The Kinocidin Interleukin-26 Shows Immediate Antimicrobial Effects Even to Multi-resistant Isolates

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The cationic proinflammatory cytokine Interleukin 26 (IL-26) shows antibacterial activity and inhibits the replication of cytomegalovirus and hepatitis C virus. This study evaluates the early microbicidal activities of IL-26 against major bacterial species including multi-resistant variants and Candida albicans. Recombinant IL-26 was bacterially expressed and studied for its microbicidal effects in culture. We show that IL-26 has strong 90% bactericidal activities against Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus, and Acinetobacter baumannii. Similarly, IL-26 sensitivity was also detectable in vancomycin-resistant Enterococcus species, methicillin-resistant S. aureus, and carbapenem-resistant A. baumannii clinical isolates. Additionally, a significant, albeit weak fungicidal effect against Candida albicans was observed. Activities against Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa were not detectable. The proinflammatory cytokine and kinocidin IL-26 shows strong bactericidal activities against A. baumannii and, almost selectively, against Gram-positive bacteria.

Keywords: kinocidin, interleukin-26, antimicrobial peptide, bactericidal activity, multi-resistant bacteria

INTRODUCTION

The highly cationic proinflammatory cytokine Interleukin 26 (IL-26) is a member of the IL-10 family of cytokines. IL-26 is produced by different cell types, such as activated T cells or inflammatory fibroblasts, and stimulates the production of other proinflammatory cytokines in various cell types (Knappe et al., 2000; Fickenscher et al., 2002; Hör et al., 2004; Corvaisier et al., 2012; Braum et al., 2013; Che et al., 2014, 2017; Larochette et al., 2019). IL-26 uses a specific, heterodimeric cytokine receptor consisting of IL-20R1 and IL-10R2 on epithelial cells, whereas its action on other cell types is independent of this receptor (Hör et al., 2004; Sheikh et al., 2004; Corvaisier et al., 2012). Accordingly, elevated IL-26 levels were found in inflamed tissues or plasma of patients with different inflammatory diseases (Dambacher et al., 2009; Corvaisier et al., 2012; Che et al., 2014, 2017; Miot et al., 2014; Meller et al., 2015; Konradsen et al., 2016; Fujii et al., 2017; Heftdal et al., 2017; Poli et al., 2017; Caiazzo et al., 2018; Larochette et al., 2019; Scala et al., 2019; Brilland et al., 2021). Moreover, IL-26 inhibits cytomegalovirus and hepatitis C virus replication and promotes the replication of vesicular stomatitis virus (Braum et al., 2013; Miot et al., 2014). IL-26 is a highly cationic protein with amphipathic helices, which are typical for cationic cell-penetrating peptides (Knappe et al., 2000; Meller et al., 2015). IL-26 shares structural similarity with antimicrobial peptides (AMP)
and bacteriostatic activities of IL-26 against both Gram-positive and Gram-negative bacteria have been demonstrated (Meller et al., 2015; Agak et al., 2018; Woetmann et al., 2018; Scala et al., 2019). Recombinant IL-26 has a molecular weight of 19 kDa and the formation of dimers, oligomers, and multimers has been described. Since IL-26 combines cytokine functions with antimicrobial activities, it belongs to the group of kinocidins (Yount et al., 2004; Yeaman et al., 2007; Laroche et al., 2019). This project targets the rapid, microbicidal activities of IL-26.

**MATERIALS AND METHODS**

**Production of Recombinant Protein**

Induction of IL-26 protein synthesis was achieved in *Escherichia coli* strain XL1blue, which is capable of producing IL-26 in inclusion bodies, by adding 1 mM isopropyl β-D-1-thiogalactopyranoside and cultivation for 6 h (Knappe et al., 2000; Hör et al., 2004). First, the bacteria were disrupted mechanically in 6 M guanidinium chloride with 0.1 M NaH₂PO₄, 0.01 M Tris, and 100 mM β-mercaptoethanol. Bacterial debris was removed by centrifugation for 30 min at 8,873 × g and by sterile filtration. Nickel-chelate affinity chromatography was used for protein purification under denaturing conditions (Ni Sepharose 6 Fast Flow, GE Healthcare Life Sciences, Buckinghamshire, UK). Particle-bound IL-26 was eluted under increasing imidazole concentrations (40 mM, 500 mM, and 600 mM). Eluate fractions were tested for 19 kDa bands in Coomassie-stained protein gels. Renaturation of relevant eluate fractions was achieved by dialysis for 24 h twice, at 4°C (20 mM HEPES, 1 mM MgCl₂, 20 mM KCl, 0.1 mM EDTA, 1% glycerol, 1 mM oxidized glutathione, and 5 mM reduced glutathione, pH 8.0).

**Testing IL-26 for Functionality**

The physiological STAT3 phosphorylation activity of the newly produced IL-26 was tested with the human colon carcinoma cell line COLO-205. IL-26 (5 μg/ml) was added for 45 min at 37°C. Cell lysates were separated on protein gels and Western blots were probed with anti-phospho-STAT3 and anti-STAT3 antibodies (anti-phospho-Tyr705 STAT3, #9131, rabbit; anti-STAT3, #9132, rabbit) and horseradish peroxidase-conjugated anti-rabbit IgG antibodies (#7074, goat, Cell Signaling Technology, Beverly, United States) as secondary reagent. Signals were detected by chemiluminescence (Figure 1) using a charged-coupled device camera (LAS-3000, Fujifilm, Tokyo, Japan). If phospho-STAT3 bands were detectable, the respective IL-26 fraction was considered biologically active.

**Testing for Antimicrobial Activity**

Bacteria and *C. albicans* were initially grown on Columbia sheep blood agar at 37°C overnight and then for 20 h at 37°C in 10 ml tryptic soy broth (TSB) medium with 10 mM NaCl. Then, 50 μl of the culture was added to 10 ml of TSB/10 mM NaCl medium and incubated for 3 h at 37°C for reaching the logarithmic phase of growth and the optical density at 600 nm was determined. We diluted the culture with 10 mM NaCl to reach target concentrations of 10⁶ CFU/ml. Next, 100 μl of the microbial suspension was added to 100 μl of different concentrated IL-26 solutions (100, 30, 10, 3, and 1 μg/ml) to achieve final IL-26 concentrations in the wells of 50, 15, 5, 1.5, and 0.5 μg/ml. We always ran negative controls with pure dialysis buffer instead of the IL-26 solution, as well as positive controls with bacteria that were previously shown to be highly sensitive to IL-26, when species with low or lacking IL-26 sensitivity were examined.

Immediately after the start of the treatment, and after 1, 2, 3, and 4 h, 20 μl from each culture was sampled and diluted in a 0.85% NaCl solution and 100 μl of that dilution was pipetted on two lysogeny broth (LB) agar plates each. The plates were incubated overnight at 37°C and the colonies were counted. The numbers of colony forming units per milliliter (CFU/ml) were calculated after averaging of the counting results of the two separate plates in consideration of the dilution factor. Totally, 15,000 LB agar plates were plated by hand for 66 independent experiments.

**Statistical Evaluation**

Statistical evaluation was performed with GraphPad PRISM 8 (GraphPad Software, Inc., San Diego, CA, United States). Values of *p* were determined by using the independent two-sample *t*-test. We used linear regression for calculating the minimal concentration for 90% bactericidal or fungicidal effects (MBC/ MFC), respectively, the lethal dose or concentration for 90% killing (LD₉₀/LC₉₀). All results of this study are given as MBC/ MFC, even though the use of LD₉₀/LC₉₀ is also common in AMP research. The use of the terms MBC/MFC may be more precise from a microbiological point of view. Results with values of *p* ≤ 0.05 were rated significant. As far as not given in numbers, significances are labeled as followed: *p* ≤ 0.05; **p** ≤ 0.01; ***p*** ≤ 0.001; and ****p*** ≤ 0.0001, ns = not significant.
RESULTS

IL-26 was tested for bactericidal activities against different Gram-positive and Gram-negative strains of different antibiotic resistance phenotypes, as well as for fungicidal activities against C. albicans. All experiments were performed with a standardized protocol and were highly reproducible. The anti-infective activities were analyzed quantitatively for the minimal concentration for 90% bactericidal or fungicidal effects (MBC\textsubscript{90}/MFC\textsubscript{90}).

Activity Against Enterococi

Directly after adding 50 μg/ml IL-26 to Enterococcus faecalis ATCC 29212 cultures, an immediate and highly significant (p ≤ 0.0001) reduction of the number of colony forming units (CFU) of approximately 98.4% was detected. After 1 h, significant effects were found from 5 μg/ml on. Complete eradication of all bacteria was reached at 50 μg/ml IL-26, after incubation for at least 1 h. The CFU numbers after 2, 3, and 4 h of incubation were reduced by 97.3, 92.3, and 96.1% at 15 μg/ml IL-26 (Figure 2A).

Similar to the antibiotic-sensitive type strain, a vancomycin-resistant clinical isolate of E. faecalis showed an instant significant effect. After 1 h, significant CFU number reductions were found starting from 0.5 μg/ml, whereas the strongest results were observed at 15 μg/ml (reduction of 72.5%) and 50 μg/ml (99.6%). The reduction levels stayed constant over time. After 4 h, the incubation with 50 μg/ml IL-26 resulted in a highly significant decline of CFU numbers by 99.95% (Figure 2B).

Likewise, Enterococcus faecium ATCC 6057 showed a prompt significant drop of the CFU numbers at 15 μg/ml by 70.6% and at 50 μg/ml by 99.5%. This effect increased, when the incubation was performed for 1 h. Reduction values were 81.3% for 5 μg/ml, 99.6% for 15 μg/ml, and a complete killing of all bacteria was achieved for 50 μg/ml. After 2 and 4 h, complete eradication were reached at 15 μg/ml. At 3 h, reductions amounted to 99.98% for 15 μg/ml and 100% for 50 μg/ml (Figure 2C).

Subsequently, we checked for IL-26 sensitivity of vancomycin-resistant E. faecium DSM 17050 (Deutsche Sammlung von Mikroorganismen, German Collection of Microorganisms, Hannover, Germany). Again, a significant immediate decline by 99.1% was achieved at 50 μg/ml IL-26. After 1 h, complete eradication of all bacteria was reached at 50 μg/ml, whereas 99.89% were killed at 15 μg/ml. These effects were stable (± 0.2%) at 15 μg/ml for 2, 3, and 4 h incubation time. After 3 h, an additional significant but weaker effect was found at 5 μg/ml, which lost its significance after 4 h (Figures 2D, 3).

Activity Against Staphylococcus aureus

In the case of S. aureus ATCC 6538, we found an instant reduction of the CFU numbers of approximately 23.2% at 50 μg/ml IL-26 (p = 0.37), which increased after 1 h to a highly significant reduction by 94.6% (p < 0.0001). Further increments were achieved upon ongoing incubation. CFU reductions at 50 μg/ml reached 95.6, 98.0, and 93.4% after 2, 3, and 4 h. After 2 and 3 h, significant but weak effects were detectable at 15 μg/ml (Figure 2E).

In contrast to the antibiotic-sensitive S. aureus ATCC 6538, the MRSA strain ATCC 33593 showed highly significant CFU reductions immediately after adding IL-26 to the cultures at 15 μg/ml with a decline by 90.1% and at 50 μg/ml by 99.3%. These effects increased after 1 h with CFU reductions by 53.3% at 1.5 μg/ml, 81.4% at 5 μg/ml, 99.8% at 15 μg/ml, and a complete eradication of all bacteria at 50 μg/ml. After 2 h, the effects at 5 μg/ml and 15 μg/ml were stable (± 0.1%) and complete killing at 50 μg/ml was still achieved. When incubating for 3 h, reductions were found at 0.5 μg/ml (33.5%), 1.5 μg/ml (36.5%), 5 μg/ml (83.9%), 15 μg/ml (99.8%), and 50 μg/ml (100%), of which all were significant, besides at 1.5 μg/ml. Last, after 4 h, declines by 63.6% at 5 μg/ml, 98.6% at 15 μg/ml, and complete eradication at 50 μg/ml were observed (Figure 2F).

Activity Against Multi-resistant Gram-positive Bacteria

The MBC\textsubscript{90} for immediate effects ranged from 38.5 μg/ml (MRSA ATCC 33593) to 57.5 μg/ml (vancomycin-resistant E. faecalis), except for the methicillin-sensitive S. aureus ATCC 6538 with an unusually high MBC\textsubscript{90} of 136 μg/ml. Two groups of similar MBC\textsubscript{90} ranges were defined. In the first group, E. faecalis ATCC 29212, E. faecium ATCC 6057, vancomycin-resistant E. faecium ATCC 17050, and MRSA ATCC 33593 had average MBC\textsubscript{90} values between 12.5 μg/ml and 13.4 μg/ml IL-26 for 1 to 3 h and 19.3 μg/ml after 4 h. In the other group, the average MBC\textsubscript{90} for 1 to 4 h for vancomycin-resistant E. faecalis and methicillin-sensitive S. aureus ATCC 6538 ranged between 43.7 μg/ml and 45.5 μg/ml (Table 1). Hence, we were able to show for the first time that IL-26 functions as a highly active bactericidal agent against different Gram-positive bacteria. These effects are independent of the antibiotic resistance phenotypes since multi-resistant strains of S. aureus, E. faecalis, and E. faecium were as sensitive as or even more sensitive than their antibiotic-sensitive counterparts.

Activity Against Acinetobacter baumannii

Concerning Gram-negative species, we first tested A. baumannii ATCC 19606. Similar to all tested Gram-positive bacteria, an initial albeit weak CFU reduction was observed. After 1 h of incubation, a highly significant decline of 99.8% was detected at 50 μg/ml IL-26 and stayed constant for 4 h (± 0.3%). Additionally, we found a 50% non-significant reduction at 15 μg/ml (Figure 2G).

Moreover, two highly resistant A. baumannii CR isolates were analyzed, which solely were sensitive for colistin. The first one was isolated in 2014 from a patient from a local outbreak at the University Hospital Schleswig-Holstein in Kiel, Germany. An immediate reduction was not observed, but, again, highly significant CFU reductions were measureable at 1, 2, 3, and 4 h (99.4, 99.3, 99.7, and 99.7%) at IL-26 concentrations of 50 μg/ml (Figure 2H). The other A. baumannii CR isolate resulted from a patient from 2018 with previous hospitalization in a country with high prevalence of colonization with CR A. baumannii. Here, we detected a significant immediate
reduction of 31.7% at 50 μg/ml IL-26. Furthermore, weaker and non-significant reductions were detectable at 0.5 μg/ml, 5 μg/ml, and 15 μg/ml. After 1 h, the decline of the CFU count at 50 μg/ml IL-26 was 98.7% and increased in the course of the experiments to 99.4% (2 h), 99.8% (3 h), and 99.6% (4 h; Figure 2I). The mean MBC_{90} values for the three A. baumannii
strains (ATCC 19606, 2014, 2018) were 374.6 µg/ml for 0 h, 46.91 µg/ml for 1 h, 48.09 µg/ml for 2 h, 46.8 µg/ml for 3 h, and 47.22 µg/ml for 4 h (Table 1).

A. baumannii was the only tested Gram-negative species with IL-26 sensitivity. In contrast, we were unable to detect any antimicrobial effects against E. coli ATCC 11775, K. pneumoniae ATCC 4352, or P. aeruginosa ATCC 27853 for IL-26 concentrations up to 50 µg/ml (Figures 2J–L).

Activity Against Candida albicans

Last, the sensitivity of C. albicans ATCC 24433 against IL-26 was investigated. Immediate effects were not detected at up to 50 µg/ml IL-26. The CFU reduction values at 50 µg/ml IL-26 were 29% after 1 h (non-significant), 67.5% after 2 h, 58.2% after 3 h, and 78.5% after 4 h (all significant). The MFC values ranged from 54.7 to 81.41 µg/ml (Table 1). Hence, we were able to show a reproducible and significant, albeit weak fungicidal activity of IL-26 against C. albicans.

Thus, the cytokine IL-26 with its proinflammatory, bactericidal, antiviral, and fungicidal activities can be attributed to the group of kinocidins which was defined for cytokines with direct antimicrobial effects, such as human mammalian platelet factor 4 (hPF-4; Yount et al., 2004; Yeaman et al., 2007; Larochette et al., 2019).

DISCUSSION

Whereas all tested Gram-positive bacterial strains were highly sensitive to IL-26, we observed a remarkable difference between A. baumannii strains and all other Gram-negatives (enterobacteria and P. aeruginosa). Thus, the questions arise why all other Gram-negative species are non-sensitive for IL-26 and what the essential factor is for the sensitivity of A. baumannii. The lipopolysaccharides (LPS) and especially the O-antigen, which is the outer chain of the LPS, are two known factors for AMP resistance in Gram-negative bacteria (Silhavy et al., 2010; Joo et al., 2016). Acinetobacter species are unable to produce complete LPS due to the absence of O-antigen-ligase activity and the lipooligosaccharide (LOS) core, the lipid A, is remaining (Weber et al., 2016). Thus, O-antigen might be responsible for the IL-26 resistance of Gram-negative species.

Concerning the mode of IL-26 action, the direct interaction with the bacterial cell membrane including pore formation seems likely, similarly to other AMP (Patel and Akhtar, 2017). Due to its high cationic charge, IL-26 binds to glycosaminoglycans of the surface of eukaryotic cells (Hör et al., 2004), as well as to LPS and lipoteichoic acid of the surface of bacteria (Meller et al., 2015). Based on electron micrographs of P. aeruginosa ATCC 27853, bleb-formation followed by membrane disruption was described as the mode of IL-26 action (Meller et al., 2015). However, this needs to be interpreted with caution, since exactly the same P. aeruginosa strain has been classified as IL-26 resistant in this study.

The initial publication concerning antimicrobial activities of IL-26 described bacteriostatic effects at 50% level for 5 to 10 µM IL-26 against P. aeruginosa ATCC 27853, E. coli ATCC 11775, K. pneumoniae O1:K2, and S. aureus ATCC 6538 but no detectable effects against E. faecalis ATCC 29212 and C. albicans ATCC 24433 (Meller et al., 2015). In contrast, our study was able to demonstrate strong bactericidal activities at 90% level against the Gram-positive strains E. faecalis ATCC 29212, vancomycin-resistant E. faecalis, E. faecium ATCC 2014, vancomycin-resistant E. faecium DSM 17050, S. aureus ATCC 6538, and MRSA ATCC 33593. Furthermore, we were able to show strong effects on naturally O-antigen deficient A. baumannii independently of carbapenem
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resistance, and weak effects on C. albicans ATCC 24433 after at least 2h of incubation. Effects against E. coli ATCC 11775, K. pneumoniae ATCC 4352, and P. aeruginosa ATCC 27853 were not detectable. This goes in line with a study in which IL-26 exhibited bacteriostatic activities against S. aureus ATCC 6538 but not against P. aeruginosa ATCC 27853 (Scala et al., 2019). Another published study did not show immediate effects for S. aureus and E. coli; however, bactericidal effects were detected after 4 to 24h of incubation with 1μg/ml IL-26 (Agak et al., 2018). In a third publication, an activity against biofilm formation of S. aureus was detected which was more pronounced for IL-26 than for the AMP LL-37 (Woetmann et al., 2018). Regarding Mycobacteria (M.) IL-26 has been shown to inhibit growth and reduce viability of M. leprae and M. tuberculosis in axenic cultures as well as within macrophages, probably by inducing lysis by bleb-formation after interaction with lipoarabinomannan (Dang et al., 2019; Hawerkamp et al., 2020).

The reason for these functional differences might be due to the different sources and qualities of commercially available IL-26 (Knappe et al., 2000; Hör et al., 2004). In order to ensure high quality and functionality, we used self-produced IL-26 (Hawerkamp et al., 2020). Besides potential pharmacotechnological and galenic challenges in the production of this instable kinocidin, additional immunologic effects of the proinflammatory IL-26 need to be considered in the case of a possible therapeutic application. In published experiments, IL-26 or LL-37 was applied nasally in mice after nasal application of bacteria and a certain reduction of the CFU number (factor 10–100) was seen (Meller et al., 2015). However, these experiments were performed with K. pneumoniae, which was classified as IL-26 resistant in this study.

Compared to other AMP, which usually have a broad activity spectrum against Gram-positive and Gram-negative bacteria, protozoa, and fungi (Ebbensgaard et al., 2015), IL-26 has its main target in Gram-positive bacteria, which has not yet been described for other AMP. Thus, IL-26 is a unique member of the family of cationic AMP. In summary, we showed for the first time that IL-26 is a proinflammatory kinocidin with bactericidal and fungicidal activities, which is also active against A. baumannii and C. albicans and kills Gram-positive bacteria almost selectively.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

BTH wrote the manuscript, designed and performed the experiments, and prepared the figures and tables. GM contributed to the experiments, reviewed and edited the manuscript, and supervised the project. RP gave advice for the experimental design and reviewed and edited the manuscript. HF wrote the manuscript, gave advice for the experimental design, and supervised the project. All authors contributed to the article and approved the submitted version.

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**TABLE 1 | MBC$_{90}$/MFC$_{90}$ values for all IL-26-sensitive species.**

| Species                     | MBC$_{90}$/MFC$_{90}$ |
|-----------------------------|-----------------------|
|                             | 0          | 1          | 2          | 3          | 4          | h     |
| E. faecalis, ATCC 29212     | 45.58      | 14.16      | 14.23      | 14.40      | 14.51      | μg/ml |
| E. faecalis (VRE)           | 54.48      | 40.04      | 41.59      | 40.67      | 42.38      | μg/ml |
| E. faecium, ATCC 6057       | 46.75      | 11.72      | 13.58      | 13.79      | 13.41      | μg/ml |
| E. faecium (VRE), DSM 17050 | 42.84      | 12.94      | 14.02      | 12.75      | 13.02      | μg/ml |
| S. aureus, ATCC 6538        | 136.00     | 47.31      | 46.19      | 47.15      | 48.54      | μg/ml |
| S. aureus (MRSA), ATCC 33593| 38.48      | 11.51      | 11.87      | 11.42      | 36.20      | μg/ml |
| A. baumannii, ATCC 11775    | 149.40     | 46.12      | 48.42      | 46.21      | 43.36      | μg/ml |
| A. baumannii (CF), 2014     | 832.10     | 47.15      | 47.90      | 48.18      | 49.97      | μg/ml |
| A. baumannii (CF), 2018     | 142.30     | 47.46      | 47.96      | 46.02      | 48.52      | μg/ml |
| C. albicans, ATCC 24433     | –          | –          | 81.41      | 77.77      | 54.74      | μg/ml |

Data calculated by linear regression; n = 3 per species; “–” = not calculable.
