Morin Moderates the Biotoxic Activity of Pneumococcal Pneumolysin by Weakening the Oligomers’ Formation

Xiaoran Zhao, Yonglin Zhou, Guizhen Wang, Dongxue Shi, Yonghong Zha, Pengfei Yi, and Jianfeng Wang

Key Laboratory of Zoonosis, Ministry of Education, College of Veterinary Medicine, Jilin University; Changchun 130062, China; and Department of Food Quality and Safety, Jilin University; Changchun 130062, China.
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Streptococcus pneumoniae (pneumococcus) is an important causative agent of acute invasive and non-invasive infections. Pneumolysin is one of a considerable number of virulence traits produced by pneumococcus that exhibits a variety of biological activities, thus making it a target of small molecule drug development. In this study, we aimed to evaluate the effect of morin, a natural compound that has no antimicrobial activity against S. pneumoniae, is a potent neutralizer of pneumolysin-mediated cytotoxicity and genotoxicity by impairing oligomer formation, and possesses the capability of mitigating tissue damage caused by pneumococcus. These findings indicate that morin could be a potent candidate for a novel therapeutic or auxiliary substance to treat infections for which there are inadequate vaccines and that are resistant to traditional antibiotics.

Key words Streptococcus pneumoniae; pneumolysin; morin; oligomerization; genotoxicity

Streptococcus pneumoniae is an opportunistic pathogen and is responsible for multiple diseases, including otitis media, pneumonia, meningitis and bacteremia. Pneumococcal infections are asymptomatic nasopharyngeal diseases in most cases. In developed countries worldwide, bacterial pneumonia is a considerable cause of childhood mortality along with malnutrition. Since the twentieth century, penicillin has been the drug of choice for the treatment of pneumococcal infections, yet the incidence of penicillin-resistance in S. pneumoniae has also increased significantly. This situation has become a matter of concern for global health. Moreover, the increased incidence of multidrug-resistant bacteria in clinical isolates makes the treatment of S. pneumoniae infections tougher. Generalized antibiotic use is a powerful selective pressure contributing to the expansion of drug-resistant S. pneumoniae, which focuses global research on new therapeutic approaches for bacterial invasive diseases.

Among the many virulence factors of S. pneumoniae, pneumolysin is one of the most notable toxins. Pneumolysin is a cytolsin released from lysed bacteria that belongs to the family of cholesterol-dependent cytolsin (CDC), which is the largest-known group of pore-forming proteins. Commonly, pneumolysin inserts itself into the cholesterol-rich cell membranes; then, many monomers oligomerize to form a large pore leading to the rupture of the host cells. Experiments in vitro and in vivo have confirmed that pneumolysin exerts potent cytotoxic effects on host cells to cause inflammation, necrosis and tissue damage and provides conditions to subvert mechanical host defenses as well as to resist complement and immune responses. Recent studies have shown that pneumolysin is capable of inducing DNA double stranded breaks (DSBs) and cell cycle arrest. Taken together, pneumolysin seems to be an ideal target for adjuvant therapy or a single treatment for localized and invasive pneumococcal infections.

Morin (3,5,7,2',4'-pentahydroxyflavone) (Fig. 1A) is a flavonoid derivative and a light yellow pigment that is extracted from old fustic (Chlorophora tinctoria), osage orange (Maclura pomifera) and members of the Moraceae family such as mulberry figures. The affinal drug and diet plants that contain abundant morin are widely used as complementary food sources in traditional Chinese herbal medicine. Previous studies have found that morin exhibits a wide range of beneficial pharmacological activities, including antioxidant, anti-mutagenic, anti-diabetic, anti-inflammatory and anti-carcinogenic properties. It has also been shown that morin can modify transcription factor activation by producing reactive oxygen species and restraining the proliferation of a variety of tumor cells. Moreover, it is also capable of advancing urate excretion in the urine and reducing hematuria during in vivo experiments. However, none of these studies have addressed the impact of morin on exogenous microbes and bacterial virulence.

Due to the prevalence of antibiotic-resistant strains and the development of new antimicrobial agents that are related to existing drugs, the treatment of bacterial infectious diseases has become more challenging. Antibiotics impose a strong selective pressure on bacteria, thereby increasing the probability of the emergence of resistance mechanisms. Existing antibacterial agents act on the essential processes of growth in pathogens that hinder their multiplication or directly lyse the cells that can release cellular contents, particularly toxins that can damage host tissue. Based on these observations, we investigated the cytotoxicity of morin against S. pneumoniae pneumolysin in vitro and its impact on S. pneumonia induction in a pneumonia mouse model.

Experimental Reagents and Cells Culture Morin was obtained from Herbpurify (CAS No. 480-16-0, Chengdu, China). Dimethyl sulfoxide (DMSO) and Triton X-100 were obtained from
Sigma Chemical Co. (St. Louis, MO, U.S.A.). Alveolar epithelial (A549) cells were obtained from ATCC (Manassas, VA, U.S.A.). Dulbecco’s modified Eagle’s medium (DMEM)/High Glucose and trypsin–ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (CA, U.S.A.), and 10% fetal bovine serum (FBS) (Biological Industries, Israel) was added in DMEM for cells culture.

Cloning, Expression and Purification of Pneumolysin Primers design, vector construction, protein expression and purification of the specific operations and reagents selection, are referred to the previous experimental methods. 21) Hemolytic Test Hemolytic activity was estimated as presented previously. 22) Shortly, the reaction mixture was incubated for 10 min at 37°C including 25 µL defibrinated rabbit red blood cells (v/v 2.5%), 1.0 µL of purified pneumolysin (0.2 mg/mL) with a series of different concentrations of morin and phosphate-buffered saline (PBS) was added to bring the volume to 1 mL. Then the absorbance at 543 nm of each cell-free supernatant was measured to evaluate the hemolytic activity of the specimens. 1% Triton X-100 was mixed in as a positive group and the control group only contained PBS and erythrocytes in the reaction system.

Determination of Minimal Inhibitory Concentrations (MIC) The minimal inhibitory concentration of morin for S. pneumoniae was determined by the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Simply put, morin was diluted in several test tubes and the concentrations ranges of 2–1024 µg/mL. Following 5×10^5 CFU/mL bacteria were inoculated and cultured overnight in THB in each tube at 37°C. The lowest concentration inhibiting microbial growth was defined as its MIC.

Live/Dead and Cytotoxic Test A549 cells were propagated in culture and then digested with trypsin, seeded in a 96-well plate at a density of 2×10^4 cells in each well, and incubated overnight. The cells were subjected to isolated incubation for 6 h with pneumolysin and increasing concentrations of morin in a 37°C incubator. After that, the cells were treated with live/dead (green/red) reagent (Invitrogen, Carlsbad, CA, U.S.A.) to evaluate cell viability. Live cells emitted green fluorescence and dead cells emitted red fluorescence. Microscopic images of stained cells were captured using a confocal laser scanning microscope (Nikon, Tokyo, Japan). Lactate dehydrogenase (LDH) activity was measured using the instructions of the Cytotoxicity Detection kit (Roche, Mannheim, Germany). The 96-well plate was centrifuged (1000 rpm, 10 min), and the supernatants were mixed with the reagents. After 30 min of
reaction in the dark, LDH activity was measured on a micro-
plate reader (Tecan, Salzburg, Austria).

**Immunofluorescence Detection** A549 cells were trans-
fected to a 24-well plate with a cell climbing piece (2×10^5 
cells per well). After incubation with pneumolysin (0.5 µg/mL) 
for 6h, the cells were treated with 4% paraformaldehyde for 
15min in sterile PBS, permeabilized with 0.3% Triton X-100 
for 15 min, and blocked with 5% BSA for 2h. Primary anti-
body was goat anti-rabbit IgG (H+L) labeled with fluorescein 
isothiocyanate (FITC) (TransGen, Beijing, China) that was 
used at 1:100 dilutions in blocking solution and incub-
ated overnight at 4°C with cover slips. The secondary anti-
body was goat anti-rabbit IgG (H+L) labeled with fluorescein 
isothiocyanate (FITC) (TransGen, Beijing, China) that was 
used at 1:500 dilutions. Cell nuclei were stained by anti-
fae 4′-6-diamidino-2-phenylindole (DAPI) (Invitrogen). All 
of the stained slides were analyzed under a confocal micro-
scope (Olympus, Tokyo, Japan), and the images were captured 
under 100× magnification, zoom 1.5×.

**Mouse Model of S. pneumoniae Pneumonia** Female 
C57BL/6J mice that had grown for 8 weeks and weighed 
20±2g were allowed to rest for one week to acclimatize be-
fore the experiments. The experimental methods for inducing 
mouse pneumonia models have previously been published.23) 
Mice were randomly divided equally into three experimen-
tal groups with each group containing 10 mice. The mice were 
anesthetized with chloral hydrate and held in the vertical 
position, and 20 µL of S. pneumoniae strain D39 suspension 
containing 5×10^7 CFU was instilled into the left nare until the 
suspension was inhaled into the lungs. Morin was dissolved 
DMSO as a stock solution. The infected mice were injected 
subcutaneously with DMSO or 50 mg/kg morin two hours 
after infection and then at 12h intervals. Mice were eutha-
nized three days post-infection. The lungs of the experimental 
animals were removed and placed in 1% formalin. Formalin-
fixed tissues were processed, stained with hematoxylin and 
esin, and visualized by light microscopy.

**Measurement of Viable Bacteria** In separate exper-
iments, the mice were sacrificed and the lungs harvested. 
The tissue was disrupted in 2 volumes of sterile PBS with 
10% homogenizer 4°C.24) Serial dilutions in sterile PBS were 
made from these homogenates, and 10-µL suspensions were 
smearied onto THB agar plates and incubated overnight at 
37°C. The different plates were used to count the number of 
viable bacteria in the target organs.

**Statistical Analysis** Student’s t-tests were implemented to 
determine the significance of changes in groups. The level of 
significance was set at *p<0.05, **p<0.01.

**Results**

**Morin Inhibits Hemolytic Activity of Pneumolysin** To 
verify that morin inhibits the cytolytic activity of pneumo-
lisin, we set up a series of experiments with increasing morin 
concentrations that contained the same amount of protein. 
The degree of erythrocyte dissolution was determined after incuba-
tion (Figs. 1B, C). In the negative group, only isotonic PBS and 
red blood cells were present, while all of the erythrocytes 
treated with morin were lysed by 0.2% Triton X-100. In each 
column, the pneumolysin concentration was kept the same, 
while the concentration of morin was varied. When no drug 
was mixed in the sample, the degree of cell lysis was similar to 
that in the positive group, indicating that the toxin could 
rupture all of the red blood cells. When the concentration of 
morin reached 4 µg/mL, the supernatant exhibited a macro-
scopic change, as shown in Fig. 1B. After the addition of the 
drug, the histogram height dropped sharply, suggesting that 
2 µg/mL morin protected approximately 38% of the erythro-
cytes from lysing and caused significant inhibition of pneu-
olysin hemolytic activity as well. When the concentration of the 
compound reached 32 µg/mL, the absorbance values at 543 nm 
were very close to the controls (Fig. 1C). Therefore, we con-
clude that morin can restrict pneumolysin-induced hemolysis 
in a dose-dependent manner.

**Oligomerization of Pneumolysin Affected by Morin** 
Oligomerization analysis was conducted to determine whether 
morin alleviated the biological toxicity by affecting the forma-
tion of pores. Earlier evidence revealed that a high concentra-
tion of pneumolysin can spontaneously aggregate to oligomers 
without the aid of cholesterol and cytomembranes.25) but the 
oligomers are not as stable as the monomers when loaded 
into SDS-PAGE. Therefore, the concentration of pneumolysin 
(2 mg/mL) used in oligomerization analysis was higher than 
that in the hemolytic test. The purified cytolysin incubated 
with varying doses of morin was placed at a suitable tempera-
ture and given sufficient time for the reaction to proceed. A 
pattern without the addition of the compound was used as a 
control. The developed photograph is presented in Fig. 1D. 
At high concentrations of morin, the bands of oligomers became 
thinner and lighter, while the monomer strips grew thicker 
and more distinct. Regarding to the phenotypic differences, 
though 8 µg/mL of morin could approximately completely 
inhibit the hemolysis, pneumolysin oligomers still could be 
detected at the same dose, due to the different concentrations 
of protein. Taken together, it is evident to claim that the ex-
perimental results are consistent with our point that morin is 
capable of setting a limit to cytotoxicity by interfering with 
the oligomerization of pneumolysin.

**Morin Attenuates Pneumolysin-Induced Loss of Cell Vi-
ability** We determined the cell necrosis effect of pneumolysin 
on A549 cells. The total volume per well was 200 µL, and 
2×10^6 cells were incubated with 3 µL of purified pneumolysin 
in DMEM in each well. Cells in the general media, DMEM, 
displayed long fusiform shapes, and the morphology of the 
cells was clear and complete (Fig. 2A). The pneumolysin-
treated cells were necrotic and exhibited red fluorescence. 
Cell morphology disappeared, and the cells became spherical 
(Fig. 2B). The number of live cells increased when 8 µg/mL of 
morin was added, and most of the live cells maintained their 
normal morphology. When the drug concentration reached 
64 µg/mL, the number of dead cells decreased significantly to a 
level approaching that of the DMEM group (Figs. 2C, D).

In the LDH release study, we measured the absorbance 
values at 560 nm of the supernatants, including those of the 
0.2% Triton X-100 lysed and DMEM groups and those of 
the pneumolysin-treated samples, with varying drug concentra-
tions. When the concentration of morin reached 2 µg/mL, 
the release value dropped from 99.37 to 93.60% compared with 
that of the non-drug group. Despite statistically significant dif-
fferences, the inhibitory effect of this concentration of morin 
on cytotoxicity was not satisfactory. With the increase in the 
concentration of the drug, the LDH release value dropped to 
7.09% when the morin concentration was 64 µg/mL (Fig. 2E). 
This experiment proved that morin was able to inhibit pneu-
Pneumolysin was treated with morin for 6 h. The live cells and the dead cells could be distinguished by confocal laser scanning microscopy, and the viability of the cells could be obtained by estimating the LDH release rates. (A) Ordinary A549 cells. (B) Cells treated with cytolysin without the compound. (C and D) Experimental cells were cultured in the medium containing 8 and 32 µg/mL of morin, respectively. (E) Morin reduces the cytotoxicity of pneumolysin. With the increase of the morin concentration, the LDH release decreased, revealing the reduction in cell death (*p<0.05, **p<0.01).

Cells were exposed to pneumolysin for 6 h, and then the γH2AX foci generated in nucleuses. However, no γH2AX foci were inspected in nucleuses of the morin-disposed set (8 µg/mL) whose manifestation was similar to that of the control group.

Morin Prevents DNA Damage Induced by Pneumolysin

In DNA double stranded breaks (DSBs), histone family 2A variant (H2AX) undergoes different modifications, including...
phosphorylation, acetylation, methylation and ubiquitination. When DSBs occur in cells, the highly conserved sequence Ser–Gln–Glu (SQE) at the C-terminal of the polypeptide rapidly phosphorylates to generate γH2AX. In our study, A549 cells were exposed to purified pneumolysin (0.5 µg/mL), and the number of γH2AX foci substantially increased after 6 h compared to the medium-only cells (shown by arrows in Fig. 3). For the treatment group, the cells treated with pneumolysin in the presence of morin (8 µg/mL) had a reduced number of positive γH2AX foci. Only slight green fluorescence caused by the non-specific binding could be seen (Fig. 3). Our observation of the photos revealed that pneumolysin could induce cytotoxicity via DSBs, and morin could neutralize its biological activity to reduce the DNA damage induced by pneumolysin. This conclusion also provides a basis to use the drug to alleviate the injury to the host caused by this toxic protein.

**Effect of Morin on Mitigating S. pneumoniae Pneumonia**

Previous studies have shown that the isogenic pneumolysin-deficient S. pneumoniae are significantly less pathogenic and lethal than wild-type S. pneumoniae, while the purified pneumolysin treated group could cause mortality similar to that of the wild-type strain group. Based on these findings, we performed additional studies on the therapeutic performance of morin in the treatment of S. pneumoniae pneumonia in the mice model. Prior to this, we verified that the MIC of morin against the microorganism was >1024 µg/mL, which indicated that morin has no bacteriostatic ability.

Experimental animals were infected intranasally with a 20-µL strain D39 suspension following subcutaneous administration of either DMSO or 50 mg/kg morin, as described in the materials and methods. The lung tissues of the negative group mice that received only the solvent were pink and soft textured. Histological sections showed complete and fine alveolar structures, and no inflammatory cells exuded. After removing the lung tissues of the infected mice that received DMSO, we found that they displayed excessive signs of lung damage with dark red color, tight texture and extensive hyperemia, as shown in Fig. 4A. Histopathological study suggested that more devastating alveolar destruction and inflammatory cell accumulation occurred in mice with pneumonia that had been injected with solvent. In contrast, the lung tissues in the morin treatment group of mice displayed a significant reduction of congestion and pulmonary inflammation (Fig. 4A), thus revealing the effect of alleviating inflammatory cell infiltration and pneumonia injury. Additionally, the number of viable bacteria was measured to further demonstrate the effect of morin in vivo. The number of bacteria in dilute tissue homogenates cultured on THB agar plates was determined after incubation at 37°C overnight. The average viable bacteria in the untreated mice was 6.35 (log_{10} CFU/mg) compared with 6.21 (log_{10} CFU/mg) in the morin treated group (p=0.002), indicating that morin influences S. pneumoniae survival and reproduction within the target organs (Fig. 4B).

The results of our in vivo experiments demonstrate that morin is able to reduce the invasion of pathogens in tissues, inhibit the propagation of microorganisms in the target organs and lessen the inflammatory response. Thus, we believe that the compound can impair the virulence of S. pneumonia in mice with pneumonia.

Discussion

Bacteria that used to succumb to antibiotics have been mutating and evolving their own resistance to 150-plus drugs, while the warning about the looming “post antibiotic era” makes the status quo more grave. The emergence of invasive pneumococcal strains and multiple drug resistance (MDR) serotypes is a global threat that must be managed through a variety of strategies, including vaccinations, small molecule anti-virulence drugs, and continuous antibiotic monitoring. The high morbidity and mortality related to pneumococcus, the most common type of streptococcal infection, distinctly shows that there is an urgent need and underserved development in developing new anti-infective pharmacons. However, this process is burdensome due to the paucity of new options for clinical medication. Anti-virulence strategy supplements long-held scientific beliefs and provides great promise for treating an array of menacing infections, which provides humans with new weapons to battle infections.

Previous studies have noted that pneumolysin is required for the development of S. pneumoniae-induced pneumonia and bacterial survival in the blood, and pneumolysin-deficient
mutants exhibited a drastic decrease in the number of pneumococcus in the blood compared with the wild-type strain.\textsuperscript{28} Pneumolysin can directly act as a eukaryotic toxin or indirectly stimulate host immune responses in different processes.\textsuperscript{29} In addition, it is a member of the CDC family that can induce cytolysis through two crucial processes, namely, binding to cell membrane cholesterol and oligomerizing to form a large pore in the cytomembrane. In our oligomerization analysis, it is striking that the concentration of oligomers was sharply reduced in the presence of morin, suggesting that the molecule interferes with the interactions between the monomers.

Morin exhibits a wide range of beneficial pharmacological activities has been mentioned before. In our current study, we aimed to investigate the function of this molecule that inhibits the activity of pneumolysin, a virulence trait, and alleviates the tissue damage caused by pneumococcal infection. According to our data, morin can reduce the hemolytic effect when it is present in the reaction system, and the levels of inhibition were better with increasing concentrations. Morin greatly enhances the survival rate of A549 cells in vitro, indicating that, in addition to its effect on erythrocytes, the drug can also reduce the toxicity on target organ eukaryotic cells. Moreover, H2AX is the first substrate to be phosphorylated and the most sensitive molecule in nucleus that reflects DNA damage.\textsuperscript{30} The amount and size of the γH2AX foci can be detected by immunofluorescence or flow cytometry, and the number of foci correlates with DSBs within a certain range. Hence, our results also display a significant effect on reducing the production of DSBs caused by pneumolysin, suggesting that morin can reduce the number of gene mutations and carcinogenic risks. In general, the direct cytoxic effect of pneumolysin is greater than those that lead to immunomodulatory or functional interference due to the cytolysin inducing pro-inflammatory cytokine release at lower concentrations.\textsuperscript{29} Since morin inhibits the formation of the pneumolysin oligomer, it becomes difficult for the cytolysin to form an intact pore in the membrane to bring about cytotoxicity or nuclear genotoxicity. On the basis of all of our in vitro data, we attempted to verify whether it is possible for morin to display the same potent antitoxic effect in vivo. Our subsequent animal experiments confirmed its effectiveness to remedy pulmonary injuries caused by \textit{S. pneumoniae}-induced pneumonia in a murine model; meanwhile, morin also has anti-inflammatory activity and an ability to inhibit viable bacteria in organs to promote recovery.\textsuperscript{30}

For future prospects of \textit{S. pneumoniae} anti-virulence medicaments, perhaps pneumolysin and choline binding proteins (CBPs) should be prioritized,\textsuperscript{1,32} as these two targets are inextricably linked to the pathogenesis of \textit{S. pneumoniae}. Although pneumolysin participates in a wide panoply of physiological processes, the study of molecular drugs and vaccines that explicitly target pneumolysin remain in their infancy. The extensive studies that have been conducted on its biological activities and the successful elucidation of the three-dimensional pneumolysin structure,\textsuperscript{33} should facilitate experiments to improve the \textit{in vivo} stability, potency and structure optimization of the toxin-antagonists so that we can calmly surmount the threats of antimicrobial resistances now and in the future.

\textbf{Ethical Approval}  
The whole processes gained authorization and were implemented according to the stipulations of the ACUC (Animal Care and Use Committee) affiliated to the Jilin University.

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\textbf{Conflict of Interest}  
The authors declare no conflict of interest.

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