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Burkholderia Is Highly Resistant to Human Beta-Defensin 3

Hany Sahly,[1]* Sabine Schubert,[1] Jürgen Harder,[2] Peter Rautenberg,[1] Uwe Ullmann,[1] Jens Schröder,[2] and Rainer Podschun[1]

Department of Medical Microbiology and Virology[1] and Clinical Research Unit, Department of Dermatology,[2] University Hospital Schleswig-Holstein, Campus Kiel, 24105 Kiel, Germany

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The bactericidal activity of the novel beta-defensin hBD-3 against 28 species and 55 strains of gram-positive cocci and gram-negative fermentative and nonfermentative rods was tested. All strains proved to be highly or intermediately susceptible to hBD-3 (minimal bactericidal concentration [MBC], ≤50 μg/ml), except for *Burkholderia cepacia*, for all 23 tested strains of which MBCs were >100 μg/ml.

Human beta-defensins play important roles in innate immune responses in mucosal surfaces and skin, probably by virtue of their antimicrobial activity (1, 3, 6, 16, 18, 22, 24, 25). Beta-defensins 1 and 2 have been shown to have potent antibacterial activity against gram-negative but not against gram-positive bacteria (9, 24, 26). In contrast, the novel human beta-defensin-3 (hBD-3) was recently found to be potent against gram-positive and gram-negative bacteria, including *Burkholderia cepacia*, which resists both beta-defensins 1 and 2. However, only two studies have expressly evaluated the antimicrobial potential of hBD-3, and each of these considered only a restricted number of bacterial isolates (seven and eight, respectively) (4, 10).

In the present study the susceptibility to hBD-3 of a comprehensive and representative panel of reference and clinical isolates of gram-positive cocci as well as gram-negative fermentative and nonfermentative rods comprising 28 species and a total of 55 strains was tested based on the minimal bactericidal concentration (MBC) and the lethal dose for ≥50% of bacteria (LD_{50}). The strains were either reference strains obtained from the American Type Culture Collection or the National Collection of Type Cultures (London, United Kingdom) or clinical strains isolated from human tracheal secretions or from blood culture (Table 1). Additionally, isolates belonging to a *B. cepacia* complex strain panel that has been described as appropriate for diagnostic and experimental purposes with regard to this genus (17) were obtained from the Belgium Coordinated Collections of Microorganisms/Laboratorium Microbiologie (BCCM/LMG) (Table 2). hBD-3 was synthesized either recombinantly, by cloning cDNA encoding the 45 amino acids containing the natural form of hBD-3 into the pET-30c expression vector (Novagen), which contains an N-terminal His tag sequence allowing purification of the fusion protein with a nickel affinity column, or chemically (Jerini Bio Tools GmbH, Berlin, Germany) as described previously (10). Test isolates were grown for 2 to 3 h in brain heart infusion broth at 36 ± 1°C, washed three times in 10 mM sodium phosphate buffer (pH 7.4), and adjusted to 10^4 to 10^5 bacteria/ml. A 100-μl volume of the bacterial suspension was mixed with 10 μl of hBD-3 solution (range of final concentrations tested, 0.0125 to 100 μg/ml) and incubated at 36 ± 1°C. After 2 h, CFU were determined. Bacterial suspensions supplemented with 10 μl of phosphate buffer or with 10 μl of 0.01% acetic acid instead of hBD-3 served as negative controls. Results are given either as MBC (≥99.9% killing) or as LD_{50}s. Arbitrarily, a strain was defined as sensitive to hBD-3 if MBC levels were <10 μg/ml, as intermediately sensitive if MBC levels were 10 to 100 μg/ml, or as resistant at MBC levels of >100 μg/ml. hBD-3 showed broad activity against all bacterial groups tested. All tested strains of gram-positive cocci, gram-negative fermentative rods, and gram-negative nonfermentative rods were shown to be highly susceptible ((MBC and LD_{50}, ≤6.25 μg/ml) except *Serratia marcescens* and *Aeromonas hydrophila*, both of which exhibited intermediate sensitivity (MBC, 50 μg/ml) (Table 1). In contrast to the previous findings of other groups (4, 10), the genus *Burkholderia* alone was resistant to hBD-3; for all 23 *Burkholderia* strains tested, MBCs and LD_{50}s were >100 μg/ml, except for 3 isolates for which LD_{50}s were ≤12.5 μg/ml (Table 2).

Our findings suggest that hBD-3 has the potential to play an important role in the clearance of opportunistic gram-negative and gram-positive bacteria. The broad range of microbicidal activity shown by hBD-3 is very promising with regard to its utility as a therapeutic alternative to antibiotic chemotherapeutics, particularly because resistance to antimicrobial peptides in general cannot be induced under physiological conditions (8). The applicability of hBD-3, however, is restricted by the high resistance of the *B. cepacia* complex.

The resistance of the *B. cepacia* complex to hBD-3 might be in part the cause for the relatively high isolation rates of *B. cepacia* from immunocompromised patients with severe underlying diseases such as cystic fibrosis (5, 11).

The results of the present study support the hypotheses that (i) unlike defensins 1 and 2, whose microbicidal activity is directed predominantly against gram-negative bacteria (9, 15, 24), hBD-3 has broad activity against both gram-positive and gram-negative bacteria, including nonfermentative rods other than *Burkholderia* and (ii) the genus *Burkholderia* possesses an intrinsic resistance to hBD-3. These results contradict those of Garcia et al. (4) showing *B. cepacia* to be rather susceptible to

* Corresponding author. Mailing address: Department of Medical Microbiology and Virology, University Hospital Schleswig-Holstein, Campus Kiel, Brunswiker Str. 4, 24105 Kiel, Germany. Phone: 49 431 597 3316. Fax: 49 431 597 3296. E-mail: sahly@medmicrobio.uni-kiel.de.

TABLE 1. MBCs and LD_{50}s of hBD-3 against a reference panel of clinically relevant bacterial species

Strain	MBC (μg/ml)	LD_{50} (μg/ml)
Gram-positive cocci		
Staphylococcus aureus ATCC 12600	1.56	0.2
Staphylococcus aureus ATCC 33593	6.25	1.56
Staphylococcus epidermidis ATCC 14990	1.56	0.39
Streptococcus pyogenes ATCC 12344	6.25	3.1
Streptococcus pneumoniae ATCC 33400	6.25	1.56
Gram-negative fermentative rods		
Escherichia coli ATCC 25922	0.39	0.1
Klebsiella pneumoniae ATCC 13883	0.1	0.05
Enterobacter cloacae ATCC 13047	6.25	0.78
Serratia marcescens NCTC 10211	50	6.25
Citrobacter freundii NCTC 9750	0.39	0.2
Aeromonas hydrophila ATCC 7966	50	3.1
Gram-negative nonfermentative rods		
Brevundimonas diminuta ATCC 11568	0.39	0.2
Pseudomonas aeruginosa ATCC 10145	3.1	0.78
Pseudomonas aeruginosa ATCC 39324	1.56	0.39
Pseudomonas aeruginosa ATCC 11440	3.1	0.78
Pseudomonas aeruginosa ATCC 11446	3.1	0.78
Pseudomonas fluorescens NCTC 10038	0.78	0.39
Pseudomonas putida BK 4912	6.25	1.56
Stenotrophomonas maltophilia ATCC 17666	0.78	0.39
Stenotrophomonas maltophilia ATCC 10257	1.56	0.78
Stenotrophomonas maltophilia M 140	0.78	0.2
Stenotrophomonas maltophilia M 141	3.1	0.39
Stenotrophomonas maltophilia M 142	1.56	0.39
Acinetobacter baumannii ATCC 19606	0.78	0.05
Acinetobacter genospecies 3 ATCC 19004	0.39	0.2
Acinetobacter lwoffii ATCC 15309	0.2	0.1
Acinetobacter junii ATCC 17908	0.39	0.1
Acinetobacter johnsonii ATCC 17909	0.39	0.2
Acinetobacter haemolyticus ATCC 17906	0.78	0.2
Acinetobacter calcoaceticus ATCC 23055	0.78	0.39
Acinetobacter genospecies 11 ATCC 11171	1.56	0.39
Acinetobacter genospecies 10 ATCC 17924	0.78	0.2
Burkholderia cepacia ATCC 25416	>100	>100

TABLE 2. MBCs and LD_{50}s of hBD-3 against the *Burkholderia* complex[a]

Strain	MBC (μg/ml)	LD_{50} (μg/ml)
Burkholderia cepacia		
ATCC 25416	>100	>100
LMG 2161	>100	>100
LMG 16654	>100	>100
LMG 16656	>100	>100
LMG 16659	>100	>100
LMG 18821	>100	>100
LMG 18826	>100	>100
LMG 18827	>100	>100
LMG 18828	>100	>100
LMG 18832	>100	>100
Burkholderia stabilis		
LMG 14294	>100	>100
LMG 18870	>100	3.1
LMG14086	>100	>100
LMG 18888	>100	>100
Burkholderia vietnamiensis		
LMG 16232	>100	>100
LMG 18835	>100	>100
LMG 10929	>100	>100
LMG 18836	>100	>100
Burkholderia multivorans		
LMG 13010	>100	>100
LMG 16660	>100	>100
LMG17588	>100	12.5
LMG 18822	>100	>100
LMG 18823	>100	3.1

[a] See reference 17.

hBD-3 (MIC, 6.6 μg/ml). This discrepancy in the results of two working groups may be due in part to differences in the methodologies applied. While Garcia et al. measured bacterial growth by means of optical density, we quantitatively determined viable counts as a gauge of microbicidal efficacy. Furthermore, the former group measured the ability of hBD-3 to inhibit growth of the bacteria (MIC), while our study considered the bactericidal capacity (MBC). Additionally, Garcia et al. based their conclusion on results achieved with only two *B. cepacia* strains, while we studied 23 reference strains, all of which were found to be resistant to hBD-3.

Burkholderia spp. are known to resist cationic peptides, as shown for polymyxin B (19), cathelicidin peptides (23), and human beta-defensins 1 and 2 (2). It has been suggested that the resistance of gram-negative bacteria to cationic peptides is in part due to structural peculiarities of the lipopolysaccharide (LPS) anchored in the bacterial outer membrane (7, 20). As to polymyxin B, several studies have shown that the various gram-negative bacteria with a priori or acquired resistance to this antibiotic peptide exhibit extensive cationic substitution of LPS by 2-amino-4-deoxy-L-arabino-pentopyranose (L-Ara*p*4N) (12, 13, 21). This substitution produces a reduction in the net negative charge of LPS, thus impairing the affinity of the bacterial surface for positively charged cationic peptides. Interestingly, Isshiki et al. have shown that LPS of *B. cepacia* is constitutively substituted by β-L-Ara*p*4N-(1→8)-α-Kop(2→4)-KDO (14).

The question of whether the substitution of LPS by L-Ara*p*4N, which reduces the negative net charge of the outer membrane, does indeed constitute the molecular basis for the resistance of *B. cepacia* to both polymyxin B and hBD-3 needs further clarification. This could be achieved by generating a *B. cepacia* L-Ara*p*4N-deficient mutant and then testing its susceptibility to hBD-3.

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