Baicalin Protects Mice From *Staphylococcus aureus* Pneumonia Via Inhibition of the Cytolytic Activity of α-Hemolysin

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α-Hemolysin (Hla) is a self-assembling, channel-forming toxin that is secreted by *Staphylococcus aureus* and is central to the pathogenesis of pulmonary, intraperitoneal, intramammary, and corneal infections in animal models. In this study, we report that baicalin (BAI), a natural compound that lacks anti-*S. aureus* activity, could inhibit the hemolytic activity of Hla. Using molecular dynamics simulations and mutagenesis assays, we further demonstrate that BAI binds to the binding sites of Y148, P151, and F153 in the Hla. This binding interaction inhibits heptamer formation. Furthermore, when added to *S. aureus* cultures, BAI prevents Hla-mediated human alveolar epithelial (A549) cell injury. In vivo studies further demonstrated that BAI protects mice from *S. aureus* pneumonia. These findings indicate that BAI hinders the cell lysis activity of Hla through a novel mechanism of interrupting the formation of heptamer, which may lead to the development of novel therapeutics that aim against *S. aureus* Hla.

*Staphylococcus aureus* is the most commonly isolated human bacterial pathogen, with up to 20%–30% of humans persistently asymptomatically colonized and 50%–60% intermittently colonized [1]. *Staphylococcus aureus* causes many skin and soft tissue infections and invasive diseases, such as sepsis, endocarditis, pneumonia, and osteomyelitis [2]. These infections are complicated to treat due to the ability of this bacterial species to become resistant to antibiotics. Presently, methicillin-resistant *S. aureus* (MRSA) is the most commonly identified antibiotic-resistant pathogen in many parts of the world, including Europe, the Americas, North Africa, the Middle East, and East Asia [3]. Moreover, MRSA rates have expeditiously increased worldwide over the past few decades. The reduced efficacy of vancomycin and linezolid against MRSA threatens to result in incurable staphylococcal infections [4].

To cause disease, *S. aureus* relies on the production of an impressive collection of virulence factors, including cell surface proteins and extracellular toxins, which contribute to the colonization of and damage to mammalian hosts [5]. α-Hemolysin (Hla), a 33.2-kDa polypeptide encoded by *hla*, is a channel-forming cytotoxin that is secreted by the majority of *S. aureus* isolates [6]. α-Hemolysin is secreted as a water-soluble monomer, and upon binding to the membrane of susceptible host cells, such as erythrocytes, monocytes, lymphocytes, macrophages, and epithelial cells, the monomer oligomerizes to form a 232.4-kDa membrane-inserted heptamer [7]. The primary mechanisms of cell damage and death mediated by Hla heptamers are leakage of ions, water, and low molecular weight molecules into and out of the cell and cell lysis [8]. The critical role of Hla to the pathogenesis in *S. aureus* infections has
been well investigated in animal models, including models involving pulmonary, intraperitoneal, intramammary, and corneal infections [9–12].

The increasing disease burden and the declining performance of traditional antimicrobial agents to combat *S. aureus* infections have necessitated alternative therapeutic strategies. Currently, antivirulence strategies to combat bacteria-mediated disease are receiving increasing interest [13, 14]. Baicalin (BAI) (Figure 1A) is a flavonoid compound isolated from *Scutellaria baicalensis* Georgi (Huang Qin), a traditional Chinese medicinal herb. Baicalin has been proven to possess antiviral, antioxidative, antithrombotic, and anticancer activities [15–19]. In the present study, the influence of BAI on the cytolytic activity of Hla was determined, and the mechanism of action was further investigated. Moreover, we assessed the potential therapeutic effect of BAI on *S. aureus* pneumonia in a mouse model of infection.

**MATERIALS AND METHODS**

**Bacterial Strains and Chemicals**

*Staphylococcus aureus* 8325-4 and DU 1090 (Hla-deficient mutant of 8325-4) and strain USA 300 (American Type Culture Collection [ATCC] BAA-1717) were used in this study. Baicalin was commercially obtained from Sigma-Aldrich. Wogonoside (WOG) and scutellarin (SCU) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products in Beijing, China. For in vitro studies, stock solutions of BAI, WOG, and SCU at various concentrations were made in dimethyl sulfoxide (Sigma-Aldrich). For in vivo experiments, BAI was dissolved in phosphate-buffered saline (PBS), and sodium hydroxide was added to obtain a final pH of 7.4 to aid in dissolution. The BAI solution was filter sterilized with a 0.22-μm pore-size acetate syringe filter. The minimal inhibitory concentrations (MICs) of BAI for *S. aureus* were determined using the broth microdilution method according to Clinical and Laboratory Standards Institute guidelines.

**Mouse Model of Intranasal Lung Infection**

C57BL/6J male mice aged 8 weeks were obtained from the Experimental Animal Center of Jilin University. Animal experiments were approved by and conducted in accordance with the guidelines of the Animal Care and Use Committee of Jilin University.

Bacteria were grown at 37°C in tryptone soy broth to an Optical density at 600 nm of 0.5. Fifty-milliliter aliquots of the cultures were centrifuged. For mortality studies, bacteria were re-suspended in 500 μL of PBS (4 × 10⁸ colony-forming units

Figure 1. Inhibition of α-hemolysin (Hla)–induced hemolysis. A, Chemical structures of baicalin (BAI), scutellarin (SCU), and wogonoside (WOG). B and C, Hemolysis assays were performed with purified Hla added to rabbit red blood cells in phosphate-buffered saline. The addition of BAI, SCU, and WOG reduced hemolysis, as measured by the absorbance at 543 nm of cell supernatants. The mean values of the experiments (n = 3). Error bars show the standard deviation. * indicates *P*< .05, and ** indicates *P*< .01 when compared with the drug-free group. Abbreviations: OD543, optical density at 543 nm.
[CFUs] per 30 μL). For bacterial load, histopathology, and bronchoalveolar lavage (BAL) fluid experiments, bacteria were re-suspended in 1000 μL of PBS (2 x 10^8 CFUs per 30 μL). For lung infection, mice were anesthetized intraperitoneally with ketamine and xylazine and then inoculated with 30 μL of S. aureus suspension in the left nare. Each experimental group contained 30 mice. To investigate the effects of BAI treatment, mice were administered 100 μL of BAI subcutaneously 2 hours after infection with S. aureus and then at 12-hour intervals. The control mice were treated with 100 μL of sterile PBS on the same schedule.

To determine the pathological correlations of staphylococcal pneumonia, infected mice were euthanized with anesthesia followed by cervical dislocation. The lungs were weighed and homogenized for calculation of bacteria burden via serial dilution and plating method. For histopathologic analysis, the lungs were placed in 1% formalin. Formalin-fixed tissues were processed, stained with hematoxylin and eosin, and visualized by light microscopy.

**Bronchoalveolar Lavage Fluid Collection and Analysis**

Bronchoalveolar lavage fluid collection was performed twice by intratracheal instillation of 500 μL of PBS. The lavage fluid was centrifuged, and the supernatants were used for cytokine measurements. Cell pellets were resuspended in 1 mL of PBS and used for total and differential cell counts. The total cell number in BAL fluid was counted using a hematology counter (Sysmex SF 3000; Sysmex Co.). Neutrophils in BAL fluid were counted on cytospin preparations (centrifuged preparations stained with the Kwik-Diff staining set; Thermo Fisher Scientific Inc.). A total of 300 cells were counted per cytospin.

Detailed methods on Western blot assay, hemolysis assay, live/dead and cytotoxicity assays, homology modelling of Hla, molecular docking, molecular dynamics (MD) simulations, calculation of binding free energy, mutagenesis of the Hla protein, determination of the binding affinities, oligomerization assay, and pharmacokinetics study are presented in the Supplementary materials.

**Statistical Analysis**

The statistical significance of the mortality studies was assessed using Fisher’s exact test; the significance of hemolysis, lactate dehydrogenase release assay results, bacterial burden, percentage of neutrophils, and cytokine levels was calculated using the 2-tailed Student t test. Differences were considered statistically significant when P < .05.

**RESULTS**

**Baicalin Inhibits the Hemolytic Activity of Hla**

Our previous studies and reports from other groups have shown that many natural compounds inhibit the expression of Hla by S. aureus and thereby reduce bacterial hemolytic activity [20, 21]. Baicalin has no anti-S. aureus activity with MICs >2048 μg/mL. In this study, we demonstrated by Western blot analysis that BAI does not influence the expression of Hla (Figure 2). However, when BAI was mixed with purified Hla, the hemolytic activity was attenuated in a dose-dependent manner (Figure 1B and 1C). In the absence of BAI, Hla causes 76.5% rabbit erythrocyte lysis, whereas the percent cell lysis was reduced to 9.9% in the presence of 16 μg/mL of BAI. The 50% inhibitory concentration (IC50) of BAI that inhibits the hemolytic activity of Hla was 6.63 μg/mL. Consequently, we may presume that BAI directly effects on Hla.

**The Binding Mode of BAI with Hla**

To explore the mechanism of BAI against Hla, we determined the preferential binding sites of the Hla water-soluble isolated protomer by MD simulation with BAI, based on the docking results. (The resulting homology modelling structure of the soluble monomer was used for the docking of Hla–BAI, and the root-mean-square deviation values with respect to the initial structure are shown in Figure 3A). In the simulation, BAI represents a ligand that can bind to Hla via hydrogen bonding and hydrophobic interactions.

Over the time course of the simulation, BAI localized in the binding cavity (P103 to T109, K147 to I156), which is also reported to participate in crucial protomer–protomer interactions during Hla self-assembly and is important for heptamer formation and cell lysis [22, 23]. The predicted binding mode of BAI with Hla is illustrated in Figure 3B. The stabilization at the binding cavity of Hla in the Hla–BAI complex was mostly due to residues Y148, P151, and F153 (Figure 3B).

**Identification of Binding Site Y148, P151, and F153 in Hla–BAI Complex**

To gain more information about the residues surrounding the binding site and their contribution to the whole system, the electrostatic, Van der Waals, solvation, and total contributions of the residues to the binding free energy were calculated with the molecular mechanics generalized born surface area (MM–GBSA) method [24, 25]. In the Hla–BAI complex, Y148 and P151 have an appreciable Van der Waals contribution.
Figure 3. The mechanism analysis of baicalin (BAI) against α-hemolysin (Hla) by molecular dynamics (MD) simulation. A, Root-mean-square deviation (RMSD) calculated on protein position over 60 ns MD simulation with respect to its initial structure for Hla. It can be seen that monomer Hla obtained from the homology modelling was found to reach equilibrium at 15 ns. B, Final conformation of the binding site of Hla–BAI. The structure of the binding mode of BAI (yellow) with WT–Hla shows the interaction of BAI with Y148, P151, and F153. C, The effects of BAI binding on Hla. Comparison of the distances (in Å) between the Cα atoms of Y148 and T155 in the free Hla and Hla–BAI complex is shown in the superimposition of structures of free Hla (green) and Hla–BAI complex (blue). The average distance of the complex is 14.6 Å. In the absence of BAI, the average distance between the defined points is 17.0 Å. D, Superimposition of structures of BAI (yellow), wogonoside (WOG) (white), and scutellarin (SCU) (blue) in the binding site of Hla. E, The distances between the Cα atoms of Y148 and T155 as a function of time. The black, red, blue, and green lines represent the distance between the same atoms in free Hla and the complexes of Hla–BAI, Hla–WOG, and Hla–SCU, respectively.
In fact, residues Y148 and P151 are close to the biphenyl group of BAI. In this case, 2 hydrophobic interactions exist (not solvated in the hydrophobic binding pocket), leading to a strong interaction between Hla and BAI. The conjugate ring of BAI serves as a hydrophobic anchor. In addition, residue F153 has a strong electrostatic interaction with the ligand because of the close proximity between the backbone oxygen of F153 and the carboxylate oxygen of BAI, which promotes strong hydrogen bonding in the complex.

For a more precise analysis of the Hla–BAI complex binding mode, the complexes of P151A-Hla and F153A-Hla mutants with BAI were used as preliminary structures for MD simulations. The MD trajectories were successively analyzed through the MM-GBSA method. The calculated binding free energy was −15.23 kcal/mol for wild-type (WT) Hla. The MM-GBSA calculation predicted that P151A-Hla and F153A-Hla bound weaker with BAI than WT-Hla (−10.26 kcal/mol for P151A-Hla and −10.48 kcal/mol for F153A-Hla). In the experiment, the binding efficiency of BAI to biological macromolecules at the ground state is described by the static fluorescence quenching constant, which is measured by the fluorescence spectroscopy quenching method. The static fluorescence quenching constants of WT-Hla, P151A-Hla, and F153A-Hla are 104.58, 63, and 74.93 mL/mg, respectively. The binding constants (K_a) of the interaction between BAI and Hla are 5.12 × 10^5, 1.12 × 10^5, and 3.64 × 10^5 L/mol and
Baicalin Bound to Hla Restrains Conformation Change of the Binding Cavity

In addition to yielding the binding modes of BAI on Hla, the MD simulation illuminates how structural motions in the binding cavity may affect protomer–protomer interactions during the formation of the heptameric transmembrane pore. Analysis of the cavity dynamics found that the conformation of the binding cavity is more rigid in the Hla–BAI complex than in the free protein. In particular, fluctuations in the side chain Y148 greatly altered the binding cavity conformation. The distance between the Cα of Y148 and the Cα of T155 ranged 13–15.5 Å over the time course of the simulation (average distance, 14.6 Å) in the complex (Figure 3C and 3E). In the absence of BAI, the distance between the defined points was 16–18 Å (average distance, 17.0 Å). In the crystal structure of Hla heptamer, which is available in the Protein Data Bank under the accession number 7AHL, the distance between the Cα of Y148 and the Cα of T155 is 17 Å. Dynamic fluctuations in the distance between the Cα of Y148 and the Cα of T155 are likely to dictate that the conformation of the binding cavity is restrained when BAI binds to Hla and BAI is depicted as a “molecular spring.”

Taken together, these findings led us to propose 1 possible inhibiting mechanism: the binding of BAI on conformation of the binding site (Y148, P151, and F153) leads to the inhibition of the self-assembly of the heptameric transmembrane pore, which decreases the cytotoxic activity of Hla. This was confirmed by a deoxycholate-induced oligomerization assay. Baicalin prevents the assembly of Hla into a sodium dodecyl sulfate stable oligomer (Hla7) in a dose-dependent fashion (Figure 5).

Comparison of the Binding Modes in the Structures of Hla–BAI, –WOG, and –SCU Complexes

The preferential binding sites of Hla with WOG and SCU were determined by MD simulations, based on the docking results. To compare the binding modes of the WOG substrate and the SCU substrate in the Hla binding pocket, MD simulation structures of WOG and SCU were superimposed on the MD simulation structure of BAI (Figure 3D). The glucuronide of WOG lies in the same site as the benzopyran moiety of BAI. This binding site is surrounded by residues Y148, V149, Q150, and P151. Thus, the stabilization in the Hla–WOG complex was mostly due to contributions from residues Y148, V149, Q150, and P151. However, compared with BAI, the benzopyran moiety of WOG is distant from residue F153, which indicates that F153 contributes minimally to the binding energy. On the other hand, according to the MD simulation, the binding site of SCU is distant from the residues of Y148, P151, and F153, which indicates that residues Y148, V149, Q150, P151, and F153 have no contribution to the binding energy (Figure 3D). These results were confirmed by calculating the energy contributions of the residues to the binding free energy using the MM-GBSA method (Figure 4B and 4C). In addition, analysis of the cavity dynamics showed that the distance between the Cα of Y148 and the Cα of T155 in the binding cavity where WOG and SCU bind is more flexible in these complexes than in the Hla–BAI complex. The distance between the Cα of Y148 and the Cα of T155 ranged 14–16 Å and 16–18 Å for Hla–WOG and Hla–SCU complexes, respectively (Figure 3E).

In this context, it is worth noting that the extent to which the flexibility of the conformation of the binding cavity is restrained follows the trend BAI > WOG > SCU. Based on the mechanism mentioned above, the inhibitory activity of BAI, WOG, and SCU for Hla also follows the trend BAI > WOG > SCU. This conclusion is consistent with the experimental evidence. The IC50 values of BAI and WOG that inhibit the hemolytic activity of Hla were 6.63 and 10.27 μg/mL, respectively; SCU has no substantial influence on Hla (Figure 1B and 1C).

The qualitatively good correlation between the simulation results and the experimental activities of the small molecules constitutes an encouraging validation of the target and of the use of information from signal transduction analysis in the detection of putative binding sites.

Baicalin Protects Mice From Staphylococcus aureus Pneumonia

The data from live/dead and cytotoxicity assays demonstrate that BAI could protect alveolar epithelial cells against cell death.
death caused by Hla (see Supplementary results and Supplementary Figure 1).

Based on these in vitro findings, we further investigated the protective effects of BAI in vivo on mouse *S. aureus*-related pneumonia. We first assessed the pharmacokinetic characteristics of BAI in mice. The maximum concentrations of BAI in plasma (C_{max}) were 9.98, 23.01, and 37.5 μg/mL for single subcutaneous doses of 25, 50, and 100 mg/kg, respectively (Figure 6A).

Further, we monitored mortality of staphylococcal pneumonia caused by *S. aureus* 8325-4 over a 72-hour time course. Mice that received 100 mg/kg of BAI were significantly protected from *S. aureus* pneumonia at 24, 48, and 72 hours (*P* < .04); mice given 50 mg/kg of BAI were significantly protected from mortality only until 72 hours postinfection (*P* = .003); at 25 mg/kg, the protective effect was not prominent (Figure 6B). Consistent with previous reports from other groups, the *S. aureus* strain lacking Hla production results in little mortality (Figure 6B). The bacterial burden in the lungs was quantified to assess the influence of BAI on *S. aureus* survival within the lungs. The CFUs 24 hours postinfection from mice that were treated with 100 mg/kg BAI were significantly lower than the CFUs in the PBS group (*P* = .0002) (Figure 6P).

To evaluate the impact of BAI treatment on pathological manifestations of lung injury, we performed histopathologic analysis of lungs 24 hours after infection from mice that received either 100 mg/kg of BAI 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 BAI, the lung tissue of infected mice was light pink and fungous (Figure 6D). As shown in Figure 6E, there were significant accumulations of inflammatory cells (dark blue or purple) in alveolar space in the group infected with *S. aureus* 8325-4. Treatment with BAI resulted in a marked alleviation of pulmonary inflammation, as indicated by less accumulation of cellular infiltrates in the alveolar space.

To clarify the extent of inflammation, we also analyzed the BAI fluid from infected mice. Mice that were intranasally infected with *S. aureus* 8325-4 had markedly neutrophil flow into the airway 24 hours postinfection, and the neutrophils accounted for 73.2% of total leukocytes in the BAI fluid (Figure 6G). Following treatment with 100 mg/kg of BAI, the percentage of neutrophils was reduced to 18.1%. The BAI fluid of mice infected with *S. aureus* DU 1090 contained few neutrophils (7.6%). Further, we detected the interleukin 1β (IL-1β), interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α) level in the BAL fluid of infected mice. Mice that received 100 mg/kg of BAI showed a significant decrease in BAL fluid IL-1β, IFN-γ, and TNF-α concentrations at 24 hours postinfection (*P* = .02, .04, and .001, respectively) (Figure 6H–I).

Furthermore, BAI was also effective in mice infected with clinically relevant isolate. As shown in Figure 6C, BAI could prevent mortality from *S. aureus* pneumonia caused by strain USA 300. In particular, a significant decrease in mortality was observed at all indicated time points in mice treated with 100 mg/kg of BAI (*P* < .002).

**DISCUSSION**

Due to the continuing rise in antibiotic resistance and a decline in the discovery of new antibiotics, we are now entering the postantibiotic era, with limited treatment options available for many bacterial infections, including MRSA [26]. However, many alternative anti-infective strategies are presently being investigated, including antivirulence therapy [13, 14]. Such strategies are based on the inhibition of virulence rather than bacterial viability. In contrast to classical antibiotics, which aim to kill microbes, this approach might apply a milder selective pressure for the development of resistance because most virulence factors are not indispensable for bacterial growth. Furthermore, the specific effect of antivirulence compounds should preserve the bacteria that constitute the normal flora [27].

*Staphylococcus aureus* produces ≥30 exoproteins [28]. However, when evaluated in animal models of disease, mutations that eliminate the expression and secretion of only 1 of these proteins do not lead to significant reductions in pathogenicity [12]. Nevertheless, there are some exceptions to this unified viewpoint. 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 significantly lower than that of mice infected with *hla*− *S. aureus* [9, 29]. Based on these findings, Hla may represent an important antivirulence target to treat *S. aureus* pneumonia. Recent research regarding the disruption of Hla function by a number of distinct immunization strategies has demonstrated that this approach protects against *S. aureus* pneumonia in a murine model [30, 31].

Recently, guided by the availability of a crystal structure of an Hla heptamer [22], more attention has been given to manipulating the properties of the fully assembled pore. For example, some researchers have designed the noncovalent molecular adaptor beta-cyclodextrin (or its derivative compounds) to block the lumen of the Hla heptameric pore and thereby compromise toxin function [32, 33]. An examination of a beta-cyclodextrin derivative (named IB201) in murine *S. aureus* pneumonia revealed that the administration of this compound protects against mortality [34]. However, such strategies could have some drawbacks. Apparently, this approach may be somewhat hysteretic and passive because the heptameric channels that mediate cell damage and death have already formed. Furthermore, beta-cyclodextrin does not significantly impact pathological manifestations of lung injury in staphylococcal pneumonia. Widespread inflammation...
Figure 6. Baicalin (BAI) protects against *Staphylococcus aureus* pneumonia. A, Serum concentrations of BAI in mice after a single subcutaneous dose of BAI. B and C, Percentage mortality of mice infected with *S. aureus* 8325-4 and USA 300 after treatment with BAI. Each group contained 30 mice. Gross pathological changes (D) and histopathology (E) of *S. aureus*-infected lung tissue from mice 24 hours after infection. Tissues were stained with hematoxylin and eosin (original magnification, ×400). F, Bacterial burden in the lungs of infected mice 24 hours postinfection. Percentage of neutrophils (G) and interleukin 1β (IL-1β) (H), interferon γ (IFN-γ) (I), and tumor necrosis factor α (TNF-α) (J) concentrations 24 hours postinfection in the bronchoalveolar lavage (BAL) fluid of infected mice that either received phosphate-buffered saline (PBS) or 100 mg/kg of BAI. Experimental results shown in panels B–E are representative of the results from 3 independent experiments. Data in panels F–J are means ± standard deviations (n = 30). * indicates *P* < .05, and ** indicates *P* < .01. CFUs, colony-forming units.
persisted in the pulmonary alveoli of mice treated with beta-cyclodextrin, as indicated by the accumulation of cellular infiltrates. It has been suggested that the Hla oligomer itself may induce cellular inflammatory responses, independent of the integrity of the pore [34]. Consequently, a treatment that precludes the full assembly of the heptameric toxin may alleviate lung injury more potently than beta-cyclodextrin derivatives.

In this study, we demonstrate that BAI binds to Hla, leading to the inhibition of the formation of the heptameric transmembrane pore, which results in a decrease in the biological activity of Hla. This mode of action may be superior to the functional blockage of the pore, as supported by the in vivo data that demonstrate that BAI not only reduces mortality but also alleviates the pathological features of lung injury resulting from *S. aureus* pneumonia.

Previous studies have shown that BAI has weak or no antibacterial activity against *S. aureus* [35, 36]. However, BAI acts synergistically with beta-lactam antibiotics oxycycline and tetracycline, thus enhancing its antimicrobial activity against MRSA [35, 37]. It is well known that subinhibitory concentrations of beta-lactam antibiotics may induce the expression of Hla in *S. aureus*, suggesting that the symptoms of *S. aureus* infections may be aggravated when treated with these antibiotics [38]. Collectively, based on the findings of our study and previous reports, it is reasonable to infer that BAI may potentially be useful for the treatment of MRSA infections when combined with beta-lactam antibiotics.

**Supplementary Data**

Supplementary materials are available at the *Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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