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A Derivative of the Thiopeptide GE2270A Highly Selective against *Propionibacterium acnes*

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A chemical derivative of the thiopeptide GE2270A, designated NAI003, was found to possess a substantially reduced antibacterial spectrum in comparison to the parent compound, being active against just a few Gram-positive bacteria. In particular, NAI003 retained low MICs against all tested isolates of *Propionibacterium acnes* and, to a lesser extent, against *Enterococcus faecalis*. Furthermore, NAI003 showed a time- and dose-dependent killing of both a clindamycin-resistant and a clindamycin-sensitive *P. acnes* isolate. Gel shift experiments indicated that, like the parent compound, NAI003 retained the ability to bind to elongation factors Tu (EF-Tus) derived from *Escherichia coli*, *E. faecalis*, or *P. acnes*, albeit with reduced efficiency. In contrast, EF-Tus derived from the NAI003-insensitive *Staphylococcus aureus* or *Streptococcus pyogenes* did not bind this compound. These results were confirmed by *in vitro* studies using a hybrid translation system, which indicated that NAI003 can inhibit most efficiently protein synthesis driven by the *P. acnes* EF-Tu. *P. acnes* mutants resistant to NAI003 were isolated by direct plating. With one exception, all analyzed strains carried mutations in the *tuf* gene, encoding EF-Tu. Because of its selective effect on *P. acnes* in comparison to resident skin flora, NAI003 represents a promising candidate for the topical treatment of acne, which has already completed a phase 1 clinical study.

Acne vulgaris, a complex disease of the pilosebaceous units of the face and upper trunk, is the most common skin condition seen by physicians. Although it affects almost 100% of adolescents to various degrees and generally wanes as adolescence ends, the disease may persist into adulthood, and it has been estimated that more than 17 million people in the United States are affected by acne (1, 2). At least four factors contribute to the development of acne: increased sebum production by the sebaceous glands, follicular hyperkeratinization, colonization of the sebaceous follicles with *Propionibacterium acnes*, and inflammation (3, 4).

Inflammatory acne is the result of the host response to *P. acnes*, a pleomorphic, anaerobic rod belonging to the phylum *Actinobacteria*. *P. acnes*, a component of the normal skin flora, is a usually harmless commensal largely incapable of tissue invasion or serious infection. The organism catabolizes sebaceous triglycerides, using the glycerol moiety as a carbon source without catabolizing the fatty acids. While this species has been advocated to play a fundamental role in the etiogenesis of acne (5, 6), a significant controversy remains as to how important a role *P. acnes* plays (4, 7). Recent metagenomic studies have confirmed it to be the major inhabitant of pilosebaceous units (8). Nonetheless, *P. acnes* is a normal skin commensal not necessarily associated with acne, although a correlation between disease and *P. acnes* phylotypes has been proposed (8). It is also worth pointing out that *Staphylococcus epidermidis* can produce catabolites that inhibit *P. acnes* growth (9), highlighting the importance of an appropriate balance of the microflora for a healthy skin. On the basis of the above considerations, a reduction in the population of *P. acnes* without affecting other commensal flora may constitute a plausible approach to an effective therapy of acne. However, no antibacterial agent possessing such an antibacterial spectrum has been described so far.

The thiopeptide GE2270A 1, produced by the actinomycete *Planobispora rosea*, is extremely active against numerous Gram-positive pathogens, including methicillin-resistant strains of *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* spp. (10). It has been demonstrated that GE2270A binds to domain II of elongation factor Tu (EF-Tu), making contacts with residues 215 to 230, 256 to 264, and 273 to 277 (11). These interactions alter the conformation of EF-Tu, so as to increase its electrophoretic mobility (12) and to inhibit EF-Tu–GTP–aminoacyl-tRNA (aa-tRNA) ternary complex formation. As a result, GE2270A interferes selectively with the functioning of the elongation factor in protein synthesis.

During a program aimed at generating analogs of GE2270A by semisynthesis, we observed that one of the derivatives, designated NAI003, showed a surprisingly restricted antibacterial spectrum, mostly limited to *P. acnes*. Here, we report the antibacterial properties of this compound, investigate the molecular basis for its reduced activity against other Gram-positive bacteria, and provide evidence that NAI003 targets EF-Tu in *P. acnes*.

MATERIALS AND METHODS

Chemistry. GE2270A (5 g, 3.87 mmol), prepared as described previously (10), was dissolved in dioxane-water-formic acid (10:1:1) and left overnight at 80°C under stirring. After cooling, the solvent was evaporated and concentrated to dryness, and the resulting residue was incubated for 1 h at

Received 28 December 2014 **Returned for modification** 4 February 2015
Accepted 11 May 2015

Accepted manuscript posted online 18 May 2015

Citation Fabbretti A, He C-G, Gaspari E, Maffioli S, Brandi L, Spurio R, Sosio M, Jabes D, Donadio S. 2015. A derivative of the thiopeptide GE2270A highly selective against *Propionibacterium acnes*. Antimicrob Agents Chemother 59:4560–4568. doi:10.1128/AAC.05155-14.

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doi:10.1128/AAC.05155-14

room temperature in 0.5 M sodium carbonate. The reaction mixture was then diluted with cold water and brought to pH 6.5 with HCl. The thiopeptides were extracted from the aqueous phase with ethyl acetate and then precipitated from the concentrated organic phase by adding petroleum ether. The resulting solid (1.3 g, 1 mmol) was dissolved in dimethylformamide (DMF) (10 ml), and 4-amino-*N*-benzylpiperidine (1.2 mmol), triethanolamine (TEA) (1.4 mmol), and diphenyl phosphorazidate (1.2 mmol) were added at 0°C. The temperature was allowed to rise to room temperature, and stirring was continued for about 4 h. The reaction mixture was acidified to pH 3 with 1 N HCl and then diluted with water to complete precipitation of the product. The wet solid was dried in air and then purified by flash chromatography (silica gel 60, 230 to 400 mesh; ASTM; Merck) with elution with 3 to 5% methanol in chloroform. Fractions containing NAI003 were pooled and evaporated to dryness as a white solid. The data are as follows: ^{1}H nuclear magnetic resonance (NMR), dimethyl sulfoxide (DMSO)-d_6 0.86 (d, 3H); 0.90 (d, 3H); 1.91 to 1.70 (n, 2H); 2.26 to 2.05 (m, 2H); 2.60 (s, 3H); 2.91 to 2.69 (m, 4H); 3.40 (s, 3H); 3.51 (br s, 2H); 3.95 to 3.75 (m, 2H); 4.30 (dd, 1H); 4.99 (s, 2H); 7.41 to 7.18 (m, 2H); 8.28 (s, 1H); 8.45 (s, 1H); 8.66 (s, 1H).

Bacterial strains and media. Bacterial strains were from public collections (ATCC) or from the Naicons collection of pathogenic strains (designated by "L" or "ND" prefixes). In particular, the *P. acnes* strains with an "L" or "ND" prefix were independent clinical isolates collected before 2003 and in 2011, respectively. Brucella broth (BB) and brucella agar (BA), Middlebrook 7H9 broth (7H9B), Mueller-Hinton broth (MHB), Todd-Hewitt broth (THB), and Wilkins-Chalgren agar (WCA) were from Difco Laboratories (Detroit, MI, USA). Cation-adjusted MHB (CAMHB), prepared by adding 20 mg/ml $CaCl_2$ and 10 mg/ml $MgCl_2$ to MHB, was used for all aerobic bacteria except for streptococci, which were grown in THB; *Corynebacterium* sp., which was grown in CAMHB with 5% lysed horse blood; and *Mycobacterium smegmatis*, which was grown in 7H9B supplemented with 0.5 g/liter Tween 80 and 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (BD, MD, USA). *P. acnes* was grown in BB or BA supplemented with 5% lysed horse blood, 5 μg/ml hemin, and 1 μg/ml vitamin K_1 (supplemented BB). All liquid media contained 0.02% bovine serum albumin, unless otherwise noted. All cultures were grown at 37°C.

Determination of MICs. MICs were determined by the broth microdilution methodology (13, 14); bacteria were inoculated at 5×10^5 CFU/ml. Assays were performed in sterile 96-well microtiter plates with round-bottomed wells. Plates were read after 20 to 24 h (aerobic bacteria) or 72 h (*Mycobacterium smegmatis*; *P. acnes*, anaerobic atmosphere). GE2270A and its derivatives were dissolved in dimethyl sulfoxide. Clindamycin and erythromycin (Sigma-Aldrich) were dissolved in water and in 95% ethanol, respectively. Appropriate dilutions were made with the required culture medium immediately before testing.

Time-kill assays. *P. acnes* colonies from 72-h BA plates were resuspended in BB at approximately 1×10^5 to 2×10^5 CFU/ml and incubated for 24 h in supplemented BB. Then, 0.5 ml of the culture was added to fresh 5 ml supplemented BB in glass vials with plastic caps containing the desired concentration of antibiotic. For this experiment, a series of antibiotic solutions were made at 100× the desired final concentration in either DMSO (for NAI003) or water (for clindamycin). In all experiments, an extra vial, supplemented with just 50 μl DMSO, was used as a growth control. Cultures were incubated at 37°C, and anaerobic conditions were achieved with 1:25 (vol/vol) Oxyrase (Oxyrase Inc., Mansfield, OH, USA). At different time points, 0.2-ml aliquots were withdrawn and serially diluted 1:10 with 0.9% NaCl, and 25 μl of each dilution was spread onto two to four BA plates. Colonies were counted after 48 to 72 h at 37°C under an anaerobic atmosphere and average values used in time-kill curves (variations in colony numbers were usually within ±30% of average counts).

Resistance analysis. The occurrence of spontaneous resistant mutants of *P. acnes* ATCC 6922 was determined by plating 0.1 ml of a stationary-phase culture on WCA containing NAI003 or GE2270A at 1 or 10 μg/ml. Plates were incubated in an anaerobic atmosphere, with colonies scored after 5 and 12 days. Colonies emerging on the plates were cultured in BB, centrifuged, and stored at −80°C in nutrient broth supplemented with 20% glycerol for MIC determination and analysis of the *tuf* genes, encoding EF-Tu. From selected strains, a cell lysate was made by heating at 95°C for 5 min and used for PCR amplifying the entire *tuf* gene with primers 5′-GTGGCAAAGGCCAAGTTCG-3′ and 5′-TCACTTGATGATCTTGGTGACTC-3′. PCR products were directly sequenced (Primm Srl, Milan, Italy). The sequenced segment from the parental strain ATCC 6922 was found to be identical to the *tuf* gene from *P. acnes* KPA171202 (15) (GenBank NC_006085.1).

TABLE 1 Oligonucleotide primers used for sticky-end PCR

tufA gene source	Sequence (5′-3′)[a]
S. aureus	F: CATGGCAAAAGAAAAATTCGATCGTTC
	R: CTTATTTAATGATTTCAGTAACAACGC
	F: GCAAAAGAAAAATTCGATCGTTCTAAAG
	R: GATCCTTATTTAATGATTTCAGTAACAACG
E. faecalis	F: CATGGCAAAAGAAAAATTTGACCGTTC
	R: CTTATTTAATGATTTCAGTAACAACGC
	F: GCAAAAGAAAAATTTGACCGTTCTAAATC
	R: GATCCTTATTTAATGATTTCAGTAACAACG
S. pyogenes	F: CATGGCAAAAGAAAAATACGATCGTAG
	R: GATCCTTAAGCTTCGTTTCTGAAACG
	F: GCAAAAGAAAAATACGATCGTAGTAAAC
	R: CTTAAGCTTCGATTTCTGAAACGATACC
P. acnes	F: CATGGCAAAGGCCAAGTTCGAGCGG
	R: GATCCTCACTTGATGATCTTGGTGACTCG
	F: GCAAAGGCCAAGTTCGAGCGGACC
	R: CTCACTTGATGATCTTGGTGACTCGACC

[a] F, forward; R, reverse. The methodology employed (16) requires two forward and two reverse primers for each amplification.

Biochemical methods. Buffers used were the following: buffer A, 25 mM Tris-HCl (pH 7.1), 5% glycerol, 700 mM NaCl, 6 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzamidine; buffer B, 25 mM Tris-HCl (pH 7.1), 300 mM NaCl, 5% glycerol, 20 mM imidazole, 6 mM β-mercaptoethanol, 0.1 mM PMSF, and 0.1 mM benzamidine; buffer C, 25 mM Tris-HCl (pH 7.1), 300 mM NaCl, 5% glycerol, 300 mM imidazole, 6 mM β-mercaptoethanol, 0.1 mM PMSF, 0.1 mM benzamidine; buffer D, 20 mM Tris-HCl (pH 7.1), 100 mM NH_4Cl, 5% glycerol, 6 mM β-mercaptoethanol, 0.1 mM EDTA; buffer E, 10 mM Tris-HCl (pH 7.7), 10 mM magnesium acetate, 60 mM NH_4Cl.

Cloning, overexpression, and purification of EF-Tus. The strategy used to clone the *tuf* genes from genomic DNA of *S. aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29922), *Streptococcus pyogenes* (ATCC 700294), and *P. acnes* (ATCC 6919) was essentially as reported by Zeng (16). Briefly, four different phosphorylated primers (Table 1) carrying NcoI and BamHI restriction sites were used in two separate DNA amplification reactions. Equal volumes of the two PCR mixtures were combined and subjected to a 5-min denaturation at 95°C and a 15-min renaturation at room temperature. Of the resulting final products, 25% are expected to carry NcoI and BamHI cohesive ends, which can be ligated with the appropriately restricted expression vector pETM11. The ligation products were then transformed into the *Escherichia coli* BL21 strain.

E. coli BL21(DE3)pLysS cells harboring the pETM11 derivatives were grown in LB medium at 37°C up to an optical density at 600 nm (OD_{600}) of 0.7, when 1 mM isopropyl-β-D-1-thiogalactopyranoside was added. After 4 h at 37°C, the cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C and the pellet, resuspended in buffer A, was stored at −80°C. After thawing, the cells were lysed by sonication, and the resulting cell extract was cleared by centrifugation at 13,000 rpm for 45 min at 4°C. The cell extract was subsequently loaded on a 5-ml nickel-nitrilotriacetic

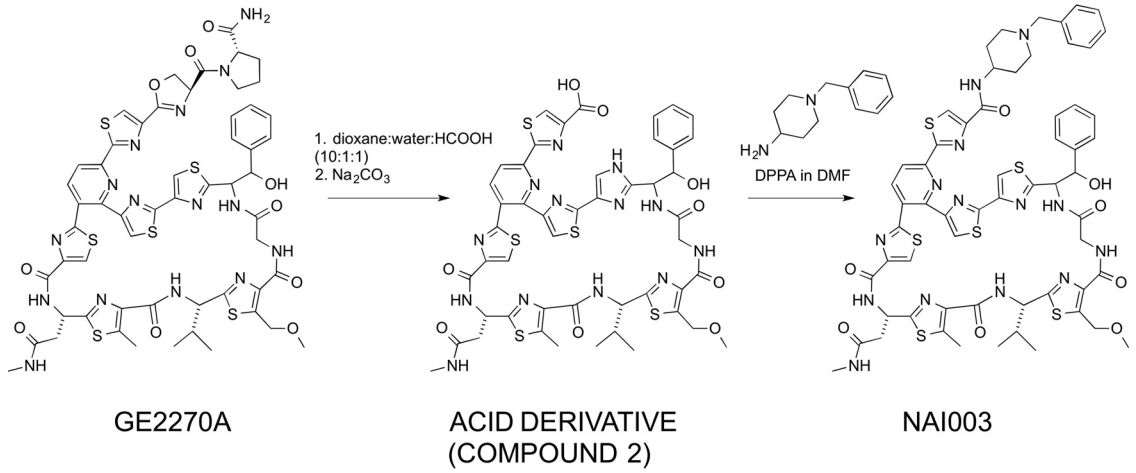

FIG 1 Synthetic route to NAI003. DPPA, diphenyl phosphorazidate.

acid (Ni-NTA) chromatographic column equilibrated in buffer A. The column was initially washed with 10 bed volumes of buffer A, followed by an additional wash with 5 bed volumes of buffer B. EF-Tu was eluted from the column with buffer C. Fractions containing EF-Tu were dialyzed to reduce salt concentration against buffer C containing 100 mM NaCl (without imidazole) and digested by tobacco etch virus (TEV) protease (enzyme/substrate ratio, 1:40) overnight at 20°C, with 20% extra TEV protease added for an additional 3 to 4 h. The reaction mixture was again loaded on the Ni-NTA resin, and the cleaved protein, without the His tag, was recovered in the flowthrough of the column. EF-Tu was concentrated by centrifugation at 4,000 rpm for 60 min at 4°C in Amicon Ultra-3 centrifugal filter devices, and after dialysis at 4°C against buffer D, it was stored at −80°C in small aliquots.

***In vitro* tests.** 30S and 50S ribosomal subunits, 027-IF2Cp(A) mRNA, EF-G, and *E. coli* cell extract were prepared as described previously (17, 18). *In vitro* mRNA translation driven by 027-IF2Cp(A) mRNA was carried out as described previously (17). Native polyacrylamide (12%) gel electrophoresis of EF-Tu preincubated with 1 mM GTP in the presence of increasing concentrations (0.1, 0.5, 1, 5, 10, 50, and 100 μM) of GE2270A or NAI003 was performed in Tris-glycine buffer (pH 8.3) as described previously (11).

Poly(U) hybrid translation system. Poly(U) translation was carried out in 30 μl of buffer E containing 3 mM phosphoenolpyruvate, 0.05 μg/ml pyruvate kinase, 1 mM GTP, 0.15 μg/μl poly(U), 10 μM [^{3}H]Phe-tRNA, and 0.2 μM *E. coli* 30S, 50S, and EF-G. Twenty picomoles of purified EF-Tu (from *E. coli*, *S. aureus*, *E. faecalis*, *S. pyogenes*, or *P. acnes*) was added to the mixture after a 5-min incubation at room temperature in the presence of increasing concentrations of GE2270A or NAI003. After 30 min at 37°C, the level of poly(U) translation was quantified from the amount of acid-insoluble [^{3}H]Phe-tRNA incorporated.

RESULTS

Synthesis of NAI003. The oxazolidine-linked L-Ser-L-Pro-NH_2 side chain of GE2270A undergoes, on acid treatment, an N-O acyl shift, forming a diketopiperazine ester that can be easily removed by mild basic treatment (19, 20). The newly formed carboxylic acid 2 can thus be effectively amidated, as for example with 4-amino-*N*-benzylpiperidine, to afford NAI003 (Fig. 1).

Antibacterial spectrum of NAI003. The parent compound GE2270A is highly active against Gram-positive bacteria but not effective against Gram-negative species (10). Table 2 shows the activity of GE2270A against *Staphylococcus*, *Streptococcus*, and *Enterococcus* spp., with MICs in the ranges of ≤0.015 to 0.25, 0.06 to 2, and 0.008 to 0.015 μg/ml, respectively. The acid derivative 2 maintains substantially the same spectrum as GE2270A, although MICs were 32- to 64-fold and 4- to 16-fold higher against staphylococci and streptococci, respectively (Table 2). Surprisingly, NAI003 had lost potency against most of the tested strains: measurable MICs were observed against *Enterococcus* spp., with MICs in the 0.5- to 16-μg/ml range, and against *Mycobacterium smegmatis*. Interestingly, in the latter case, compounds 1, 2, and 3 were found to be equally active, with MICs of 4 to 8 μg/ml (Table 2). It should be noted that all four *Corynebacterium* strains tested in this experiment were equally insensitive to the three compounds, suggesting that this genus may be insensitive to this class of thiopeptides (Table 2).

We then wondered whether, among the pathogenic *Actinobacteria*, some species might have retained sufficient sensitivity to NAI003. Preliminary experiments indicated that *P. acnes* was sufficiently sensitive to this compound to warrant further investigation. The results of these evaluations are reported in Table 3, where it can be seen that *Propionibacterium* spp., including *P. acnes*, were highly sensitive to NAI003, with MIC values in the range of 4 to 250 ng/ml. In contrast to most bacterial species in Table 2, *P. acnes* appears to be equally sensitive to all of the three thiopeptides tested in this study (Table 3). Among the tested *P. acnes* isolates, one strain was highly resistant to clindamycin and erythromycin, an increasingly frequent phenotype among *P. acnes* isolates (21). As expected from their different targets, NAI003 and GE2270 retained activity against this strain.

Killing effect on *P. acnes*. To our knowledge, a single study has been performed to evaluate the effect of topical treatment on the number of *P. acnes* cells on the forehead of healthy volunteers: a reduction of approximately 1 log CFU was observed after 1 or 2 weeks of daily applications of 1% clindamycin (22, 23).

We thus wondered whether NAI003 had any effect *in vitro* on the number of viable *P. acnes* cells in comparison with clindamycin. To this end, we tested the effect of the two antibiotics when added to exponentially growing cells. The results are illustrated in Fig. 2A. Under the experimental conditions used, *P. acnes* reached stationary phase (10^8 CFU/ml) after approximately 48 h, with an apparent duplication time of 4 to 5 h. When these cultures were exposed to clindamycin at 1×, 10×, or 100× the MIC, a slow decline in viable counts was observed during the first 48 h, fol-

TABLE 2 MICs of GE2270A, compound 2, and NAI003 against selected Gram-positive bacteria

Species and strain	MIC (μg/ml) of antibiotic:			
	GE2270A	Compound 2	NAI003	Vancomycin
Staphylococcus aureus				
ATCC 29213	≤0.015	0.5	>128	1
ND041009	≤0.015	0.5	>128	1
ND018107	≤0.015	1	>128	0.25
ND018107	0.03	1	>128	0.5
ND055710	0.03	1	>128	0.5
ND055910	0.03	0.5	>128	1
Staphylococcus epidermidis				
ND006607	≤0.015	1	>128	1
ND015007	0.125	2	>128	2
ND020708	0.03	0.5	>128	2
ND021208	0.03	2	>128	2
ND024908	≤0.015	1	>128	2
ND025708	0.03	1	>128	2
ND026508	0.06	4	>128	1
Staphylococcus capitis				
ND004307	0.03	2	>128	2
ND018807	0.25	8	>128	2
Streptococcus agalactiae				
ND050210	2	>128	>128	0.5
L310	2	32	>128	0.5
Streptococcus pyogenes				
L1303	0.5	2	>128	0.5
L800	0.5	4	>128	0.5
Streptococcus pneumoniae ATCC 49619	0.06	0.5	>128	0.25
Enterococcus faecalis				
VanS L559	0.008	NT[a]	0.5	0.5
VanA L560	0.008	NT	0.5	>128
Enterococcus faecium				
VanS L568	0.015	NT	16	2
VanA L569	0.015	NT	4	>128
Mycobacterium smegmatis mc^2155	4	8	4	NT
Micrococcus luteus L108	≤0.015	1	>128	0.125
Corynebacterium jeikeium				
L2508	>128	>128	>128	1
L2645	>128	>128	>128	0.5
Corynebacterium sp.				
L689	>128	>128	>128	1
L691	>128	>128	>128	0.5

[a] NT, not tested.

lowed by an apparent faster decline afterwards, resulting in viable counts below the detection limit of the assay (around 20 CFU/ml). In contrast, the NAI003 killing rate was relatively constant with time, leading after 6 days to a 2- and 3-log reduction in viable counts at 10× and 100× the MIC. In the presence of 1× the MIC, after an approximately 24-h lag, the strain was eventually able to grow, albeit at a reduced rate (Fig. 2A).

A similar experiment was then performed with strain ND063311, which is resistant to clindamycin (MIC, 64 μg/ml) but equally sensitive to NAI003 (Table 2). While NAI003 could be used again at 1×, 10×, and 100× the MIC, we could test clindamycin only at 1× (i.e., 64 μg/ml) and 4× (i.e., 256 μg/ml) the MIC. The results are shown in Fig. 2B. Neither clindamycin nor NAI003 at 1× the MIC had a significant effect on the viable counts of the strain but simply resulted in growth retardation and 1-log-lower CFU per milliliter at stationary phase. At higher concentrations, NAI003 was able to decrease the number of viable counts in a dose-dependent manner, with again a 3-log reduction seen after 6 days. Interestingly, the behaviors of NAI003 and clindamycin at intermediate concentrations (10× and 4× the MIC, respectively)

TABLE 3 MICs of GE2270A, compound 2, and NAI003 against *P. acnes*

Propionibacterium acnes strain	MIC (μg/ml) of antibiotic:				
	GE2270A	Compound 2	NAI003	Erythromycin	Clindamycin
ND062411	≤0.0018	0.03	0.015	0.03	0.06
ND062511	≤0.0018	0.06	0.015	0.06	0.125
ND062611	≤0.0018	0.06	0.06	0.06	0.06
ND062711	≤0.0018	0.06	0.015	0.03	0.06
ND062811	≤0.0018	0.06	0.015	0.03	0.06
ND062911	≤0.0018	0.03	0.03	0.06	0.03
ND063011	≤0.0018	0.03	0.007	0.06	0.5
ND063111	≤0.0018	0.06	0.015	0.06	0.06
ND063211	≤0.0018	0.03	0.015	0.06	0.06
ND063311	0.0009	0.03	0.015	>128	64
L627	≤0.015	NT[a]	0.06	NT	NT
ATCC 6919	0.008	NT	0.06	NT	NT
ATCC 6922	0.03	NT	0.13	NT	NT
ATCC 25746	0.03	NT	0.25	NT	NT
L1559	0.008	NT	0.06	NT	NT
L1560	0.015	NT	0.06	NT	NT
L1561	0.015	NT	0.06	NT	NT
L1562	0.008	NT	0.06	NT	NT
L1563	0.008	NT	0.06	NT	NT
L1564	0.015	NT	0.06	NT	NT
L1565	0.015	NT	0.06	NT	NT

[a] NT, not tested.

were similar, with some decrease in viable counts up to 3 to 4 days, followed by a tendency of viable counts to rebound (Fig. 2B).

In conclusion, these experiments indicate that NAI003 at 100× the MIC (i.e., 1.5 μg/ml) can decrease the number of viable counts to similar extents for both a clindamycin-sensitive (strain ND062711) and a clindamycin-resistant (ND063111) strain. In contrast, killing by clindamycin can occur against the sensitive strain at lower MIC multiples and concentrations (i.e., 0.06 μg/ml) than those for NAI003. However, with the clindamycin-resistant strain tested here, 256 μg/ml clindamycin led to a lower reduction in viable counts than 1.5 μg/ml NAI003.

NAI003 binds to and inhibits the function of EF-Tu from *Escherichia coli*. To confirm that the molecular target of NAI003 is EF-Tu, the assay described by Anborgh and Parmeggiani was initially used (12). This test takes advantage of the different mobilities of free *E. coli* EF-Tu–GTP and of the EF-Tu–GTP–GE2270A complex in native polyacrylamide gel electrophoresis (12, 24).

The results shown in Fig. 3 indicate that NAI003 is indeed able to alter the electrophoretic mobility of *E. coli* EF-Tu, thereby confirming that this elongation factor is also the target of this molecule. It is also interesting that the difference in the electrophoretic

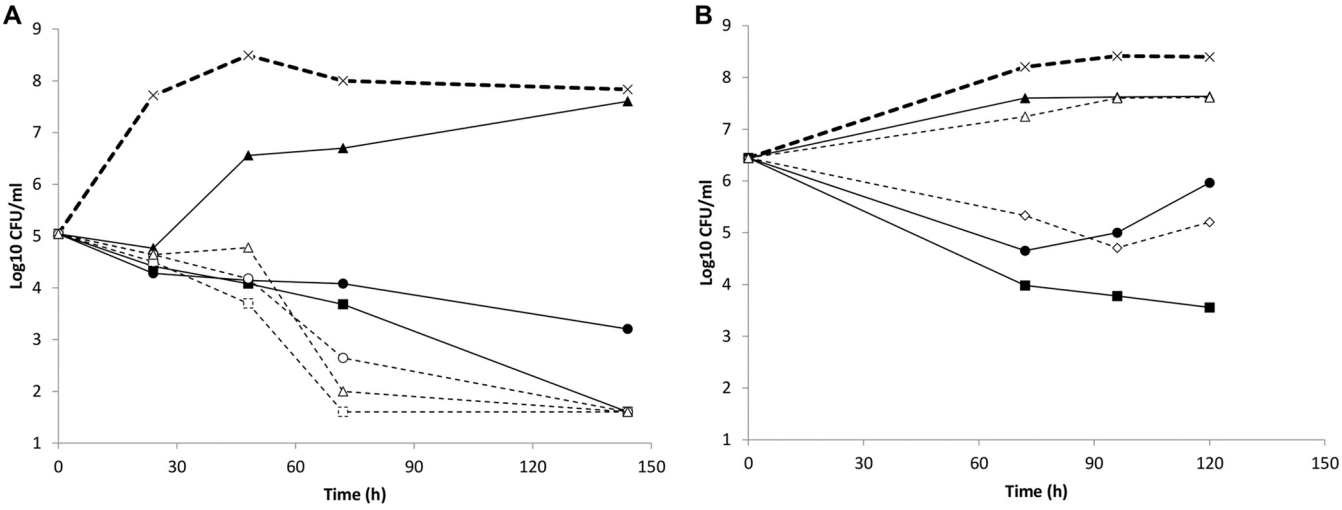

FIG 2 Killing kinetics of *P. acnes*. Effect of NAI003 (closed symbols, solid lines) and clindamycin (empty symbols, dashed lines) on the viability of *P. acnes*, using the clindamycin-sensitive ND062711 (A) and clindamycin-resistant ND06311 (B) isolates. Compounds were added at 1× MIC (triangles), 10× MIC (circles), or 100× MIC (squares). In panel B, clindamycin was used at only 1× (open triangles) and 4× (open diamonds) the MIC. Growth controls are shown for both panels by a thick dashed line.

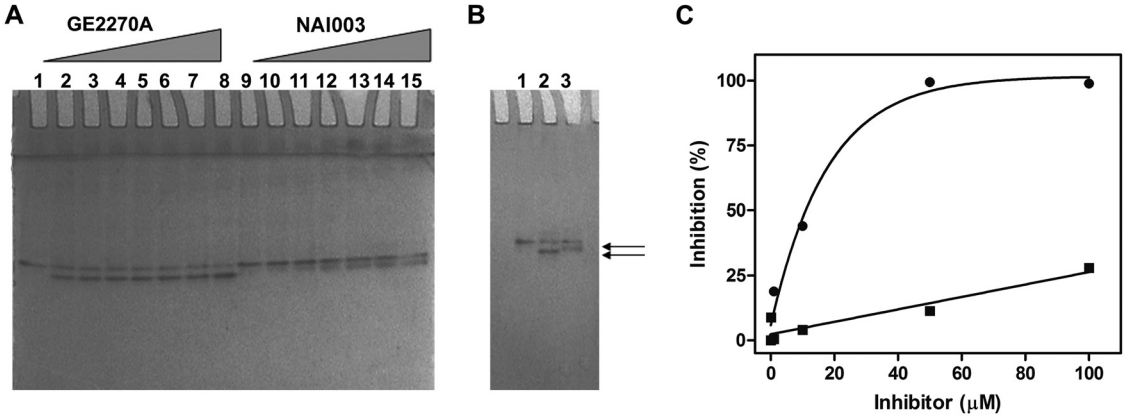

FIG 3 Effects of GE2270A and NAI003 on the electrophoretic mobility of EF-Tu and on *in vitro* translation. (A) Migration on native polyacrylamide gel of *E. coli* EF-Tu (preincubated with GTP) in the presence of increasing concentrations (from left to right, 0.1, 0.5, 1, 5, 10, 50, and 100 μM) of GE2270A (lanes 2 to 8) or NAI003 (lanes 9 to 15). (B) Electrophoretic mobility difference between EF-Tu–GTP alone (lane 1) and in the presence of 1 μM GE2270A (lane 2) or of 10 μM NAI003 (lane 3). The two arrows indicate the different migrations of EF-Tu in complex with GE2270A or with NAI003. (C) Inhibition by GE2270A (●) or NAI003 (■) in a protein synthesis system based on an *E. coli* extract programmed with 027-IF2Cp(A) mRNA.

mobilities between free and antibiotic-bound EF-Tu is smaller for NAI003 than for GE2270A (Fig. 3B). This finding suggests that the interaction of the two antibiotics with EF-Tu induces different conformational changes of the elongation factor. From the gel shift experiments, it can be surmised that low concentrations (0.5 to 1 μM) of GE2270A increase the mobility of more than 50% of EF-Tu, while greater concentrations (approximately 10 μM) of the derivative are required to shift the same amount of protein. In addition, from the gel shift experiments it can be observed that low concentrations (0.5 to 1 μM) of GE2270A increase the mobility of EF-Tu by more than 50%, while greater concentrations (approximately 10 μM) of the derivative are required to shift the same amount of protein.

GE2270A is known to inhibit *in vitro* protein synthesis in an *E. coli*-based cell-free system. When the effect of NAI003 was compared to that of GE2270A in an *E. coli in vitro* translation system programmed with the 027-IF2Cp(A) mRNA (17), both antibiotics proved to interfere with protein synthesis but to different extents: whereas GE2270A caused 50% inhibition of protein synthesis at a 10 μM concentration, a 10-fold-higher concentration of NAI003 was necessary to achieve 25 to 30% inhibition only (Fig. 3C).

From the results of the *in vitro* translation assays and from those obtained with the gel shift experiments, it is possible to deduce that both GE2270A and NAI003 form a stable interaction with elongation factor EF-Tu that causes an altered electrophoretic mobility of the protein in a native gel. However, the chemical modification of GE2270A which gives rise to NAI003 reduces by almost 1 order of magnitude the affinity of the latter antibiotic for EF-Tu and also reduces its efficiency as a protein synthesis inhibitor in *E. coli* (Fig. 3C).

Effect of NAI003 on different EF-Tus. To understand whether the difference in the antibacterial spectra of GE2270A and NAI003 might correlate with differences in affinities for the corresponding EF-Tus, *in vitro* experiments were carried out with EF-Tu purified from various organisms, namely, the NAI003-insensitive *S. aureus* and *S. pyogenes*, the moderately sensitive *E. faecalis*, and the highly sensitive *P. acnes*. To do so, the *tuf* genes encoding the EF-Tus from these organisms were amplified and cloned in the expression vector pETM-11, following essentially the method described by Zeng (16). This procedure and the presence of an N-terminal oligohistidine tail afforded sufficient amounts of protein from the four microorganisms after affinity chromatography. After purification, the oligohistidine tail was removed by treatment with the TEV protease and the affinities of these factors for NAI003 and for GE2270A were compared by gel shift assays as described above.

As seen in Fig. 4, the different EF-Tus bind GE2270A with approximately the same affinities, at least within the resolution limits of the experimental technique used, but display appreciable differences in their capacities to bind NAI003. Indeed, whereas the

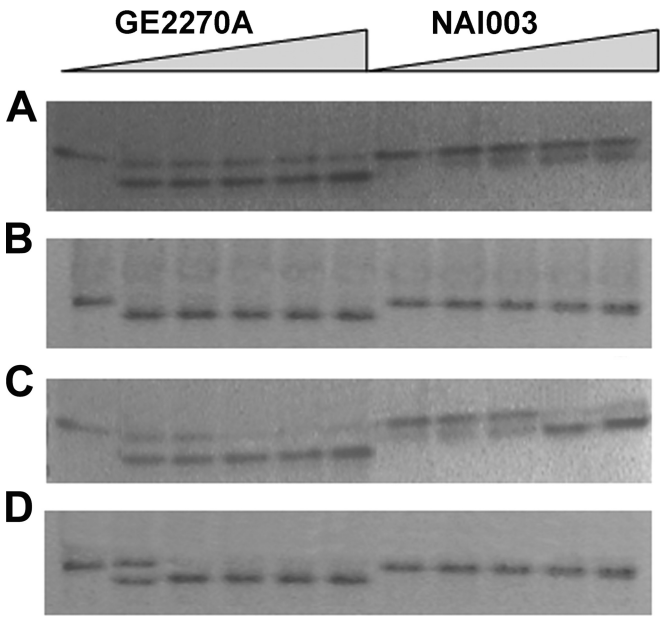

FIG 4 Effect of GE2270A and NAI003 on the electrophoretic mobilities of different EF-Tus. Migration on native polyacrylamide gels of EF-Tu from *E. coli* (A), *S. aureus* (B), *P. acnes* (C), and *S. pyogenes* (D) in the presence of increasing concentrations (1, 4, 19, 50, and 100 μM, respectively) of GE2270A (lanes 2 to 6) or of NAI003 (lanes 7 to 11).

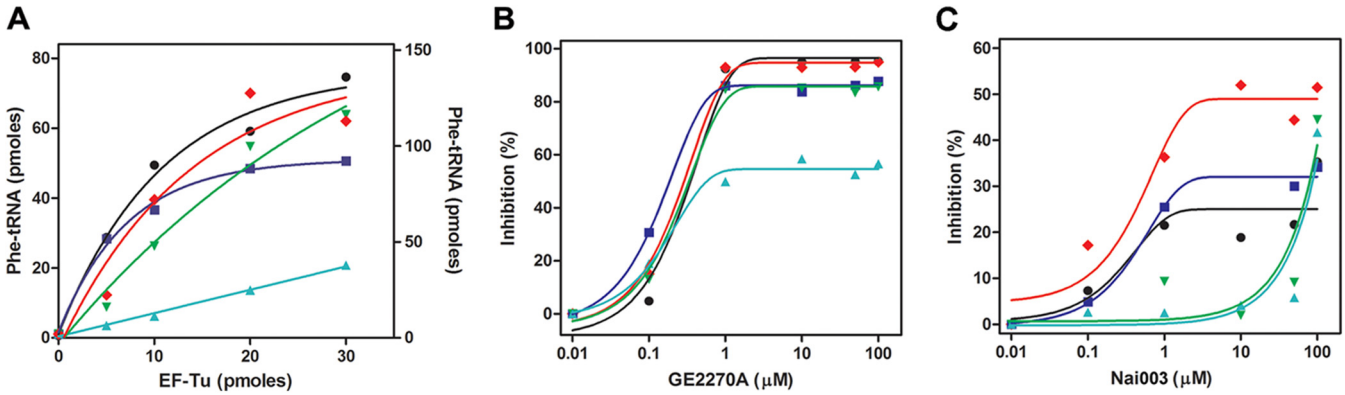

FIG 5 Effects of GE2270A and NAI003 on *in vitro* protein synthesis. (A) Poly(U)-dependent incorporation of [^{3}H]Phe in a hot-trichloroacetic acid-insoluble product in the presence of increasing concentrations of different EF-Tus. The amounts of Phe incorporated with *E. coli* EF-Tu are indicated on the right ordinate, while those obtained with EF-Tu from *P. acnes*, *S. aureus*, *E. faecalis*, or *S. pyogenes* are indicated on the left ordinate. (B and C) Effects of GE2270A (B) and NAI003 (C) on *in vitro* translation with different EF-Tus. Symbols for EF-Tu are as follows: black circles, *E. coli*; red diamonds, *P. acnes*; purple squares, *E. faecalis*; green inverted triangles, *S. aureus*; turquoise triangles, *S. pyogenes*.

proteins from *E. coli* and *P. acnes* can clearly bind NAI003, as judged from their different electrophoretic mobilities in the presence of increasing concentrations of the antibiotic, the same behavior was not observed for the factors from *S. aureus* and *S. pyogenes*, whose mobilities are hardly affected by the presence of NAI003.

The electrophoretic assay used in the experiments shown above yields qualitative but not sufficiently quantitative data to permit an estimation of the binding constants of the antibiotics for the various types of EF-Tu. In fact, the altered mobility is caused by the net electrical charge displayed on the protein surface as a result of antibiotic-induced conformational change. Different ligands can induce different changes on EF-Tu, and some of these alterations may not trigger variations of the electrical charge. Furthermore, the lack of a mobility shift does not constitute an absolute proof of the lack of an interaction between the elongation factors and the antibiotic. For these reasons, *in vitro* translation tests were carried out to detect a possible inhibition of EF-Tu function. Since EF-Tu is involved in the elongation stage of protein synthesis, these experiments were performed in a poly(U)-dependent poly(Phe) synthesis test, a translational assay that does not depend upon the complex initiation pathway of protein synthesis (17). The tests were carried out using a hybrid system in which ribosomes and EF-G purified from *E. coli* were incubated with [^{3}H]Phe-tRNA and a poly(U) template in the presence of an appropriate energy-regenerating system and of the appropriate EF-Tu protein.

When increasing amounts of EF-Tu originating from the selected bacteria were added to the heterologous cell-free system, the response was similar to that observed upon the addition of the homologous *E. coli* EF-Tu. The addition of 10 pmol of *E. coli* EF-Tu promoted the incorporation of ~90 pmol of [^{3}H]Phe in a hot-acid-insoluble product while the incorporation promoted by the same amount of EF-Tu from *P. acnes*, *S. aureus*, and *E. faecalis* was ~40 pmol. The only exception was found with *S. pyogenes* EF-Tu, whose efficiency was about 15% of that of *E. coli* (Fig. 5A). These preliminary data indicate that the EF-Tus from the selected bacteria are compatible with the *E. coli* components in the heterologous translation system. In turn, these data indicate that this type of test can be used to identify EF-Tu inhibitors, even in the case of the less efficient *S. pyogenes* EF-Tu-dependent system, and to compare the inhibitions by GE2270A and by NAI003 on the different EF-Tus.

The graph presented in Fig. 5B shows that protein synthesis was fully inhibited by GE2270A independently of the source of EF-Tu, with a 50% inhibitory concentration (IC_{50}) between 0.2 and 0.5 μM. With the exception of translation in the presence of *S. pyogenes* EF-Tu, at ~1 μM GE2270A inhibition reached 100% (Fig. 5B). The results obtained in the same test with NAI003 indicate that at ~1 μM this antibiotic caused ~25 to 30% inhibition when translation depended upon *E. coli* or *E. faecalis* EF-Tu but had no effect on the systems containing *S. aureus* or *S. pyogenes* EF-Tus, which are only slightly inhibited at very high NAI003 concentrations (Fig. 5C). On the other hand, the translational systems dependent on *P. acnes* EF-Tu proved to be the most sensitive to NAI003 inhibition, translation being reduced by >50% at ~1 μM NAI003 (Fig. 5C). These findings are overall consistent with the conclusions drawn from the data presented in Fig. 4 and allow for the conclusion that the replacement of the C-terminal moiety in GE2270A with the 4-amino-*N*-benzylpiperidine moiety present in NAI003 results in a weaker or different interaction of the latter molecule with EF-Tu and a general reduction of its inhibitory activity. However, this same chemical modification resulted in a molecule endowed with an increased selectivity in the inhibition of EF-Tu from *P. acnes*.

Resistance to NAI003 is due to mutations in *P. acnes* EF-Tu. The frequency of resistance to this class of thiopeptides was evaluated in parallel experiments with NAI003 and GE2270A. When *P. acnes* was plated on medium containing 1 or 10 μg/ml antibiotic, colonies appeared at frequencies of 6.3×10^{-11} or 3.1×10^{-11}, respectively, for GE2270A and at frequencies of 1.5×10^{-10} and 9.4×10^{-11}, respectively, for NAI003.

Twelve independent NAI003^r isolates were evaluated and characterized for the possible presence of mutations in the *tuf* gene, encoding EF-Tu, as well as for their susceptibilities to GE2270A, NAI003, and compound 2. With one exception, all resistant colonies harbored one mutation in the *tuf* gene for a total of 8 independent changes at 6 different positions (Table 4). Some of the observed mutations (G260R and G260C) occur at a residue (G257 in *E. coli* and *Bacillus subtilis* EF-Tu) previously reported to confer

TABLE 4 Genotypes and phenotypes of *P. acnes* NAI003^r mutants

Strain	*tuf* mutation[a]	EF-Tu		MIC (μg/ml) of antibiotic:		
		Substitution[b]	Domain[c]	NAI003	GE2270A	Compound 2
ATCC 6922				0.015	0.007	≤0.06
L1015R13	C207A	H68Q	I	8	0.015	0.5
L1015R51	T245C	V81A	I	32	≤0.06	1
L1015R6	G300T	Q99H	I	8	0.015	1
L1015R5	G300T	Q99H	I	2	0.007	0.5
L1015R7	G300T	Q99H	I	1	0.003	0.25
L1015R24	G781C	G260R	II	>128	0.125	0.25
L1015R8	G781T	G260C	II	32	0.125	2
L1015R96	T791C	M263T	II	4	≤0.06	4
L1015R73	A830G	N276S	II	1	0.007	0.125
L1015R72	A830G	N276S	II	2	0.007	0.25
L1015R21	C831G	N276K	II	1	0.03	0.25
L1015R86	None	None		128	0.25	4

[a] Nucleotide numbering is based on *tuf* sequence from *P. acnes* KPA171202 (15).
[b] Amino acid numbering is based on EF-Tu sequence from *P. acnes* KPA171202 (15).
[c] As defined by Parmeggiani et al. (11).

GE2270A resistance through the G257S substitution (11, 25, 26). Interestingly, in the three-dimensional (3D) structure of the complex between *Thermus thermophilus* EF-Tu, GTP, and GE2270A, a Gln residue (equivalent to Q99 in the *P. acnes* protein) makes direct contact with the antibiotic. Consistently, three independent *P. acnes* isolates carry the Q99H mutation. Finally, two independent mutations (N276K and N276S) were observed at an Asn residue (equivalent to N273 in the *E. coli* protein) that also makes direct contact with the antibiotic (11). When transposed to the crystal structure of *T. thermophilus* EF-Tu, the other observed mutations (Table 4) map to domain I (H68Q and V81A) or II (M263T), although they do not appear to be directly involved in antibiotic binding.

In terms of resistance levels, some mutations (i.e., V81A, G260C, and G260R) confer elevated protection to NAI003, while mutant strains harboring the other mutations exhibit intermediate resistance (Table 4). Remarkably, although the different mutations afforded an increased resistance to GE2270 or to compound 2 with respect to the parental strain, none of them was sufficient to confer resistance levels equivalent to those achieved against NAI003 (Table 3). It should be noted, however, that we sequenced the *tuf* gene only and the existence of additional mutations elsewhere in the genome cannot be ruled out. Indeed, one mutant (L1015R86) did not show any mutation in the *tuf* gene, despite its high level of resistance to NAI003 (Table 4). Thus, further studies will be necessary to understand the full range of mutations conferring resistance to NAI003.

DISCUSSION

The results presented here demonstrate that NAI003 binds to the EF-Tu from *P. acnes* with an affinity comparable to that of GE2270A and that its binding alters the electrophoretic mobility to the same extent as that observed with GE2270A. Furthermore, the translation test performed demonstrates that NAI003 inhibits *in vitro P. acnes* EF-Tu-dependent translation to a level similar to that obtained with GE2270. That EF-Tu is the target of NAI003 *in vivo* is confirmed by the observation that most spontaneous resistant mutants of *P. acnes* carry mutations in the *tuf* gene, corresponding to residues already established to confer resistance to or to bind GE2270A (11).

GE2270A is a better inhibitor of *P. acnes* EF-Tu than its derivative NAI003. Consistently, GE2270A MICs tend to be lower than those of NAI003. Nonetheless, we were surprised to observe that mutations in EF-Tu that confer complete resistance to NAI003 *in vivo*, some of which correspond to residues previously demonstrated to be critical for GE2270A binding, afford only partial protection to GE2270A in *P. acnes*. Further studies will be necessary to explain this observation.

We have demonstrated that the lack of sensitivity of many Gram-positive bacteria to NAI003 is correlated with a lower inhibition of their EF-Tus by this antibiotic. In this respect, the observed extent of EF-Tu inhibition (Fig. 5C) well correlates with the MIC values for the cognate microorganism (Table 1). It should be noted that GE2270A is able to effectively inhibit all EF-Tus tested so far, with natural resistance documented only in the producer strain *Planobispora rosea* (25) and in the *Corynebacterium* species tested here (Table 2). This is consistent with the fact that EF-Tu is a highly conserved protein with limited variation among divergent bacterial taxa. Despite these features, GE2270A has been converted by a relatively simple chemical modification into a derivative with a dramatically reduced antibacterial spectrum. This has resulted from the fortuitous occurrence of two factors: the highly reduced susceptibility of EF-Tus from the three *Firmicutes* species analyzed here and the lack of permeability of *E. coli* (and presumably of other Gram-negative bacteria) to this class of thiopeptides, which protect an otherwise partially sensitive EF-Tu. In light of these results, it would be interesting to conduct further experiments aimed at elucidating the 3D structure of the *P. acnes* EF-Tu complexed with NAI003 and compare it to the EF-Tu–GE2270A complex. This would provide insights into the nature of the different EF-Tu conformations induced by the two antibiotics and enable predictive models of which bacterial strains may be sensitive to NAI003 or to other GE2270A derivatives.

NAI003 was found to be equally active against 21 independent *P. acnes* strains, including one highly resistant to clindamycin and erythromycin (Table 3). Additional testing confirmed that this GE2270A derivative retains activity against several independent clindamycin- and erythromycin-resistant *P. acnes* clinical isolates (D. Jabes, unpublished data). This observation is not surprising,

since clindamycin and erythromycin resistance in *P. acnes* mostly involves mutation in 23S RNA (27–29), a site unrelated to the interaction of EF-Tu with the ribosome.

Antibacterial agents with an extremely narrow spectrum hold the promise of curing infections with minimal disturbance of commensal flora. This might be particularly true for topical applications, where a diverse and body-specific flora is expected to contribute to skin health (30–32). The compound previously designated BIK 0376 has already completed a clinical study in healthy volunteers as a topical application (32, 33). A clinical study will be necessary to establish its efficacy.

ACKNOWLEDGMENTS

This work was partially supported by a grant from Regione Lombardia (Progetto ATP).

We thank Gabriella Romanò and Paola Guglierame for early contributions to this project and Cristina Brunati for performing the time-kill assays. We are also grateful to Claudio O. Gualerzi for valuable advice and insights into this work.

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