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ORIGINAL PAPER

Phloretin derived from apple can reduce alpha-hemolysin expression in methicillin-resistant *Staphylococcus aureus* USA300

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Abstract Methicillin-resistant *Staphylococcus aureus* (MRSA) has become increasingly important because it is the most common cause of hospital-acquired infections, which have become globally epidemic. Our study specifically focused on the MRSA strain USA300, which was shown in 2014 to be responsible for the most current pandemic of highly virulent MRSA in the United States. We aimed to evaluate the in vitro effect of phloretin on USA300. Susceptibility testing, western blotting assays, hemolysis assays and real-time RT-PCR were employed to examine the in vitro effects of phloretin on alpha-hemolysin (Hla) production when the bacterium was co-cultured with phloretin. The protective effect of phloretin against the USA300-mediated injury of human alveolar epithelial cells (A549) was tested using the live/dead analysis and cytotoxicity assays. We showed that sub-inhibitory concentrations of phloretin have no effect on bacterial viability; however, they can markedly inhibit the production of Hla in culture supernatants and the transcriptional levels of *hla* (the gene encoding Hla) and *agrA* (the accessory gene regulator). Phloretin, at a final concentration of 16 μg/ml, could protect A549 cells from injury caused by USA300 in the co-culture system. Our study suggests that phloretin might have a potential application in the development of treatment for MRSA infections.

Keywords Methicillin-resistant *Staphylococcus aureus* · USA300 · Alpha-hemolysin · Phloretin · Virulence factor

Introduction

Staphylococcus aureus (*S. aureus*) is a dangerous gram-positive pathogen that is responsible for a large number of infections worldwide, including skin and soft tissue infections, toxic shock syndrome, pneumonia and sepsis (Lowy 1998). The continued emergence of methicillin-resistant *S. aureus* (MRSA) makes combating *S. aureus* very crucial. MRSA emerged only 2 years after methicillin was recommended as a treatment for penicillin-resistant *S. aureus* (Enright et al. 2002). MRSA is a critical public health problem in the United States, particularly the MRSA strain USA300 (Diekema et al. 2014).

The epidemiology of MRSA in the United States shows that among clinically significant *S. aureus* infections, 51 % are MRSA and the most common type is USA300 (61 %) (Diekema et al. 2014). Meanwhile, humans are natural receivers and carriers of *S. aureus*, and asymptomatic infection and persistent colonization have been described as risk factors for subsequent infection. Compared with patients who have methicillin-susceptible infections, patients with MRSA suffer from an increased mortality risk, more expensive treatment costs, longer hospital stays, and more likely to die (Reed et al. 2005). The performance of traditional antibiotic therapy is controversial, and poor clinical outcomes are not uncommon with MRSA infections (Fowler et al. 1999; Stevens et al. 2007). The development of effective alternatives for the treatment of MRSA

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infections is important and urgent. Anti-virulence therapies have recently attracted interest because previous studies have shown that natural compounds could target bacterial virulence factors and suppress their activity (Shoham 2011; Wang et al. 2015a).

Staphylococcus aureus produces a variety of toxins as molecular weapons to facilitate the establishment of infections, such as colonization, evasion of the immune system and cell damage (Cheung et al. 2011). One of these virulence factors, alpha-hemolysin (Hla), a water-soluble virulence factor that is secreted late in the exponential phase of growth by most *S. aureus* strains, is encoded by the *hla* gene and controlled by the Agr two-component system. As a β-barrel, pore-forming cytotoxin, cellular lysis is the prominent consequence of the biological activity of Hla. A number of host cell types are targeted by Hla, such as erythrocytes, platelets and monocytes (Berube and Bubeck Wardenburg 2013). Moreover, Hla plays a significant role in skin and soft tissue infections and even lethal diseases in animal models of staphylococcal infection (Rauch et al. 2012; Bubeck Wardenburg et al. 2007). The currently epidemic the USA300 strain of MRSA exhibits more virulent in experimental models (Montgomery et al. 2008). Considering the crucial role of Hla in the pathogenesis of human disease, Hla is a promising candidate as a drug target for the treatment of MRSA disease. Previous researches have demonstrated that inhibitors targeting Hla were able to prevent MRSA infection suggesting a promising strategy for combating deadly staphylococcal pneumonia (Ragle et al. 2010).

According to previous studies, some chemical compounds, such as savirin, quorum-quenching agents, luteolin, and morin, have been identified as anti-*S. aureus* infection inhibitors (Sully et al. 2014; Kuo et al. 2015; Qiu et al. 2011; Wang et al. 2015b). Dihydrochalcone phloretin (Fig. 1) derived from exclusively in apples, which are regularly consumed by humans (Barreca et al. 2014). The concentration is approximate 80–420 mg/kg in the peel of apples and 16–20 mg/ml in the pulp (Barreca et al. 2014). Phloretin exhibits diverse pharmacological activities, such as anti-inflammatory, antioxidative, and anticancer (Lin et al. 2014). In this study, the inhibitory effect of phloretin on the production of Hla by USA300 was evaluated. The results indicate that sub-inhibitory concentrations of phloretin could attenuate Hla production in culture supernatants partly via down-regulation of the transcription of *hla* and provide significant protection against *S. aureus*-mediated A549 cell injury.

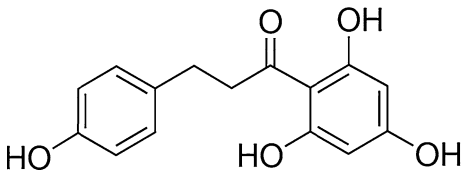

Fig. 1 Chemical structure of phloretin (CAS No. 60-82-2)

Materials and methods

Bacterial strains, reagents and growth conditions

CA-MRSA strain USA300 was obtained from the American Type Culture Collection (ATCC). For the hemolysis assay, western blot analysis and real-time RT-PCR testing, USA300 samples were grown at 37 °C in tryptic soy broth (TSB) to a post-exponential growth phase (OD_{600nm} = 2.5), and the culture supernatant was pelleted (1 min, 1000×*g*, 4 °C). For the cell assay, USA300 was grown at 37 °C in TSB to OD_{600nm} = 0.6 and washed in PBS; subsequently, 5 ml of the culture was prepared, as described above. The suspension was suspended in 10 ml of Dulbecco's modified Eagle's medium (DMEM), and 100 µl of the suspension was used per assay well. A stock solution of phloretin (Sigma-Aldrich) was prepared by dissolution in dimethyl sulfoxide (DMSO).

Susceptibility testing

The broth microdilution method was performed as previously described to determine the minimal inhibitory concentrations (MICs) of phloretin for USA300 (CLSI 2005).

Growth curve assay

Staphylococcus aureus strain USA300 was grown overnight at 37 °C in 5 ml of TSB, and the cultures were diluted 1:100 in 500 ml of fresh TSB media. After reaching an OD_{600nm} of 0.3, 100 ml volumes of the culture were liquated into five 250 ml Erlenmeyer flasks, followed by the addition of phloretin at sub-inhibitory concentrations of 2, 4, 8 and 16 µg/ml. The culture without phloretin was used as a control. In the presence of phloretin, the bacteria were further cultured until OD_{600nm} = 2.5, with shaking at 37 °C. The cell growth was measured at OD_{600nm} every 30 min.

Hemolysis assay

For measuring the hemolytic activity, 500 µl of *S. aureus* supernatant was harvested by centrifugation (5000×*g*, 5 min), using a 0.2-µm filter to remove the residual cells. Then, 100 µl of culture supernatants was increased to 975 µl by the addition of sterile PBS buffer, and 25 µl of defibrinated rabbit red cells was then added to the samples.

Following incubation for 15 min at 37 °C, the samples were centrifuged (10,000×*g*, room temperature, 1 min). The hemolytic activity was determined by measuring the absorbance of the samples at OD_{543nm}.

To investigate whether phloretin was able to directly inhibit the hemolytic activity of *S. aureus* culture supernatant, 100 µl of bacterial culture supernatants were mixed with 875 µl PBS buffer, and different concentration of phloretin. The mixtures were incubated at 37 °C for 25 min. Then, 25 µl of defibrinated rabbit red cells was added to the samples. The mixtures were also incubated at 37 °C for 15 min. Lysis of the cells was determined as described above. PBS treatment was used as a negative control, and 1 % saponin (Sigma-Aldrich) served as a positive control. The percent of hemolysis was calculated by comparing each sample to the positive control in both assays.

Western blot assay

Briefly, the culture supernatants (25 µl) were resolved via 12 % SDS-PAGE, and the proteins were transferred to PVDF membranes. After blocking in 5 % bovine serum albumin in PBS for 2 h, the membranes were incubated with an antibody to Hla (Sigma-Aldrich), diluted 1:8000, for 2 h, followed by horseradish peroxidase-conjugated anti-rabbit antiserum (Sigma-Aldrich), the secondary antibody, which was diluted 1:4000, for 2 h. The proteins of interest were then detected using ECL Western blotting-detection reagents (GE Healthcare).

Real-time RT-PCR

MRSA strain USA300 was cultured in TSB with/without certain concentrations of phloretin to $OD_{600} = 2.5$. The total RNA from the bacteria was isolated, as described previously (Qiu et al. 2011). The total RNA was reverse transcribed into cDNA using the Takara RNA PCR kit (AMV), ver. 3.0 (Takara, Kyoto, Japan). The PCR reactions were performed in 25-µl volumes using SYBR Premix Ex Taq TM (Takara), according to the manufacturer's instructions. The PCR amplification was assessed by the 7000 Sequence Detection System (Applied Biosystems, Courtaboeuf, France). All samples were analyzed in triplicate, and the housekeeping gene, *gyrBRNA*, served as an endogenous control to normalize the changes in the transcription levels between the samples. The primer pairs used for this assay as described previously (Qiu et al. 2011).

Live/dead and cytotoxicity assays

Hla has been demonstrated to be an essential factor that mediates A549 cells injury and death (Wang et al. 2015b). Therefore, A549 cells were grown in DMEM (Sigma-Aldrich), supplemented with 10 % fetal calf serum (PAA). The cells were rinsed with sterile PBS, plated at a density of 2.0×10^4 cells per 96-well, and allowed to grow at 37 °C in 5 % CO_2 for 12 h. The A549 cells were incubated in triplicate with 100 µl of suspension per well in DMEM medium in the presence of graded concentrations of phloretin in triplicate. At 5 h after incubation at 37 °C, the therapeutic effect of phloretin on cell survival was assessed by using live/dead (green/red) reagent (Roche) and by measuring the lactate dehydrogenase (LDH) release (Roche) at OD_{490nm} using a microplate reader as recommended by the manufacturer.

Statistical analysis

The cytotoxicity assays were analyzed for significance using an independent Student's *t* test. The statistical analyses were performed using the SPSS 13.0 statistical software and a *P* value of <0.05 was considered significant.

Results

Phloretin has no influence on USA300 growth

The MIC value of phloretin against USA300 was 64 µg/ml, which indicated that phloretin has little anti-bacterial activity. The growth curves of USA300 (Fig. 2) suggested that the presence of phloretin at graded subinhibitory concentrations from 2 to 16 µg/ml had no significant inhibitory effect on the growth of USA300.

Phloretin decreases the levels of Hla production in culture supernatant

One hallmark of Hla is hemolysis, and rabbit red blood cells have been reported to be exquisitely sensitive to Hla-

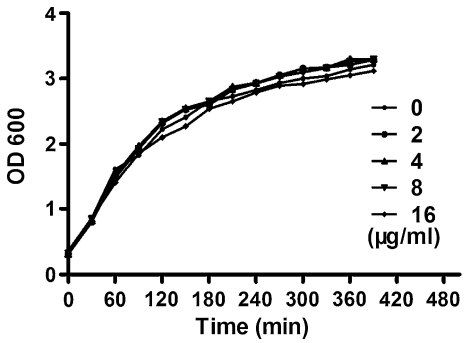

Fig. 2 The growth curve for USA300 cultured with or without phloretin. *filled circle*, *filled square*, *filled triangle*, *filled inverted triangle*, and *filled diamond* represent USA300 grown in TSB with 0, 2, 4, 8 and 16 µg/ml of phloretin, respectively

mediated hemolytic activity (Berube and Bubeck Wardenburg 2013). A hemolysis assay was employed to assess the effect of phloretin on the hemolytic activity of bacterial cultural supernatants. Phloretin inhibited the hemolytic activity in the culture supernatants in a concentration-dependent manner. Compared with the drug-free sample, the treatment with 8 μg/ml of phloretin led to a significant decrease in the hemolytic activity (from 76 to 24 %), and approximately no hemolytic activity was detected in the culture supernatants co-cultured with 16 μg/ml of phloretin (Fig. 3a). Additionally, phloretin, at the concentrations tested in this study, was not able to neutralize the Hla-induced hemolysis directly (Fig. 3b).

A western blot assay was further performed to evaluate whether the decrease of hemolytic activity in the culture supernatants was caused by the reduced production of Hla. As expected, a dose-dependent reduction of Hla levels in the samples was observed in the presence of phloretin. No Hla band was detected in the sample treated with 16 μg/ml of phloretin (Fig. 3c). These results are in good agreement with those of the hemolysis assay, indicating that phloretin inhibited the production of Hla in culture supernatants and, subsequently, diminished the hemolytic activity of the supernatants.

Because the Hla protein encoding gene, *hla*, is positively regulated by the Agr regulatory system, and *hla* transcription is upregulated by phosphorylated *agrA* indirectly, we performed real-time RT-PCR to determine the effect of phloretin on the transcription of *hla* and *agrA* in MRSA USA300. The data showed that the transcriptional levels of *hla* and *agrA* were down-regulated by phloretin in a dose-dependent manner. The transcription levels of *hla* and *agrA* in USA300 were both decreased significantly when exposed to 16 μg/ml of phloretin. Based on these observations, we might infer that the mode of action by which phloretin reduces the production of Hla might partially be through the inhibition of the transcription of *agrA* (Fig. 3d).

Phloretin protects A549 cells from USA300-mediated cell injury

Hla has been demonstrated to have critical cytotoxicity for causing injury to A549 cells, as the Hla mutant caused almost no cell injury to the *S. aureus* and A549 cell co-culture system (Wang et al. 2015b). Thus, based on these results, we evaluated the potential protective effect of phloretin on USA300-indcued A549 cells injury in the co-

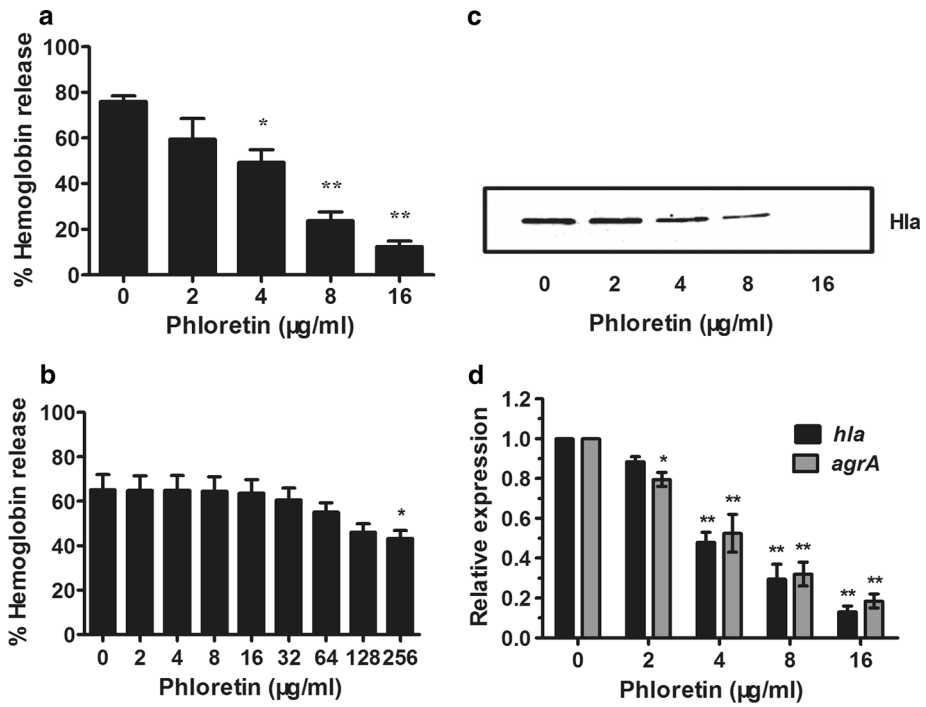

Fig. 3 Phloretin decreases Hla secretion. **a** The hemolytic activity of Hla produced by USA300 co-culture with subinhibitory concentrations of phloretin. **b** Phloretin neutralizes the Hla-induced hemolysis directly. **c** Western blot of the Hla expression in the culture supernatants with or without phloretin. The specificity of the Hla antibodies and rabbit antibodies interaction enables a target Hla to be identified in the culture supernatants. **d** The relative expression of *hla* and *agrA* in USA300 after growth with graded concentrations of phloretin. The data in **a**, **b** and **d** from three independent experiments are displayed. The data shown are representative of three independent experiments. $*P < 0.05$ and $**P < 0.01$, compared with the phloretin-free culture

culture system. The uninfected A549 cells retained green fluorescence (Fig. 4a).The vast majority of the cells exposed to a 5-h infection with USA300 had damaged membranes and were stained red (Fig. 4b).Treatment with 4 and 16 μg/ml (Fig. 4c, d) of phloretin both lead to a reduction in cell injury.

The cell viability was quantified using an LDH release assay kit. LDH was used as an indicator of cell death, and the results are presented as percentages of cell death. The level of cell death was 79 % for the drug-free group; when exposed to 16 μg/ml of phloretin, the level of cell death was decreased to 15 %. A dose-dependent reduction was observed for the samples in the presence of the indicated concentrations of phloretin, which indicated that cell death was diminished (Fig. 4e).

In light of the existing observations, these data suggest that at the subinhibitory concentrations tested in our study, phloretin has no influence on the growth of bacteria; however, the treatment with phloretin significantly inhibited the production of Hla in the culture supernatant, downregulated the *hla* transcription and prevented A549 death in the co-culture system.

Discussion

Antibiotic agents appeared to be promising, and the global use of antibiotic therapy has saved many lives. It was hypothesized that most infectious bacterial diseases could be overcome by antibiotics; however, the notable ability of *S. aureus* to acquire resistance has become a serious problem (Abdelhady et al. 2015). Severe MRSA diseases, which are associated with high morbidity and mortality, are dramatically increasing, whereas the antibiotic agents against these diseases are extremely limited, highlighting the urgent need for alternative treatments to combat *S. aureus* infection (Kreisel et al. 2011; Seidl et al. 2011).Previous reports have demonstrated that most virulence factors are not essential for bacterial survival and that they could target host cells and cause disease (Berube and Bubeck Wardenburg 2013; Lowy 1998). Anti-virulence therapy combats disease by interfering with the virulence factors of the pathogen while having no effect on the bacterial viability, which offers an alternative strategy in the development of new anti-infection drugs (Daly et al. 2015). This strategy would apply milder selective pressure on the target

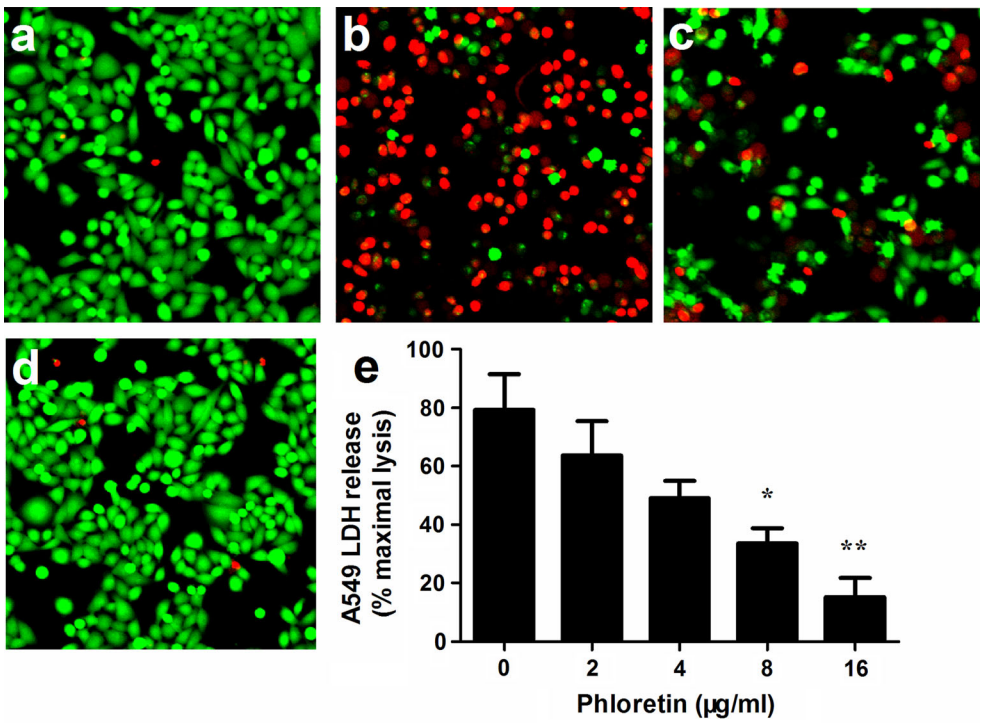

Fig. 4 Phloretin alleviates A549 human alveolar epithelial cell injury caused by USA 300. Live/dead reagent-stained A549 was observed with fluorescent imaging (×100), using calcein AM and ethidium homodimer-1 (EthD-1), respectively. The cells with intact membranes are stained fluorescent green, and those with damaged membranes are stained fluorescent red; **a** the uninfected A549 cells; **b** the USA300-infected cells in the absence of phloretin; treatment with 4 μg/ml **c** and 16 μg/ml **d** phloretin; **e** the LDH release by A549 cells was quantified at 490 nm using a micro-plate reader. The samples were tested after exposure to certain concentrations of phloretin. The data are displayed as the means from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$

bacteria (Rasko and Sperandio 2010; Khodaverdian et al. 2013); this differs from antibiotic therapy, which is aimed at limiting bacterial growth or killing the pathogens directly.

Diep et al. (2008) showed that USA300 displays enhanced virulence because of the presence of various virulence factors. Hla, one of the virulence factors produced by *S. aureus*, is a multifunctional protein that could cause cells lysis, tissue injury and the evasion of the immune system by bacteria. Many studies have shown that only a small number of mice succumbed to infection when inoculated with the *hla* mutant strain (Ragle and Bubeck Wardenburg 2009; Bubeck Wardenburg et al. 2007). In their recent study, Kobayashi et al. (2011) developed a rabbit skin infection model with USA300 and its *hla* mutant and identified that the mutant strain leads to the formation of abscesses with significantly less volume and area. These findings suggest that phloretin could become an anti-virulence agent by inhibiting Hla production. For the purposes of this study, we screened an effective Hla inhibitor, phloretin, as an anti-virulence drug candidate for *S. aureus* infection. Despite the fact that phloretin has little anti-*S. aureus* activity, it could significantly inhibit the production of Hla in culture supernatants by down-regulating the transcriptional levels of *hla* in a dose-dependent manner. In our co-culture system, the addition of phloretin conferred robust protection against *S. aureus*-mediated A549 cells injury. Our results in this study indicated that phloretin has the potential to be a leading compound as an anti-virulence agent for the treatment of *S. aureus* infection specifically targeting Hla.

The exposure of *S. aureus* to sub-inhibitory concentrations of β-lactams leads to the strong induction of Hla, indicating that the inadvertent use of β-lactam antibiotics against MRSA infections could facilitate the further aggravation of resistant disease during clinical treatment (Ohlsen et al. 1998). Interestingly, Kuo et al. (2015) showed combination therapy of anti-virulence agents with β-lactam antibiotics to which MRSA is resistant in monotherapy sensitized MRSA to these antibiotics. Our study has demonstrated that phloretin, at sub-inhibitory concentrations ranging from 2 to 16 μg/ml, could decrease the production of Hla and protect A549 cells from cell damage in vitro; those beneficial effects might partially correlate with *hla* and *agrA* suppression. Consequently, the combination of phloretin and β-lactams would provide an improved clinical outcome, extend the lifespan of antibiotics and lessen the usage of each agent, decreasing the costs and slowing the development of resistance.

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Conflict of interest The authors declare that they have no conflict of interest.

Human and Animals Rights This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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