Insights into the Mode of Action of Novel Fluoroketolides, Potent Inhibitors of Bacterial Protein Synthesis

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Ketolides, the third generation of expanded-spectrum macrolides, have in the last years become a successful weapon in the endless war against macrolide-resistant pathogens. Ketolides are semisynthetic derivatives of the naturally produced macrolide erythromycin, displaying not only improved activity against some erythromycin-resistant strains but also increased bactericidal activity as well as inhibitory effects at lower drug concentrations. In this study, we present a series of novel ketolides carrying alkyl-aryl side chains at the C-6 position of the lactone ring and, additionally, one or two fluorine atoms attached either directly to the lactone ring at the C-2 position or indirectly via the C-13 position. According to our genetic and biochemical studies, these novel ketolides occupy the known macrolide binding site at the entrance of the ribosomal tunnel and exhibit lower MIC values against wild-type or mutant strains than erythromycin. In most cases, the ketolides display activities comparable to or better than the clinically used ketolide telithromycin. Chemical protection experiments using *Escherichia coli* ribosomes bearing U2609C or U754A mutations in 23S rRNA suggest that the alkyl-aryl side chain establishes an interaction with the U2609-A752 base pair, analogous to that observed with telithromycin but unlike the interactions formed by cethromycin. These findings reemphasize the versatility of the alkyl-aryl side chains with respect to species specificity, which will be important for future design of improved antimicrobial agents.

Macrolides represent a large family of protein synthesis inhibitors that have already demonstrated their medical importance since the introduction of erythromycin (Fig. 1A) into clinical practice in the 1950s (1–4). However, the widespread usage of macrolides has resulted in the selection of resistant strains, providing a strong incentive for the development of newer macrolide drugs that can overcome such resistance mechanisms (5–9). This has led to the development of a new generation of macrolides, namely, ketolides, with the most distinguished members being telithromycin, cethromycin, and solithromycin (Fig. 1B to D) (7, 10–13). These ketolides are semisynthetic derivatives of erythromycin, where a keto group at the C-3 position of the lactone ring replaces the cladinose sugar present in erythromycin. In addition, ketolides possess a cyclic carbamate fused at positions C-11 and C-12 of the lactone ring, as well as a heteroaromatic side chain that is linked via a flexible alkyl-linker to the C-11–C-12 cyclic carbamate (telithromycin and solithromycin, Fig. 1B and D) or via an “O-bridge” at the C-6 position (cethromycin, Fig. 1C).

For a long time, macrolide antibiotics have been considered general inhibitors of translation by plugging the ribosomal tunnel and thereby preventing the synthesis of the nascent polypeptide chain. In contrast to this prevailing view, Kannan and coworkers recently demonstrated that the mode of action of these drugs is sequence specific (14). With the macrolide antibiotic bound within the tunnel, the sequence and structure of the N terminus of the nascent polypeptide chain determine whether protein synthesis is aborted, stalled, or continued. For the majority of proteins, the binding of the drug within the tunnel causes synthesis to be aborted when the nascent peptide chain reaches a length of between 5 and 10 amino acids, which in turn leads to dissociation of the peptidyl-tRNA (drop-off) from the ribosome (15–17). A small number of specific short nascent peptides, such as those encoded in the regulatory cistrons of macrolide resistance genes, can induce ribosome stalling, which retains the peptidyl-tRNA but prevents peptide bond formation with the incoming aminoacyl-

tRNA (18–20). In both of these scenarios, the nascent chain cannot bypass the antibiotic obstacle in the tunnel and protein expression is curtailed. In contrast, some peptide sequences have the ability to thread through the antibiotic-occupied tunnel, in a process termed bypassing, which leads to the synthesis of long polypeptides on drug-bound ribosomes (14). Macrolides, such as erythromycin, appear to allow fewer proteins to bypass than ketolides, such as telithromycin (14). Consistently, crystallographic structures of ribosome-macrolide complexes show that macrolides do not completely block the tunnel but leave an opening that may provide a passage for the nascent peptide (21–24). Moreover, the passage is slightly wider in the case of ketolides, which lack the cladinose sugar present in erythromycin, which may explain the larger number of bypass proteins observed with ketolides. This partial inhibition of protein synthesis by ketolides, rather than the more complete inhibition caused by macrolides, is likely to cause more cellular deregulation, which could explain the higher bactericidal activity of ketolides than macrolides (14).

Such fundamental insights into the mode of action of macrolide antibiotics offer novel directions for drug discovery. These new directions may include optimizing the tunnel-bound antibiotic for inhibiting defined proteins necessary for the cell and therefore improving the outcome of antibiotic therapy. With these new classes of macrolide potency, we present here four new fluoroketolide antibiotics developed by Kosan Bioscience (now Bristol-Myers Squibb) which carry an extended alkyl-heteroaryl

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FIG 1 Chemical structures of erythromycin (A), telithromycin (B), cethromycin (C), solithromycin (D), and fluoroketolides K-1602 (E), K-1636 (F), K-1835 (G), and K-1804 (H).

side chain at the O-6 position of the lactone ring, analogous to cethromycin, but in addition bear one or two fluorine atoms attached to the C-2 or C-13 position of the lactone ring (K-1602, K-1636, K-1835, K-1804; Fig. 1E to H). According to our genetic and biochemical tests involving a number of wild-type and mutant resistant bacterial strains, the new fluoroketolides showed better *in vivo* activity (expressed as MIC values) than erythromycin or telithromycin. *In vitro* studies using a coupled transcription-translation system and competition experiments with [14 C]erythromycin as well as chemical protection experiments indicated that the new ketolides bind in the nascent peptide exit tunnel at a site previously described as the site of macrolide antibiotics, while their heterocyclic side chain interacts with the U2609-A752 base pair and promotes the formation of specific interactions with helix 35 of 23S rRNA, causing an additional interaction with the ribosomes that may be responsible for the effectiveness of the new ketolides.

MATERIALS AND METHODS

Materials. Erythromycin, GTP, ATP, poly(U), poly(A), tRNA from *Escherichia coli* strain W, dimethyl sulfate (DMS), and sodium wolframate were purchased from Sigma (St. Louis, MO). 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene (CMCT) and kethoxal were purchased from Fluka Biochemicals and MP Biomedicals, respectively. L-[2,3,4,5,6- 3 H]-phenylalanine and L-[4,5- 3 H]-lysine were obtained from Amersham Pharmacia Biotech (Piscataway, NJ), whereas [14 C]erythromycin and [γ - 32 P]ATP were purchased from PerkinElmer (United States) and Izotop (Hungary), respectively. Avian myeloblastosis virus reverse transcriptase, deoxynucleoside triphosphates (dNTPs), and dideoxynucleotides were obtained from Roche Diagnostics GmbH (Mannheim, Germany), and T4 polynucleotide kinase was purchased from TaKaRa (Japan). Telithromycin was kindly supplied by Sanofi-Aventis Inc. to D. L. Kalpaxis. Fluoroketolides K-1602, K-1636, K-1835, and K-1804 were provided by Kosan Bioscience Inc. (U.S. patent 7595300 B2), now Bristol-Myers Squibb.

Biochemical preparations. 70S reassociated ribosomes were prepared from the *E. coli* K-12 strain as described previously (25) and kept in buffer

containing 20 mM HEPES-KOH (pH 7.6), 50 mM $\text{CH}_3\text{COONH}_4$, 6 mM $(\text{CH}_3\text{COO})_2\text{Mg}$, and 4 mM β -mercaptoethanol. The S-100 fraction was prepared as previously described (26) and was treated with DE-52 cellulose in order to remove tRNAs and RNases.

Poly(U)-dependent poly(Phe) synthesis. The assay was carried out in buffer A [20 mM HEPES-KOH (pH 7.6), 150 mM $\text{CH}_3\text{COONH}_4$, 4.5 mM $(\text{CH}_3\text{COO})_2\text{Mg}$, 4 mM β -mercaptoethanol, 2 mM spermidine, and 0.05 mM spermine]. In a first incubation, tight-coupled 70S ribosomes (0.5 μM) were preincubated for 10 min at 37°C with each antibiotic at the appropriate concentration. Then in a total volume of 15 μl , 25 μg of poly(U) mRNA was mixed with [3 H]phenylalanine (5 nmol, 50 dpm/pmol), 1 A_{260} unit tRNA bulk (*E. coli*), 3 mM ATP, 1.5 mM GTP, 5 mM acetyl phosphate, and an optimized amount of S-100 fraction to maximize the picomole of phenylalanine polymerized per picomole of 70S ribosomes. After 30 min of incubation at 37°C, hot trichloroacetic acid (TCA) precipitation was performed and polypeptides were filtrated through glass fiber filters (27). The radioactivity retained on the filters was measured in a liquid scintillation counter. The Phe/ribosome ratio represents the picomole of Phe incorporated into the polypeptide chain per picomole of 70S ribosomes as a function of time (28). Edeine, at the appropriate concentration, was used as a positive control.

Poly(A)-dependent poly(Lys) synthesis. This assay was performed as described for poly(U)-dependent poly(Phe) synthesis, except that poly(A) was used instead of poly(U) and [3 H]lysine (3 nmol, 500 dpm/pmol) was the source of amino acid. The incubation was performed at 37°C for 1 h, and the TCA solution used for precipitating polylysine peptides was treated with 0.25% sodium wolframate before use.

Competition of [14 C]erythromycin binding. Reassociated 70S ribosomes (0.20 μM final concentration) were incubated in buffer A with [14 C]erythromycin (150 dpm/pmol) at the appropriate concentration. After 10 min of incubation at 37°C, the mixture was diluted to a total volume of 3 ml with cold buffer A, and the erythromycin bound on ribosome was determined by nitrocellulose filtration (Millipore filters, 0.45- μm pore size). Next, the binding of [14 C]erythromycin was studied in competition with cold erythromycin or fluoroketolides using a constant [14 C]erythromycin concentration (0.6 μM) and increasing concentrations of nonradioactive competitors (29).

TABLE 1 Susceptibility of the new ketolides against Gram-positive and Gram-negative bacteria

Antibiotic	MIC ($\mu\text{g/ml}$)				
	<i>E. coli</i> wild type ^a	<i>E. coli</i> U2609C ^a	<i>E. coli</i> U754A ^a	<i>S. aureus</i> ^b	<i>S. pyogenes</i> ^b
Erythromycin	128	128	128	1	2
Telithromycin	16	64	32	0.25	0.25
K-1602	16	32	>16	0.25	1
K-1636	8	8	16	0.03	0.25
K-1835	8	8	4	0.03	0.25
K-1804	16	8	<16	0.03	0.25

^a rRNA either in wild-type or mutant *E. coli* ribosomes were expressed in the TA531 strain from plasmid-borne genes that were the single source of rRNA in the cell. Wild-type cells contained plasmid pKK3535, the U2609C mutant contained plasmid pKK2609C (34), and the U754A mutant contained plasmid pKK754A, which is identical to pKK3535 except for the presence of a T754A mutation in the 23S rRNA gene (46).

^b Macrolide susceptible.

MIC determination. Cells were grown overnight in Luria-Bertani (LB) medium supplemented with 100 $\mu\text{g/ml}$ of ampicillin when need. The overnight cultures were subsequently diluted 100-fold into fresh ampicillin-containing LB medium and allowed to grow again until exponential phase. At this stage, cultures were newly diluted to a final A_{600} of 0.050 and then incubated with the appropriate antibiotic at 37°C, first for 15 h without shaking and then for 3 h with shaking. The MIC represents the lowest concentration of the drug that caused culture growth arrest.

In vitro coupled transcription-translation assay. All coupled transcription-translation experiments were performed using the *E. coli* lysate-based system for the expression of the green fluorescence protein (GFP) type cyc3 in the presence and absence of antibiotics, as described elsewhere (28, 30). Each reaction mixture contained 2.4 μl *E. coli* lysate, 2 μl reaction mix, 2.4 μl amino acids, 0.2 μl methionine, 1.8 μl reconstitution buffer, 0.2 μl pIVEX2.2-GFPcyc3 (1 mg/ μl), and 1 μl antibiotic or control buffer. After incubation at 30°C with shaking (900 rpm) for 4 to 5 h, two microliters of each reaction was diluted with 50 ml of buffer containing 10 mM HEPES-KOH (pH 7.8), 10 mM MgCl_2 , 60 mM NH_4Cl , and 4 mM β -mercaptoethanol and then transferred into black 96-well chimney flat-bottom microtiter plates. The GFP fluorescence was read on a Tecan Infinite M1000 (excitation wavelength of 395 nm and emission of 509 nm). Images were then quantified using ImageQuantTL (GE Healthcare) and graphically represented using SigmaPlot (Systat Software, Inc.). All measurements were repeated in triplicate and had a standard deviation of less than 10%.

Antibiotic probing and chemical modification. Aliquots of 70S ribosomes, 50 pmol per tube, were incubated with or without antibiotic at 37°C. The incubation took place in buffer containing 80 mM HEPES-KOH (pH 7.8), 20 mM MgCl_2 , 100 mM NH_4Cl , and 1.5 mM dithiothreitol for 10 min, followed by 10 min of further incubation at 20°C. After cooling on ice, chemical modification of ribosomes was carried out by using DMS, CMCT, or kethoxal, as described previously (31, 32). The reactions were quenched, and then the ribosomes were precipitated by ethanol. The pellets were resuspended in 50 μl of buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl , 5 mM EDTA, and 0.5% SDS, and the rRNA was extracted with equal volumes of phenol, phenol-chloroform, and chloroform. The rRNA was precipitated by ethanol and resuspended in water.

Primer extension. The modifications in the 23S rRNA were monitored by primer extension analysis using avian myeloblastosis virus reverse transcriptase and 5'-labeled primer. DNA primers (18 to 20 nucleotides [nt]) were labeled at the 5' end using T4 polynucleotide kinase and [γ - ^{32}P]ATP. The cDNA products of the primer extension reactions were separated on 6% polyacrylamide-7 M urea sequencing gels. Gels were scanned with a PhosphorImager-type Fujifilm (FLA-3000; Berthold) and

analyzed with ImageQuant software AIDA (Raytest). The stop positions during the cDNA synthesis were identified by reference to dideoxy sequencing reactions performed in parallel on 23S rRNA (31).

Induction of *erm*(C) Expression by fluoroketolides. Disc-based assay of the pERMZ α reporter induction in *E. coli* JM109 cells was performed as described previously (33). All plates were photographed after 24 h of incubation.

RESULTS

The inhibitory activity of the four fluoroketolides was first tested in cell cultures, and the MIC values were determined for the Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus pyogenes*, as well as for the Gram-negative *Escherichia coli* strain TA531 (Table 1). The *E. coli* TA531 strain lacks the chromosomal *rrn* alleles but is complemented with a plasmid expressing either a wild-type or mutated form of the 23S rRNA (34). As presented in Table 1, the fluoroketolides display excellent activity against both Gram-positive *S. aureus* and *S. pyogenes* strains, with MIC values ranging between 0.03 and 1 $\mu\text{g/ml}$, which was generally better than erythromycin (1 to 2 $\mu\text{g/ml}$) and comparable to or better than telithromycin (0.25 $\mu\text{g/ml}$). As expected, the MIC values were significantly worse against Gram-negative *E. coli* strains, particularly those for erythromycin (128 $\mu\text{g/ml}$). Telithromycin displayed a MIC of 16 $\mu\text{g/ml}$ against wild-type *E. coli* strains but was less effective against the U2609C and U754A mutants, as reported previously (35). In contrast, the fluoroketolides were all at least as effective as telithromycin against wild-type *E. coli* (8 to 16 $\mu\text{g/ml}$) and, with the exception of K-1602, did not appear to be significantly affected by the U2609C or U754A mutant strains (4 to 16 $\mu\text{g/ml}$). These findings suggested that the fluoroketolides may have improved ribosome binding properties compared to those of telithromycin, particularly with respect to ribosomes bearing the erythromycin resistance mutations U2609C or U754A.

To explore the ribosome binding properties of the fluoroketolides, competition assays were employed using radioactive [^{14}C]erythromycin. As seen in Fig. 2, all compounds compete with erythromycin for ribosome binding following simple saturation

FIG 2 Competition of [^{14}C]erythromycin binding to vacant ribosomes by nonradioactive erythromycin (●) or fluoroketolides: K-1602 (■), K-1636 (▲), K-1835 (▼), and K-1804 (◆). The binding of radioactive erythromycin in the absence of competing ligand was assigned as 100%. The graph indicates the percentage of binding versus the concentration of competing antibiotic.

TABLE 2 IC_{50} for inhibition of the bound radioactive erythromycin, and IC_{50} (μM)^{GFP} for GFP inhibition of the new ketolides^b

Antibiotic	IC_{50} (μM)	IC_{50} (μM) ^{GFP}	K_i (nM)
Erythromycin	2.52		15
Telithromycin		1.25	8.33 ^a
K-1602	0.68	1.8	4.05
K-1636	0.25	1.45	1.49
K-1835	3.66	0.9	39.64
K-1804	2.21	0.5	13.15

^a According to reference 37.^b IC_{50} was measured from Fig. 2, IC_{50} (μM)^{GFP} was measured from Fig. 3, and the values of K_i were calculated from IC_{50} values according to reference 29.

curves, which is compatible with occupation either of the same or mutually exclusive sites of erythromycin on the ribosome. Measuring the 50% inhibitory concentration (IC_{50}) from Fig. 2 (see Table 2), namely, the concentration of each ketolide necessary to replace half of the bound radioactive erythromycin from the ribosome, enabled us to calculate the K_i constants for each antibiotic according to previous publications (29, 36). According to the K_i values, each fluoroketolide binds to the *E. coli* wild-type ribosome tighter than erythromycin, except for K-1835. Telithromycin has a previously reported K_i of 8.33 nM (37), thus leading to the following hierarchy of binding affinities: K-1636 > K-1602 > telithromycin > K-1804 > erythromycin > K-1835.

To corroborate the inhibitory effect of the fluoroketolides on ribosome function, we employed an *in vitro* *E. coli* lysate-based coupled transcription-translation system to determine the expression level of protein in the presence of each compound (30, 38). Specifically, the ability of the fluoroketolides to inhibit the synthesis of green fluorescent protein (GFP) was assessed by monitoring the fluorescence of GFP in the presence of increasing concentrations of each fluoroketolide, with telithromycin used as a control. According to Fig. 3, all fluoroketolides were excellent inhibitors of

FIG 3 Inhibitory effect of fluoroketolides on *in vitro* translation. Fluorescence produced by translation of GFP in an *in vitro* *E. coli* transcription-translation assay in the absence or presence of increasing concentrations of telithromycin (●), K-1602 (▲), K-1636 (▼), K-1835 (■), and K-1804 (◆). The fluorescence of GFP in the absence of antibiotic was assigned as 100%. **FIG 4** Effect of fluoroketolides on poly(Phe) and poly(Lys) synthesis. (A) Poly(Phe) formation in the absence or in the presence of antibiotics. Edeine was used as a positive control. The concentration of each antibiotic was 10 μM , and the reaction mixture was incubated at 37°C for 30 min. The final product was expressed as picomole of incorporated amino acid per picomole of ribosomes during the time of incubation. (B) Poly(Lys) synthesis in the absence or in the presence of erythromycin and new ketolides. Experimental conditions and antibiotic concentrations were the same as before, except that the incubation time was 1 h.

translation, with an IC_{50} value similar to that of telithromycin [values are presented in Table 2, termed IC_{50} (μM)^{GFP}]. Certainly, the inhibition measurement used does not provide any indication for the length of peptides inhibited, since the fluorescence is derived only from the fully folded active fraction of GFP protein. Thus, this assay does not determine the stage of inhibition, namely, the peptide length when elongation was interrupted. In addition, we assessed the ability of each compound to inhibit the *in vitro* synthesis of poly(U)-dependent poly(Phe) as well as poly(A)-dependent poly(Lys) (Fig. 4A and B). In the absence of any compound, ribosomes incorporated ~700 Phe/ribosome (Fig. 4A) or ~60 Lys/ribosome (Fig. 4B). The presence of 10 μM each compound did not show any inhibitory effect on poly(Phe) synthesis, whereas edeine at the same concentration inhibited the synthesis almost completely (Fig. 4A). The lack of effect of high

FIG 5 Chemical probing of interactions of fluoroketolides, erythromycin, and telithromycin with the wild-type ribosomes of *E. coli* in domain V of 23S rRNA. (A) DMS probing of drug interactions mainly with bases A2058, A2059, and A2062; (B) kethoxal probing of drug interactions with G2505. All derivatives were used at a final concentration equal to 50 μ M. Lanes are marked as follows: U, A, G, and C (panel A) and A, U, G, C (panel B), sequencing lanes; control, unmodified ribosome; no drug, ribosome modified with chemical reagent (either DMS or kethoxal) and modified in the presence of antibiotics; Ery, erythromycin; Tel, telithromycin; and K-1602, K-1636, K-1835, K-1804.

concentrations of macrolides on poly(Phe) formation is in accordance with previous reports (29, 36) and is suggested to arise because poly(Phe) does not pass through the ribosomal tunnel (39). In contrast, poly(Lys) was strongly inhibited by both macrolides and ketolides (Fig. 4B), consistent with the poly(Lys) nascent chain passing through the ribosomal tunnel. The effect of the fluoroketolides on poly(Lys), but not poly(Phe) formation analogous to other macrolides, supports the notion that they occupy a similar binding position within the ribosomal tunnel.

To examine in more detail the interaction of the fluoroketolides within the ribosomal tunnel, we performed the chemical footprinting of the 23S rRNA in the presence and absence of erythromycin, telithromycin, and the four fluoroketolides. In addition to wild-type *E. coli* 70S ribosomes, we also performed chemical footprinting experiments on *E. coli* 70S ribosomes bearing 23S rRNA mutations U2609C and U754A. Based on the crystal structures of ribosomes in complex with erythromycin and telithromycin (7, 21–24, 40, 41), we focused our investigations on the regions of the tunnel that comprise the macrolide binding pockets, namely, nucleotides in proximity of A2058, A2059, A2062, and G2505 within domain V. In addition, we also probed the area surrounding nucleotides A751 and A752 within domain II, which has been shown to establish interactions with the alkyl-aryl side chain of ketolides, such as telithromycin and cethromycin (22–24, 37, 40). As expected, all compounds tested strongly protected bases A2058 and A2059 from DMS modification (Fig. 5A), as well as G2505 from modification by kethoxal (Fig. 5B). The protection patterns of the

FIG 6 DMS probing of interactions of antibiotics with the domain II (helix 35) of 23S rRNA in wild-type *E. coli* ribosomes (A) or ribosomes with the U2609C mutation (B) or the U754A mutation (C), indicating the differences on protection of the A752 base of 23S rRNA between wild-type and the other two mutant *E. coli* ribosomes. All antibiotics were used at a final concentration equal to 50 μ M. The lanes are numbered as following: lanes 1 to 4, U, A, G, C, sequencing lanes; lane 5, untreated 23S rRNA; lane 6, 23S rRNA modified by DMS; lanes 7 to 12, 23S rRNA modified with DMS in the presence of erythromycin, telithromycin, K-1602, K-1636, K1835, and K-1804, respectively.

fluoroketolides were identical to those observed for the macrolide erythromycin and the ketolide telithromycin, in agreement with the crystal structures showing that the desosamine sugar of both ketolides and macrolides establishes the same interactions with residues A2058, A2059, and G2505 of the ribosomal tunnel (21–24, 40–43). Collectively, these findings suggest that the lactone ring and attached desosamine sugar of the fluoroketolides also bind analogously to the ribosomal tunnel as all other macrolides and ketolides.

To investigate the interaction of the alkyl-aryl side chain, we monitored the modification by DMS of domain II (A752 region) in the absence and presence of erythromycin, telithromycin, and the four fluoroketolides. As reported previously (44, 45), erythromycin which lacks any heterocyclic side chain does not protect but rather enhances the reactivity of A752 to DMS modification (Fig. 6A). In contrast, telithromycin protects A752 from DMS modification (Fig. 6A), as reported previously (37, 46) and in agreement with the stacking of the alkyl-aryl side chain of telithromycin on the base pair formed between A752 and U2609 observed in the recent *E. coli* 70S crystal structure (24). Analogous to telithromycin, all four fluoroketolides also protect A752 from DMS modification (Fig. 6A), implying that the alkyl-aryl ring of these ketolides may also stack upon the U2609-A752 base pair in *E. coli* 70S ribosomes.

We also repeated the above-described experiments using *E. coli* ribosomes bearing the U2609C and U754A mutations (Fig. 6B and C). The resulting protections were quantified and are pre-

TABLE 3 Reactivity of nucleotides against chemical probes in the presence of fluoroketolides^b

<i>E. coli</i> strain	Antibiotic	Result ^a				
		A2058/ A2059	A2062	A752	U2609	G2505
Wild type	Ery	++++	0	—	0	+
	Tel	++++	—	+++	+	+
	K-1602	++++	—	+++	++	+
	K-1636	++++	—	+++	++	+
	K-1835	++++	—	+++	++	+
	K-1804	++++	—	+++	+	+
U2609C	Ery	++++	0	—	0	+
	Tel	++++	—	0	0	+
	K-1602	++++	—	—	0	+
	K-1636	++++	—	0	0	+
	K-1835	++++	—	0	0	+
	K-1804	++++	—	0	0	+
U754A	Ery	++++	0	—	0	0
	Tel	++++	0	++	0	0
	K-1602	++++	0	++	0	0
	K-1636	++++	0	++	0	0
	K-1835	++++	0	++	0	0
	K-1804	++++	0	++	0	0

^a The effect of modification is assigned as follows: +++++, very strong protection; +++, strong protection; ++, moderate protection; +, weak protection; —, very strong enhancement of modification; —, strong enhancement of modification; 0, no modification. Quantification of the band intensities was made using Image-Quant software AIDA, Raytest.

^b Erythromycin (Ery) and telithromycin (Tel) were used for the sake of comparison.

sented in Table 3, with a comparison to the results obtained with wild-type *E. coli* ribosomes from Fig. 6A. In all cases, the drugs protected A2058 and A2059 equally well on mutant ribosomes as on the wild-type ribosomes, indicating that the lactone ring and desosamine sugar appear to interact similarly on the mutant ribosomes and on the wild-type ribosomes. In contrast, the observed strong protection of A752 by telithromycin and the four fluoroketolides was absent in the U2609C mutation (Fig. 6B), suggesting that interaction between the alkyl-aryl side chain and A752 is perturbed in these cases. In the U754A mutant, the protection of A752 is reduced in the absence of antibiotic, suggesting that this mutation leads to conformational changes within the neighboring area. Nevertheless, telithromycin and the four fluoroketolides reduce the protection of A752 further.

Finally, we studied another important aspect of macrolide antibiotics, the induction of *erm* genes. We used the pERMZa reporter in the *E. coli* JM109 strain to test the ability of our compounds to activate the expression of the inducible *erm*(C) gene (33). With the exception of K-1602, all compounds induced the *erm*(C) gene at a concentration of 1 to 2 μ g/ml, after 18 h of incubation (Fig. 7). This is in agreement with previous data supporting the induction of *erm*(C) by ketolides, although in a narrow range of concentrations and a low rate of induction compared to those for macrolides (33). The fact that K-1602 did not induce the expression of the *erm*(C) gene under the conditions tested here must be explored carefully in order to clarify if this is a property of the compound *per se* or whether it is related to the specificity of the test, as was previously found to be the case for azithromycin (33).

FIG 7 Induction of expression of the pERMZa reporter (33) in the *E. coli* JM109 strain by fluoroketolides. Telithromycin was tested as the positive control. The plates contained ca. 1.5×10^8 cells plated in 0.6% LB agar on top of 1.5% LB agar containing ampicillin, isopropyl- β -D-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). Fluoroketolides and telithromycin were tested at 1, 2, 5, 10, 20, 50, 100, and 200 μ g/ml, and the plates were incubated for 24 h at 37°C.

FIG 8 Interaction of macrolide and ketolides on the ribosome. (A and B) Binding position of the macrolide erythromycin (Ery; olive) (A) and the ketolide telithromycin (Tel; pale green) (B) on the *E. coli* 70S ribosome from reference 24 (PDB identifiers 3OFR and 3OAT, respectively). (C) Model for the binding of the fluoroketolide K-1804 (yellow) to the *E. coli* ribosome; (D) binding position of the ketolide cethromycin (Cet; pink) on the *D. radiodurans* 50S subunit from reference 43 (PDB identifier 1NWX).

DISCUSSION

The successful introduction of telithromycin to the market at the beginning of this century had prompted many labs to develop novel versions of ketolide compounds to combat the rising tide of multidrug-resistant bacteria (2, 6, 8, 9). Such a version of new compounds has been developed by Kosan Bioscience, namely, the four fluoroketolides which we have studied as new possible antibacterial agents. According to our data, the new ketolides occupy the known site of macrolides on the large ribosomal subunit, just at the entrance of the tunnel (47). This is supported by the competitive inhibition of radioactive erythromycin binding, which is completely reversible and fits perfectly to the competition formula (29). Additionally, this fitting is in agreement with the binding of one molecule of antibiotic per ribosome, as was expected from previous 14-membered macrolides and in contrast with azithromycin, for which there is evidence for two molecules per ribosome in *Deinococcus radiodurans* (43, 48). The strong protection of bases A2058 and A2059 by all compounds is also consistent with the ketolides occupying the classic macrolide binding site, where the desosamine sugar interacts with these bases (Fig. 8A to D). The lack of effect of all compounds on poly(Phe) and the strong inhibition on poly(Lys) formation is also in accordance with the classical macrolide site and their inhibitory effects (29, 30, 36, 39). With the exception of K-1835, all compounds tested bind to the ribosome tighter than erythromycin, and all exhibited lower MICs than erythromycin, including K-1835 (Table 1).

The improved properties of these ketolides compared to those of erythromycin, and in some cases even telithromycin, are most likely due to the stronger contacts that the alkyl-aryl side chain of these ketolides established with domain II of 23S rRNA (Fig. 8B and C). Indeed, like telithromycin, all four fluoroketolides strongly protected base A752 in the loop of H35 of 23S rRNA from DMS modification (Fig. 6A). In contrast, erythromycin, which

lacks such an extension (Fig. 1A), enhances the accessibility of A752 to DMS (Fig. 6A), suggesting that although erythromycin does not interact directly with this region (Fig. 8A), drug binding induces conformational changes in domain II (44, 45). In *E. coli*, nucleotides A752 and U2609 form a base pair that bridges domains II and V in the 23S rRNA (49). In the *E. coli* 70S-telithromycin structure (24), this base pair provides a surface for the entire face of the alkyl-aryl arm to engage in a stacking interaction that favors drug binding, suggesting that the protection of A752 from chemical probing by telithromycin results through stabilization of the base pair. Although the alkyl-aryl side chain of the fluoroketolides is attached indirectly to the C-6 position rather than the C-11 position, as holds for telithromycin, molecular modeling indicates that the flexibility and length of the alkyl-linker are sufficient to enable stacking interactions between the aryl-heterocyclic side chain and the U2609-A752 base pair (Fig. 8C). The C-6 alkyl-aryl side chain of cethromycin, which is identical to that of the fluoroketolides (Fig. 1), bound to the *D. radiodurans* 50S subunit, adopts a distinct conformation compared to that observed in *E. coli* (Fig. 8D). This is most likely because *D. radiodurans* has C752 instead of A752 and therefore cannot form a canonical base pair with U2609.

Domain II appears to be an important determinant for fluoroketolide accommodation, since mutation of these two bases has direct influence both on MICs and the protection pattern from DMS modification. Based on the protection data, mutation of U2609C appears to abolish the base pair, as would be expected, and in doing so increases the MIC for telithromycin. In contrast, U2609C appears to have less effect on the interaction of the fluoroketolides, which we suggest may be because of their linkage to the C-6 position, which enables them to maintain a stacking interaction, even with a shifted position of the mutant C2609. U754A mutations affect the MIC of both telithromycin and the

fluoroketolides. U754 is located within H35 of 23S rRNA, where it forms Watson-Crick base pairs with A743. Thus, mutation of A754 would cause a disturbance in the helix that would indirectly affect neighboring bases, such as A752. This would explain the distinct banding pattern within this area compared to that of the wild-type ribosomes. The lack of reactivity does not necessarily suggest that the A752-U2609 base pair is disrupted. However, the loss of protection by telithromycin and the fluoroketolides suggests that even if the base pair is intact, it does not have an orientation suitable for stacking interactions by the respective alkyl-aryl side chains of these ketolides.

In relation to the fluorine atoms of the fluoroketolides, some early reports questioned the importance of fluorine at the C-2 lactone position for the activity of ketolides (50), and previous data had shown that 2C-fluorine participates directly positioned near the glycosidic bond (atom N-1) of C2611 and, thus, can potentially contribute to the drug binding (7). Based on the telithromycin structure, we envisage that the C-13 position of the fluoroketolides could contribute to binding by forming hydrogen interactions with the Lys90 side chain of ribosomal protein L22 (Fig. 8C). Therefore, ketolides with two fluorine atoms (Fig. 1G and H) could be expected to be more effective compared to mono-fluorine compounds due to a possible synergistic effect. This is true according to their MIC values (Table 1) or GFP inhibition (Table 2) but is not in agreement with erythromycin binding inhibition and inhibition constant K_i (Table 2). While the mono-fluoroketolides had improved ribosome binding properties compared to those of telithromycin, bis-fluoroketolides did not. This may suggest that the nature of the side chain (position of the nitrogen atom) may act together with the C-13 fluorine to collectively determine binding efficiency. In addition, the low MIC of the fluoroketolides, specifically against the U2609C and U754A *E. coli* mutant strains compared to telithromycin (see Table 1), suggests that the fluorine atoms may improve other chemical properties of the drugs, such as solubility or cellular uptake.

Collectively, our results show that the new fluoroketolide compounds are strong antimicrobial agents acting on the 50S ribosomal subunit where they occupy the known macrolide binding site. The protection assays and molecular modeling studies support an interaction of the C-6 alkyl-aryl side chain with the U2609-A752 base pair in *E. coli* ribosomes. The fluoroketolides display improved MIC values compared to those of erythromycin and telithromycin against both Gram-positive and Gram-negative cells, particularly against strains bearing mutations in 23S rRNA U2609C and U754A, indicating that they have the potential for future development as clinically relevant antimicrobial agents.

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