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Antimicrobial Susceptibility Studies

In vitro spectrum of pexiganan activity; bactericidal action and resistance selection tested against pathogens with elevated MIC values to topical agents

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ABSTRACT

Pexiganan, in Phase 3 clinical development for topical use, exhibited bactericidal activity *in vitro* against Gram-positive and -negative isolates and was also shown to have a low potential for resistance development in broth serial passage experiments. Susceptibility studies were performed against bacterial isolates (110 total from 2004 to 2013; primarily from skin and soft tissue infections) selected for elevated MIC values (non-wildtype [WT] distributions) to bacitracin, polymyxin B, neomycin, mupirocin, retapamulin, fusidic acid, or gentamicin. A narrow range of pexiganan MIC values (4–32 μg/mL) against *Staphylococcus aureus* was observed (MIC_{50} and MIC_{90} values, 16 μg/mL) with a pexiganan mode and MIC_{50} value for the subsets of isolates with non-WT MIC values to bacitracin and neomycin ($n = 14$), fusidic acid ($n = 11$), mupirocin ($n = 12$) and retapamulin ($n = 11$) at 16 μg/mL. For coagulase-negative staphylococci (CoNS), the pexiganan mode and MIC_{50} values were 4 μg/mL. The pexiganan mode and MIC_{50} for each non-WT CoNS subset was also 4 μg/mL. Pexiganan MIC values for *Enterococcus faecium* was 8 μg/mL, but *E. faecalis* isolates exhibited MIC values that ranged from 128–256 μg/mL. Pexiganan was active against β-hemolytic streptococci including non-WT subsets (MIC range, 4–64 μg/mL). MIC values for pexiganan varied by species for viridans group streptococci, with highest values occurring for *Streptococcus oralis*. The broad bactericidal spectrum of pexiganan activity and low potential for resistance selection offers the possibility that this experimental agent may be able to play an important role in the current environment of emerging multi-drug resistant pathogens.

1. Introduction

Magainins are broad-spectrum cationic peptides which selectively damage bacterial membranes through mechanisms that make the development of resistance to this agent difficult (Boman, 1995; Hancock, 1997; Hancock et al., 1995; Nicolas and Mor, 1995). These peptides disrupt the outer membrane of Gram-negative bacteria. The damaged membrane allows the passage of a variety of molecules and promotes the uptake of the peptide (Hancock, 1997). The mechanism of bacterial killing for Gram-positive and Gram-negative bacteria is the formation of channels in the cytoplasmic membrane (Falla et al., 1996; Hancock et al., 1995).

Pexiganan is a 22-amino-acid synthetic analogue of peptide magainin II undergoing Phase 3 development as a topical agent (pexiganan cream 0.8% [8000 μg/mL pexiganan free base]) for treatment of mild infections of diabetic foot ulcers (NCT01590758, NCT01594762). The Phase 3 clinical studies are randomized, double-blind, multicenter, superiority, placebo-controlled trials of pexiganan cream 0.8% applied twice daily for 14 days in the treatment of adults with mild infections of diabetic foot ulcers. The goal of these studies is to establish the clinical superiority and the safety of topical pexiganan cream plus standard local wound care as compared to placebo cream plus standard local wound care.

In this *in vitro* study, the activity of pexiganan and its potential for resistance development were investigated against a collection of isolates including antimicrobial-resistant phenotypes. Additionally, pexiganan was evaluated against bacterial strains primarily selected as having elevated MIC values to currently used, or under investigation for topical use, topically applied antibiotics including bacitracin (BAC), polymyxin B (PMB), neomycin (NEO), mupiricin (MUP), retapamulin (RET), fusidic acid (FA), and gentamicin (GEN).

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2. Materials and methods

2.1. Organisms

In order to measure the bactericidal activity of pexiganan (MBC/MIC), a total of 21 isolates from Gram-positive and -negative bacterial

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species including multidrug-resistant (MDR) phenotypes were selected for testing. For resistance development studies, 10 isolates were chosen for serial passage determinations. To further characterize the activity of pexiganan, bacterial strains having elevated MIC results (compared to wild-type [WT] distribution) to currently available topical agents were chosen (110 total isolates [2004–2013] primarily from skin and soft tissue infections). These strains included 21 isolates with elevated MICs to PMB (MIC, >4 μg/mL), 22 isolates with elevated MICs to BAC (MIC, >100 μg/mL), 24 isolates with elevated MICs to NEO (MIC, >16 μg/mL), 20 isolates with elevated MICs to MUP (MIC, >8 μg/mL), 21 isolates with elevated MICs to FA (streptococcal MIC, >16 μg/mL and staphylococcal/enterococcal MIC, >4 μg/mL) and 22 isolates with elevated MICs to RET (MIC, >4 μg/mL). Among these isolates were 20 strains which exhibited elevated MIC results to both BAC and NEO. There was one high-level GEN non-susceptible isolate of *Staphylococcus aureus* (GEN MIC, 512 μg/mL).

2.2. Antimicrobial agents

For the MBC/MIC and serial passage experiments, the investigational compound (pexiganan) was provided by Dipexium Pharmaceuticals, (New York, New York, USA). Levofloxacin and FA were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA). PMB, BAC, NEO, GEN, and MUP were obtained from US Pharmacopeial Convention (Rockville, MD, USA). Pexiganan was tested over a 12 $\log_2$ dilution range (8192–4 μg/mL) and levofloxacin over 12 $\log_2$ dilution steps (16–0.008 μg/mL). For the evaluation of isolates with elevated MIC values to topical agents, the following MIC ranges were tested: pexiganan, 512–≤0.25 μg/mL; PMB, 31.3–≤0.015 μg/mL; BAC 338–≤0.17 μg/mL; NEO, 219–≤0.11 μg/mL; triple antibiotic ointment (TAO): PMB, 31.3; BAC, 338; NEO, 219 μg/ml–PMB, ≤0.015; BAC, ≤0.17; and NEO, ≤0.11 μg/mL; MUP, 256–≤0.12 μg/mL; FA, 32–≤0.06 μg/mL and GEN, 1024–≤0.5 μg/mL.

2.3. Susceptibility testing

Broth microdilution MIC and MBC testing were performed according to reference broth microdilution methods (CLSI, 1999, 2015a) using cation-adjusted Mueller-Hinton broth (supplemented with 2.5-5% lysed horse blood for streptococci). MBC determinations for pexiganan and levofloxacin were assessed by plating the entire broth content from the MIC broth microdilution well and those 5 or more $\log_2$ dilutions above the MIC onto appropriate agar growth medium. Quantitative colony counts were performed on the initial inoculum. The lowest concentration of each compound that killed ≥99.9% of the starting test inoculum was defined as the MBC endpoint (Moody and Knapp, 2004). Bactericidal activity was defined when the MBC/MIC ratio was ≤2 (CLSI, 1999; Moody and Knapp, 2004). Interpretive criteria and quality control (QC) were those as published by the Clinical and Laboratory Standards Institute (CLSI, 2015b). QC strains included: *S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Streptococcus pneumoniae* ATCC 49619.

2.4. Serial passage

Stepwise development of resistance to pexiganan was determined by passaging strains using the broth microdilution test over a period of 7 days (Montgomery et al., 2014). For each of 7 consecutive days, after the MIC was read, broth was taken from the microtiter well one dilution below the well where the MIC occurred. That broth was grown/adjusted to achieve a density equivalent to a 0.5 McFarland and an MIC test was performed. This procedure provided for continuous exposure of the bacterium to sub inhibitory concentrations of the antimicrobial in the wells that were below the MIC. Strains demonstrating increased resistance to pexiganan above the baseline MIC were selected and re-tested by broth microdilution against pexiganan upon completion of serial passaging analysis in order to confirm resistance and evaluate the emergence of resistance. The stability of developed mutants was assessed by subculturing mutant colonies twice onto drug-free medium to determine pexiganan MIC values after exposure.

3. Results

3.1. Bactericidal activity

Pexiganan demonstrated bactericidal activity (Table 1). The pexiganan MBC/MIC ratio was 1:1 (MBC = MIC) for 13 (61.9%) isolates and 2:1 for 7 (33.3%) isolates. One isolate had a pexiganan MBC value (16 μg/mL), however the MBC/MIC ratio could not be calculated because the MIC value was off-scale (≤4 μg/mL, MBC/MIC ratio at ≥4; Table 1). Only 5 isolates exhibited both MIC and MBC values on-scale for the tested range of levofloxacin. Among those 5 strains, levofloxacin MBC values were identical to MIC (MBC = MIC) for 3 strains, one-doubling dilution higher (MBC/MIC ratio of 2) for one strain, and 2 doubling dilutions higher (MBC/MIC ratio of ≥4) for one strain (Table 1).

3.2. Serial passage

Seven of 10 isolates tested showed no major variation (more than +/− 1 $\log_2$ dilution) of the starting pexiganan MIC after 7 consecutive daily passages in the subinhibitory concentrations of pexiganan (Table 2). The highest increase in the pexiganan MIC values was observed with a SHV-12 producing strain of *Klebsiella pneumoniae.* Pexiganan MIC value increased from 512 μg/mL to 8192 μg/mL (16-fold) after 7 passages. The MIC value decreased to 2048 μg/mL following 2 consecutive passages in drug-free media (Table 2). The *K. pneumoniae* KPC-2 producing strain exhibited an 8-fold increase in the pexiganan MIC value (from 32 to 256 μg/mL), and the community-acquired-MRSA USA300 strain showed a 4-fold increase (from 16 to 64 μg/mL; Table 2).

3.3. Activity against Gram-positive species

The MIC distributions for pexiganan when tested against staphylococci and β-hemolytic streptococci with elevated (non-WT) MIC values against the topical antimicrobial agents BAC, FA, MUP, NEO and RET are presented in Table 3. A narrow range of MIC values for pexiganan (4–32 μg/mL) against all 48 *S. aureus* strains was documented. The pexiganan MIC mode, MIC_{50} and MIC_{90} values were 16 μg/mL. The mode, MIC_{50} and MIC_{90} value for the subsets of isolates with non-WT MIC values to BAC and NEO ($n = 14$), FA ($n = 11$), MUP ($n = 12$) and RET ($n = 11$) were also at 16 μg/mL (Table 3). Six isolates were gentamicin resistant (MIC, ≥32 μg/mL) including one isolate with a MIC value at 512 μg/mL. The pexiganan MIC values for these isolates were 8–16 μg/mL (data not shown). The concentration of pexiganan free base in the experimental topical cream (8000 μg/mL) was 500-fold greater than the MIC_{90} for *S. aureus* (Table 3).

For coagulase-negative staphylococci (CoNS), the pexiganan MIC mode, MIC_{50} and MIC_{90} were 4, 4, and 8 μg/mL, respectively (Table 3). The mode for each of the subsets with non-WT MIC values was also 4 μg/mL. An exception was one isolate with elevated MIC results to both BAC and NEO (MIC to pexiganan, 8 μg/mL). The β-hemolytic

Table 1
Summary of bactericidal activity.

Antimicrobial (no. isolates)	No. of strains (%) with MBC/MIC ratio of:		
	1	2	≥4
Pexiganan (21)	13 (61.9)	7 (33.3)	1 (4.8)[a]
Levofloxacin (21)[b]	3 (14.3)	1 (4.8)	1 (4.8)

[a] MIC value of ≤4 μg/mL and MBC value of 16 μg/mL.
[b] MBC/MIC ratios for levofloxacin could not be determined for 16 isolates due to MIC and/or MBC values that were above or below the concentration range tested.