Isoalantolactone protects against *Staphylococcus aureus* pneumonia

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Abstract

*Staphylococcus aureus* is a versatile pathogen that can cause life-threatening infections. The growing emergence of methicillin-resistant *S. aureus* strains and a decrease in the discovery of new antibiotics warrant the search for new therapeutic targets to combat infections. *Staphylococcus aureus* produces many extracellular virulence factors that contribute to its pathogenicity. Therefore, targeting bacterial virulence as an alternative strategy to the development of new antimicrobials has gained great interest. α-Toxin is a 33.2-kDa, water-soluble, pore-forming toxin that is secreted by most *S. aureus* strains. α-Toxin is essential for the pathogenesis of pneumonia, as strains lacking α-toxin display a profound defect in virulence. In this report, we demonstrate that isoalantolactone (IAL), a naturally occurring compound found in *Inula helenium* (Compositae), has no anti-*S. aureus* activity as per MIC evaluation in vitro. However, IAL can markedly inhibit the expression of α-toxin in *S. aureus* at very low concentrations. Furthermore, the in vivo data indicate that treatment with IAL protects mice from *S. aureus* pneumonia.

**Introduction**

*Staphylococcus aureus*, a ubiquitous and virulent pathogen, causes significant morbidity and mortality from a variety of infectious syndromes, ranging from minor skin and soft-tissue infections to life-threatening deep tissue infections (Lowy, 1998). Along with bacteremia, *S. aureus* pneumonia is one of the most prevalent *S. aureus*-mediated diseases, and it occurs in approximately 13.3% of all invasive staphylococcal infections (Klevens et al., 2007). The pathogenicity of *S. aureus* is largely dependent on extracellular virulence factors, including α-toxin, toxic shock syndrome toxin 1, and enterotoxins.

α-Toxin is a pore-forming toxin that possesses cytolytic, hemolytic, and dermonecrotic activities. A number of mammalian cells, including erythrocytes, monocytes, lymphocytes, and endothelial cells, are susceptible to α-toxin (Song et al., 1996). The toxin is primarily expressed in the stationary phase and is secreted as a 33.2-kDa water-soluble monomer (Gouaux, 1998). Upon binding to the membrane of a susceptible cell, the monomer oligomerizes to form a 232.4-kDa membrane-inserted heptamer (Song et al., 1996). McElroy et al. (1999) reported that α-toxin could damage the air–blood barrier of the lung in a rat model of *S. aureus*-induced pneumonia. Additionally, Bubeck Wardenburg et al. (2007) have also defined a central role of α-toxin in *S. aureus*-related pneumonia, as strains lacking α-toxin displayed a profound defect in virulence in a murine model of disease.

In the last 20 years, methicillin-resistant *S. aureus* (MRSA) has spread throughout the world. Kuehnert et al. (2005) reported that 53% of the staphylococcal pneumonia isolates are classified as MRSA. The treatment options for *S. aureus* pneumonia are limited; vancomycin and linezolid are recommended empirical and definitive therapies (Mandell et al., 2007). However, clinical failures are common when treating *S. aureus*-related pneumonia. For example, Wunderink et al. (2003) reported that, in the clinic, treatment with linezolid and vancomycin cures 59% and 35.5% of MRSA nosocomial pneumonia cases,
respectively. Therefore, novel antimicrobial agents are urgently required to improve outcomes.

Unfortunately, over the last 20 years, there has been a decline in the discovery of new antibiotics, creating a pressing need for the development of alternative therapies (Liu et al., 2008). Recently, targeting bacterial virulence factors as an alternative approach to the development of new antimicrobials is gaining increased interest (Rasko & Sperandio, 2010). It has been reported that the production of α-toxin in *S. aureus* could be affected by some natural compounds (Shah et al., 2008; Qiu et al., 2010). In the present study, we demonstrated that isalantolactone (IAL) (Fig. 1), a naturally occurring compound found in *Inula helenium* (Compositae), had no anti-*S. aureus* activity but could substantially inhibit the production of α-toxin by *S. aureus* at very low concentrations. Furthermore, we demonstrated its protective effects against *S. aureus*-related pneumonia in a mouse model.

**Materials and methods**

**Bacterial strains, culture, and reagents**

The bacterial strains used in the study are listed in Table 1. For hemolysis, Western blot, and real-time RT-PCR assays, *S. aureus* strains were cultured in tryptic soy broth (TSB) at 37 °C with the indicated concentrations of IAL until the bacteria reached the postexponential phase (OD₆₀₀ nm of 2.5, 2.0, 2.0, 2.5, and 2.5 for strains ATCC 29213, Wood 46, BAA-1717, 8325-4, and DU 1090, respectively). For cytotoxicity studies and *in vivo* studies, the *S. aureus* (8325-4 and DU 1090) used for the infection of mice was grown at 37 °C in TSB to an OD₆₀₀ nm of 0.5. Fifty milliliters of culture aliquots was centrifuged and washed with phosphate-buffered saline (PBS) prior to resuspension. For mortality studies, *S. aureus* 8325-4 and DU 1090 were resuspended in 500 μL PBS (4 × 10⁸ CFU per 30 μL). For histopathology experiments, *S. aureus* 8325-4 and DU 1090 were resuspended in 1000 μL PBS (2 × 10⁸ CFU per 30 μL). For cytotoxicity studies, 5 mL of culture prepared as described above was resuspended in 10 mL of DMEM medium (Invitrogen, CA). A 100 μL suspension was used per assay well.

IAL was commercially obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). For *in vitro* studies, IAL stock solutions of various concentrations were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO). For *in vivo* assays, IAL was suspended in sterile PBS.

**Susceptibility testing**

The minimal inhibitory concentrations (MICs) of IAL for *S. aureus* were determined using the broth microdilution method according to CLSI guidelines (CLSI, 2005). Oxacillin was used as a positive control.

**Hemolysis assay**

Hemolytic activity was assessed as described previously (Worlitzsch et al., 2001). Briefly, 100 μL of washed rabbit erythrocytes (5 × 10⁸ mL⁻¹) was added to 96-well V-bottom plates, filled with 100 μL of serially diluted bacterial culture supernatants and incubated for 20 min at 37 °C. One percent saponin (Sigma) was used as a positive control, and PBS served as a negative control. Following centrifugation, the OD₄₅₀ nm of the supernatant fluid was determined. One unit of hemolytic activity was defined as the amount of test solution able to liberate half of the total hemoglobin from the erythrocytes.

**Western blot assay**

After boiling in Laemmli sample buffer, 25 μL of culture supernatant was loaded onto a 12% sodium dodecyl sulfate–polyacrylamide gel (Laemmli, 1970). Protein was then transferred to polyvinylidene fluoride membranes. The membranes were blocked for 2 h using 5% bovine serum albumin in PBS. An antibody to α-toxin was purchased from Sigma-Aldrich and diluted 1 : 8000, and horseradish peroxidase-conjugated anti-rabbit antisemur (Sigma-Aldrich) diluted 1 : 4000 was used as the secondary antibody. The blots were developed using Amersham ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

**Real-time RT-PCR**

*hlα* and *RNAIII* expression was detected using real-time RT-PCR. *Staphylococcus aureus* 8325-4 was cultivated in TSB with or without graded subinhibitory concentrations of IAL until the postexponential growth phase (OD₆₀₀ nm of 2.5). The RNA was isolated as described by Samban-thamoorthy et al. (2006). Briefly, the cells were harvested by centrifugation (5000 g for 5 min at 4 °C) and resuspended in TES buffer containing 100 μg mL⁻¹ lysostaphin (Sigma-Aldrich). The samples were incubated at
37 °C for 10 min, and total bacterial RNA was isolated using Qiagen RNeasy Maxi columns according to the manufacturer’s instructions. RNase-free DNase I (Qiagen, Hilden, Germany) was used to remove contaminating DNA. The quality, integrity, and concentration of the purified RNA were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) according to the manufacturer’s protocol. The primer pairs used for real-time RT-PCR are listed in Table 2. cDNA was synthesized from total RNA using the Takara RNA PCR kit (AMV) Ver. 3.0 (Takara, Kyoto, Japan) according to the manufacturer’s instructions. The PCRs were performed in 25-μL reactions using SYBR Premix Ex Taq™ (Takara) as recommended by the manufacturer. PCR amplification was carried out using the 7000 Sequence Detection System (Applied Biosystems, Courtaboeuf, France). All samples were analyzed in triplicate, and the housekeeping gene gyrB RNA was used as an endogenous control. In this study, relative quantification based on the expression of the target gene relative to gyrB RNA was used to determine changes in transcription levels between samples.

**Live/dead and cytotoxicity assays**

A549 human lung epithelial cells (ATCC CCL 185) were cultured in DMEM medium supplemented with 10% fetal bovine serum (Invitrogen). Cells were seeded in 96-well plates at a density of 5.0 × 10^4 cells per well. For both assays, A549 cells were cultured in triplicate with 100 μL of staphylococcal suspension per well in DMEM medium with the indicated concentrations of IAL. Following incubation at 37 °C for 6 h, cell viability was measured either using live/dead (green/red) reagent (Invitrogen) or by measuring lactate dehydrogenase (LDH) release using a Cytotoxicity Detection kit (LDH) (Roche, Basel, Switzerland) according to the manufacturer’s directions. Microscopic images of stained cells were obtained using a confocal laser scanning microscope (Nikon, Japan). LDH activity was measured on a microplate reader (TECAN, Austria).

**Pharmacokinetics study**

All animal studies were conducted according to the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at Jilin University. Eight-week-old C57BL/6J mice were obtained from the Experimental Animal Center of Jilin University (Changchun, China).

For pharmacokinetics study, mice were administered a single subcutaneous dose of 10, 20, or 50 mg kg^{-1} IAL in sterile PBS. Groups of three mice were sacrificed in a CO2 chamber 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h after dosing. Blood samples were collected by cardiac puncture. Serum concentrations were determined using the WINNONLIN program (Pharsight, Mountain View, CA).

**Mouse model of intranasal lung infection**

For lung infection, mice were anesthetized intraperitoneally with 50 μL of rodent III anesthetic and then inoculated with 30 μL of S. aureus suspension in the left nare. Animals were held upright to allow recovery and then observed over the time course indicated in the figures. Each experimental group contained 20 mice.
To investigate the effects of IAL treatment, mice were administered 100 μL of IAL subcutaneously 2 h after infection with \textit{S. aureus} and then at 12-h intervals thereafter for a total of six doses. The control mice were treated with 100 μL of sterile PBS on the same schedule. For histopathologic analysis, mice were euthanized with anesthesia followed by cervical dislocation. The lungs were placed in 1% formalin. Formalin-fixed tissues were processed, stained with hematoxylin and eosin, and visualized by light microscopy.

**Statistical analysis**

The experimental data were analyzed with SPSS 12.0 statistical software. An independent Student’s \( t \)-test was used to determine statistical significance, and a \( P \) value < 0.05 was considered to be statistically significant.

**Results**

**IAL has no influence on \textit{S. aureus} growth**

As presented in Table 1, the MIC values for IAL that were tested against \textit{S. aureus} strains were > 1024 μg mL\(^{-1}\), which indicates that IAL does not inhibit the growth of \textit{S. aureus}.

**IAL in vitro reduces \textit{S. aureus} α-toxin production**

Four α-toxin-producing \textit{S. aureus} strains were cultured with increasing concentrations of IAL, and the culture supernatants were tested for the ability to perform hemolysis. As shown in Fig. 2a, treatment with IAL repressed the hemolytic activity in culture supernatants. The hemolytic units (HUs) in drug-free culture fluids were 42.4, 38.2, 110.7, and 46.4 for \textit{S. aureus} ATCC 29213, BAA-1717, Wood 46, and 8325-4, respectively. When 8 μg mL\(^{-1}\) of IAL was added to the media, the HUs were 1.2, 4.5, 14.1, and 0.6, respectively. Notably, a dose-dependent (1–8 μg mL\(^{-1}\)) attenuation of hemolysis was observed in all the tested strains. Furthermore, drug-free culture supernatants preincubated with 8 μg mL\(^{-1}\) of IAL exhibited no difference in HUs, indicating that the reduction in hemolytic activity was not owing to direct interaction of IAL on α-toxin (data not shown).

α-Toxin is the major toxin produced by \textit{S. aureus} and can cause hemolysis of rabbit erythrocytes. Therefore, \textit{S. aureus} culture supernatants were subjected to Western blot analysis to determine whether the reduced hemolytic activity was attributed to a decrease in the production of α-toxin. Ten nanograms of purified α-toxin was used as a positive control. As expected, IAL reduced the production of α-toxin (data not shown).

**Important notes:**

- Hemolytic activity of \textit{S. aureus} culture supernatants grown in the presence of increasing concentrations of IAL was significantly reduced compared to the IAL-free culture. \( *P < 0.05 \) and \( **P < 0.01 \) compared to the IAL-free culture.

**Fig. 2.** The influence of IAL on \textit{Staphylococcus aureus} α-toxin production. (a) Hemolytic activity of \textit{S. aureus} culture supernatants grown in the presence of increasing concentrations of IAL. (b) Western blot of α-toxin production. Culture supernatants of \textit{S. aureus} strains 8325-4 (lanes 1–5) and DU 1090 (lane 7) grown in the absence or in the presence of IAL were electrophoresed and blotted onto polyvinylidene fluoride membranes, and α-toxin was detected with specific rabbit antibodies against α-toxin. Lane 6, 10 ng of purified α-toxin. (c, d) Relative expression of \textit{hla} and \textit{RNAIII} in \textit{S. aureus} 8325-4 as determined by real-time RT-PCR. (a, c, d) Data are presented as the mean value ± SD of three independent experiments. \( *P < 0.05 \) and \( **P < 0.01 \) compared to the IAL-free culture.
of α-toxin in a dose-dependent manner (Fig. 2b). The addition of 1 μg mL⁻¹ IAL resulted in an undistinguished reduction in α-toxin; however, at 8 μg mL⁻¹, no immunoreactive α-toxin antigen could be detected in the supernatants of the tested strains. The results were confirmed with hemolysis assay.

Transcription of hla in S. aureus 8325-4 was measured using real-time RT-PCR. The expression of virulence factors in S. aureus is controlled by several global regulatory systems such as Agr, Sar, Sae, and Rot (Cheung & Zhang, 2002). The accessory gene regulator (Agr) is one of the best-characterized global regulatory systems and is known to regulate α-toxin. To evaluate the hypothesis that agr expression contributes to the differential expression of hla in IAL-treated and IAL-free S. aureus cultures, we measured the expression of RNAIII, the effector molecule of the agr response, which ultimately interacts with target genes to regulate transcription (Novick et al., 1993). As shown in Fig. 2c and d, expression of hla and RNAIII was inhibited by IAL in a dose-dependent manner. Remarkably, when S. aureus was exposed to 8 μg mL⁻¹ of IAL, the transcriptional levels of hla and RNAIII were reduced by 12.5- and 8.6-fold, respectively. The mode of action by which S. aureus controls α-toxin expression is fairly intricate and involves an interactive, hierarchical, regulatory cascade, which includes the products of Sar, Agr, and other components (Chan & Foster, 1998). Therefore, this result indicates that the reduced α-toxin levels may be partly attributable to inhibition of the Agr two-component system by IAL.

**IAL attenuates α-toxin-mediated injury of human lung cells**

Human A549 alveolar epithelial cells have been commonly used as a model for human pulmonary epithelia in a variety of biological and physiological studies (Nizet et al., 1996; Hirst et al., 2002). Bubeck Wardenburg & Schneewind (2008) have demonstrated the critical role of α-toxin in human alveolar cell injury; for example, S. aureus strains lacking α-toxin do not cause cellular injury. Furthermore, Liang et al. (2009) have also demonstrated that wild-type α-toxin causes significant death in epithelial cells (A549) in a dose-dependent manner. The addition of as little as 0.1 μg mL⁻¹ α-toxin resulted in the death of approximately 50% of cells (Liang et al., 2009). In this study, A549 cells were co-cultured with S. aureus 8325-4 in the presence of increasing concentrations of IAL; the amount of cell death was determined using live/dead (green/red) reagent. As shown in Fig. 3a, the uninfected A549 cell revealed a green fluorescent. Upon co-culturing with S. aureus 8325-4, cell death was apparent, as indicated by an increase in the number of red fluorescent dead cells and a change in the cellular morphology of the live cells (Fig. 3b). However, the addition of 8 μg mL⁻¹ of IAL caused a marked decrease in

![Fig. 3. IAL protects human alveolar epithelial cells from Staphylococcus aureus-induced injury. Live (green)/dead (red) staining of A549 alveolar epithelial cells was imaged using confocal laser scanning microscopy 6 h after infection with S. aureus 8325-4 or DU 1090. Cells were uninfected (a) or co-cultured with S. aureus 8325-4 in medium (b); co-cultures in the presence 8 μg mL⁻¹ of IAL (c); IAL-free co-culture plus 1% DMSO (d); and co-culture with S. aureus DU 1090 (e). (f) LDH release by A549 cells was determined using cells co-cultured with S. aureus 8325-4 in medium supplemented with the indicated concentrations of IAL. The data shown in panels a to e are representative of three independent experiments. The values in panel f represent the means ± SD of three independent experiments. **P < 0.01 compared to the IAL-free co-culture.](image-url)
A549 cell injury (Fig. 3c). The drug-treated co-culture contained 1% DMSO; therefore, the effect of DMSO on A549 cell viability was examined. As shown Fig. 3d, the addition of 1% DMSO resulted in the similar amount of cell death as in the IAL-free co-culture. The effect of the S. aureus DU 1090, an α-toxin-deficient mutant of S. aureus 8325-4, on cell viability was also investigated and resulted in no cell death (Fig. 3e). This result was consistent with a previous study that indicated that S. aureus strains lacking α-toxin did not cause cell injury in A549 cells (Bubeck Wardenburg & Schneewind, 2008).

Additionally, cellular injury in this system was also quantitated by an LDH release assay, and the results are presented as percent cell death. Treatment with IAL conferred a dose-dependent protection with concentrations ranging from 1 to 8 μg mL⁻¹ in co-cultures of A549 cells (Fig. 3f). As indicated above, IAL does not inhibit the growth of S. aureus; therefore, it can be concluded that IAL did not decrease S. aureus CFUs, which then led to a decrease in A549 cell injury.

IAL protects mice from S. aureus pneumonia

The in vitro results show that low concentrations of IAL inhibit the production of α-toxin by S. aureus and attenuate α-toxin-mediated injury of human lung cells, which indicates that IAL has potential therapeutic relevance.

To investigate the in vivo protective effects of IAL on mouse S. aureus-related pneumonia, we first assessed its pharmacokinetic characteristics in mice. Time–concentration profiles of plasma for three single subcutaneous IAL doses are presented in Fig. 4. The maximum concentrations of IAL in plasma (C_max) were 6.16, 15.67, and 32.66 μg mL⁻¹ for doses of 10, 25, and 50 mg kg⁻¹, respectively. The area under each of the concentration–time curves (AUC) for plasma was calculated from 0.25 to 24 h and was 29.73, 82.69, and 206.31, for doses of 10, 25, and 50 mg kg⁻¹, respectively.

Mice were infected via the intranasal route with 4 × 10⁸ CFUs of S. aureus 8325-4. Following treatment with IAL as described in the Materials and methods, mortality was monitored over 72 h. As a control, the mortality following infection with an hla⁻ S. aureus strain DU 1090 was also determined. As shown in Fig. 5a, mice that received 50 mg kg⁻¹ of IAL were significantly protected from S. aureus pneumonia (P < 0.05); however, the mortality was much higher than that in mice infected with S. aureus DU 1090. The protective effect was less evident in mice that received 25 mg kg⁻¹ of IAL, and little protective effect was observed in mice that were given 10 mg kg⁻¹ of IAL.

To evaluate the impact of IAL treatment on pathological manifestations of lung injury, we performed histopathologic analysis of lungs from S. aureus-infected mice that received 50 mg kg⁻¹ of IAL or PBS as a control. Gross inspection indicated that the lung tissue of infected mice was crimson and had a tight texture. Following treatment with IAL, the lung tissue of infected mice was light pink and fungous (Fig. 5b). As shown in the Fig. 5c, there were significant accumulations of inflammatory cells (dark blue or purple) in alveolar space in the group infected with S. aureus 8325-4. Notably, treatment with IAL resulted in a marked alleviation of pulmonary inflammation; treated mice had less accumulation of cellular infiltrates in the alveolar space.

Discussion

The increase in resistance of S. aureus to β-lactam antibiotics as well as the decreased clinical performance of vancomycin and linezolid (Mandell et al., 2007; Nguyen & Graber, 2010), combined with a decrease in the discovery of new antibiotics (Liu et al., 2008), warrants the search for new therapeutic targets to combat infections caused by S. aureus. Currently, targeting bacterial virulence as an alternative strategy to the development of new antimicrobials is gaining interest (Rasko & Sperandio, 2010). These methods disrupt the pathogenesis of bacterial infections without affecting bacterial growth. Therefore, the evolutionary development of resistance may decrease, as most virulence traits are not essential for bacterial viability. In contrast, antibiotics that kill microbes exert a strong selective pressure, which results in the emergence of drug-resistant strains (Levy et al., 1976).

Staphylococcus aureus secretes a number of extracellular virulence factors that contribute to its pathogenicity. Moreover, many recent studies have demonstrated a rapid
evolution of virulence in MRSA strains, which may lead to more severe and widespread disease (Holden et al., 2004; Li et al., 2009). Consequently, the clinical therapeutic values of antimicrobial agents selected for the treatment for S. aureus infections are evaluated not only for their respective bactericidal or bacteriostatic activities but also for their effect on virulence factors (Bernardo et al., 2004). On the other hand, virulence factors may potentially serve as targets for the development of new drugs. However, previous reports have indicated that mutations that abolish the expression of only one of S. aureus extracellular virulence factors do not cause a significant decrease in pathogenesis when measured in animal models of disease (O’Reilly et al., 1986; Patel et al., 1987). Nevertheless, there are some exceptions; intranasal infection of mice with hla− S. aureus resulted in substantially less lung injury and inflammation than an infection with hla+ S. aureus, and the mortality of mice infected with hla− S. aureus was much lower than that of mice infected with hla+ S. aureus (Bubeck Wardenburg et al., 2007; Bartlett et al., 2008). Disruption of the toxin function by a number of distinct immunization strategies has been shown to provide protection against S. aureus pneumonia (Bubeck Wardenburg & Schneewind, 2008; Ragle & Bubeck Wardenburg, 2009).

Targeting virulence factors is a promising strategy that relies on newly discovered synthetic or natural small organic compounds to inhibit the expression or secretion of virulence factors (Hung et al., 2005; Rasko et al., 2008). Based on our results that IAL in vitro inhibits the production of α-toxin by S. aureus and in vivo protects mice from S. aureus pneumonia, the structure of IAL could potentially be used as a basic structure for the development of drug that aimed against S. aureus α-toxin.

Use of antivirulence drugs in combination with established or novel antimicrobials is suggested and may extend the life span of these drugs (Cegelski et al., 2008; Paul & Leibovici, 2009). It has been shown that subinhibitory concentrations of β-lactam antibiotics can strongly increase the production of α-toxin, which may aggravate disease (Ohlsen et al., 1998; Worlitzsch et al., 2001). Therefore, the data presented here suggest that IAL is potentially useful for the treatment for S. aureus pneumonia when used in combination with β-lactam antibiotics.

In conclusion, the findings in the present study support the views of antivirulence as a new antibacterial approach for chemotherapy, and the pathogenicity of S. aureus in pneumonia could be decreased by inhibiting the production of α-toxin.

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Fig. 5. IAL protects against *Staphylococcus aureus* pneumonia. Mice were infected via the intranasal route with S. aureus 8325-4 or DU 1090. (a) Percent mortality of mice infected with S. aureus after treatment with IAL. The statistical analysis of mortality studies was performed by comparing each condition and time point with the equivalent PBS alone control, *P* < 0.05. (b) Gross pathological changes and (c) histopathology of S. aureus-infected lung tissue from mice that were treated with either PBS or IAL. Experimental results shown in panels a, b, and c are representative of the results of three independent experiments.
Authors’ contribution

J.Q., M.L., and J.W. contributed equally to this work.

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