Potential Toxicity of Polymyxins in Human Lung Epithelial Cells

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ABSTRACT Inhaled polymyxins are of considerable utility in achieving optimal exposure in the respiratory tract for the treatment of lung infections caused by multidrug-resistant Gram-negative pathogens. Current inhaled polymyxin therapy is empirical, and often large doses are used that may lead to potential pulmonary adverse effects. This study aimed to investigate the effect of polymyxins on human lung epithelial (A549) cells. The viability of A549 cells was examined after treatment with polymyxins by flow cytometry. Activation of caspases 3, 8, and 9, expression of Fas ligand (FasL), loss of mitochondrial membrane potential, and mitochondrial oxidative stress induced by polymyxin B were evaluated. The concentration of polymyxin B required to induce 50% of maximal cell death was 1.74 mM (95% confidence interval, 1.60 to 1.90 mM). Colistin was at least 2-fold less toxic than polymyxin B, while colistimethate was nontoxic. With 2.0 mM polymyxin B, 30.6%/H11006 11.5% (mean H11006 standard deviation) of the cells were apoptotic at 8 h and this increased to 71.3% 3.72% at 24 h. Concentration- and time-dependent activation of caspases 3, 8, and 9 was evident, while the activation of caspase 9 was more dramatic. Furthermore, polymyxin B caused concentration- and time-dependent FasL expression, production of mitochondrial reactive oxygen species, and changes in mitochondrial membrane potential. This is the first study to demonstrate that both extrinsic death receptor and intrinsic mitochondrial pathways are involved in polymyxin-induced toxicity in A549 cells. This knowledge base is critical for the development of novel strategies for the safe and effective inhalation therapy of polymyxins against Gram-negative “superbugs.”

KEYWORDS polymyxin, pulmonary delivery, respiratory toxicity, mitochondria, apoptosis

Antibiotic resistance has presented a major global health challenge (1), and lung infections caused by multidrug-resistant (MDR) Gram-negative bacteria are particularly problematic (2). Because of the new antibiotic discovery void, polymyxins are used as a last-line therapy against Gram-negative “superbugs” (3). However, current dosing recommendations of parenteral polymyxins are suboptimal (4–6), in particular for lung infections because of very limited drug exposure at the infection site (7, 8). Unfortunately, polymyxin resistance has been increasingly reported worldwide, very likely because of suboptimal use. Moreover, nephrotoxicity is the dose-limiting factor and can occur in up to 60% of patients (9–11). Therefore, parenteral administration of larger doses may not be able to produce polymyxin exposure in the airways for bacterial eradication. Pulmonary delivery of polymyxins thus holds great promise for the treatment of lung infections (12). Aerosolized polymyxins display antimicrobial...
efficacy superior to that of intravenous administration in the treatment of patients with ventilator-associated pneumonia (13) and other nosocomial pneumonia (14). Despite the aforementioned advantages, current inhaled polymyxin therapy is empirical and has never been optimized by using pharmacokinetics/pharmacodynamics (PK/PD). Excessively large doses of inhaled colistin in the form of colistimethate (CMS) (6 million IU [i.e., 180 mg colistin base activity or 480 mg of CMS]/day) are comparable to the recommended intravenous doses and may cause unnecessary local adverse effects (15, 16). Additionally, potential adverse effects of inhaled high-dose antibiotics often lead to poor patient compliance and potential emergence of resistance (16). There is a dearth of information on the potential toxicity of inhaled polymyxins, which is critical for the determination of the therapeutic index for lung delivery. Polymyxin B-induced apoptosis in kidney tubular cells involves mitochondrial, death receptor, and endoplasmic reticulum pathways (17, 18). However, there is no mechanistic information available in the literature on the potential pulmonary toxicity of polymyxins at the cellular level. Our study aimed to elucidate the mechanism of potential toxicity of polymyxins in human lung epithelial cells and provide key information for the optimization of inhaled polymyxins.

RESULTS

Concentration- and time-dependent polymyxin-induced apoptosis in A549 cells. Apoptotic (early and late), necrotic, and viable cells were differentiated by fluorescein isothiocyanate-annexin V and propidium iodide double staining (see Fig. S1 in the supplemental material). Both polymyxin B and colistin caused concentration-dependent apoptosis in A549 cells. The concentration of polymyxin B required to induce 50% of maximal cell death (50% effective concentration [EC50]) in A549 cells after 24 h of treatment was 1.74 ± 0.02 mM; the EC50 values of colistin and CMS could not be calculated because of the lack of a plateau (Fig. 1A). Polymyxin B at 2.0 mM caused apoptosis in ~70% of the cells following treatment for 24 h, and this concentration was employed to investigate the time-dependent apoptosis caused by polymyxin B (Fig. 1B). Minimal induction of apoptosis was observed within 1 h, while 15.7% ± 5.2% of the cells were in early and late apoptosis at 4 h because of polymyxin B treatment. Apoptotic cell death induced by colistin was significantly lower than that due to polymyxin B and failed to reach a plateau, while CMS treatment did not induce any significant cell death (≤20% at 0.5 to 5.0 mM CMS versus <10% of the control). Less than 5% of the CMS at 0.5 to 5.0 mM in the cell culture medium was converted to colistin at 24 h.

Comparison of the polymyxin B sensitivities of A549 and HK-2 cells. After treatment with 100 μM polymyxin B for 24 h, the viability of HK-2 cells decreased to approximately 55%, whereas the viability of A459 cells was not affected (Fig. 2A). Polymyxin B at 250 μM induced death in >80% of the HK-2 cells at 24 h, while >90% of the A549 cells remained viable. Staining of cells with a polymyxin B-specific mono-
clonal antibody (MAb) showed substantially more polymyxin B accumulation in HK-2 cells than in A459 cells at 24 h (Fig. 2B).

**Polymyxin B-induced activation of caspases and expression of FasL.** Polymyxin treatment of A549 cells induced concentration- and time-dependent activation of three major caspases (Fig. 3 to 5) associated with apoptotic cell death. Activation of caspase 9 increased ∼31-fold at 24 h ($P < 0.0001$) because of 2.0 mM polymyxin B treatment, whereas activation of caspases 3 and 8 increased approximately 9- and 13-fold, respectively. Time course data revealed that 2.0 mM polymyxin B activated caspases 3, 8, and 9 even at 4 and 8 h, whereas activation of caspases 3, 8, and 9 by 1.0 mM...
Polymyxin B was mainly observed at 24 h (Fig. 3D, 4D, and 5D). Polymyxin B treatment activated the death receptor apoptosis pathway in A549 cells and increased Fas ligand (FasL) expression in a concentration- and time-dependent manner (Fig. 6). At 24 h, the proportion of FasL-positive cells increased to 31.6% ± 1.11% and 79.0% ± 2.25% following treatment with 1.0 and 2.0 mM polymyxin B, respectively (Fig. 6A and B). It is evident that 2.0 mM polymyxin B induced significant FasL expression even at 4 h (Fig. 6C and D).

Polymyxin B-induced oxidative stress and loss of mitochondrial membrane potential. Concentration-dependent mitochondrial oxidative stress was also evident following polymyxin B treatment. Significantly more oxidative stress, measured by MitoSOX red fluorescence intensity, was observed in cells treated with 1.0 mM (~2.0-fold) and 2.0 mM (~4.3-fold) polymyxin B than in untreated control cells (Fig. 7A and B). Polymyxin B-induced oxidative stress was also time dependent (Fig. 7C and D). After 8 h of treatment, the cellular oxidative stress increased around 1.9- and 3.8-fold at 1.0 and 2.0 mM polymyxin B, respectively, and increased to 2.6- and 4.7-fold at 24 h, correspondingly. Similarly, a concentration-dependent increase in oxidative stress was observed in colistin-treated cells (Fig. 8A and B), whereas in CMS-treated cells, no oxidative stress was detected even at 24 h.

Polymyxin B treatment led to a concentration-dependent loss of mitochondrial membrane potential, as evidenced by a decrease in tetramethylrhodamine ethyl ester (TMRE) fluorescence intensity (Fig. 9A and B). Compared to that of the untreated control, TMRE fluorescence intensity decreased to 41.3% ± 3.05% and 2.14% ± 0.57% with 1.0 and 2.0 mM polymyxin B at 24 h, respectively. We also observed a time-dependent loss of mitochondrial membrane potential because of polymyxin B treat-
ment (Fig. 9C and D). TMRE fluorescence decreased significantly in cells treated with 2.0 mM polymyxin B even at 4 h (Fig. 9C and D).

DISCUSSION

Pulmonary delivery of polymyxins has offered a great promise for the treatment of lung infections (13, 14) caused by MDR Gram-negative pathogens, as intravenous administration leads to poor drug disposition at the infection site (7) and nephro-toxicity can occur in up to 60% of patients (9–11). However, the mechanism of polymyxin-associated pulmonary toxicity has not been examined. A549 is a well-established cell line of alveolar epithelial type 2 pneumocytes that constitute the pulmonary epithelial barrier (19, 20). Polymyxin B induced apoptosis in A549 cells in a concentration- and time-dependent manner (Fig. 1). It appears that the hydrophobicity of position 6 in the polymyxin core structure is key for the apoptotic effect on A549 cells. As position 6 is the only difference between polymyxin B and colistin, the more hydrophobic D-Phe at position 6 of polymyxin B (21) may explain the greater magnitude of cell death (Fig. 1A) and mitochondrial toxicity (Fig. 7) than that caused by colistin (D-Leu at position 6) (Fig. 8). The net positive charge of polymyxins also appears important for the apoptotic effect on A549 cells. Minimal cellular toxicity and mitochondrial oxidative stress in A549 cells were observed with the negatively charged prodrug CMS even at 6.0 mM (Fig. 8). Confirmed by high-performance liquid chromatography (HPLC), the conversion of CMS to colistin was minimal (<5%) at 24 h. Therefore, negatively charged CMS is much less toxic to A549 cells than polymyxin B and colistin. Our finding of a minimal toxic effect of CMS on A549 cells supports the safety of inhaled CMS in patients with cystic fibrosis (e.g., 2 million IU every 8 h), which has been widely used in Europe over the last few decades (22). Inhaled polymyxin B
(e.g., 50 mg/12 h) also appears safe (23) for patients with pneumonia caused by MDR Gram-negative pathogens. Our cell culture data (Fig. 1A and B) indicate that PK/PD optimization of inhaled polymyxin B in patients is important to avoid any potential adverse effects. Compared to HK-2 cells (EC50 = 0.35 mM) (24), which were employed as a positive control in the present study, A549 cells (EC50 = 1.74 mM) were significantly less susceptible to polymyxin B (Fig. 2A). Hence, the accumulation of polymyxin B in both A549 and HK-2 cells was determined (25) by using an anti-polymyxin B MAb. Interestingly, substantially less accumulation of polymyxin B was observed in A549 cells than in HK-2 cells (Fig. 2B). This result is encouraging, as it indicates that lung epithelial cells are much more tolerant of polymyxins than kidney tubular cells. The notable difference in polymyxin B susceptibility between A549 and HK-2 cells may be due to the differential expression of membrane transporters such as megalin, which exists in the latter but not in lung epithelial cells (26). Very likely, uptake of polymyxins is mainly by PEPT2 in A549 cells (27) and by both PEPT2 and megalin in HK-2 cells (28, 29).

In the present study, we discovered that polymyxin treatment activated three major caspases involved in apoptosis pathways in A549 cells (Fig. 3 to 5). Caspase 3, 8, and 9 are a family of aspartate-specific cysteine proteases that serve as the primary mediators of apoptosis (30). Activation of caspases can be triggered by two potentially interacting pathways, the mitochondrial pathway (intrinsic) and the cell death receptor pathway (extrinsic) (31). The concentration- and time-dependent activation of all three caspases in A549 cells by polymyxin B shows that both death receptor and mitochondrial pathways were involved in polymyxin-induced apoptosis (Fig. 3 to 5). The interaction between the cell death surface receptor Fas and plasma membrane-anchored FasL is a key component of the cell death receptor apoptosis pathway (32, 33). Binding of FasL to Fas triggers the activation of the initiator caspase 8, which subsequently activates the executioner caspase 3, culminating in apoptotic cell death (34). To this
end, our results revealed that polymyxin B induced the expression of FasL in A549 cells both concentration and time dependently (Fig. 6). A steady increase in FasL surface expression was evident with time, beginning as early as 4 h and continuing over 24 h. The FasL time course data are consistent with the observed activation of caspase 8 (Fig. 4), both of which showed that the cell death receptor pathway is a mediator of polymyxin B-induced apoptosis in A549 cells.

Caspase 9 is mainly activated through proapoptotic proteins released via the intrinsic mitochondrial pathway (35). Mitochondrial stress induces release of cyto-

**FIG 7** Polymyxin B-induced mitochondrial oxidative stress in A549 cells. (A, B) Concentration-dependent (0.25, 1.0, and 2.0 mM) polymyxin-induced mitochondrial oxidative stress was detected with MitoSOX. (C, D) Time-dependent mitochondrial oxidative stress at 1.0 mM (black bars) or 2.0 mM (gray bars) polymyxin B. Scale bars, 50 μm. Results are presented as the mean ± SD (n = 3). **, P < 0.01; ****, P < 0.0001 compared with control samples. In panel B, the concentration-dependent data represent the 24-h time point.

**FIG 8** Colistin- and CMS-induced mitochondrial oxidative stress in A549 cells. (A, B) Concentration-dependent (2.0, 4.0, and 6.0 mM) mitochondrial oxidative stress was detected with MitoSOX only in the colistin group at 24 h. Scale bar, 50 μm. Results are presented as the mean ± SD (n = 3). **, P < 0.01; ****, P < 0.0001 compared with control samples.
chrome c, which promotes the activation of caspase 9 (36). The impact of polymyxin B on the activation of caspase 9 was significant, with an ~31-fold increase following treatment at 2.0 mM for 24 h and a 13-fold increase with caspase 8 under the same conditions (Fig. 5A and B). Our results showed that the mitochondrial pathway plays a key role in polymyxin-induced apoptosis in A549 cells, which is different from that in NRK-52E cells, in which the death receptor pathway is predominantly activated by polymyxins (3.6-fold with caspase 8 versus 1.9-fold with caspase 9) (17). The exact mechanism of this difference is not clear and is currently being examined by a systems pharmacology approach in our laboratory. The loss of mitochondrial membrane potential and mitochondrial superoxide formation are highly associated with the regulation of cellular functions and are commonly observed to be mediators of apoptotic events (37–41). Mitochondrial stress caused by polymyxin B in A549 cells was clearly demonstrated by the concentration- and time-dependent generation of reactive oxygen species (Fig. 7) and loss of mitochondrial membrane potential (Fig. 9). Our findings suggest that there might be cross talk between the death receptor and mitochondrial pathways, as the activation of both caspases 8 and 9 was observed in polymyxin B-treated cells. It has been reported that death receptor-activated caspase 8 may propagate apoptotic signals by activating downstream caspases or causing the release of cytochrome c from mitochondria, thereby triggering the mitochondrial pathway through the activation of caspase 9 (42).

To the best of our knowledge, this is the first study to reveal that polymyxins induce apoptosis in human lung epithelial cells in a concentration- and time-dependent manner. Our findings also demonstrate that the apoptotic events are triggered via the extrinsic death receptor and intrinsic mitochondrial pathways. This
study sheds new light on the cell death pathways that mediate potential polymyxin-induced toxicity in the lungs. The fundamental knowledge obtained will facilitate the development of novel strategies for the effective and safe use of inhaled polymyxins in patients to treat life-threatening pulmonary infections caused by Gram-negative “superbugs.”

MATERIALS AND METHODS

Chemicals and reagents. Polymyxin B (sulfate; catalog no. 86-40302; lot 20120204; minimum potency, 95%) and colistin (sulfate; catalog no. 86-41620; lot 20120719; minimum potency, 95%) were purchased from BetaPharma (Shanghai, China). CMS (catalog no. C1511) and etoposide (catalog no. E1383; potency, ≥98%) were obtained from Sigma-Aldrich (NSW, Australia). Mouse anti-polymyxin B IgM MAb (Invitrogen, catalog no. MA1-40133) and goat anti-mouse IgM conjugated with Alexa Fluor 647 conjugated with Alexa Fluor 647 (A-21238) were purchased from Thermo Fisher Scientific Australia Pty. Ltd., Melbourne, Australia.

Cell culture. A549 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Life Technologies, Victoria, Australia). A549 cells (1.5 × 10^5/well) were used to seed 12-well plates and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. The medium was then removed by aspiration, and the cells were washed twice with phosphate-buffered saline (PBS) (pH 7.4; Invitrogen). Treatments with polymyxins were conducted with DMEM supplemented with 1% FBS.

Concentration- and time-dependent apoptosis studies. Measurement of apoptosis was conducted as described previously (24). Briefly, A549 cells were cultured in 12-well plates and incubated with and without polymyxins (polymyxin B, colistin, and CMS; the concentrations examined are presented in Table 1) for 24 h to evaluate the concentration-dependent effect. The EC₅₀ values of polymyxins were calculated by fitting a Hill function by using unweighted nonlinear least-squares regression analysis in GraphPad Prism (v6.0; GraphPad Software, San Diego, CA, USA) (24). Time-dependent induction of apoptosis was measured in the presence of polymyxin B (2.0 mM) at 1, 2, 4, 8, 16, and 24 h. Cells treated with etoposide (340 μM) were employed as the positive control (43). For the other experiments described below, polymyxin B was employed as the representative. Concentrations of colistin formed in the CMS-treated samples at 24 h were measured by HPLC (44). Excellent linearity (r², >0.996) was obtained for the calibration curve with concentrations ranging from 0.10 to 8.0 mg/liter; the accuracy and reproducibility of the assay were within 16%.

Toxicity and cellular disposition of polymyxin B in A549 cells compared to HK-2 cells. The effect of polymyxin B on the (percent) viability of A549 cells was compared with its effect on that of HK-2 cells as described above. HK-2 cells (1.5 × 10^5/well of a 12-well plate) were grown in complete keratinocyte serum-free medium at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. Both HK-2 and A549 cells were treated with polymyxin B (100 and 250 μM) for 24 h. For determination of the intracellular disposition of polymyxin B (12.5 μM for 24 h), A549 and HK-2 cells were grown for 24 h on sterile glass coverslips (Thermanox; ThermoFisher Scientific) at a density of 1.5 × 10^5/well in six-well plates. For immunostaining, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 15 min at room temperature. Cells were washed twice with PBS for 5 min between steps. Nonspecific binding site was blocked by treatment of 1% goat serum for 2 h. Cells were then washed twice with cold PBS and incubated with goat anti-mouse IgM conjugated with Alexa Fluor 647 (1:500 in blocking buffer) at room temperature for 1 h in a dark and humid chamber. Cells were then incubated with 2.0 ml of Hoechst 33342 (2 μg/ml) in PBS for 10 min to stain the nuclei and then washed with PBS only for 5 min. A Leica SP8 inverted confocal microscope (Leica, Wetzlar, Germany) was used for fluorescence imaging with a 63× oil immersion objective (numerical aperture [NA] 1.4).

Activation of caspases and expression of the plasma membrane receptor FasL. To assess the activation of caspases 3, 8, and 9 by polymyxin B, cell membrane-permeating, fluorogenic, caspase-specific substrates were employed in accordance with the manufacturer’s instructions (CaspGLOW red active caspase 3, 8, and 9 assay kit [BioVision, USA]; excitation and emission wavelengths of 561 and 566 to 685 nm, respectively) (17). A549 cells on chamber slides were treated with 0.25, 1.0, or 2.0 mM polymyxin B for 24 h. Cells treated with growth medium were employed as a control. Cells were incubated with 340 μM etoposide for 24 h and used as a positive control. For the negative control, cells were exposed to 1.0 mM polymyxin B for 24 h and treated with the caspase inhibitor Z-VAD-FMK (BioVision, USA) before incubation with the fluorogenic substrates. Hoechst 33342 (2 μg/ml; Invitrogen;
excitation and emission wavelengths of 405 and 410 to 551 nm, respectively) was used as a nuclear stain. Fluorescence was quantified with a Leica SP8 laser scanning confocal microscope (63× PlanApo, NA 1.4, oil immersion objective). NIH ImageJ was employed to quantify the background-subtracted average intensity per cell by counting Hoechst 33342-positive nuclei (45, 46). Three random locations with at least 50 cells per sample were selected for each analysis.

Flow cytometry (47) was employed to determine polymyxin B-induced FasL expression in A549 cells. A549 cells were cultured on 12-well plates (Corning, USA) and treated with 0.25, 1.0, or 2.0 mM polymyxin B for 24 h. For the assessment of time-dependent FasL expression, cells were treated with 1.0 or 2.0 mM polymyxin B for 0, 4, 8, or 24 h. Cells were then washed and detached from the plates by trypsinization, followed by neutralization of the trypsin with DMEM. The cell suspension was centrifuged, and the cell pellets were resuspended in PBS. The cells were then washed and incubated with Alexa Fluor 647 (AbD Serotec, Germany)-conjugated anti-CD178 (FasL) MAb (hamster anti-mouse CD178) for 45 min at room temperature. After incubation, cells were centrifuged, washed with PBS, and resuspended in 2% FBS-supplemented PBS. FasL expression was measured by fluorescence-activated cell sorter analysis (excitation and emission wavelengths of 650 and 668 nm, respectively).

Mitochondrial superoxide formation and loss of mitochondrial membrane potential. A549 cells (0.5 × 10^5/ml) were used to seed sterilized eight-well chamber slides with a 1.5H (170 ± 5 µm) polymer coverslip-like bottom (ibidi, Germany) in DMEM and grown overnight at 37°C. Cells were treated with 0.25, 1.0, or 2.0 mM polymyxin B for 24 h, and concentration-dependent superoxide formation was evaluated. The effects of colistin and CMS treatments (2.0, 4.0, and 6.0 mM) on mitochondrial superoxide formation were also determined. For evaluation of time-dependent superoxide formation, cells were treated with 1.0 and 2.0 mM polymyxin B for 0, 4, 8, and 24 h. To assess the formation of mitochondrial superoxide, cells were incubated with 5.0 µM MitoSOX Red dye (Invitrogen; excitation and emission wavelengths of 514 and 531 to 622 nm, respectively) at 37°C for 45 min. The loss of mitochondrial membrane potential was measured by incubating cells in the dark with 50.0 nM TMRE (Invitrogen; excitation and emission wavelengths of 561 and 568 to 690 nm, respectively) for 30 min at 37°C. Nuclei were stained with 2 µg/ml Hoechst 33342 (Invitrogen; excitation and emission wavelengths of 405 and 410 to 551 nm, respectively) to allow cell counting. Fluorescence intensity was quantified by confocal laser scanning microscopy (Leica SP8 inverted microscope equipped with a 63× oil immersion objective). The average fluorescence intensity per cell for each treatment was calculated by using ImageJ (45, 46).

Three random fields containing at least 50 cells per treatment condition were analyzed, and the TMRE fluorescence in untreated control cells was set as 100%.

Statistical analysis. All experiments were conducted with three replicates, and results are presented as the mean ± standard deviation (SD). One-way analysis of variance and Tukey’s test with a significance level of P < 0.05 were performed with GraphPad Prism (v6.0).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02690-16.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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We have no conflicts of interest to declare.

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