Isolation and Characterization of a Lytic Staphylococcus aureus Phage WV against Staphylococcus aureus Biofilm

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Keywords
Staphylococcus aureus · Phage · Biofilm · Antibiotic

Abstract
Background: Staphylococcus aureus is a Gram-positive, pathogenic bacterium that causes a wide range of symptoms in humans and can form biofilm, which is a multicellular community of microorganisms that attaches to nonbiological and biological surfaces. Methods: Here, we aimed to isolate and characterize an S. aureus phage and examine the bactericidal activity alone and in conjunction with streptomycin treatment. Results: We isolated a virulent phage, WV, from a slaughterhouse in Jiangsu, China. This strain belonged to the family Myoviridae and presented a genome size of 141,342 bp. The optimal pH of the preservation buffer was 6–7, optimal growth temperature was 37°C, and optimal multiplicity of infection was 0.01. Phage WV can sterilize most clinical strains of S. aureus that had been isolated from clinical patients in the First People’s Hospital of the Yunnan Province. Against low-concentration S. aureus culture, streptomycin demonstrated a greater antibiofilm effect than that of phage WV. By contrast, in high-concentration S. aureus culture, phage WV demonstrated greater antibiofilm effect than that of streptomycin. The use of phage WV and streptomycin together had a substantially greater overall antibiofilm effect than that achieved using either component alone. Conclusion: This study provides strong evidence for the effectiveness of phage application for the reduction of S. aureus biofilm growth and suggests that phages can be considered as a viable alternative to antibiotics in clinical settings.

Introduction
Staphylococcus aureus is a Gram-positive, pathogenic bacterium that causes a wide range of symptoms such as gastroenteritis, clinical mastitis, mastitis, and device-related infections [1–6]. Abuse of antibiotics, such as methicillin, vancomycin, trimethoprim, and sulfamethoxazole, has led to the emergence of highly antibiotic-resistant strains of S. aureus [7, 8]. Human wound infections have a strong association with the formation of S. aureus biofilms [9]. Furthermore, human organs and medical devices have also been shown to support biofilm growth.

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Fortunately, phages have a strong ability to remove biofilms [10, 11].

A bacterial biofilm is a multicellular community of microorganisms that form a self-produced, highly hydrated extracellular matrix that attaches to both biological and nonbiological surfaces [12–14]. The extracellular polymeric substances of the biofilm matrix act as a barrier against the penetration of antimicrobial agents into the interior of the biofilm [15, 16]. Biofilms are highly resistant to desiccation, antibiotics, acidic condition, and heat [17]. Thus, bacteria in biofilms are approximately 10- to 1,000-fold less sensitive to antimicrobial agents than planktonic bacteria because the extracellular polymeric substances of the biofilm can prevent contact with antimicrobial agents [18, 19]. Complete elimination of the biofilm in areas such as husbandry and food industry and in clinical settings has been typically impossible [20].

Abuse of antibiotics has resulted in the formation of multidrug resistant (MDR) bacteria. In 2003, there were an estimated 80,000 annual deaths caused by antibiotic abuse in China [21]. Phages and their derivatives are ideal candidates for replacement of, or in addition to, treatments with potential antibiotic resistance issues in the future [22]. Phages have the ability to sterilize bacteria [23, 24] and appear to be a suitable alternative to antimicrobials and disinfectants to kill bacteria. In addition, phages only infect bacteria and are not harmful to humans, making them a safe application in clinical and food products [25]. A recent study found that phages are highly effective at reducing and controlling bacterial biofilms on various surfaces formed by species such as Escherichia coli, Salmonella sp., Pseudomonas aeruginosa, and Listeria monocytogenes [26–30].

In this article, we isolated and characterized a lytic S. aureus phage, WV. Prevention and control of contamination caused by the S. aureus biofilm is of considerable importance from economic, health, and medical perspectives. Next, we studied the basic features of the S. aureus phage WV in order to apply this for the reduction of S. aureus biofilm formation or to control mature biofilms. Our aim was to determine whether the phage could be used as an alternative therapeutic agent against MDR bacterial strains, specifically S. aureus strains.

**Materials and Methods**

**Bacterial Strains and Growth Conditions**

*S. aureus* was isolated from a patient at Yunnan First People’s Hospital, China. This *S. aureus* isolate was used as a host bacterium for the further isolation of relevant phages. The host strain and phage host range determination strains were also isolated from patients at Yunnan First People’s Hospital, China. Strains were grown aerobically on BHI plates or in BHI broth (Difco, Detroit, MI, USA) and incubated at 37°C. Soft top agar containing 0.5% (w/w) agar of BHI broth for plaque plaque confirmation and BHI agar plates containing 1.8% (w/w) agar were used. All *S. aureus* strains were stored at −80°C (Difco, Detroit, MI, USA) with the addition of 20% (v/v) glycerol.

**Phage Isolation and Purification**

*S. aureus*-targeting phages were isolated from a pig slaughterhouse. The isolation method for phages was modified as follows [31]: in brief, 10 g of pig farm trashcan sewage was mixed with 30 mL of sterile normal saline (0.9% NaCl) buffered in a 50-mL sterile centrifuge tube and then shaken in an incubator at 180 rpm for 3 h at room temperature. Samples were then centrifuged at 4,500 g for 10 min and were filtered with a 0.22-μm filter membrane; 15 mL of each filtered medium was added to 35 mL of BHI broth containing 1% (v/v) *S. aureus* overnight culture and was incubated for 2 days. Next, cultures were centrifuged at 7,000 g for 10 min, and the supernatant was filtered with a 0.22-μm filter membrane. The filtrate was diluted 10-fold in series and mixed with 6 mL of molten BHI soft agar containing 200 μL *S. aureus* (2 × 10⁸ colony-forming units [cfu]/mL) and immediately added to a BHI plate. Following overnight incubation, the formation of culture plaques was observed. A single phage plaque was selected for phage purification, and this was repeated 3 times.

**Optimal pH, MOI, Growth Curve, and Thermotolerance of Isolated Phage**

The phage WV stock was diluted to 1 × 10⁸ pfu/mL with BHI broth. Liquid buffer (0.99 mL) with a pH of 3, 4, 5, 6, 7, 8, 9, 10, and 11 (50 mmol/L citrate buffer for pH 3, 4, and 5; 50 mmol/L phosphate buffer for pH 6, 7, and 8; 50 mmol/L Tris-HCl buffer for pH 9; and 50 mmol/L sodium carbonate buffer for pH 10 and 11) was placed in 2-mL sterile centrifuge tubes, with addition of 0.01 mL of diluted phage WV with a titer of 1 × 10⁸ pfu/mL in each tube. These were placed at room temperature for 1.5 h, following which the titer of phage WV in the different pH buffers was detected. The experiments were repeated 3 times. Thermotolerance detectors were placed in 1.5 mL diluted phage WV in a temperature controller at 4, 25, 37, 50, 60, and 90°C for 1.5 h. Multiplicity of infection (MOI) is the ratio of phages to host bacteria of initial infection. Phage WV was added at an MOI of 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 to *S. aureus* culture and cultured at 37°C for 10 h. Cultures were centrifuged at 12,000 g for 15 min at 4°C, and the supernatants were filtered with a 0.22-μm filter; the titer of each phage WV solution was determined using the double plate method. The experiments were repeated 3 times. The 1-step growth curve of phage WV was carried out as follows: in brief, 5 mL of midexponential phase *S. aureus* culture was harvested by centrifugation (6,000 g, 5 min, 4°C) and the pellet resuspended in 10 mL of fresh BHI to obtain an OD₆₀₀ of 1.0. Following this, 10 mL of phage WV was added to an MOI of 0.1 and allowed to adsorb for 10 min at 37°C. The mixture was centrifuged at 6,000 g for 5 min at 4°C and the pellet resuspended in 5 mL of fresh BHI. Samples were taken every 10 min for 2 h, and the supernatants were plated.
Transmission Electron Microscopy

The morphology of the phage WV particles was observed by TEM. In brief, each phage stock dilution (approximately $3 \times 10^8$–$3 \times 10^9$ pfu/mL) was deposited on copper grids with carbon-coated Formvar films and stained with 2% uranyl acetate (pH 4.0). Phage WV samples were imaged using a Philips EM 300 electron microscope, operated at 80 kV in Jiangnan University (Wuxi, China). Phage WV was classified and identified referring to the International Committee on Taxonomy of Viruses.

Phage Genome DNA Extraction, Sequencing, and Bioinformatic Analysis

First, phage WV was purified from a concentrated, high-titer stock using a 10-kDa filter (approximately $10^9$–$10^{10}$). Purified phage WV was treated with DNase and RNase at 37°C for 1.5 h. Then, purified phage WV genomic DNA was obtained using a MiniBEST viral RNA/DNA Extraction kit (Takara, cat#9766). The restriction endonucleases EcoRI, NotI, HindIII, and XhoI were used for phage WV genome digestion. The extracted phage WV genomic DNA was sequenced using an Illumina Hiseq system (Sangon Biotech, China). The original sequencing data were evaluated by FastQC and assembled using SPAdes assembler software. NCBI BLAST search was used with multiple databases (COG, KOG, CDD, NR, NT, PFAM, SwissProt, and TrEMBL) to obtain functional information from gene sequences.

Phage Lytic Spectrum and Antimicrobial Susceptibility of S. aureus

The host range of phage WV was determined by the spot test method [32]. The reference strains (all isolated from clinical patients) were tested for susceptibility to phage WV. In brief, 250 μL reference strain ($10^8$ cfu/mL) was added to 6 mL liquefied BHI soft agar (BHI broth with 0.5% (w/w) agar) and poured over a BHI 1.8% (w/w) agar plate. Four minutes later, a single drop of phage WV suspension was added and incubated at 37°C for 24 h. Antibiotic sensitivity of S. aureus strains was tested against 17 antibiotics using the minimal inhibitory concentration method. The anti-microbials tested were penicillin, streptomycin, kanamycin sulfate, ciprofloxacin, gentamicin, levofloxacin, rifampicin, vancomycin, erythromycin, teicoplanin, and tetracycline.

The Different Effects of Phage WV and Antibiotics on Biofilms

To make first-phase preparations, a 48-well cell slide was placed into a 24-well plate. Seed solution was inoculated into 100 mL BHI culture solution at 4% (v/v). Bacterial solution (1 mL) was added into a 24-well plate. The first group of cultures was treated with phage WV (added to an MOI of 1), streptomycin (at a final concentration of 10 μg/mL), or mixtures of streptomycin and phage WV, whereas control wells contained no additives; all cultures were incubated at 37°C for 24 h. In the second group, S. aureus was first cultured for 12 h, followed by the addition of phage WV, streptomycin, and mixtures of streptomycin and phage WV as indicated above and incubation under the same conditions. The cfu of each sample were measured using the plate counting method. Next, the culture was washed twice with PBS buffer and then dehydrated with an increasing ethyl alcohol gradient (15, 30, 40, 50, 70, and 100% v/v) for 15 min at each step. Next, samples were dried overnight and gilt. Images were obtained using a scanning electron microscope with an accelerating voltage of 20 kV.
S. aureus seed solution was inoculated in BHI with 4% of overnight culture. Cultures were then diluted 200-fold dilution with BHI, and samples were added to a 96-well plate (200 μL/well) in triplicate. To the first group, phage WV (to an MOI of 1), streptomycin (to 10 μg/mL final concentration), and mixtures of streptomycin or phage WV were added, whereas control wells contained no additives; cultures were incubated at 37°C for 24 h. In the second group, S. aureus was first cultured for 12 h, and then phage WV, streptomycin, and mixtures of streptomycin and phage WV were added as indicated above; the control wells contained no additives. The cultures were incubated as previously stated. The S. aureus population density (OD 600 nm) was measured using ELISA (ThermoScientific, EUA), and the bacterial solution was discarded. Each well was washed twice with PBS to remove unattached S. aureus, and this step was repeated 3 times. Next 99% methanol was added, and samples were fixed for 10 min. Methanol was then discarded, and samples were dried at room temperature. Following this, samples were stained with 2% crystal violet for 10 min. The plate was rinsed with running water until the water was colorless. After drying, the absorption at 570 nm wavelength was measured using a microplate reader. The experiment was repeated 3 times.

**Results**

*Characteristics and Morphology of Isolated Phages*

Virulent S. aureus phage WV was isolated from a pig farm trashcan in Wuxi, China. The plaques of phage WV were 1 mm in diameter after overnight incubation at 37°C (Fig. 1a). Negative staining of purified S. aureus phage WV was observed with an electron microscope. TEM revealed a phage WV virion with an icosahedral head of 80 ± 2 nm in diameter and a noncontractile tail of 200 ± 5 nm in length (Fig. 1c). This morphology placed phage WV within the family Myoviridae. The 1-step growth curves of phage WV propagated in S. aureus are shown in Figure 1b. WV had a latent period of 70 min followed by a rise period of 40 min.

Optimal Temperature, pH, and MOI of Isolated Phages

Phage WV demonstrated the highest activity levels after treatment for 1 h at 42°C. There was a noticeable decline at 60°C and complete inactivation at 90°C (Fig. 2). The results show that phage WV has low temperature adaptability, which is consistent with the optimum survival temperature of S. aureus. Phage WV produced the most plaques at pH 6–7, with further plaques at pH 10–11. Plaque levels decreased significantly at pH 3–4 (Fig. 2). These results indicated that phage WV is intolerant to alkali and acids. The optimal MOI of phage WV was 0.01. The number of plaques of WV decreased significantly beyond an MOI value of 0.1 and was the lowest at an MOI of 100 (Fig. 2).

*Characteristics and Analysis of Phage WV Genome*

The complete genome size of phage WV was 141,342 bp. We identified 204 protein-coding open reading frames within the complete genome. Phage WV DNA was digested by EcoRI and Hind III but could not be digested by NotI and XhoI. Genome analysis revealed that phage WV is a virulent phage (Fig. 1d, 3). The complete genomic sequence of phage WV was deposited into the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/nuccore): GenBank accession MT787017.
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Phage WV Lytic Spectrum and Antibiotic Resistance of S. aureus

*S. aureus* strains were isolated from clinical patients in the First People’s Hospital of the Yunnan Province. Unfortunately, these strains have a broad spectrum of resistance (Table 1); however, several strains can be killed or removed by phage WV (Table 2). First, biochemical tests and antibiotic resistance tests were carried out for the isolated pathogens. Second, they were identified again by 16S rRNA gene sequencing as belonging to different strains. All strains possessed resistance to penicillin, kanamycin sulfate, erythromycin, and tetracycline but were sensitive to streptomycin, gentamicin, ciprofloxacin, levofloxacin, and rifampicin. Lytic *S. aureus* phage WV is capable of infecting 4 strains of *S. aureus* that had been isolated from the First People’s Hospital of Yunnan

Table 1. Antibiotic resistance of *S. aureus* isolates used in this study

| Antibiotic                  | A | B | C | D | E | F | G | H | I | J | MSSA | Sau |
|-----------------------------|---|---|---|---|---|---|---|---|---|---|-----|-----|
| Penicillin                  | R | R | R | R | R | R | R | R | R | R | R   | R   |
| Streptomycin                | S | S | S | S | S | S | S | S | S | S | S   | S   |
| Kanamycin sulfate           | R | R | R | R | R | R | R | R | R | R | R   | R   |
| Gentamicin                  | S | S | S | S | S | S | S | S | S | S | S   | S   |
| Ciprofloxacin               | S | S | S | S | S | S | S | S | S | S | S   | S   |
| Levofloxacin                | S | S | S | S | S | S | S | S | S | S | S   | S   |
| Rifampicin                  | S | S | S | S | S | S | S | S | S | S | S   | S   |
| Vancomycin                  | S | S | S | S | S | S | S | S | S | S | S   | S   |
| Erythromycin                | S | S | S | S | S | S | S | S | S | S | S   | S   |
| Tetracycline                | S | S | S | S | S | S | S | S | S | S | S   | S   |

Fig. 3. Line map of the phage WV genome. In the WV track, genes colored red encode lysozyme, genes colored yellow encode tail proteins, and genes colored purple encode endonuclease. The arrows represent the ORFs and point of direction of transcription. ORFs, open reading frames.
Province, China (Table 2). This analysis underlined the wide host range of isolated phage WV.

**Comparison of the Effects of Phage WV and Streptomycin on Host Biofilm**

Scanning electron microscopy was used to assess *S. aureus* biofilm formation on a round coverslip after treatment with phage WV (MOI = 0.1) and streptomycin (10 μg/mL). The bactericidal effects of phage WV and streptomycin were compared by determining the OD₅₇₀ values for the *S. aureus* biofilm and scanning electron microscopy of bacterial culture solutions inoculated with *S. aureus* at 4% (v/v), with phage WV (MOI = 0.1) and streptomycin (10 μg/mL) added immediately, followed by culturing for 24 h. This analysis demonstrated that streptomycin had a greater bactericidal effect than that of phage WV (Fig. 4–6). Phage WV had a greater bactericidal effect than that of streptomycin when phage WV (MOI = 0.1) and streptomycin (10 μg/mL) were added to a 12-h culture of *S. aureus* that was then cultured for a further 12 h (Fig. 4–6). In addition, joint treatment of the 12-h culture of *S. aureus* with phage WV and streptomycin for a further 12 h had an improved bactericidal effect than that of either phage WV or streptomycin (Fig. 4–6). The result of the *S. aureus* cfu analysis indicated that joint treatment with phage WV and streptomycin had a great-

**Table 2. Host range analysis of phage WV**

| Strain                        | WV |
|-------------------------------|----|
| Staphylococcus aureus-A       | −  |
| Staphylococcus aureus-B       | −  |
| Staphylococcus aureus-C       | −  |
| Staphylococcus aureus-D       | −  |
| Staphylococcus aureus-E       | −  |
| Staphylococcus aureus-F       | −  |
| Staphylococcus aureus-G       | −  |
| Staphylococcus aureus-H       | +  |
| Staphylococcus aureus-I       | −  |
| Staphylococcus aureus-J       | +  |
| Staphylococcus aureus-MSSA    | +  |
| Staphylococcus aureus-Sau     | +  |

**Fig. 4.** Scanning electron micrograph of *S. aureus* colonization before and after phage WV (MOI = 0.1) and streptomycin (10 μg/mL) application for treatment of the biofilm formed on round coverslips. **a** *S. aureus* cultured for 24 h. **b** Phage WV (MOI = 0.1) added to *S. aureus* culture. **c** Streptomycin (10 μg/mL) added to *S. aureus* culture. **d** *S. aureus* cultured for 12 h prior to addition of phage WV (MOI = 0.1) with continued culture for a further 12 h. **e** *S. aureus* cultured for 12 h prior to addition of streptomycin (10 μg/mL) with continued culture for a further 12 h. **f** *S. aureus* cultured for 12 h prior to addition of phage WV (MOI = 0.1) and streptomycin (10 μg/mL) with continued culture for a further 12 h (magnification, ×5,000). All *S. aureus* cultures were inoculated at 4% (v/v) with a seed solution.
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Discussion

S. aureus strains used in this study were isolated from the First People’s Hospital of Yunnan Province, China, and all strains demonstrated resistance to penicillin, kanamycin sulfate, erythromycin, and tetracycline. The emergence of MDR strains such as these necessitates the need for new measures to inhibit these pathogens. Abuse of antibiotics has resulted in MDR bacteria and super bacteria that represent a significant public health concern. Phages have been shown to be effective agents at combating this resistance issue. The isolated S. aureus phage WV is an ideal substitute for antibiotics because of the strong endolysin performance. Furthermore, phage WV has a greater bactericidal effect than that of streptomycin in high-concentration culture when these treatments are examined separately. Joint treatment with phage WV and streptomycin demonstrated an improved bactericidal effect than that of either phage WV or streptomycin (Fig. 6). In addition, incubation of the 12-h S. aureus culture for a further 12 h with phage WV (MOI = 0.1) had a greater bactericidal effect than that of streptomycin (10 μg/mL) (Fig. 6).

Fig. 5. Effects of phage WV and streptomycin (10 μg/mL) on biofilms. a, b Effects on S. aureus cultured in the presence of phage WV (MOI = 0.1) and streptomycin (10 μg/mL) for 12 or 24 h (OD600). c, d Effects on S. aureus cultured for 12 h prior to addition of phage WV and streptomycin (10 μg/mL) with continued culture for a further 12 or 24 h (OD600). e, f Effects on the S. aureus biofilm cultured in the presence of phage WV and streptomycin (10 μg/mL) for 12 or 24 h (OD570). g, h Effects on the S. aureus biofilm cultured for 12 h prior to addition of phage WV and streptomycin (10 μg/mL) with continued culture for a further 12 or 24 h (OD570). All S. aureus cultures were inoculated at 4% (v/v) with a seed solution.

Fig. 6. Effects of phage WV and streptomycin (10 μg/mL) on cfu of S. aureus. a Effects of phage WV and streptomycin (10 μg/mL) on S. aureus inoculated at 4% (v/v) and cultured for 24 h. b Effects of phage WV and streptomycin (10 μg/mL) added to a 12-h culture of S. aureus inoculated at 4% (v/v) with culturing continued for a further 12 h. cfu, colony-forming units.
tomycin treatment alone in a low-concentration culture (Fig. 4–6).

The isolated *S. aureus* phage WV belonged to the family Myoviridae and had a genome size of 141,342 bp. Comparatively, the genome size of *S. aureus* phage fRuSau02 is 148,464 bp and that of DRA88 is 141,907 bp [33, 34]. The genome characteristics of phage WV revealed the presence of a small endolysin with high lytic ability (Fig. 3).

When comparing antibiotics and phages, phage endolysin has many advantages as an antibacterial agent for clinical patients and for combating food spoilage [35]. Phage endolysin is a one-use enzyme with a short half-life [36]. Guo et al. [37] found that *S. aureus* phage endolysin contains a catalytic domain of Ch-type lysozyme at the N-terminus and that this functional area was able to cleave the 6-O-acetylated peptidoglycans that are present in the *S. aureus* cell wall. Comparatively, endolysin is an alternative to antibiotics and phages for inhibition of pathogens. At the same time, there are several challenges for long-term control of robust *S. aureus* strains in the clinical and food industries [38, 39].

The ability to form biofilm on different surfaces increases the risk of microorganism cross-contamination, particularly in poultry products, and this has been a serious issue for food industries and clinical settings and for overall public health [40–42]. *S. aureus* biofilms in catheter sites and wounds can lead to significant problems for patients such as drug resistance, morbidity, and mortality [33]. The complete elimination of such pathogenic biofilms has been challenging [43]. Until now, a suitable technology for biofilm control has not been available, and new strategies for biofilm control were constantly being sought and recommended [44]. In this study, we demonstrated that phage WV has superior properties than those of streptomycin for reduction *S. aureus* biofilm formation (Fig. 4–6).

The results show that phage WV and streptomycin have the ability to reduce *S. aureus* biofilm formation and progression. Streptomycin has greater antibiofilm effects than phage WV in low-concentration *S. aureus* culture (Fig. 4–6). In contrast, phage WV has greater antibiofilm effects than streptomycin in high-concentration *S. aureus* culture (Fig. 4–6). Our data provide strong evidence to suggest that the application of phage WV could reduce the growth of the *S. aureus* biofilm, which would have significant beneficial implications for public health.

In this study, the novel *S. aureus* phage WV demonstrated positive bactericidal and antibiofilm properties due to the presence of an endolysin consisting of only 57 amino acids (LNDLIKGNKYYHKVRAGETLWTISKNYVDIJKKLQELNNIKSVSRTSLEYVLVCE). This study provides a solid theoretical basis for the study of *S. aureus* phage endolysin.

In conclusion, we first isolated and characterized a lytic *S. aureus* phage, WV, and identified its biological properties. Next, we found that the combined use of phage WV and streptomycin yielded significantly better antibiofilm and bactericidal effects against *S. aureus* than those achieved using streptomycin or phage WV alone. The data herein provide strong evidence that application of phage WV could reduce the growth of the *S. aureus* biofilm, which would have significant beneficial implications for public health.

**Statement of Ethics**

All participants involved in this paper signed written informed consent. Ethical approval was obtained from the Institutional Ethics Committee (The First People’s Hospital of Yunnan Province, Kunming, Yunnan, China). The study protocol was in accordance with the Declaration of Helsinki for Human Research of 1974 (last modified in 2000). Written informed consent was received from each patient before sample collection.

**Conflict of Interest Statement**

The authors declare no conflicts of interest.

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**Author Contributions**

Conceived and designed the experiments: Y.X.J. and L.M.J. Performed the experiments: Y.X.J. and L.M.J. Analyzed the data: L.M.J. and Q.X. Contributed reagents/materials/analysis tools: R.Z. and L.M.J. Wrote the manuscript: L.M.J. All authors read and approved the final manuscript.
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