Aloe-emodin Attenuates Staphylococcus aureus Pathogenicity by Interfering With the Oligomerization of α-Toxin

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α-toxin, an essential virulence factor secreted by Staphylococcus aureus (S. aureus), is a critical exotoxin in multiple infections. In this study, we found that aloe-emodin (AE), a natural compound lacking anti-S. aureus activity, could inhibit the hemolytic activity of α-toxin. Oligomerization assays, molecular dynamics simulations, and fluorescence-quenching analyses were used to determine the mechanism of this inhibition. The oligomerization of α-toxin was restricted by the engagement of AE with K110, T112, and M113 of the toxin, which eventually resulted in inhibition of the hemolytic activity. Lactate dehydrogenase and live/dead assays demonstrated that AE decreased the injury of human lung epithelial cells (A549) and mouse lung macrophages (MH-S) mediated by S. aureus. Furthermore, treatment with AE showed robust protective effects in mice infected by S. aureus. These findings suggest that AE effectively inhibited the pore-forming activity of α-toxin and showed a protective effect against S. aureus virulence in vitro and in vivo, which may provide a new strategy and new antibacterial agent for clinical treatment of S. aureus infections.

Keywords: Staphylococcus aureus, α-toxin, aloe-emodin, antibiotic-resistant, pneumonia

INTRODUCTION

Staphylococcus aureus (S. aureus), one of the most common pathogens, is a critical cause of many local and systemic infections ranging from pneumonia, sepsis, and endocarditis, to osteomyelitis. Among these diseases, S. aureus pneumonia is one of the most serious ventilator-associated infections, with ~10–25% mortality and, for some secondary infections, the rate can even reach 75% (Gillet et al., 2002; Del Giudice et al., 2011; Li et al., 2011; Chastre et al., 2014). Although lactams, aminoglycosides, tetracyclines, sulfonamides, and other major antimicrobial drugs have been commonly used in the past century, we still cannot effectively inhibit the S. aureus pneumonia observed in the clinic most of the time. However, the abuse of antibiotics has led to many resistant strains, and treatment of S. aureus infections has cost at least 450 million dollars due to the increasing resistance (Parvizi et al., 2010; Song et al., 2010). In Europe, ~10–25% of S. aureus isolated from hospitals were observed to be methicillin-resistant S. aureus (MRSA), and the proportion has reached 50% in some regions (Commun, 2011). Even worse, the proportion of MRSA appeared to reach the highest level in years in parts of east Asia, such as Taiwan and South Korea, with an average rate of 77.6% (Chen and Huang, 2014). Since the twentieth century,
the multiresistance of MRSA has become more complicated, which typically results in a delay in clinical treatment (Mendes et al., 2013). Currently, vancomycin is the most commonly used drug to treat MRSA-associated pneumonia (Wunderink et al., 2003). However, the sensitivity of MRSA to vancomycin has been gradually decreasing for years and, given the current trends, the time required for the spread of resistant strains is much less than the time required for research and application of a new medicine. Accordingly, no treatments may be available for MRSA pneumonia in the future, and we need a new treatment strategy to replace the old antibiotic use regimens. Several studies have reported that targeting virulence factors typically results in weak pathogenicity of pathogens, suggesting that this may be a promising strategy in the treatment of S. aureus pneumonia (Qiu et al., 2012a,b; Wang et al., 2016).

During infection, a variety of virulence factors are secreted for invasion and colonization, including exotoxin and surface-associated protein (Vandenesch et al., 2012). α-toxin is one of the most important exotoxins produced by S. aureus and plays a key role in the course of multiple diseases as a pore-forming protein. It is a 33.2 kDa water-soluble monomer encoded by hla and can oligomerize into a 232.4 kDa membrane-inserted heptamer that penetrates the membrane (Gouaux, 1998; Nguyen and Kamio, 2004). The oligomer comprises seven monomers and consists of three major domains, including the cap domain, the rim domain, and the stem domain, which forms the transmembrane channel (Gouaux et al., 1994; Song et al., 1996). Many types of mammalian cells, including monocytes, erythrocytes, macrophages, and epithelial cells, are sensitive to α-toxin (Gouaux, 1998; Nygaard et al., 2012). For S. aureus pneumonia, studies have reported the destructive effect of α-toxin on the air-blood barrier, and a mutant strain lacking α-toxin showed decreased toxicity in animal models (McElroy et al., 1999; Xu et al., 2015). Therefore, targeting α-toxin is a promising therapeutic strategy for S. aureus infections, particularly MRSA pneumonia.

Aloe-emodin [AE; 1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone] (Figure 1A) is a common active compound derived from the leaves of Aloe vera and Rheum officinale (Dutta et al., 2007) that has been reported to possess antimicrobial, antiviral, and hepatoprotective activities (Eshun and He, 2004) as well as anticancer activity toward hepatoma cells, lung squamous cell carcinoma, and neuroectodermal tumors (Pecere et al., 2000; Lee, 2001; Kuo et al., 2002). In this study, we observed that AE can inhibit the hemolytic activity of S. aureus without decreasing the expression of α-toxin. In addition, we evaluated the protective effect of AE against MRSA in vitro and in vivo.

**MIC Determination**

The MIC of AE against USA300 was assessed via the broth microdilution method according to Clinical and Laboratory Standards Institute guidelines. The MIC value was defined as the lowest concentration of AE at which no visible growth of the microorganism could be observed.

**Hemolysis Assay**

Hemolysis assays were used to determine the inhibitory effect of AE on the hemolytic activity of α-toxin. The USA300 supernatants were harvested by centrifugation at 10,000 rpm for 1 min at 4°C, and then 0.1 ml of each supernatant was added to 1 ml of PBS buffer. The mixture was preincubated with AE at concentrations of 2, 4, 8, and 16 µg/ml at 37°C for 20 min. Next, 25 µl of rabbit erythrocytes (5 × 10^6 cells/ml) was added to each tube. Following an additional 30 min incubation at 37°C, the unlysed erythrocytes were removed by centrifugation at 6,000 rpm for 1 min, and the hemolytic activity was assessed by determining the OD_{543} values of the supernatant. For the control group, 25 µl of rabbit erythrocytes was added to 975 µl distilled water, and the supernatant served as the 100% hemolysis control. The % hemolysis of the AE-treated samples were calculated by comparing the OD_{543} values of the samples with that of the control cultures.

We also compared the effect of AE on the wild-type and mutant forms of α-toxin. Purified proteins (100 ng/ml) were preincubated with various concentrations of AE at 37°C for 20 min. Next, rabbit erythrocytes were added to the mixtures and incubated for another 30 min at 37°C. Erythrocyte lysis was determined as described above.

**Western Blot Assay**

For the Western blot assay, USA300 was grown in TSB at 37°C with shaking at 200 rpm to an OD_{600} of 0.3. Next, the cultures were equally divided into five 50-ml Erlenmeyer flasks with or without AE at concentrations of 2, 4, 8, and 16 µg/ml and then were cultured with shaking at 37°C until reaching the postexponential growth phase (OD_{600} of 2.5). Subsequently, the bacterial cultures were centrifuged at 10,000 rpm for 1 min, and the supernatants were boiled in Laemmli sample buffer and loaded on a 12% sodium dodecyl sulfate-polyacrylamide gel. The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The membranes were blocked with 5% bovine serum albumin (Wako) in PBS at 4°C overnight. Next, a rabbit antibody against α-toxin (diluted to 1:8,000; Sigma-Aldrich) was incubated...
with membranes at 37°C for 2 h, followed by an incubation with a horseradish peroxidase-conjugated secondary antibody (diluted to 1:4,000; Sigma-Aldrich) under the same conditions. The blots were detected with Amersham ECL. Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK) and visualized on Tanon-5200 Chemiluminescent Imaging System (Tanon Science & Technology Co., Ltd., Shanghai, People's Republic of China).

Deoxycholate-Induced Oligomerization Assay
To assess the deoxycholate-induced oligomerization of α-toxin, 500 ng of purified α-toxin was added to 5 mM deoxycholate and incubated with AE at concentrations of 2, 4, 8, and 16 μg/ml at 22°C for 20 min. Then, the samples were placed in Laemmli sample buffer without β-mercaptoethanol at 55°C for 10 min, and the effect of AE on protein oligomerization was assessed by Western blot assays as described above.

Molecular Modeling
The initial structure of α-toxin was obtained from the 3D X-ray structure (PDB code: 4YHD). To obtain the starting structure of the AE/α-toxin complex for MD simulation, we used a standard docking procedure for a rigid protein and a flexible ligand using AutoDock 4 (Morris et al., 2009; Hu et al., 2010). Subsequently, the MD simulation of the complex systems was performed, and the detailed processes of the computational analyses were described in a previous report (Dong et al., 2013; Niu et al., 2013).

Binding Affinity Determination of Ligands With Proteins
To investigate the importance of Lys110, Tyr112, and Met113 in the binding of α-toxin and AE, we mutated these amino acids to...
alanine. Next, the WT α-toxin and its variants (K110A, Y112A, and M113A mutant proteins) were expressed and purified as described by Qiu et al. (2012a), with the primers used for mutagenesis listed in Table 1. The fluorescence-quenching method was used to measure the binding constants ($K_d$) of AE with the proteins. A 280-nm excitation wavelength with a 5-nm bandpass and a 345-nm emission wavelength with a 10-nm bandpass were used for the measurements. Details of performing the measurements were described previously (Bandyopadhyay et al., 2002; Jurasekova et al., 2009).

**Live/dead Assays and LDH Release**
A549 human lung epithelial cells (ATCC CCL-185) and mouse lung macrophages (MH-S, ATCC CRL-2019) were plated in 96-well plates at a density of 2.0 × 10^4 cells/well and were cultured overnight. These cells were subsequently treated with 200 μl of bacteria at an MOI of 500 with or without AE and then were incubated for 5 h at 37°C. For the live/dead assays, the cells were stained using a live/dead (green/red) reagent (Invitrogen) and detected with a confocal laser scanning microscope (Nikon, Tokyo, Japan). For the LDH assay, the supernatants of the wells were assessed with a Cytotoxicity Detection Kit (LDH, Roche, Basel, Switzerland).

**Animal Infection**
Six-to-eight-week-old C57BL/6J female mice were purchased from Jilin University Experimental Animal Center. Animal experiments were approved by and conducted in accordance with the guidelines of the Animal Care and Use Committee of Jilin University. This study was approved by the Animal Welfare and Research Ethics Committee of Jilin University. The mice in the control group were treated with DMSO at the same dosage. Each experimental group in this study contained 10 mice.

USA300 was cultured in TSB with shaking at 200 rpm at 37°C to an OD_{600} of 0.8, and the cells were then harvested by centrifugation for 10 min at 3,000 rpm. The bacteria were resuspended in physiological saline and quantified at OD_{600}. The mice were anesthetized with ether and then were administered nasal drops with 20 μl of suspended bacteria (1 × 10^10 CFUs/mL). The mice in the treatment group were subsequently injected with 100 mg/kg AE by hypodermic injection 2 h after infection and were continually treated for 72 h at 8-h intervals. The mice in the control group were treated with DMSO at the same dosage. Each experimental group in this study contained 10 mice.

**RESULTS**

**AE has no Effect on S. aureus Growth**
The minimum inhibitory concentration (MIC) determination and growth curve assays were performed to determine the antibacterial activity of AE for *S. aureus*. The MIC of AE for the *S. aureus* strain USA300 was >1,024 μg/mL, suggesting that this compound, as a therapeutic agent, lacks antibacterial activity against USA300. Furthermore, USA300 grown with 2–16 μg/mL of AE showed no difference from the control group cultures without AE (Figure 1B).

**AE Inhibits the Hemolytic Activity of α-toxin**

Then, the potential inhibitory effect of AE against α-toxin activity was evaluated at the concentrations without antibacterial activity against *S. aureus*. The results of hemolysis assays showed that AE notably inhibited the hemolytic activity of the *S. aureus* supernatant in a dose-dependent manner when cocultured with bacteria. Consistent with previous studies, 80.97% of rabbit erythrocytes were lysed by α-toxin in the control group without AE, and the activity was significantly decreased (to 22.19%) when cultured with 8 μg/mL of AE. Furthermore, only 4.00% of erythrocytes were lysed in the supernatants treated with 16 μg/mL of AE (Figure 1C). The 50% inhibitory concentration (IC_{50}) of AE determined to be 4.81 μg/mL.

**TABLE 1 | Primers used in this study.**

| Primer  | Oligonucleotide primer sequence (5′-3′) |
|---------|---------------------------------------|
| WT-F    | CGCGGATCCGCGCAATCTCTGATTAATTTAAAC     |
| WT-R    | CGCGCTCAAGTTTTGCTTTTCTTTTCTTTTCT      |
| K110A-F | CCAGAAGATCTTGCCTTACTTTTAC             |
| K110A-R | GTCGACGACTTTTACAGCTTTTCTTTTCT         |
| T112A-F | GATTTGATACAAAAGAGTTAGTATCATTTG        |
| T112A-R | GAGTACAGTTTTTACCTCGCTTGTTTAGTACAT    |
| M113A-F | GATTTGATACAAAAGAGTTAGTATCATTTG        |
| M113A-R | CCAAGAAATTCGATTGATACAGCGGAGTATATGAGTACTTTAAC |

**Figure 2** | AE interferes with the oligomerization of α-toxin. α-toxin was incubated with 5 mM deoxycholate with various concentrations of AE (0, 2, 4, 8, and 16 μg/mL) at 22°C for 20 min. The oligomerization of toxin was evaluated via Western blot assays.
Western blot assays were used to further determine whether the decreased hemolytic activity was due to a reduction in α-toxin. However, the secretion of α-toxin was barely affected in the supernatants after treatment with various concentrations of AE (Figure 1D). Therefore, we hypothesized that AE may act on α-toxin directly to inhibit its hemolytic activity. To validate this, we used recombinant purified α-toxin in hemolysis assays. Consistent with the hemolysis described above, the hemolytic activity of purified α-toxin was significantly inhibited in the presence of AE (Figure 1E). Taken together, our results suggested that AE inhibits the hemolytic activity of α-toxin by direct interaction with the toxin.

AE Inhibits the Oligomerization of α-toxin

Deoxycholate-induced oligomerization assays were used to determine whether the loss of hemolytic activity was due to the inhibition of oligomerization, which is critical for the hemolytic activity of α-toxin. As expected, formation of the α-toxin heptamer was significantly inhibited in the sample treated with 4 µg/ml of AE, and the oligomerization was almost completely inhibited when 16 µg/ml of AE was added to the reaction system (Figure 2). Taken together, these results indicate that the loss of hemolytic activity may be due to AE-mediated inhibition of the oligomerization of α-toxin.

Molecular Dynamics Simulation for α-toxin-AE

Computational biology assays were further performed to characterize the detailed mechanisms of this inhibition. First, computational biology analyses were used to explore the potential binding mode of AE with α-toxin in the active site. AE binds to α-toxin, and the binding mode is shown in Figure 3A. AE can bind to α-toxin via hydrogen bonding and hydrophobic interactions. During the time course of the simulation, AE could localize to the “stem” region of α-toxin (residue 100 to 150). In detail, the binding model of AE with α-toxin revealed that the side chain of AE can form strong interactions with Lys110, Tyr112, and Met113. As shown in Figure 3B, the complex was found to reach equilibrium at 50 ns based on the analysis of the root-mean-square deviations (RMSD) of backbone Cα atoms, which indicated that the complex system reached the equilibrium.

To explore the energy contributions from residues of the binding sites in the α-toxin-AE complex, we calculated the energy

![Figure 3](https://example.com/figure3.png)

**Figure 3** | The 3D structure determination of the α-toxin with AE via molecular modeling method by using Gromacs 5.1.0 software. (A) The 3D structure of α-toxin binding with AE obtained via molecular modeling. (B) The root-mean-square deviations (RMSDs) displayed by the backbone atoms of the protein during MD simulations of α-toxin-AE is presented by using Gromacs 5.1.0 software. The RMSDs as a function of the molecular dynamics simulation time. (C) Decomposition of the binding energy on a per-residue basis in the binding sites of the α-toxin-AE complex.
decomposition for the α-toxin-AE complex system. As shown in Figure 3C, Tyr112 makes a strong contribution to the total binding energy, with an \( \Delta E_{\text{total}} \leq -2.0 \) kcal/mol. In addition, the Lys110 and Met113 residues also have appreciable total binding energy contributions, with \( \Delta E_{\text{total}} \) values of \( \leq -0.5 \) kcal/mol. The results suggest that these three residues are key residues for AE.

To confirm these theoretical results, we calculated the total binding free energy for the α-toxin-AE complex and their detailed energy contributions according to the MM-PBSA approach, which are summarized in Table 2. According to the calculation results, the binding free energy, \( \Delta G_{\text{bind}} \), of the interaction between AE and the protein decreased in the following order: WT-α-toxin > mutants, indicating that the WT-α-toxin had the strongest AE-binding ability. By fluorescence spectroscopy quenching, we measured the \( \Delta G_{\text{bind}} \) and the number of binding sites between AE and the three mutants, and these results were consistent with those obtained by computational methods (Table 2). Another hemolysis assay was used to determine the effect of AE on the hemolytic activity of the mutant proteins. As expected, the sensitivity of these mutants to AE was much lower than that of the WT protein, indicating that the mutations affected the binding of AE to α-toxin (Figure 4).

Taken together, these results indicate that the molecular dynamics (MD) simulation of the α-toxin-AE complex is reliable and that binding of the AE inhibitor at the active site (residues Lys110, Tyr112, and Met113) of α-toxin leads to inhibition of its activity.

### Table 2

|            | WT-α-toxin | K110A | Y112A | M113A |
|------------|------------|-------|-------|-------|
| Computational method | −12.6 ± 1.1 | −8.2 ± 0.8 | −7.7 ± 1.0 | −8.1 ± 0.9 |
| \( K_A (1 \times 10^6) \) L·mol\(^{-1} \) | 8.9 ± 0.8 | 5.8 ± 0.6 | 6.1 ± 0.9 | 7.1 ± 0.7 |

#### Figure 4

The effect of AE on the hemolytic activity of α-toxin and its three mutants. Recombinant α-toxin was preincubated with various concentrations of AE and the hemolytic activity of toxin was determined using hemolysis assay as described in Figure 1E. Bars represent the mean values of the experiments (* indicates \( P < 0.05 \) and ** indicates \( P < 0.01 \) compared with the AE-free group; two-tailed Student’s t-test).

### AE Protects A549 Cells From USA300-mediated Cell Injury

Live/dead cell staining and lactate dehydrogenase (LDH) release assays were performed to assess the protective effects of AE on S. aureus-mediated A549 cell injury in the coculture system of S. aureus and cells with or without AE. After incubation for 5 h, the cells were stained with live/dead (green/red) reagent (Figure 5A). As shown in Figure 5B, most of the cells were dead (red fluorescence) in the control group without AE. However, the cell injury was decreased with increasing concentrations of AE, and almost no dead cells could be observed under a confocal laser scanning microscope when the concentration of AE reached 16 \( \mu \)g/ml (green fluorescence for live cells, Figure 5C). Furthermore, the cells treated with 16 \( \mu \)g/ml of AE only showed a normal cell morphology, with almost no dead cells (Figure 5D), suggesting that no toxicity could be observed at therapeutic concentrations.

LDH can be released into culture medium as an indicator of cell lysis. Thus, LDH release assays of A549 cells and mouse lung macrophages (MH-S) were used to quantify cell death. As shown in Figure 5E, ~74.4% of A549 cells and 36.1% of MH-S cells were dead after incubation with USA300 for 5 h without AE. Significantly, when AE was added at 16 \( \mu \)g/ml, the rate of cell death was decreased to 14.31 and 8.75%, respectively (Figure 5E). Taken together, the results indicated that AE protected against cell injury mediated by α-toxin in both A549 cells and MH-S cells in vitro.

### AE Protects Mice From S. aureus Pneumonia

Following the determination of the protective effect of AE against S. aureus virulence in vitro, we further investigated whether this effect occurred in vivo by establishing a mouse model of S. aureus pneumonia. C57BL/6j mice were infected with USA300 as previously described (Qiu et al., 2012a,b) and treated with AE or DMSO for 72 h. The mortality of infected mice that received AE was significantly decreased compared with that of mice injected with DMSO (Figure 6A). The lungs of the mice were pathologically analyzed. The redness of the lungs from the AE-treated group was much lighter than that of the control group when observed by the naked eye (Figure 6B), and the alveolar space was obliterated by inflammatory cell infiltrates in the control group as shown by microscopy (Figure 6C). Furthermore, we quantified the colonization of bacteria in the lungs. As shown in Figure 6D, the quantity of bacteria in lungs treated with AE was much lower than that of the control group. Taken together, our results indicated that AE is an effective candidate for protecting mice from S. aureus pneumonia.

### DISCUSSION

Over the past 100 years, the application of antibiotics has saved countless lives, and scholars once believed that human beings could completely eradicate diseases caused by bacteria. However, the abuse of antibiotics has gradually increased resistance, which has finally led to the elimination of antibiotics after decades of
application and has resulted in many multidrug resistant strains that are insensitive to conventional antibacterial strategies, such as MRSA (Rasko and Sperandio, 2010). The use of antibiotics is typically related to the survival of bacteria and always leads to the selection of resistant strains that gradually become dominant and eventually results in resistant flora. Thus, it is possible that
most antibiotics may eventually induce drug resistance due to the excellent ability of bacteria to adapt to the environment (Cegelski et al., 2008). Therefore, new treatment strategies are needed that are less effective at selecting for resistance. In this study, we observed that AE did not affect the viability of S. aureus at the concentrations tested (2–16 µg/ml). These results indicate that AE would not put selective pressure on S. aureus.

In recent years, many studies have suggested the targeting virulence factors as an alternative strategy and have shown some impressive results (Qiu et al., 2012a,b). For S. aureus infections, many virulence factors are employed during the invasion, and most of them, such as α-toxin, are not correlated with bacterial survival. Based on previous studies, the destruction of the air-blood barrier by α-toxin is one of the most important factors in S. aureus pneumonia (McElroy et al., 1999; Xu et al., 2015). Accordingly, in this study, we attempted to treat S. aureus-induced pneumonia by targeting α-toxin. We identified AE as a potential inhibitor of α-toxin, as it directly interferes with the hemolytic activity of α-toxin without affecting the production of α-toxin by S. aureus or bacterial viability. By means of MD simulation, we found that AE could localize to the catalytic pocket of α-toxin (residue 100–150), which is very close to the binding site of substrate, by directly engaging residues K110, T112, and M113. These interactions blocked the binding of α-toxin and its substrate and finally led to a loss of biological activity of α-toxin. Furthermore, we also demonstrated that AE had protective effects in a tissue infection model and animal model of S. aureus pneumonia. These results indicated that AE is a potential treatment for S. aureus infections, especially MRSA infections. In addition, our results confirmed the feasibility of the anti-virulence strategy.

Interestingly, Aloe vera, Rheum officinale, and semen cassiae torae, which are the major sources of AE, have been used as traditional Chinese medicines in the treatment of lung infections for thousands of years. Although this study cannot show that traditional Chinese medicines in the treatment of lung infections, it provides support for the theory of traditional Chinese medicine at the molecular level.

Previous studies have reported that α-toxin can also damage the membranes of B cells and T cells, which may lead to an attenuated immune response and create a stable and lasting bacterial colonization environment (Nygaard et al., 2012). As reported in several studies, subinhibitory concentrations of beta-lactam antibiotics may increase α-toxin expression, indicating that infections may be uncontrollable when treated with beta-lactams (Ohlsen et al., 1998). Therefore, targeting α-toxin by AE may support the therapeutic effects of immune cells and antibiotics, but this strategy requires further confirmation. Finally, our results indicated that AE is a promising candidate for S. aureus infection by targeting α-toxin.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Six-to-eight-week-old C57BL/6J female mice were purchased from Jilin University Experimental Animal Center and were handled according to the standards approved by the Animal Welfare and Research Ethics Committee of Jilin University.

AUTHOR CONTRIBUTIONS

ZT, JW, and LJ conceived and designed the experiments. LJ, TY, and ZS performed the experiments. LJ analyzed the data. ZT and JW wrote the paper.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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