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Activity of the Novel Macrolide BAL19403 against Ribosomes from Erythromycin-Resistant *Propionibacterium acnes*▽

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BAL19403 is a macrolide antibiotic from a novel structural class with potent activity against propionibacteria in vitro. The antibacterial spectrum of BAL19403 covers clinical isolates with mutations in the 2057 to 2059 region of 23S rRNA that confer resistance to erythromycin and clindamycin. The basis of this improved activity was investigated by ribosome binding assays and by a coupled transcription and translation assay. The latter was specifically developed for the use of ribosomes from *Propionibacterium acnes*. BAL19403 inhibited protein expression by ribosomes from erythromycin-sensitive and erythromycin-resistant *P. acnes* with similar potencies if the resistance was due to G2057A or A2058G mutations. BAL19403 showed a >10-fold higher activity than erythromycin against ribosomes from a strain with the *erm*(X) gene. Erm(X) confers high levels of macrolide and lincosamide resistance by dimethylation of A2058. Assays with such ribosomes showed that BAL19403 was potent enough to inhibit half of the total activity with a 50% inhibitory concentration very close to the value measured with erythromycin-sensitive ribosomes. We concluded from our data that the *P. acnes* strain with the *erm*(X) gene had a mixed population of ribosomes, with macrolide-sensitive and macrolide-resistant species.

Propionibacterium acnes is a skin-colonizing gram-positive bacterium associated with sebum-excreting pilosebaceous follicles (20, 23, 24). *P. acnes* is involved in the development of inflamed lesions in the course of acne (3). The observed inflammatory response is presumably directed against propionibacterial antigens (15, 18, 35). Mild to moderate inflammatory acne is often treated with antibiotics, usually erythromycin (ERY) or clindamycin (CLI), but sometimes with tetracycline (34). Antibiotic treatment of acne is nowadays challenged by an increasing incidence of antibiotic-resistant *P. acnes* (5, 11, 19, 32). A worldwide survey revealed that about 50% of clinical *P. acnes* isolates were resistant to ERY (Ery^R) and about 20% were resistant to tetracycline (11). Apart from playing a role in acne, propionibacteria can also be the main pathogen in severe infections, especially after surgery or in immune-compromised patients. A survey covering 13 European countries revealed that 15.1% of *P. acnes* clinical isolates from different systemic infections were resistant to CLI (Cli^R) and 17.1% were Ery^R (27).

Macrolide antibiotics interact with the 50S ribosomal subunit and block bacterial translation. Several mechanisms, including target alteration, are known for conferring macrolide resistance. Mutations of G2057, A2058, and A2059 (*Escherichia coli* numbering) in domain V of the 23S rRNA are the most frequently encountered mechanisms in *P. acnes* (26, 30, 31, 33). Mutations of G2057 reportedly cause low levels of ERY resistance in *P. acnes* (MICs of 1 to 2 μg/ml), whereas mutations of A2058 or A2059 lead to high-level ERY resistance, with MICs of >128 μg/ml (29–31). The expression of the Erm(X) methylase leads to dimethylation of A2058, resulting in high-level resistance to both ERY and CLI. The *erm*(X) gene was found in less than 10% of Ery^R clinical isolates collected in different European countries (26, 32).

BAL19403, a new macrolide from our antibacterial research programs, was found to have pronounced activity against Ery^R and Cli^R propionibacteria and to possess good anti-inflammatory properties (16, 17, 22) (Fig. 1). The molecular basis for the good antibacterial activity against Ery^R propionibacteria was the subject of this study. Here, we report on the interaction of BAL19403 with ribosomes isolated from Ery^S and Ery^R strains of *P. acnes*.

MATERIALS AND METHODS

Antibiotics and bacterial strains. ERY was bought from Sigma, and [^{14}C]ERY (55 mCi/mmol, 0.1 mCi/ml) was from Amersham Pharmacia, Inc. BAL19403 was synthesized in-house as described in reference 17. The properties of the bacterial strains used in this work are summarized in Table 1 and are described in reference 16.

Preparation of S30 and S100 fractions and ribosomes from *Escherichia coli*. The protocol was based on procedures described in references 1 and 2. A 2-liter culture of *E. coli* in LB broth was grown to an optical density at 600 nm (OD_{600}) of about 0.6. Cells were harvested by centrifugation (4,200 × *g* for 20 min at 4°C), and the pellet was washed with 300 ml of S70 buffer (20 mM HEPES-KOH, pH 7.5, at 4°C, 6 mM magnesium acetate, 30 mM NH_4Cl, 4 mM 2-mercaptoethanol). The cells were pelleted again and stored at −80°C or taken up in S70 buffer (2 ml/g [wet weight]) and disrupted by two passages through a French press at 138 MPa. The cell lysate was centrifuged (30,600 × *g* for 20 min at 4°C), and the supernatant (S30 fraction) was kept for further use.

Ribosomes were pelleted from the S30 fraction by ultracentrifugation at 100,000 × *g* for 2 h at 4°C. The pellet was taken up in 5 ml of ribosome buffer (10 mM HEPES-KOH, pH 7.5, at 4°C, 10 mM magnesium acetate, 30 mM NH_4Cl, 4 mM 2-mercaptoethanol). The OD_{260} was determined, and aliquots were frozen at −80°C. The supernatant was stored at −80°C as the S100 fraction.

Isolation of ribosomes from *P. acnes*. Aliquots of Wilkins-Chalgren broth (Difco) were inoculated with *P. acnes* cells that had been grown for 48 to 72 h on a plate of Wilkins-Chalgren agar (Oxoid). The liquid cultures were incubated without shaking for 72 h at 37°C under anaerobic conditions. Cells were harvested from 5 liters of pooled cultures by centrifugation (12,200 × *g* for 30 min

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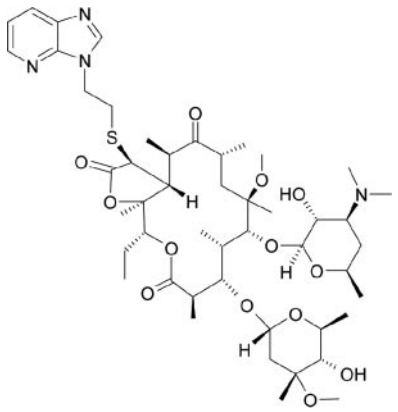

FIG. 1. Chemical structure of BAL19403.

at 4°C). The cell pellets were either stored at −80°C or taken up in S70 buffer (5 ml/g [wet weight]). The resuspended cells were incubated with 1 mg/ml lysozyme (Pharmacia) for 1 h at 37°C on a shaker (130 rpm). A protease inhibitor mix (EDTA-free complete cocktail; Roche Applied Science) was added. Ribosomes were prepared as described for *E. coli*.

Ribosome binding assay. The assay was adapted from references 6, 8, 25, and 28. Two-hundred-microliter reaction mixtures in assay buffer (50 mM Tris, pH 7.8, 4 mM $MgCl_2$, 10 mM NH_4Cl, 100 mM KCl) containing ribosomes (final OD_{260} of 4), test compound, and [^{14}C]ERY were incubated at room temperature for 1 h in 96-well plates. Ninety-six-well filter plates with mixed cellulose ester membranes (Millipore, Inc.) were prepared by passing 150 μl of H_2O and 200 μl of 0.5% polyethyleneimine in H_2O (Sigma) per well and 150 μl of H_2O two times per well. The assay mixtures were transferred to the filter plate and immediately filtered by vacuum. Each well was washed three times with 150 μl of cold (4°C) wash buffer (100 mM Tris, pH 7.2, 50 mM KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol). One hundred microliters Microscint 20 scintillation cocktail (Perkin Elmer) was added to each well, and the plates were shaken for at least 2 h before they were measured in a Packard Top Count scintillation counter. $[ERY]_{bound}$ (amount of ERY bound to ribosomes) was plotted against $[ERY]_{free}$ (amount of unbound ERY), and the K_d^{app} (apparent dissociation constant) for ERY was determined by fitting equation 1 to the experimental data. "Capacity" in equation 1 indicates the saturation level of bound ERY. Curve fits were done with GraFit software (GraFit version 5; Erithacus Software Ltd., Horley, United Kingdom).

$$[ERY]_{bound} = \frac{\text{capacity}\,[ERY]_{free}}{K_d^{app} + [ERY]_{free}} \quad (1)$$

The K_d for BAL19403 was calculated by fitting equation 2 to a plot of K_d^{app} versus the BAL19403 concentration.

$$K_d^{app} = K_d^{ERY} + \frac{K_d^{ERY}}{K_d^{BAL19403}}[BAL19403] \quad (2)$$

where K_d^{ERY} and $K_d^{BAL19403}$ are the K_d for each drug.

In vitro transcription and translation. The transcription and translation assay was done according to the protocol of the supplier (*E. coli* S30 extract system for circular DNA; Promega Corp.). Where indicated below, S30 was replaced by a 1:1 (vol/vol) mixture of S100 and ribosomes. The plates were sealed and incubated at 37°C in a plate Thermomixer (Eppendorf). After the desired time, a luciferin mix was added, and luminescence was measured in an Analyst AD microtiter plate reader (Molecular Devices Corp.). Inhibition curves were derived from at least three different measurements per concentration of inhibitor. The uninhibited reaction was determined as the average of 12 measurements. For biphasic inhibition curves, the data points around the first inflection point and the data points at the high-concentration ends represent the average values from six measurements. The 50% inhibitory concentrations (IC_{50}s) and their standard errors were obtained by curve fitting.

Equation 3 was used for curve fits to biphasic dose-response patterns. y represents the measured luciferase activity, and x is the inhibitor concentration.

$$y = \frac{a}{1 + \left(\frac{x}{IC_{50-a}}\right)^{S_a}} + \frac{b}{1 + \left(\frac{x}{IC_{50-b}}\right)^{S_b}} \quad (3)$$

One part (a) of the total activity is inhibited with an IC_{50-a} and the slope s_a. The inhibition of the remaining activity (b) is described by IC_{50-b} and the slope s_b.

RESULTS

ERY-sensitive (Ery^S) ribosomes were isolated from *E. coli* BL21 and from *P. acnes* EG7NS. The *E. coli* ribosomes had a slightly higher affinity for [^{14}C]ERY than those isolated from *P. acnes* (Table 1). The binding of BAL19403 to ribosomes from *E. coli* BL21 was measured in competition experiments with [^{14}C]ERY and led to a K_d in the same range as the values obtained with ERY. The measurements could not be extended to Ery^R ribosomes, which did not efficiently bind radiolabeled ERY. The binding curves from control experiments with ribosomes from Ery^R *P. acnes* P95 showed a weak binding of [^{14}C]ERY, and the K_d was estimated as >2,100 nM.

We set up a coupled in vitro transcription and translation assay to assess the influence of macrolides on gene expression. A luciferase gene on a plasmid served as the template, and the luciferase produced was quantified by its enzymatic activity. S30 bacterial cell extracts from *E. coli* BL21 showed slightly different susceptibilities toward ERY than toward inhibitor BAL19403. ERY inhibited the expression of luciferase with an IC_{50} of 2.13 ± 0.16 (mean ± standard deviation) μM, whereas BAL19403 inhibited luciferase production with an IC_{50} of 0.85 ± 0.26 μM.

S30 extracts prepared from *P. acnes* cultures did not support the expression of luciferase from the provided plasmid. The assay was adapted to the use of *P. acnes* ribosomes by mixing S100 from *E. coli* BL21 with ribosomes from *P. acnes* cultures. Control reactions showed that luciferase was made above background levels only by S100 in combination with ribosomes and not by the individual components alone (Fig. 2).

BAL19403 showed basically the same activity as the comparator ERY against Ery^S ribosomes from either *E. coli* or *P. acnes* (Table 1). The situation changed when ribosomes from

TABLE 1. Activities of BAL19403 and ERY

Strain	Resistance mechanism	ERY			BAL19403		
		MIC (μg/ml)	IC_{50} (μM)	K_d (nM)	MIC (μg/ml)[a]	IC_{50} (μM)	K_d (nM)
E. coli BL21	Outer membrane	128	0.76 ± 0.041	11 ± 3.8	128	0.42 ± 0.072	26 ± 6.2
P. acnes EG7NS		≤0.06	0.17 ± 0.0096	29 ± 15	≤0.06	0.11 ± 0.012	
P. acnes P95	G2057A	4	2.2 ± 0.14	>2,100	≤0.06	0.12 ± 0.0054	
P. acnes SW54EA	A2058G	>512	87 ± 13		0.5	0.26 ± 0.0050	
P. acnes GE4E	Erm(X)	>512	1.9 ± 0.62 and >500		≥256	0.19 ± 0.014 and >50	

[a] MICs for *P. acnes* strains are taken from reference 16.

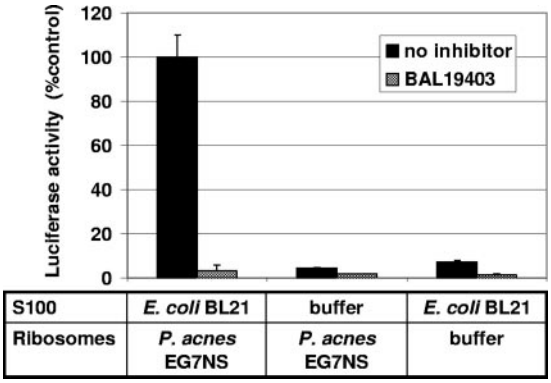

FIG. 2. Activity of luciferase made in vitro. Reaction mixtures contained either no or 10 μM BAL19403. Error bars show standard deviations.

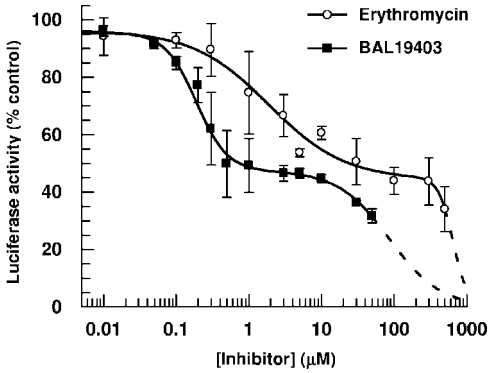

FIG. 3. Inhibition of methylated ribosomes. Luciferase was expressed in standard reaction mixtures with ribosomes from *P. acnes* GE4E and S100 from *E. coli* BL21. Inhibition of protein expression was determined by measuring luciferase activity as a function of increasing BAL19403 or ERY concentration. Results are expressed as percent uninhibited signal, and curves were fitted to data points using equation 3. Error bars represent standard deviations, and the dotted parts of the curves indicate curvatures for IC_{50}s of >50 μM and >500 μM, respectively.

Ery^R strains were investigated. While BAL19403 kept its activity towards ribosomes from *P. acnes* strains P95 (G2057A) and SW54EA (A2058G), the activity of ERY decreased by about 10-fold against *P. acnes* P95 ribosomes and by more than 100-fold against those from *P. acnes* SW54EA. Dose-response curves measured with ribosomes from *P. acnes* SW54EA had a sigmoidal shape with a single inflection point.

Both BAL19403 and ERY were only partially active against ribosomes from *P. acnes* GE4E. This strain expresses the Erm(X) methylase, which confers high-level ERY resistance. ERY up to 500 μM and BAL19403 up to 50 μM did not fully inhibit the activity of ribosomes isolated from *P. acnes* GE4E. Higher concentrations could not be tested because of the limited solubility of the agents. The dose dependence of the detected inhibition appeared to be biphasic for both ERY and BAL19403 (Fig. 3). Equation 3 describes a model that comprises two independent steps for the total inhibition of *P. acnes* GE4E ribosomes. IC_{50} values for the two steps were obtained by fitting curves as described by equation 3 to the measured data. ERY inhibited 52% of the ribosomal activity with an IC_{50} of 1.9 ± 0.62 μM and the remaining activity with an IC_{50} of >500 μM. BAL19403 inhibited 48% of the total ribosomal activity with an IC_{50} of 0.19 ± 0.014 μM and the remaining activity with an IC_{50} of >50 μM.

DISCUSSION

BAL19403 was selected from a series of investigational antibacterial macrolides for its potency against *P. acnes*. A 4-azabenzimidazole side chain linked to a unique macrolactone core resulted in substantially improved antibacterial activity in comparison to that of ERY (16). BAL19403 was active against *P. acnes* that was resistant to ERY and CLI due to mutations in the 2057 to 2059 region of domain V of the 23S rRNA (*E. coli* numbering). To understand the molecular basis for the enhanced activity, BAL19403 was tested in vitro for its ability to interfere with ribosome activity. Ery^S ribosomes isolated from *E. coli* BL21 or from *P. acnes* EG7NS bound [^{14}C]ERY and BAL19403 with comparable affinities (Table 1). The K_d of 11 nM for ERY binding to *E. coli* ribosomes agreed with published values covering the range of 1 to 100 nM (4, 6–9, 12–14, 25, 28). Thus, the different antibacterial activities of ERY and BAL19403 against *E. coli* and *P. acnes* could not be explained by the binding studies alone. We implemented an in vitro protein expression assay using S30 bacterial extracts to make luciferase in vitro. *E. coli* S30 extracts worked well, but they could not be replaced by S30 extracts prepared from *P. acnes*. The propionibacterial expression machinery may not recognize the promoter which controls transcription of the luciferase gene in this assay. Alternatively, *P. acnes*, with DNA of high GC content, employs a codon usage different from that of *E. coli*. However, luciferase synthesis could be achieved by replacing the *E. coli* S30 with a mixture of *E. coli* S100 and *P. acnes* ribosomes. The adapted assay protocol permitted studies of propionibacterial ribosome activity in vitro. The inhibition of activity found in those studies (IC_{50}) corresponded well to the observed growth inhibition (MIC) for the respective strains. ERY inhibited protein expression with an IC_{50} below 1 μM only when ribosomes from Ery^S *E. coli* or *P. acnes* cells were used. A 10-fold increase of the IC_{50} in the in vitro transcription and translation assay reflected the elevated MIC of 4 μg/ml against *P. acnes* P95. The inhibition of ribosomes from highly Ery^R *P. acnes* was achieved only with high ERY concentrations, resulting in about 100-fold-increased IC_{50} values.

BAL19403 also inhibited protein expression by ribosomes from Ery^S *P. acnes* with an IC_{50} lower than 1 μM. In contrast to ERY, the IC_{50} for BAL19403 remained at this low level even when ribosomes from the Ery^R strains P95 and SW54EA were used. This agreed with the improved in vitro antipropionibacterial activity of BAL19403, as seen by MICs of ≤0.06 and 0.5 μg/ml, respectively. Dose-response analysis indicated that the ribosome preparations contained single ribosome species. This was noteworthy in the case of 23S rRNA mutations because *P. acnes* contains three copies of the rRNA operon. Our data agreed with the earlier findings of Ross et al. (30), who analyzed a collection of 37 *P. acnes* isolates, including 11 strains with the A2058G mutation and 6 with the G2057A mutation. They did not find evidence for heterozygosity in the 23S rRNA gene sequences in any strain.

Both BAL19403 and ERY strongly inhibited *E. coli* ribosomes but showed no activity against whole cells (MIC, >128 μg/ml). Ribosome binding studies suggested that the lack of activity against *E. coli* was not caused by inactivity towards the target but may be due to cell permeation barriers.

Both ERY and BAL19403 were inactive against *P. acnes* GE4E. This strain harbors an *erm*(X) gene which codes for an rRNA methylase. Dimethylation of the adenine at position 2058 (*E. coli* numbering) is known to confer high-level macrolide resistance. The inhibition of ribosomes from *P. acnes* GE4E followed a biphasic dose-response curve. Such a result could be explained by a heterogeneous ribosome population composed of ribosomes that are susceptible and others that are resistant to a given drug. The experimental data could be well described by a model incorporating two independent inhibition events. In such a model, the susceptible ribosomes are inhibited at a low drug concentration, whereas inhibition of the resistant species would require high concentrations. Our results suggested that about half of the *P. acnes* GE4E ribosomes were susceptible to BAL19403 and a lesser fraction was susceptible to ERY. BAL19403 inhibited this more-susceptible part of the population with an IC_{50} of 0.19 μM, whereas ERY acted with an IC_{50} of 1.9 μM. Inhibition of the remaining activity was seen only at high concentrations of ERY or BAL19403, indicating the presence of highly resistant ribosomes. In analogy to our other data, these activities should translate into MICs of ≤0.06 μg/ml for BAL19403 and about 4 μg/ml for ERY if 50% inhibition of ribosomal activity were sufficient for activity against whole cells. The fact that the measured MICs were much higher than 0.06 μg/ml suggested that more than 50% of the ribosomes have to be inhibited in order to see antibacterial activity.

Previous work on rRNA methylases has shown that monomethylation of A2058 conferred intermediate levels of resistance to ERY and that dimethylation increased resistance levels (10, 21, 36, 37). Other data suggested that a large proportion of the rRNA had to be dimethylated in order to achieve high resistance to the ketolide telithromycin (10). If we assume a similar situation for *P. acnes*, then the Ery^S fraction of *P. acnes* GE4E ribosomes consisted of unmethylated and monomethylated ribosomes. The IC_{50} of BAL19403 for the macrolide-sensitive part of *P. acnes* GE4E ribosomes was about the same as for ribosomes from *P. acnes* EG7NS. This is consistent with an activity of BAL19403 toward monomethylated ribosomes comparable to that of telithromycin. Dimethylated ribosomes would account for the highly resistant ribosomes and were most likely responsible for the high IC_{50} values determined for complete inhibition. Irrespective of the ribosomal methylation pattern, *P. acnes* GE4E was resistant to ERY and BAL19403 without prior induction of *erm*(X) expression by macrolides. All our ribosome preparations were made from cells grown in medium devoid of antibiotics. Based on the resistance phenotypes and the in vitro ribosome inhibition data, we concluded that uninduced *P. acnes* GE4E expressed Erm(X) at a level high enough to confer macrolide resistance.

The correlation of the in vitro translation inhibition data with the MIC data strongly suggests that enhanced activity at the ribosome level is the basis for the improved activity of BAL19403 against Ery^R *P. acnes*.

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