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Antimicrobial susceptibility of anaerobic bacteria

Susceptibility of *Cutibacterium acnes* to topical minocycline foam

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ABSTRACT

FMX101 4% minocycline foam (FMX101 4%) is a novel, topical minocycline formulation for treatment of acne vulgaris. We report that FMX101 4% had an MIC_{90} of 0.25 μg/ml and was ≥4-fold more active than comparator antimicrobials against a panel of 98 clinical *Cutibacterium acnes* isolates. The panel was diverse by clonal complex and sequence type, having 20 novel multi-locus sequence types including clonal complexes and sequence types associated with acne (CC1, CC3, and CC4; ST1 and ST3). Some isolates were phenotypically resistant to clindamycin (6.1%), erythromycin (14.3%), and tetracycline (2.0% intermediate resistance). Six isolates (6.4%) carried a mutation in the quinolone resistance-determining region of *gyrA*. With *C. acnes*, spontaneous resistance to FMX101 4% occurred at frequencies ranging from $\leq 5 \times 10^{-9}$ to $<1 \times 10^{-8}$; mutations were identified in *rpsJ*, a gene encoding 30S ribosomal protein S10. No mutant exhibited a minocycline MIC above 0.5 μg/ml. No second-step mutation in previously isolated mutants or strains containing *rpsJ* ± 16S rRNA mutations was detected following minocycline challenge. Minocycline retained antibacterial activity against *C. acnes* over 15 multiple passages; thus, no selective growth advantage for minocycline-resistant mutants occurred under the experimental conditions. FMX101 4% has the potential to retain the favorable resistance profile of minocycline in diverse *C. acnes* isolates while providing the benefits of a topical formulation for treatment of acne vulgaris.

1. Introduction

Acne vulgaris (AV) is a common skin disease affecting approximately 50 million persons in the United States; it is the most prevalent skin disease of adolescence [1,2]. *Cutibacterium acnes* (previously called *Propionibacterium acnes*) [3] and inflammatory mechanisms play key roles in the development of AV [1]. Minocycline and doxycycline are effective in treating AV due to their antibacterial and anti-inflammatory properties [1,2,4]. These tetracycline antibiotics are highly recommended (Grade A recommendation) as treatment options for moderate-to-severe AV by the American Academy of Dermatology [1]. Only oral formulations of doxycycline and minocycline are currently available. Although effective, oral antibiotics are associated with potentially serious systemic effects, an increased risk of bacterial resistance, and consequent treatment failure [1,2,5,6].

Topical formulations with clindamycin and erythromycin are available and are approved to treat AV, but their use has been limited by increasing reports of resistance [1,7]. In contrast, a lower prevalence of resistance in *C. acnes* has been observed for tetracyclines [8–10]: within the tetracycline class, minocycline has the lowest minimal inhibitory concentration (MIC) among resistant *C. acnes* isolates [11,12]. Minocycline is approved, and commonly used, for the treatment of acne in Japan [9]. A 2017 Japanese study, which examined 69 clinical isolates from patients with acne, found that minocycline exhibited the lowest MIC with the tetracycline-resistant isolates (≤8 μg/ml), followed by doxycycline (≤16 μg/ml) and tetracycline (≤32 μg/ml) [12]. These isolates had at least 3 genetic modifications [12]. Thus, with respect to resistance, minocycline appears to be the best choice for development of a new topical antibiotic for acne.

The challenge in developing minocycline as a topical agent was

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finding a formulation that restricted minocycline degradation due to intrinsic photosensitivity, ease of oxidation, and inactivation upon contact with water [13]. Since minocycline is one of the more lipophilic of the tetracycline family, that property was used to formulate an oleaginous foam vehicle that also contained stabilizers [13]. The result was a novel, foam-based minocycline, FMX101 4%, for treatment of moderate-to-severe AV that would leverage minocycline's anti-inflammatory [14] and bacteriostatic properties [1] while also minimizing, primarily, systemic adverse events associated with oral administration. In this regard, a Phase 1 maximum-use safety and pharmacokinetics study has demonstrated that once-daily topical administration of up to 4 g of FMX101 4% for 21 days resulted in systemic exposure ~750 times lower than that following a single oral dose of minocycline at the recommended dose of approximately 1 mg/kg [15].

To examine FMX101 4% as a potential treatment for AV, we investigated susceptibility to FMX101 4% foam and comparator antibiotics with a phenotypically diverse set of clinical isolates of *C. acnes* and assessed the potential for the emergence of resistance. *C. acnes* isolates used in the susceptibility studies were subjected to whole-genome sequencing (WGS) and multi-locus sequence typing (MLST) to characterize the panel's genotypic diversity and to identify resistance to antibiotics, including tetracycline-resistance mutations associated with the *rpsJ* and 16S ribosomal genes (these mutations were previously identified as contributing to reduced susceptibility to doxycycline in *C. acnes*) [12]. The present resistance studies were carried out utilizing a diverse set of *C. acnes* isolates that included intrinsically tetracycline-susceptible and tetracycline-resistant isolates.

2. Materials and methods

Bacterial isolates. Clinical isolates (n = 98) of *C. acnes* isolated between 2007 and 2018 were obtained from the freezer (−80 °C) collection of International Health Management Associates (IHMA) (Schaumburg, IL). Countries of origin were primarily the United States (n = 76, 77.6%) and Australia (n = 18, 18.4%). Sources of infection were skin/wound (n = 36, 36.7%), eye (n = 19, 19.4%), and other (n = 43, 43.9%).

Additional isolates included the facial acne isolate *C. acnes* ATCC 6919, our designated reference strain, and quality control (QC) strains *Bacteroides fragilis* ATCC 25285, *Clostridioides difficile* ATCC 700057, and *Eggerthella lenta* ATCC 43055 obtained from the American Type Culture Collection (ATCC; Manassas, VA).

Anaerobic procedures. Anaerobic studies were performed using Clinical and Laboratory Standards Institute (CLSI) guidances [16,17] using an anaerobic chamber Model AS-580 (Anaerobic Systems, Morgan Hill, CA) with a controlled atmosphere of 5% H_2, 10% CO_2, and 85% N_2. Briefly, all media (broth or agar) were made and reduced in an anaerobic chamber at 36 °C ± 1 °C prior to experimental use. Brucella broth (Fisher Scientific™, Hampton, NH) supplemented with hemin (5 μg/ml), vitamin K_1 (1 μg/ml), and 5% (v/v) lysed horse blood and Brucella agar (Fisher Scientific™, Hampton, NH) supplemented with hemin (5 μg/ml), vitamin K1 (1 μg/ml), and 5% (v/v) laked sheep blood were used for all experiments and will be referred to as sBrucella broth and sBrucella agar, respectively. Preparation of circular 15 × 100 Petri plates was done by adding 1 ml of laked sheep blood and 2 ml of 10 × antimicrobial agent solution to 17 ml of molten Brucella agar (maintained at 48−50 °C) that had been previously supplemented with hemin and vitamin K_1. Broth macrodilution tubes were prepared similarly. Broth microdilution plates were prepared by mixing 50 μl of inoculum with wells containing an equal volume of 2-fold serial dilutions of an antibiotic (2 × final concentration) in sBrucella broth.

Antibiotics and *in vitro* susceptibility testing. FMX101 4%, FMX101 vehicle, and micronized minocycline were provided by Foamix Pharmaceuticals, Inc. USA (Bridgewater, NJ). Minocycline powder was obtained from Sigma (North Liberty, IA) for the larger MIC_{90} study. Other antibiotics were purchased from the following companies: tetracycline (United States Pharmacopeia [USP], Rockland, MD, or Sigma, North Liberty, IA), doxycycline (USP, Rockland, MD), clindamycin (USP, Rockland, MD), erythromycin (Fisher Scientific™, Hampton, NH, or USP, Rockland, MD), bacitracin (Fisher Scientific™, Hampton, NH, or TOKU-E, Burlington, NC), neomycin (Fisher Scientific™, Hampton, NH, or USP, Rockland, MD), fusidic acid (Fisher Scientific™, Hampton, NH, or TOKU-E, Burlington, NC), benzoyl peroxide (Sigma, North Liberty, IA) or Proactiv® and mupirocin (Selleckchem, Houston, TX, or TOKU-E, Burlington, NC).

Drugs were prepared as recommended by CLSI [16] except for FMX101 4% and FMX101 foam vehicle, which went through a degassing procedure consisting of gentle heating (40 °C) and stirring to remove propellant. The resulting products were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 8 mg/ml using a starting concentration of 40 mg/g as supplied by the manufacturer. This solution was further dissolved in sBrucella broth per CLSI 2012 guidance [17] and serially diluted into prepared sBrucella broth or agar, with a final DMSO concentration <0.1%.

Strains were grown anaerobically at 36 ± 1 °C on pre-reduced s*Brucella* agar or broth unless otherwise noted. For susceptibility testing, a direct colony suspension, obtained from isolated colonies on an sBrucella agar plate, equivalent to a 0.5 McFarland standard following growth for 36−42 h, was prepared in sBrucella broth. The inoculum was diluted 1:150 into sBrucella broth. For agar dilution methodology, an aliquot of each suspension was placed into the corresponding well in a Steer's replicator (CMI-Promex, Inc., Pedricktown, NJ) inoculum block. The agar plates were marked for orientation of the inoculum spots, and each inoculum (approximately 3 μl) was applied to the agar surface using the replicating device. Alternatively, 3 μl of inoculum were placed directly on the surface of previously marked agar plates using a micropipette. For broth dilution assays, the inoculum was added in equal volume to a series of tubes in which antibiotic concentration differed by increments of 2. Growth control plates or broth tubes lacking antimicrobial agents were included and incubated both aerobically and anaerobically with all experiments. All plates or broth tubes containing test materials were incubated at 36 ± 1 °C under anaerobic conditions; the final inoculum was 10^5 CFU per spot when using agar methodology and 10^6 CFU/ml when broth dilution was used. MIC endpoints were determined according to CLSI guidance for anaerobic bacteria [16,17].

Determination of MIC range for FMX101 4% and minocycline. To determine a preliminary MIC range of FMX101 4% and minocycline with the anaerobic QC strain *B. fragilis* ATCC 25285, 20 independent inocula were evaluated using the range of 0.016−8 μg/ml for FMX101 4%, 0.031−4 μg/ml for tetracycline, and 0.016−8 μg/ml for clindamycin, in both broth macrodilution and agar studies.

When a small tester panel of *C. acnes* isolates (n = 8) (Table 1) was examined for antimicrobial susceptibility to minocycline, clindamycin, tetracycline, FMX101 4% foam, and FMX101 4% foam vehicle, the range of antibiotic concentration tested on sBrucella agar was 0.03−32 μg/ml. QC strain *B. fragilis* ATCC 25285 was also included.

C. acnes isolates (n = 98) from IHMA were tested for susceptibility to FMX101 4% and comparator antibiotics using the following ranges: 0.03−32 μg/ml for FMX101 4%, tetracycline, minocycline, and clindamycin; 0.12−64 μg/ml for erythromycin, bacitracin, neomycin, mupirocin, and fusidic acid; and 1−512 μg/ml for benzoyl peroxide. QC strains *B. fragilis* ATCC 25285, *C. difficile* ATCC 700057, and *E. lenta* ATCC 43055 were included in each test set.

Table 1
Susceptibility of *C. acnes* to FMX101 4% and comparators by agar dilution.

Strain	Agar MIC (μg/ml)				
	FMX101 4%	Vehicle	MIN[a]	TET[b]	CLI[c]
C. acnes 775411	0.06	≥32	0.06	0.25	0.06
C. acnes 775419	0.06	≥32	0.06	0.25	0.06
C. acnes 775454	0.25	≥32	0.25	1	0.06
C. acnes 775473	0.06	≥32	0.06	0.25	0.06
C. acnes 775484	2	≥32	2	8	≥32
C. acnes 775486	2	≥32	2	8	≥32
C. acnes 775491	2	≥32	2	8	1
C. acnes 6919	0.06	≥32	0.12	0.5	0.06
B. fragilis 25285	≤0.03	≥32	≤0.03	0.5	2

Abbreviations.
[a] MIN, minocycline.
[b] TET, tetracycline.
[c] CLI, clindamycin.

Since there were only QC ranges for tetracycline and clindamycin with the anaerobic strains, aerobic strains *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and *Enterococcus faecalis* ATCC 43055 served as QCs for minocycline. All QC strains tested within the ranges from CLSI M100 guidance [16].

Minimal bactericidal concentration (MBC) determination. MBC was determined for clinical strains of *C. acnes* using CLSI guidances [16–18]. Broth microdilution, similar to broth macrodilution, as described in the CLSI method for antimicrobial susceptibility testing of anaerobic bacteria [17], was used. MIC values determined by broth microdilution in the laboratory of the Institute for Life Science Entrepreneurship (ILSE, Union, NJ) were found to be equivalent to, or within a 2-fold difference between, the MIC values determined using agar dilution (data not shown). To establish MBC values, the total number of viable cell counts in each well was determined by plating 10 μl of culture from the broth wells for all wells above the MIC (≥5 higher antibiotic dilutions) and compared to the CFU/ml in the starting inoculum (studies performed in duplicate, with each sample plated in duplicate). MBC was interpreted as the lowest concentration that demonstrated a ≥3-log reduction in CFU/ml (99.9% kill) when compared with the initial inoculum. MBC:MIC ratios were interpreted using established criteria such that the antibiotic is considered bactericidal if the MBC:MIC ratio is ≤ 4, and bacteriostatic when the ratio is > 4 [18,19].

Bacterial genome sequencing. Total DNA was extracted from the cells obtained from approximately 25% of an agar plate covered with 48-h-old *C. acnes* growth (~2.5–5.0 × 10^9 CFU) using the DNeasy® UltraClean® Microbial Kit (Qiagen, Germantown, MD) per the manufacturer's instructions. DNA quality and purity were assessed by NanoDrop™ spectrophotometer (ThermoFisher Scientific ™, Hampton, NH) and Qubit™ fluorometer (ThermoFisher Scientific™, Hampton, NH) analysis, followed by Illumina library construction and paired-end (PE150) sequencing on an Illumina NextSeq. The resulting sequence data were subjected to an in-house-developed *de novo* assembly pipeline (ILSE, Union, NJ), parent/daughter sequence comparison, and mutational analysis (single nucleotide polymorphism [SNP], indel, and rearrangement) using the CLCBio software suite of tools (v 9.01; Qiagen). The genome sequence for *C. acnes* ATCC 6919 (Genbank accession # NZ_CP023676.1) was used as the reference *C. acnes* strain. All isolates were sequenced to >100-fold coverage across the respective genome.

MLST typing of isolates and identification of resistance factors. MLST was determined for each strain of *C. acnes* using the expanded multilocus sequence-typing scheme [20] and was assigned using pubMLST (https://pubmlst.org/cacnes/). MLSTs were denoted by sequence type_clonal complex_phylotype. Novel MLSTs were assigned sequentially in the form FMX_ST-XX. Known antimicrobial resistance factors were identified through use of an in-house-developed *de novo* annotation pipeline (ILSE, Union, NJ), incorporating the Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca/) and ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) software and databases. Previously identified resistance mutations associated with *rpsJ* and 16S ribosomal genes were identified through direct BLAST query and multi-sequence alignment.

Spontaneous resistance frequency and mutant prevention concentration (MPC). Spontaneous or single-step mutational frequency was calculated as the number of mutants that grew on an antibiotic-selective plate divided by the number of cells plated. The MPC was determined as the concentration of minocycline in which no colony grew when 10^9 cells were tested.

For preparation of plates, FMX101 4% or minocycline was added to sBrucella molten agar at 50 °C to yield 20 ml of agar at the correct multiple of the 2, 4, 8, or 16 × the respective strain's MIC for the antibiotic. Plates containing no antibiotic were also prepared and used to determine viable counts.

For preparation of inocula, growth from a 48-h plate of each bacterial strain was harvested by flooding the plate with 1 ml sBrucella broth and gently using a sterile spreader to resuspend the bacterial cells. Bacteria were collected into sterile tubes, and CFU/ml for each strain was determined by serial dilution and plating on sBrucella agar. Each inoculum was prepared and used within 15 min.

For plating of bacterial suspensions, aliquots (2 × 100 μl) of the harvested culture containing ~1 to 2 × 10^{10} CFU/ml were plated on antibiotic-containing medium. Plates were incubated anaerobically for 5 days at 36 ± 1 °C and evaluated for growth daily. Colonies that grew during this period were confirmed for reduced susceptibility by passage on agar containing antibiotic at the selective concentration and subsequently archived at −80 °C for further analysis. MIC testing of presumptive mutants was determined by agar dilution following CLSI guidelines [17].

Second-step mutants. High-density inoculum cultures of *C. acnes* strains that were nonsusceptible to minocycline by at least one known mechanism were grown and spread on antibiotic-containing agar plates as described above and incubated anaerobically for 7 days. Colony counts, passaging of resistant colonies, and calculation of mutational frequency were done as described under spontaneous resistance frequency.

Resistance development by multiple passage. Eight isolates of *C. acnes* were cultured on sBrucella agar and incubated anaerobically at 36 ± 1 °C for 42–48 h. The multiple-passage study was initiated by suspending cells to a density equivalence of 0.5 McFarland and using 20 μl to inoculate each of 5 tubes containing 2 ml of sBrucella broth with minocycline or clindamycin at concentrations bracketing the MIC for the respective strains. Each broth macrodilution tube was incubated anaerobically for 48–72 h, and 20 μl of culture suspension from the tube with the highest antibiotic concentration showing growth was used as the inoculum for the subsequent passage. The remainder of the inoculum tube was diluted with the addition of 1 ml sBrucella broth containing 60% glycerol and was archived at −80 °C. This study was conducted for 15 cycles over a period of approximately 30 days.

3. Results

Preliminary QC range FMX101 4%. As a baseline for consideration of antibiotic susceptibility in *C. acnes*, the susceptibility of the anaerobic QC strain *B. fragilis* ATCC 25285 was determined by both agar dilution and broth macrodilution to provide a preliminary QC

Table 2
FMX101 4% *in vitro* activity against 98 *C. acnes* isolates.

Antibiotic	MIC (μg/ml)			
	MIC_{50}	MIC_{90}	%S	Range
FMX101 4%	0.12	0.25	NB[a]	0.06–1
Minocycline	0.25	0.5	NB	0.06–2
Tetracycline	0.5	1	98.0%	0.5–8
Clindamycin	≤0.03	2	93.9%	≤0.03 – >32
Erythromycin	≤0.12	32	85.7[b]	≤0.12 – >64
Benzoyl peroxide	256	512	NB	128 – >512
Bacitracin	1	1	NB	≤0.12–4
Fusidic acid	8	8	NB	0.25–8
Mupirocin	>64	>64	NB	>64 – >64
Neomycin	8	8	NB	≤0.12–32

[a] NB, no breakpoint, based on CLSI guidance [16].
[b] Breakpoint determined using MIC ≥2 μg/ml [50].

range. These measurements, which were done in a single laboratory, yielded an MIC of 0.03 μg/ml for all inocula by agar (n = 20) and broth macrodilution (n = 20). The preliminary QC range of MIC values for FMX101 4% was established as 0.016–0.06 μg/ml to allow for the known ±1-log_2 doubling dilution, the normal technical variability of antimicrobial susceptibility testing [21].

Tester panel of *C. acnes* isolates. FMX101 4% was compared in 8 strains of *C. acnes* to its active ingredient, micronized minocycline powder, and to comparators tetracycline and clindamycin by agar methodology (Table 1). All MIC values of FMX101 4% minocycline foam and minocycline powder were within ±1 log_2 doubling dilution. The tetracycline MICs (8 μg/ml) for *C. acnes* 775484, 775486, and 775491 indicated intermediate sensitivity to this antibiotic according to CLSI guidance M100 [16]. The MICs of FMX101 4% minocycline foam and minocycline powder for the tetracycline-intermediate-resistant strains were 2 μg/ml, 4-fold lower than observed with tetracycline. *C. acnes* 775484 and 775486 were resistant to clindamycin (MIC ≥ 32 μg/ml), while the remaining *C. acnes* strains were susceptible to clindamycin. The vehicle foam had no detectable activity (MIC ≥ 32 μg/ml), supportive of testing only minocycline for susceptibility studies with *C. acnes*.

Susceptibility of clinical isolates of *C. acnes* (n = 98). MIC values for FMX101 4%, minocycline powder, and comparator antimicrobials were determined for 98 clinical isolates of *C. acnes* by agar methodology (Table 2). FMX101 4% exhibited the lowest MIC_{90} (0.25 μg/ml) of all compounds tested; it was one doubling dilution lower than for minocycline (MIC_{90} = 0.5 μg/ml) and 2 doubling dilutions lower than for tetracycline. MIC for FMX101 4% ranged from 0.06 to 1 μg/ml, for minocycline from 0.06 to 2 μg/ml, and for tetracycline from 0.5 to 8 μg/ml. By MIC_{90} comparisons, FMX101 4% was 4-fold more active than bacitracin and tetracycline, 8-fold more active than clindamycin, and ≥32-fold more active than neomycin, erythromycin, and fusidic acid. Mupirocin ($MIC_{50/90}$ = >64/>64 μg/ml) and benzoyl peroxide ($MIC_{50/90}$ = 256/512 μg/ml) had little activity against the strain set.

Sequence diversity analysis and resistance factors of *C. acnes* clinical isolates. MLST analysis of 94 *C. acnes* isolates identified 26 strains comprising 20 novel MLSTs, 6 strains in the CC1 clonal complex, 1 strain in CC2, 3 strains in CC3, 1 strain in CC4, 8 strains in CC5, 4 strains in CC6, and 3 strains in CC72 (Table 3). Twenty-one known sequence types (ST1, ST2, ST3, ST4, ST5, ST6, ST21, ST22, ST25, ST42, ST54, ST65, ST68, ST69, ST70, ST74, ST100, ST107, ST122, ST135, and ST137) were found, representing all 6 phylotypes (1A1, 1A2, 1B, IC, II, and III). By these criteria, the collection was diverse.

WGS data were evaluated to identify antibiotic-resistance mechanisms present within the *C. acnes* isolates (n = 94). In the panel, 4 isolates had one or more alleles with 16S rRNA G1058C (*E. coli* numbering), 10 isolates had an amino acid alteration in *rpsJ*, 11 isolates had either G2058 G/C or A2059G in 23S rRNA (*E. coli* numbering), and 6 isolates had QRDR mutations in *gyrA* (encoding fluoroquinolone resistance). Notably, the phenotype did not always match with the rRNA genotype, because *C. acnes* strains have 3 rRNA operons and phenotypic susceptibility may depend on the ratio of genes harboring a resistance mutation, similar to what has been observed with other species [22,23].

Table 3
Results of MLST analysis of *C. acnes* clinical isolates (n = 94).

MLST	Number
FMX-ST1_CC1_IA1	4
FMX-ST2_CC6_II	1
FMX-ST3_CC6_II	1
FMX-ST4_CC6_II	1
FMX-ST5_CC72_II	1
FMX-ST6_CC72_II	1
FMX-ST7_CC3_IA1	1
FMX-ST8_CC3_IA1	1
FMX-ST9_CC2_IA2	1
FMX-ST10_CC5_IB	3
FMX-ST11_CC4_IA1	1
FMX-ST12_CC5_IB	2
FMX-ST13_CC5_IB	1
FMX-ST14_CC5_IB	1
FMX-ST15_CC1_IA1	1
FMX-ST16_CC1_IA1	1
FMX-ST17_CC72_II	1
FMX-ST18_CC6_II	1
FMX-ST19_CC3_IA1	1
FMX-ST20_CC5_IB	1
ST1_CC1_IA1	14
ST2_CC2_IA2	5
ST3_CC3_IA1	7
ST4_CC4_IA1	8
ST5_CC5_IB	11
ST6_CC6_II	4
ST21_CC4_IA1	1
ST22_1A2	1
ST25_CC6_II	1
ST42_CC5_IB	2
ST54_IA1	1
ST65_CC6_II	2
ST68_CC72_II	1
ST69_CC72_II	3
ST70_CC107_IC	1
ST74_CC77_III	1
ST100_CC72_II	1
ST107_CC107_IC	1
ST122_CC1_IA1	1
ST135_II	1
ST137_II	1

Minimal bactericidal concentration (MBC). Seven *C. acnes* (Table 4) isolates were evaluated to determine the MBC of FMX101 4% and minocycline. All strains had an MBC range of >16 to >64 μg/ml for FMX101 4%. The MBC:MIC ratios were >32 μg/ml, consistent with bacteriostatic activity.

Frequency of spontaneous resistance, MPC, and antimicrobial susceptibility of mutants. *C. acnes* strains used in single-step resistance-development studies are shown in Table 5. Spontaneous resistance to FMX101 4% occurred at frequencies ranging from 1×10^{-8} to $\leq 5 \times 10^{-9}$ in *C. acnes*. Three *C. acnes* strains yielded viable colonies on medium containing concentrations of minocycline above the strain's respective MIC. *C. acnes* strains ATCC 6919 and 775411 yielded 17 and 2 colonies, respectively, with ≥4-fold decreased susceptibility to minocycline (Table 5). Isolate 775419 yielded 4 colonies with ≥2-fold reduced susceptibility on initial screening (Table 5). Minocycline MPCs were 1 μg/ml for *C. acnes* 775411 and *C. acnes* ATCC 6919, 0.25 μg/ml for *C. acnes* 775419, and

Table 4
C. acnes isolates and QC strains evaluated in MBC and second-step resistance studies.

Strain[a,b]	MLST[c]	Genotype		Source	Year Collected	MIC (μg/ml) by Agar	
		rpsJ[d]	16S rRNA[e]			MIN[f]	CLI[g]
C. acnes 775484[b]	ST3_CC3_IA1	Y58D	G1058C	Australia	2007	2	32
C. acnes 775486[b]	ST3_CC3_IA1	Y58D	G1058C	Australia	2007	2	32
C. acnes 775491[b]	ST69_CC72_II	Y58D	G1058C	Australia	2007	2	1
C. acnes 775411[a]	ST1_CC1_IA1	WT	WT	Australia	2007	0.06	0.06
C. acnes 775419[a]	ST69_CC72_II	WT	WT	Australia	2007	0.06	0.06
C. acnes 775454[a,b]	ST122_CC1_IA1	Y58D	WT	Australia	2007	0.25	0.06
C. acnes 775473[a]	ST1_CC1_IA1	WT	WT	Australia	2007	0.06	0.06
C. acnes 6919[a]	ST1_CC1_IA1	WT	WT	England	1920	0.12	0.06

[a]*C. acnes* strains utilized in determination of mutant prevention concentration studies.
[b]*C. acnes* strains used in second-step resistance studies.
[c]MLST, multi-locus sequence type: sequence type_clonal complex_phylotype.
[d]*rpsJ*: predicted wild type (WT) amino acid sequence or amino acid variant conferring reduced susceptibility to minocycline.
[e]16S rRNA: WT sequence for 16S rRNA or mutation conferring tetracycline resistance, using *E. coli* 16S rRNA numbering.
Abbreviations.
[f]MIN, minocycline.
[g]CLI, clindamycin.

0.06 μg/ml for *C. acnes* 775473 (Table 5).

When MICs for minocycline and comparator compounds were assessed, no mutant derived by spontaneous resistance exhibited a minocycline MIC above 0.5 μg/mL (Table 6). Mutations in *rpsJ*, the gene encoding 30S ribosomal protein S10, generally resulted in phenotypically minocycline-resistant mutants that had MIC ≤0.5 μg/ml and that demonstrated partial cross-resistance to other tetracycline-class antibiotics. Minocycline-resistant mutants isolated from *C. acnes* strain ATCC 6919 were ≥4-fold less susceptible to doxycycline and tetracycline relative to the parental strain. Some *C. acnes* mutants derived from ATCC 6919 and 775411 also had increased MICs to erythromycin, but no cross-resistance was observed with clindamycin or other tested antibiotics (Table 6).

Resistance determinants present in parental and single-step mutants. To determine the mutational event(s) leading to reduced susceptibility, wild-type and mutant isolates having ≥2-fold increased MIC to minocycline were subjected to WGS analysis (see Table 6). All isolates with reduced susceptibility to minocycline had acquired a mutation in *rpsJ* at amino acid positions 57 or 58 in ribosomal protein S10 (Table 6). The S10 (RpsJ) amino acid changes included K57N (11/14 mutants) and one mutant each with K57R, K57T, and Y58D. Several other mutations were identified in addition to those observed in *rpsJ*. Interestingly, all ATCC 6919 mutants, but not the parental strain, had an apparent mutation in the 23S rRNA gene (T1618C) in 30%–39% of the total sequence reads (data not shown). Since *C. acnes* has 3 copies of the rRNA operon [23], these data indicate that one copy of the 23S rRNA (one-third of the sequence reads) had likely incurred the T1618C mutation. Mutations in this region of the 23S rRNA have not been previously associated with antibiotic resistance; thus, the significance of this mutation, if any, is unknown. We also observed mutations scattered among genes that are not known to impact tetracycline resistance or synonymous mutations in ATCC 6919, 775411, or 775419. No mutation was detected within the ribosomal genes from other mutant or wild-type strains when compared to reference strain ATCC 6919.

Second-step resistance. The frequency of second-step mutations from minocycline exposure was evaluated in *C. acnes* strains that [1] already displayed reduced susceptibility to minocycline [2], were clinical isolates or mutants from single-step mutation studies with minocycline having MIC values of 0.25–0.5 μg/ml (strain 775454, Table 1; strains 775411: 8x-1, 775411: 8x-2; strain 775419: 2x-4 and strain 6919: 8x-4, Table 6), and [3] were clinical isolates from Australia having a minocycline MIC of 2 μg/ml (strains 775484, 775486, and 775491, Table 4). No second-step resistant mutant arising from strains harboring a mutation in *rpsJ* or both a mutation in *rpsJ* and 16S rRNA G1058C (*E. coli* numbering) was obtained from the 8 strains studied. The average frequency of resistance was $\leq 3.6 \times 10^{-10}$ for the 8 strains. A second attempt was made with the strains 775411: 8x-1, 775411: 8x-2, 775419: 2x-4, and 6919: 8x-4 using FMX101 4% as the selecting agent, but no mutant was isolated from 10^9 cells tested.

Multiple-passage studies. The propensity of *C. acnes* isolates, with various susceptibilities to minocycline and clindamycin, to develop resistance over the course of 15 sequential passages at subinhibitory concentrations was assessed. Of the 8 strains evaluated, 4 were wild type for the *rpsJ* and 16S rRNA genes (775411, 775419, 7754473, and ATCC 6919), and 4 contained either a *rpsJ* or a

Table 5
Frequency of resistance after single exposure to minocycline.

Strain	MIN[a] MIC (μg/ml)	No. MIN Resistant Colonies (≥4 ×)	Mutation Frequency[b]	MIN Resistant Colonies (≥2 ×)	Mutation Frequency	MPC[c] (μg/ml)
C. acnes ATCC 6919	0.12	17	5.70×10^{-8}	None[d]		1
C. acnes 775411	0.06	2	1.30×10^{-8}	None		1
C. acnes 775419	0.06	0	$\leq 3.80 \times 10^{-9}$	4	1.50×10^{-8}	0.25
C. acnes 775473	0.06	0	$\leq 5.00 \times 10^{-9}$	None		0.06
C. acnes 775454	0.25	0	$\leq 2.70 \times 10^{-9}$	None		0.5

Abbreviations.
[a] MIN, minocycline.
[b] Determined as the number of resistant colonies over the CFU screened.
[c] MPC, mutant prevention concentration; represents a threshold above which the selective proliferation of resistant mutants is expected to occur only rarely [51].
[d] No discernable colony was identified within a background haze.

Table 6
Susceptibility of *C. acnes* isolates and mutants isolated by single-step passage.

Strain[a]	*rpsJ* Genotype[b]	MIC (μg/ml)									
		ERY[c]	BCT[d]	NEO[e]	MUP[f]	BPO[g]	FA[h]	TET[i]	DOX[j]	CLI[k]	MIN[l]
C. acnes 775411	WT	≤0.12	1	32	≥128	400	2	0.25	≤0.25	0.06	0.03
775411: 8x-1[a]	K57N	0.5	1	16	≥128	200	2	0.5	0.5	0.06	0.5
775411: 8x-2[a]	Y58D	0.5	1	64	≥128	400	4	0.5	0.5	0.06	0.5
***C. acnes* 775419**	**WT**	**0.5**	**0.25**	**32**	**≥128**	**400**	**1**	**0.25**	**≤0.25**	**0.06**	**0.06**
775419: 2x-1	ND	0.25	0.25	32	≥128	200	4	0.25	0.5	0.06	0.12
775419: 2x-2	K57R	0.25	0.25	32	≥128	400	1	0.25	0.5	0.06	0.25
775419: 2x-3	K57T	0.25	0.25	32	≥128	400	2	0.5	0.5	0.06	0.25
775419: 2x-4[a]	K57N	0.5	0.25	32	≥128	200	4	1	≤0.25	0.06	0.25
***C. acnes* ATCC 6919**	**WT**	**≤0.12**	**0.5**	**16**	**≥128**	**200**	**2**	**0.25**	**≤0.25**	**0.06**	**0.06**
6919: 4x-1	K57N	2	1	8	≥128	200	4	2	1	0.06	0.5
6919: 4x-2	K57N	0.5	0.5	16	≥128	400	4	2	1	0.06	0.25
6919: 4x-3	K57N	1	0.5	16	≥128	400	4	2	1	0.06	0.25
6919: 4x-4	K57N	2	0.5	16	≥128	400	2	2	1	0.25	0.5
6919: 8x-1	K57N	≤0.12	0.5	32	≥128	200	4	2	1	0.06	0.25
6919: 8x-2	K57N	≤0.12	0.5	32	≥128	400	2	2	1	0.06	0.25
6919: 8x-3	K57N	≤0.12	1	64	≥128	400	2	2	1	0.06	0.25
6919: 8x-4[a]	K57N	≤0.12	1	32	≥128	400	4	2	1	0.06	0.25
6919: 8x-5	K57N	8	≥64	≥64	≥128	200	16	2	1	0.06	0.25

[a] Parental strains in bold; mutant convention is strain number: multiple of MIC on which mutant colony was isolated-number of clone from the agar plate.
[b] *rpsJ* gene was wild-type (WT) or contained the amino acid change indicated relative to the deduced amino acid sequence of the protein derived from the ATCC6919 reference strain.
[c] ERY, erythromycin.
[d] BCT, bacitracin.
[e] NEO, neomycin.
[f] MUP, mupirocin.
[g] BPO, benzoyl peroxide.
[h] FA, fusidic acid.
[i] TET, tetracycline.
[j] DOX, doxycycline.
[k] CLI, clindamycin.
[l] MIN, minocycline.

rpsJ/16S rRNA G1058C rRNA mutation prior to the start of the experiment. MIC was monitored during the course of passage (Fig. 1). Minocycline retained antibacterial activity against *C. acnes* regardless of parental minocycline susceptibility over the course of the 15 passages, with changes in overall MIC observed within ±1-$\log_2$ doubling dilution for all 8 strains examined (Fig. 1).

In contrast, the antimicrobial activity of clindamycin varied among tested strains: 4 of 8 cultures rapidly acquired resistance to clindamycin, with increases in MIC observed as early as passage 2. These data indicate that mutants having reduced clindamycin susceptibility have a competitive advantage over susceptible strains at sub-inhibitory concentrations of clindamycin.

4. Discussion

AV is associated with significant physical and psychological morbidity; it is one of the most common disorders treated by dermatologists [1]. Systemic and topical antibiotics have long been a mainstay of treatment for acne [10,24]. Perhaps unsurprisingly, the challenge with current treatments is that there is an increasing, global prevalence of antibiotic resistance reported for *C. acnes*, the bacterium associated with AV [10]. Oral doxycycline and minocycline are preferred over erythromycin for the treatment of acne because of their lower inherent risk for antibiotic resistance [1]. Available topical antibiotics have also been implicated in reports of rising resistance [7,8,10,11]. Their use as monotherapies has accordingly been curtailed, and it is recommended that, when used, they be combined with agents such as benzoyl peroxide to impede resistance development [1,7]. To maintain an effective, anti-inflammatory, antimicrobial treatment for AV, a topical minocycline foam has been developed (FMX101 4%). After application to the skin, the high local concentration of minocycline [13] is expected to help suppress the emergence of resistance.

To assess the activity of minocycline and FMX101 4%, susceptibility was determined with a phenotypically and genotypically diverse set of 98 clinical isolates of *C. acnes*, and the potential for resistance was assessed. FMX101 4%, which had an MIC_{90} of 0.25 μg/ml, was more active than 8 comparator antimicrobials. The frequency of spontaneous resistance was low. As expected, resistant mutants were recovered that exhibited amino acid sequence changes in small ribosomal protein S10, and the increases in minocycline MIC were 4- to 16-fold (see Table 6); however, the MIC values of the mutants were still 16-fold below the intermediate-resistant breakpoint (susceptible ≤4 μg/ml; intermediate, 8 μg/ml; resistant, ≥16 μg/ml [16]). Clinical isolates from this study and others [12] that have an elevated minocycline MIC (2 μg/ml) carry a mutation in 16S rRNA (G1058C, *E. coli* numbering) and in a variant *rpsJ* [12]. However, we were unsuccessful with *in vitro* second-step mutant and multiple-passage studies in isolating the target-based G1058C mutation or any other mutation conferring minocycline resistance in strains having a variety of genotypic and phenotypic backgrounds. Thus, minocycline, used in the topical formulation FMX101 4%, is microbiologically suitable for treatment of AV.

As with other tetracyclines [4], FMX101 4% (and minocycline) were bacteriostatic against *C. acnes* isolates. The absence of bactericidal activity is likely unimportant for patient response, since retrospective reviews of controlled trials report few instances of bactericidal drugs having better overall efficacy in the treatment of infection [25–27]. Indeed, recent clinical trials with FMX101 4% treatment in patients with moderate-to-severe acne confirmed that FMX101 4% significantly reduced both the inflammatory and noninflammatory lesion count from baseline at week 12 [28,29]. Part of the reason for this may be high minocycline concentrations at the target site, as indicated by a porcine ear skin penetration study using FMX101 4% [13]. The active ingredient, minocycline, concentrated in viable skin (epidermis and dermis, including

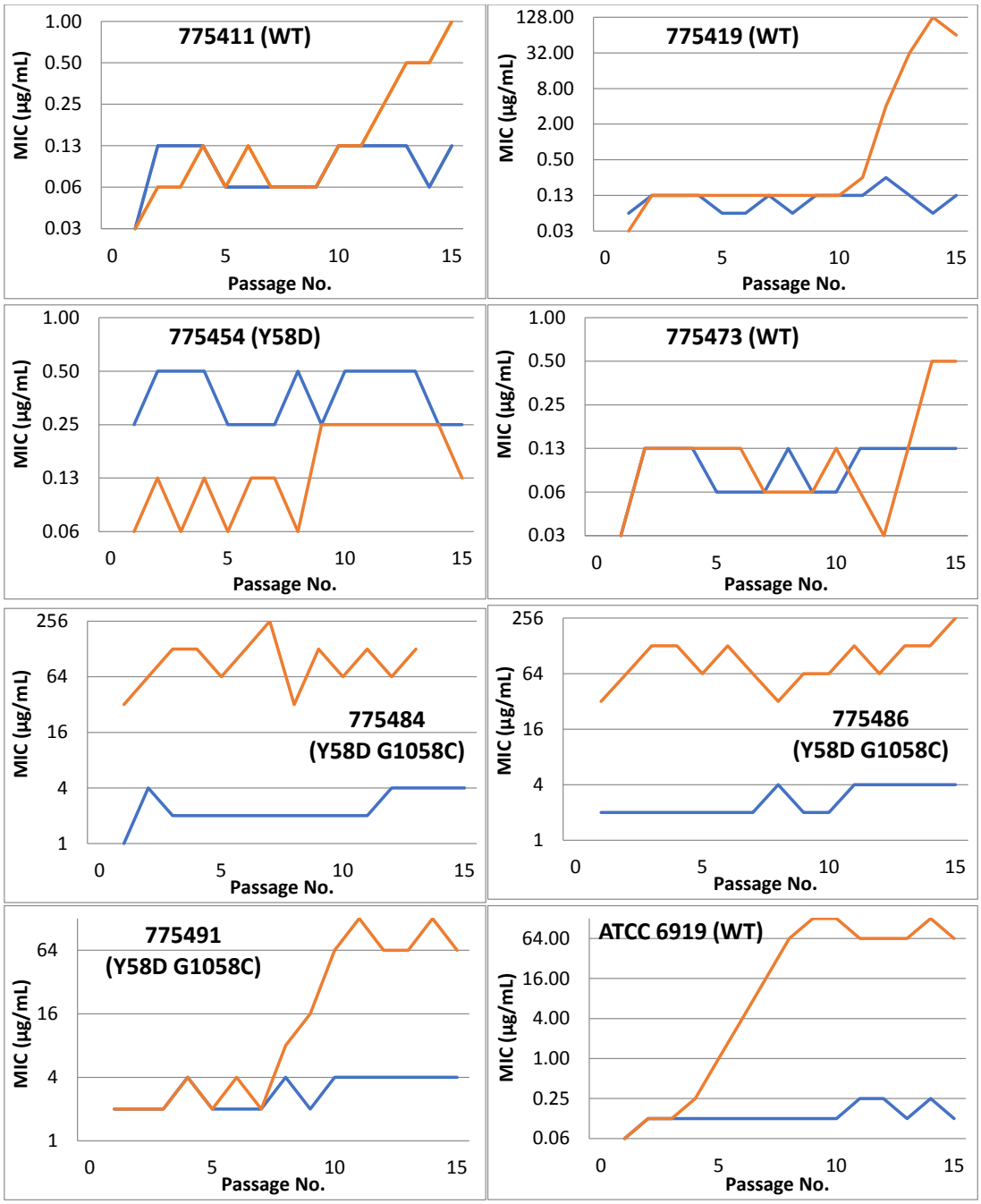

Fig. 1. Multiple-passage study of *C. acnes* in the presence of sub-inhibitory concentrations of minocycline or clindamycin. Eight strains, indicated in panels by strain numbers with relevant genotype in parentheses (WT is wild-type for *rpsJ* and contains 16S rRNA G1058; Y58D is a variant amino acid in *rpsJ*; G1058C is the variant in 16S rRNA that encodes target-based resistance to tetracycline). The multiple-passage study was initiated by suspending cells cultured on sBrucella agar for 42–48 h to a density equivalence of 0.5 McFarland; 20 µl was used to inoculate each of 5 tubes containing 2 ml of sBrucella broth with minocycline or clindamycin at concentrations bracketing the MIC for the respective strain/antibiotic combination and anaerobic incubation as described in CLSI guidances [16,17]. MIC values with minocycline (solid blue line) or clindamycin (solid orange line) were plotted by passage. All strains were evaluated twice by serial passage yielding similar results.

pilosebaceous units) at a mean content of 3.5 µg/g, or roughly 3500 µg/ml [13]. This value is > 1000-fold higher than the *C. acnes* MIC_{90} observed in this study. It is also >1000-fold higher than the MPC for selected isolates (Table 5).

With bacteriostatic agents, achieving high concentrations is central to restricting the emergence of resistance, the threshold for which is defined by the MPC [30,31]. A recent study determined the MPC of topical antimicrobial agents against *C. acnes* [32]. When we examined 4 tetracycline-susceptible isolates, we found that the minocycline MIC ranged between 0.06 and 0.12 µg/ml, and the MPC ranged between 0.06 and 1 µg/ml (Table 5). The values of MPC (mutant MIC) were well below the minocycline levels reported for the porcine ear model [13], indicating that resistance is likely to emerge only rarely. The mutant selection window (MPC/MIC) ranged widely, from 1 to 16, for reasons that are currently unknown (Table 5). We also examined 8 tetracycline nonsusceptible isolates. We were unable to obtain second-step resistance mutants: the MPC/MIC was essentially 2 (lowest concentration where no mutants were selected was 2x the MIC), with minocycline MICs ≤2 µg/ml. Given the concentration of minocycline recovered from viable

skin in the porcine model [13], it is likely that minocycline resistance will be suppressed by the high concentration of minocycline achieved with topical FMX101 4%.

When identifying strategies for restricting the emergence of resistance, there are several additional considerations, one of which is the molecular basis of resistance. In Gram-positive organisms, resistance to topical agents, such as mupirocin and chlorhexidine, is generally mediated by intrinsic efflux pumps or drug-resistance efflux pumps [33,34] that have been acquired on mobile elements (for a recent example with *S. aureus*, see Copin et al. [35]). Although tetracycline-specific and nonspecific efflux pumps exist in both Gram-positive and Gram-negative organisms [36], they have never been found to be the reason for tetracycline resistance in *C. acnes*. Likewise, there has been no report of tetracycline ribosomal protection proteins or modification enzymes in tetracycline-resistant isolates from AV patients. Indeed, *C. acnes* resistance to topical clindamycin and erythromycin products is mediated through mutations or modifications in 23S rRNA [23]. Thus, tetracycline (minocycline) resistance appears to be target-based in *C. acnes* [12].

The bacteria appear to acquire tetracycline resistance mutations in a step-wise fashion. For example, in experimental evolutionary models with several bacterial species, the first level of adaptation to tigecycline, a third-generation tetracycline, occurs in *rpsJ*, a highly conserved ribosomal protein that has a loop region located near the 16S rRNA tetracycline-binding pocket [12,36,37]. That mutation appears to precede rRNA changes that lower susceptibility in *C. acnes* based on the following evidence. In our study and a recent Japanese study, *C. acnes* G1058C (16S) rRNA mutations were found only in clinical strains that also contained *rpsJ* mutations [12]. Moreover, this same recent Japanese study determined that doxycycline, like minocycline, selected *rpsJ* mutations, but not G1058C mutations, in laboratory spontaneously resistant strains [12]. These findings, and our study, support the hypothesis that acquisition of tetracycline resistance in *C. acnes* occurs in a step-wise fashion, and that the first step of the acquisition is the *rpsJ* mutation [12]. In comparison, topical clindamycin and oral/topical macrolide use is accompanied by 23S rRNA mutations or ribosomal modification, such as A2058X, A2059X, or dimethylation of A2058 by *erm*(X), that confer co-resistance to each other [9,23].

Attempts to isolate second-step mutants from strains containing *rpsJ* mutations or from clinical isolates containing both *rpsJ* and target-based 16S rRNA G1058C (*E. coli* numbering) were unsuccessful. For example, 15 passages of clinical isolates ($n = 8$) with no known tetracycline-resistance mechanisms or with *rpsJ*, or *rpsJ* + 16S rRNA G1058C (minocycline and tetracycline MIC values of 2 and 8 μg/ml, respectively) failed to produce resistant mutants that could overgrow the wild-type population even under selective pressure. Thus, low doses were effective in this setting and did not lead to other stepwise mutations. Although FMX101 4% does deliver doses significantly above the MPC [13], low-dose strategies [38–40] targeting sub-MIC levels of antibiotic (oral doxycycline) have been used to effectively treat acne and rosacea systemically [40–43], with the goal of mitigating antimicrobial resistance. It should be noted, however, that selective pressure can begin at antibiotic concentrations significantly lower than the MIC. Gullberg et al. demonstrated in competition experiments that the minimal selective concentration of tetracycline against *Salmonella typhimurium* was approximately 100-fold below the MIC [44].

Failure to obtain second-step mutants *in vitro* does not rule out the acquisition of second-step mutational changes spontaneously or through genetic exchange with other organisms in the skin environment. Our results do, however, indicate that such secondary mutations, which might further lower susceptibility, will likely occur at a much lower frequency than the isolation of the *rpsJ* first-step mutation, which in our hands occurred at a low frequency (from 1×10^{-8} to $\leq 5 \times 10^{-9}$).

Another consideration is the diversity of *C. acnes*. Isolates examined to date consist of 5 highly distinct evolutionary lineages, known as type IA (clades $1A_1$ and $1A_2$), IB, IC, II, and III, which display differences in inflammatory properties, production of virulence determinants, and association with medical conditions [45–48]. Acne is predominantly associated with clade type IA_1, with clonal complexes CC1, CC3, and CC4 and ST1 and ST3 lineages being highly represented [20]. With the use of WGS and MLST analysis, our large collection of clinical *C. acnes* isolates was shown to be diverse based on predicted serotype and clonal complex, with all of the phylotypes represented (42 IA_1, 7 IA_2, 21 IB, 2 IC, 21 II, and 1 III) and 41 isolates belonging to CC1, CC3, or CC4. Identified antibiotic resistance markers included mutations associated with resistance to tetracyclines: 10 isolates with *rpsJ* mutations, 4 of which also contained the 16S G1058C ribosomal mutation, but even the minocycline MIC in the latter mutants did not exceed 2 μg/ml. Also uncovered were 11 isolates with 23S ribosomal mutations associated with resistance to macrolides and clindamycin; 6 isolates had mutations in the quinolone-resistance–determining region of *gyrA* expected to encode fluoroquinolone resistance [49]. Thus, FMX101 4% is likely to be effective against a range of *C. acnes* diversity seen in the clinic.

Overall, the data presented above, coupled with the results from phase 3 FMX101 4% acne studies [28,29], indicate that FMX101 4% has the antimicrobial potential to treat AV patients with genetically diverse *C. acnes* isolates at concentrations that will suppress the emergence of resistance. FMX101 4% was formulated to stabilize minocycline and to deliver high levels of minocycline to the pilosebaceous unit [13], where *C. acnes* resides. The data in this paper demonstrates that these high levels suppress emergence of resistance *in vitro*. This successful combination provides a new strategy for a topical agent for AV, and its use will provide the clinical test of the selection window hypothesis.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Joyce Sutcliffe, PhD, and Robert McLaughlin, PhD, served as investigators on the study. Guy Webster, MD, PhD, Andrew Read, PhD, and Karl Drlica, PhD, have served as consultants for Foamix Pharmaceuticals. Inc. USA. Russell Elliott, PhD, and Iain Stuart, PhD, are employees of Foamix Pharmaceuticals, Inc. USA.

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