pylori* and *S. pneumoniae* (Vester and Douthwaite, 2001). Mutations at position 2057 have also been observed in clinical isolates although with a limited frequency (Fyfe *et al*., 2016). The U2609C mutation in *E. coli* was selected for resistance to telithromycin and cethromycin (Garza-Ramos *et al*., 2002). In *E. coli*, nucleotides A752 and U2609 form a base pair that connects domains II and V in the 23S rRNA (Schuwirth *et al*., 2005). In the *E. coli* 70S-telithromycin structure (Dunkle *et al*., 2010), this base pair offers a surface for the alky–aryl arm to engage in stacking interactions that favour drug binding. The protection of A752 from chemical probing by telithromycin therefore most likely arises through stabilization of the A752–U2609 base pair. The C6 alkyl–aryl side chain of cethromycin bound to the *D. radiodurans* 50S subunit adopts a distinct conformation compared with that observed in *E. coli*, most likely because *D. radiodurans* has C752 instead of A752 and therefore cannot form a canonical base pair with U2609 (Figure 3D) (Vester and Douthwaite, 2001; Schlünzen *et al*., 2003; Franceschi *et al*., 2004). Additional mutations in *S. pneumoniae* have been reported, namely, C2610U and C2611U, that also confer macrolide resistance, whereas deletion of A752 results in high resistance to both macrolides and ketolides (Canu *et al*., 2002).

Mutations in ribosomal proteins

Mutations in genes encoding ribosomal proteins L4 and L22 can confer erythromycin resistance and reduce telithromycin susceptibility (Tait-Kamradt *et al*., 2000a; Pihlajamaki *et al*., 2002). In addition to *E. coli* laboratory isolates, a variety of clinical isolates have also been identified with ribosomal protein mutations that confer resistance to macrolides, including *S. pneumoniae* (Tait-Kamradt *et al*., 2000a, 2000b; Farrell *et al*., 2004), *S. pyogenes* (Bingen *et al*., 2002), *St. aureus* (Prunier *et al*., 2005), *H. influenzae* (Peric *et al*., 2003) and *Mycoplasma genitalium* (Shimada *et al*., 2011). In addition to the changes detailed below, a list of L4 and L22 mutations can be found in Franceschi *et al*. (2004). Changes within a highly conserved sequence of *S. pneumoniae* L4 ($_{63}$KPWRQKGTGRAR$_{74}$), resulted in decreased susceptibility to macrolides or ketolides (a 500-fold increase to a telithromycin MIC of 3.12 mg·mL^{-1} for one variation), as well as a reduction in fitness (Tait-Kamradt *et al*., 2000a, 2000b; Farrell *et al*., 2004). Mutations encoding amino acid changes in the C-terminal region of ribosomal protein L22 (e.g. G95D, P99Q, A93E, P91S, G83E, A101P and $_{109}$RTAHIT$_{114}$ tandem duplication) also led to decreased susceptibility to macrolides and ketolides, although the MICs were not greater than 1 mg·L^{-1} in *S. pneumoniae* (Farrell *et al*., 2003). In *M. pneumoniae*, all 14-membered macrolide-resistant isolates harboured a T508C mutation in L22, and for most, either an A2058G or A2059G mutation in 23S rRNA was also present (Cao *et al*., 2010; Jensen *et al*., 2014). Resistance to telithromycin in *S. pneumoniae* significantly increases when 23S rRNA methylation/mutations are combined with ribosomal protein mutations. For example, a combination of a truncated leader peptide leading to constitutive synthesis of *erm*(B) conferred a telithromycin MIC of 16 mg·L^{-1} (Wolter *et al*., 2008), whereas clinical isolates with both a constitutive *erm*(B) and a $_{69}$GTG$_{71}$ to TPS substitution in L4 (Wolter *et al*., 2007), or a combined A2058G mutation and a three-amino acid deletion in L22 (Faccone *et al*., 2005), provided high-level telithromycin resistance (256 mg·L^{-1}). It is also important to mention that, since all previous protein mutation positions are rather distal from the macrolide binding pocket (9–10 Å), it was concluded that resistance may be triggered by induction of structural changes in the rRNA nucleotides that propagate to the binding pocket of the antibiotics (Tu *et al*., 2005).

Modification by macrolide esterases

Macrolide aglycons are converted to cyclic lactones via an ester bond that is formed during the final ring synthesis step and is catalysed by the thioesterase activity module of the polyketide synthase, responsible for the ring closure step that generates 6-deoxyerythronolide B (Donadio *et al*., 1991). It is therefore expected that this key bond would have been targeted by macrolide resistance enzymes operating by reversing the ring closure reaction. The first erythromycin esterase was reported in 1984 and was isolated from a macrolide-resistant *E. coli* strain (Barthelemy *et al*., 1984; Wright, 2005). Cloning of this *ereA* gene revealed a protein of 406 amino acids with an expected mass of 44.8 kDa (Ounissi and Courvalin, 1985). Subsequently, another orthologue, *ereB*, was cloned from another *E. coli* isolate (Arthur *et al*., 1986). Ere(A) and Ere(B) both hydrolyze the lactone ring in 14-membered macrolides; however, the two enzymes are only weakly related with 25% protein sequence identity. Through the use of a genomic enzymology approach, the catalytic mechanisms of the 'erythromycin esterase superfamily' enzymes were compared (Morar *et al*., 2012). Ere(A), Ere(B) and two related enzymes from *Bacillus cereus*, Bcr135 and Bcr136, whose three-dimensional

structures had previously been determined, were studied. Only Ere(A) and Ere(B) were predicted to cleave the macrocyclic ester, and their resolved enzymic hydrolytic mechanism was shown to pass through an hemiketal intermediate, while Bcr136 was confirmed as an esterase that is, however, unable to inactivate macrolides (Morar *et al.*, 2012). The presence of these genes on mobile genetic elements implies the ability to become widespread in the microbial community, and the presence of esterases has been confirmed in at least one clinical isolate of *St. aureus* (Wondrack *et al.*, 1996) and in environmental isolates of *Pseudomonas* sp. (Kim *et al.*, 2002). Hydrolytic inactivation of macrolides by esterases specifically involves 14- and 15-membered macrolides, whereas ketolides and 16-membered macrolides, such as josamycin, midecamycin, rosaramycin and spiramycin, are not substrates (Arthur and Courvalin, 1986; Arthur *et al.*, 1987; Morar *et al.*, 2012).

Modification by kinases (or phosphotransferases)

Macrolide phosphotransferases are macrolide-inactivating enzymes widespread in Gram-negative and Gram-positive bacteria (Sutcliffe and Leclercq, 2002; Roberts, 2008; Fyfe *et al.*, 2016) that, by *in silico* analysis, are classified in the same family as aminoglycoside and macrolide protein kinases (Shakya and Wright, 2010). The first reported purifications of macrolide phosphotransferases were from macrolide-resistant *E. coli*, and this mechanism was soon shown to be prevalent in clinical isolates of *E. coli* Tf481A, in Japan (O'Hara *et al.*, 1989; Kono *et al.*, 1992; Taniguchi *et al.*, 2004). Macrolide 2′-phosphotransferases, commonly found on mobile genetic elements, are inducible (e.g. *mph*(A)) or constitutively expressed (e.g. *mph*(B)) intracellular enzymes capable of transferring the γ-phosphate of nucleotide triphosphate to the desosamine 2′-OH group of 14-, 15-, and 16-membered ring macrolide antibiotics, thereby disrupting the key interaction of macrolides with A2058 (Shakya and Wright, 2010). Mphs can be divided into two classes distinguished by differences in primary sequence and substrate specificity, while their structures in complexes with many macrolides have been resolved in atomic resolution (Fong *et al.*, 2017). Although early studies showed that Mph enzymes could use ATP, more recent work with Mph(A) has demonstrated a preference for GTP under physiologically relevant *in vitro* assay conditions (Shakya and Wright, 2010). Expression of *mph*(A) is induced by erythromycin, and recently, the structure of the MphR(A) repressor protein, a negative regulator of *mph*(A) expression, was solved, uncomplexed and complexed with erythromycin to 2.00 and 1.76 Å resolutions respectively (Zheng *et al.*, 2009). Erythromycin binds with a stoichiometry of 1:1 to each monomer of the functional MphR(A) dimer in a large hydrophobic cavern composed of residues from an α-helix of one monomer and the dimeric interface of the other monomer that appears too close around the ligand as it binds (Zheng *et al.*, 2009). Macrolide phosphotransferases are widespread in bacteria of clinical, veterinary, agricultural and environmental origins. Genes encoding Mph enzymes are usually found on mobile genetic elements containing other macrolide resistance genes and genes conferring resistance to other antibiotic classes. The most recently identified macrolide phosphotransferase, mph (G), has been found in *Vibrio* spp. and photobacteria in the seawater of fish farms (Nonaka *et al.*, 2015).

Glycosyltransferases modification

Glycosyl transfer is not a widespread mechanism of antibiotic resistance, although it certainly plays an important role in self-protection of antibiotic-producing organisms. Therefore, the antibiotic does not strongly interfere with the synthesizing machineries or the producer organism, which explains why not all antibiotic producers are resistant against their produced drug. Macrolide resistance due to 2 -glucosylation has not been reported yet in a bacterial pathogen but has been found in *Streptomyces antibioticus*, which produces the known macrolide oleandomycin (Vilches *et al.*, 1992; Fyfe *et al.*, 2016). In this macrolide self-resistance mechanism, the intracellular glycosylation inactivates the antibiotic, and after secretion is reactivated by an extracellular-glycosidase (Vilches *et al.*, 1992). The glycosylation of macrolides is mediated by glycosyltransferases, which transfer activated donor sugars to acceptor species. These enzymes are grouped into families based on their sequence, and to date they display only two major folds defined as GT-A and GT-B (Lombard *et al.*, 2014). The oleandomycin inactivation takes place by transfer of a glucose molecule from a donor/UDP-glucose to oleandomycin, a process catalysed by an intracellular glucosyl transfer (Quirós and Salas, 1995). The extracellular glucosidase that activates oleandomycin, OleR, converts the glycosylated form of oleandomycin into the active antibiotic (Quirós *et al.*, 1998). Two glycosyltransferase proteins OleI and OleD and one glucosidase OleR were isolated and studied from *S. antibioticus*, and a model was proposed for oleandomycin intracellular inactivation, secretion and extracellular reactivation (Quirós *et al.*, 1998). Unlike OleI, which only glycosylates oleandomycin, OleD displays broad acceptor specificity and hence will inactivate a wider spectrum of macrolide antibiotics, including also tylosin and erythromycin (Coutinho *et al.*, 2003).

Conclusions

The resistance of pathogens to antibiotics has become a serious and persistent therapeutic problem today, with a rapid development of new effective and safe antibiotics being the only answer to this problem. Macrolides are a family of valuable first choice antibiotics with great contribution to therapy, which is gradually becoming ineffective due to increasing resistance. Thus, the development of new generations of macrolides is required, as soon as possible. Additionally, this development has to be associated with safety issues to overcome problems related to the use of the last generation of macrolides (Kim *et al.*, 2012; *Telithromycin*, 2014). In the beginning of the previous decade, there was a revolution concerning the development of new macrolides and a great enthusiasm prevailed after approval of telithromycin by the FDA. However, telithromycin usage was accompanied by serious side effects. This led to disappointment and caused many researchers to stop working on macrolide derivatization, assuming that this procedure had reached the end. In parallel, the discovery of modular macrolide polyketide synthases initiated efforts to

alter the specificities and activities of the enzyme domains, for the purpose of changing the structure of the corresponding aglycone and/or the linked sugars (Khosla and Zawada, 1996; Khosla, 2009). This procedure permitted recombinant genes to be introduced into the macrolide producers in order to create desired changes to the structure of macrolides produced (Katz and McDaniel, 1999; McDaniel *et al.*, 2005). The few fully elaborated novel macrolides produced by genetic engineering have not yet fulfilled the original promise (Park *et al.*, 2010). Therefore, it is still too early to assess whether this avenue of discovery will be effective. The findings that many of engineered PKSs either do notproduce the expected compounds, or do so at levels too low to be useful, indicate that greater understanding of the biochemical details of polyketide biosynthesis is required before full exploitation of their chemical potential can be realized. Fortunately, this inability of genetic manipulation to rapidly produce new compounds designed on the huge amount of available crystal structure data has been recently overcome by chemical macrolide synthesis procedures, which have opened new horizons into the synthesis of novel macrolide compounds (Seiple *et al.*, 2016). On the other hand, the recent resolution of the 'ketek effects' also provided answers to the second critical issue, namely, the safety of macrolides in clinical use (Andrade and Tulkens, 2011; Fernandes *et al.*, 2016), and therefore, macrolide antibiotics are back in the forefront of science, and new, important, effective and safe macrolides are expected very soon to re-enter the market.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b).

Conflict of interest

The authors declare no conflicts of interest.

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