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Synergistic effect of proanthocyanidins and cefquinoxime sulfate on methicillin-resistant *Staphylococcus aureus*

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Abstract: To address the severe problem of methicillin-resistant *Staphylococcus aureus* (MRSA) resistance, this study identified a single component from traditional Chinese medicine that, when used in combination with existing antibiotics, enhances the therapeutic efficacy of the antimicrobial drugs. Using the micro broth dilution method and the checkerboard dilution method, susceptibility tests were conducted on ten commonly used β-lactam antibiotics against eleven strains of MRSA. It was found that cefquinome sulfate exhibits synergistic activity with PROs. The results indicate that among the ten antibiotics tested, five-potassium penicillin, ampicillin, ceftazidime, amoxicillin and CEFS had resistance rates exceeding 70%, indicating a serious resistance situation. Synergistic antibacterial effects showed that 25μg/mL of PROs could reduce the minimum inhibitory concentration (MIC) of CEFS against MRSA strains from 8μg/mL to 1μg/mL, with a fractional inhibitory concentration index (FICI) of 0.38±0.0, thus restoring the sensitivity of MRSA. These findings suggest the need for regular and continuous monitoring of the clinical distribution and resistance of MRSA in pig farms and implementing effective measures to prevent or control MRSA infections.

Keywords: PROs, synergistic effects, CEFS, MRSA.

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INTRODUCTION

With multi-drug resistant pathogens increasing year by year, it makes bacterial infections increasingly difficult to treat. For example, methicillin-resistant MRSA, which accounts for 60%-70% of hospital-based *S. aureus* infections, is one of the most invasive infections of all drug-resistant species and can cause bacteremia, skin abscesses, pneumonia and endocarditis (Chalmers & Wylam, 2020; Tabah & Laupland, 2022). Currently, MRSA infections occur not only in hospitals, but also frequently in the community (Li *et al*., 2022). β-lactam antibacterial drugs (including penicillins and cephalosporins) are the drugs of choice for the treatment of Gram-positive bacterial infections and MRSA has developed resistance to almost all available β-lactams. The development of resistance greatly restricts the clinical use of antimicrobial drugs and the rate of development of new antimicrobial drugs is much slower than the rate of resistance generation. Therefore, there is an urgent need for antimicrobial drug potentiators or new approaches to address this problem.

Combination therapy offers a promising strategy for overcoming bacterial resistance mechanisms and restoring the effectiveness of antimicrobial drugs. The use of combination therapies can expand the range of antimicrobial activity, reduce bacterial resistance, target new drug targets and reduce the toxicity and effective dose of these antimicrobial agents (Jorge *et al.*, 2017). In the development of anti-MRSA drugs, the therapeutic ability of natural compounds is increasingly recognized.

PROs are present in the flowers, nuts, fruits, bark and seeds of various plants and act as substances that resist both biotic and abiotic stress. They are oligomers and polymers in the flavonoid biosynthesis pathway. The components of PROs include catechins and epicatechins (Patanè *et al*., 2023). Proanthocyanidin dimers have been found to be effective antibacterial agents (Karioti *et al*., 2011), and PROs in combination with camellia oil have anti-inflammatory effects (Zhang *et al*., 2022). PROs in combination with mitoxantrone enhance the efficacy of colorectal cancer immunotherapy and reduce toxic side effects on immune organs (Qian *et al*., 2022).

Existing inhibitors against MRSA include: natural polyphenols, baicalein, Hibifolin, Punicalagin, and Rhodionin. Echinacoside, a natural polyphenol, when used in combination with vancomycin, is a potential SrtA inhibitor and can serve as a potential anti-virulence agent against *Staphylococcus aureus* infections (Jiang *et al*., 2023). Baicalein enhances the immune response of vancomycin against lethal pneumonia caused by methicillin-resistant MRSA through dual inhibition of

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sortase A and caseinolytic peptidase P (Wang *et al*., 2022). Hibifolin, with a synergistic index of 0.312 when combined with cefotaxime, is an SrtA inhibitor and can reduce the virulence of MRSA (Song *et al*., 2022). Punicalagin, a natural compound, is an SrtA inhibitor with an IC_{50} value of 4.23μg/mL (Song *et al*., 2022). Rhodionin, a natural flavonoid glucoside, effectively inhibits SrtA activity with an IC50 value of 22.85μg/mL (Wang *et al*., 2022). Scrophularioside A is an SrtA inhibitor (Li *et al*., 2024).

Based on the fact that PROs themselves are effective antibacterial agents and existing inhibitors against MRSA, this study initially investigates the synergistic effect of PROs in combination with CEFS, thereby restoring the sensitivity of resistant MRSA strains and addressing the issue of CEFS resistance in MRSA.

MATERIALS AND METHODS

Materials

Drugs

(104376-79-6), ceftazidime (72558-82-8), ceftiofur sodium (104010-37-9), cefadroxil (15686-71-2), CEFS (118443-89-3), cefazolin (27164-46-1), ceftiofur hydrochloride (103980-44-5) and proanthocyanidins (4852-22-6), a total of 7 kinds of 10 kinds of antibacterial drugs, all purchased from Shanghai The basic information of the drugs is shown in table 1 below.

Bacterial strains

The strains used for the test are the strains kept in this laboratory. Basic information is detailed in table 2 below.

Culture medium

MH broth medium, MH agar medium (MHA), brain heart infusion broth (BHI) were purchased from Qingdao Haibo Biotechnology Co.

Methods

Preparation of antibacterial drugs

A small amount of solvent (100μL-200μL) was used to dissolve the antibacterial drug and monomer and then the antibacterial drug was diluted with diluent to prepare a final concentration of 5120μg/mL drug stock solution, which was filtered and de-sterilized by 0.22 um filter membrane and then dispensed into sterile 2mL centrifuge tubes and stored in a -20ºC refrigerator for backup.

Preparation of bacterial solution

Pick a single colony on LB agar plate, mix well using the mixer, and then adjust the turbidity to 0.5 Mack's turbidity (concentration about 1×10^8 CFU/mL) with a bacterial turbidity meter and then MH broth will dilute the bacterial solution by 10×10 times with a concentration of about 1×10^6 CFU/mL as the test solution.

Minimum inhibitory concentration (MIC) assay

The MIC of PROs and 10 antimicrobial drugs was determined using ATCC 29213 as a quality control strain using the micro broth dilution method (Humphries *et al.,* 2021) as specified in CLSI 2020.

Combination drug sensitivity test (FIC assay)

To determine the antibacterial effect of procyanidin and 10 antimicrobial drugs in combination against drug-resistant *Staphylococcus aureus*, a checkerboard dilution method was used. ATCC 29213 was used as the quality control strain and ATCC 33591 as the test strain. The MIC results of PROs and 10 antimicrobial drugs were used as a reference and the checkerboard dilution method was used to design a 6×6 combination of two components with 1x, 1/2x, 1/4x, 1/8x, 1/16x and 1/32x MIC using 96-well sterile microtiter plates. The two combined components were diluted separately and the component diluted in the horizontal column was the primary object of investigation-herbal monomers and the component diluted in the vertical column was the secondary object-antibiotics. PROs were diluted in 96-well plates in a gradient and antibiotics were diluted in centrifuge tubes in a gradient in advance and 50μL of each was added to the wells. After the bacterial suspension was well mixed, 100μL was added to the test wells by pipetting with an eight-well pipette. The MIC, negative and positive wells of PROs and 10 antibacterial drugs alone were also set up as controls. 24h incubation at 37°C was performed and the results were observed and recorded.

The FIC index (graded inhibition concentration FICI) was calculated and determined as follows in equation (1) (Mhlongo *et al*., 2023):

FIC index = MIC A drug combination/MIC A drug alone + MIC B drug combination/MIC B drug alone (1)

FIC index reading criteria: when FIC≤0.5, it is synergistic; when 0.5<FIC≤1, it is additive; when 1<FIC≤2, it is irrelevant; when FIC>2, it is antagonistic.

Determination of minimum bactericidal concentration (MBC)

Based on the above results, the MBC of ceftiofur sodium, procyanidins and the MBC when both were used in combination were determined. 100 μL of culture from the last visible drug-containing well and each subsequent clear well were taken and evenly coated on MH agar plates and incubated at 36°C for 24h. Three parallels were set for each concentration and the test was repeated three times. The minimum bactericidal concentration is the lowest concentration capable of killing >99.9% of the bacterial content (<100 CFU/mL) (Denys *et al.,* 2011).

STATISTICAL ANALYSIS

The Inhibition concentration MIC and MBC values were calculated using the GraphPad Prism 8.2.1 t test.

RESULTS

Results of minimum inhibitory concentration (MIC) of procyanidins and antibacterial drugs

As shown in table 4, the minimum inhibitory concentrations of 11 strains of methicillin-resistant Staphylococcus aureus to procyanidin and 10 antibacterial drugs were different. The minimum inhibitory concentrations of procyanidin ranged from 200μg/mL to 400μg/mL, mostly concentrated at 256μg/mL (63.64±0.00%). 10 antimicrobial drugs had relatively serious resistance: the resistance rates of three antimicrobial drugs, namely, penicillin potassium, ampicillin and ceftazidime, were 100.00±0.00%, followed by 81.82±0.00% for amoxicillin, 72.8±0.00% for cephalosporin sulfate and 72.50±0.00% for quinoxime. The resistance rate of quinoxime was 72.73±0.00%. The lower resistance rates were ceftriaxone, CEFS, cefazolin and ceftiofur hydrochloride, with resistance rates of 27.27±0.00%, 27.27±0.00%, 18.18±0.00% and 18.18±0.00% for the three antimicrobial drugs, respectively.

Multi-resistance results of methicillin-resistant MRSA

The multi-resistance results of 11 strains of methicillin-resistant MRSA to 10 antimicrobial drugs were different. The multi-drug resistance of methicillin-resistant *S. aureus* was relatively serious. 63.64±0.00% of the 11 strains were resistant to more than 5 antimicrobial drugs. The multi-resistant strains were MRSA DY32, MRSA 2AY56-1, MRSA 2AY27, MRSA 2AY68-1, MRSA DY67-1 and ATCC33591 and their resistance profiles were PG+AM+AMX+CETS+CZ, PG+AM+AMX+CETS +CZ, PG+AM+CETS+CEFS +Among them, strain ATCC 33591 is resistant to all 10 antimicrobial drugs and is well above the range of resistance values, belonging to highly resistant strains. Therefore, ATCC 33591 was used as the standard for the initial screening for synergistic effects with procyanidins.

Synergistic results of procyanidin and CEFS

As can be seen from table 6: the MIC values of procyanidin on ATCC 33591 ranged from 100μg/mL - 256μg/mL and the MICs of 10 antibacterial drugs on ATCC 33591 ranged from 8μg/mL-512μg/mL, among which the MIC of CEFS was the lowest 8μg/mL and the MICs of penicillin potassium, ceftazidime, ampicillin and amoxicillin CEFS, ceftiofur hydrochloride and cefazolin had relatively good inhibitory effects. Next was the combined effect of procyanidin with 10 antibacterial drugs, screened by the inhibition index FIC ≤0.5 and found that only cefixime sulfate exerted synergistic effect with procyanidin, FIC=0.38±0.00, then. 100μg/mL of procyanidin reduced 8μg/mL of cefixime sulfate to 1 μg/mL, which reduced the concentration gradient of antibacterial drugs by 8 times and even restored the susceptibility of strain ATCC33591.

As shown in table 8, a total of three strains, MRSA 2AY54, MRSA DY67-1 and MRSA 2AY68-1, acted synergistically with inhibition indices FICs of 0.5, 0.375 and 0.5, respectively. 200μg/mL of MRSA 2AY54 procyanidin reduced 4μg/mL of CEFS to 1μg/mL, a 4-fold decrease. MRSA DY67-1 256μg/mL of procyanidins reduced 8μg/mL of CEFS to 2μg/mL, a 4-fold decrease. MRSA 2AY68-1 400μg/mL of procyanidins reduced 4μg/mL of CEFS to 1μg/mL, a 4-fold decrease. Meanwhile PROs all restored three strains of methicillin-resistant MRSA to susceptible status.

Minimum bactericidal concentration (MBC) results

From table 8, it can be seen that: the MBC of 3 strains of ATCC 33591, MRSA 2AY54 and MRSA 2AY68-1 PROs were all 400μg/mL, MRSA DY67-1 was 512μg/mL, while the MBC of all 4 strains of CEFS was 16μg/mL, and the value of MBC was 1 concentration gradient away from the value of MIC. The closer the MBC is to the MIC, the more effective it is, and it is likely to be a potential bactericide.

DISCUSSION

Serious analysis of MRSA resistance to 10 antimicrobial drugs commonly used in clinical practice

In this study, the resistance rates of the penicillin and cephalosporin antibiotics involved were as follows: potassium penicillin (100%), ampicillin (100%), ceftazidime (100%), amoxicillin (81.82%), and cefquinome sulfate (72.73%). The resistance rates of these five antibiotics exceeded 70%, indicating a relatively severe resistance situation. Additionally, it was found that the resistance rates of four penicillin antibiotics-penicillin, amoxicillin, ampicillin and oxacillin-were all 100%, especially penicillin, which maintained a 100% resistance rate over five years. What causes such high resistance rates? Penicillin antibiotics are derived from penicillin fungi and are naturally occurring antibiotics that are ubiquitous in the environment. This widespread presence might contribute to the high resistance rates. Cephalosporin antibiotics are synthesized from natural cephalosporin C by modifying their side chains. Ceftriaxone sodium, cefazolin sodium, and ceftazidime have high sales volumes, and their intermediates (thioctic acid, AE-active ester, triazine ring, tetrazole acetic acid, mercapto-tetrazole, and furoylamine salt) also show significant market potential (Chow *et al*., 2024). The extensive use of cephalosporin antibiotics may also be a contributing factor to their high resistance rates. Given the high resistance rates of these antibiotics, it is suggested that the pig farm discontinue the use of the five aforementioned antibiotics. Instead, it is recommended to use CEF, CEFS, cefazolin and cefotaxime hydrochloride.

Table 1: 11 kinds of drugs basic information

Classification	Name	Content	Fluids	Final concentration (μg/mL)
Penicillin	PG	≥97%	Water	5120
	AMX	96%	pH 6.0 phosphate buffer	5120
	AM	86%	0.1 mol/L phosphoric acid retardant solution at pH 8.0 was used for solubilization and then 0.1 mol/L phosphoric acid retardant solution at pH 6.0 was used for dissolution and volume fixation.	5120
Cephalosporins	CEF	≥97%	Water	5120
	CAZ	≥98%	Sodium carbonate aqueous solution	5120
	CETS	89.4%	Water	5120
	CN	≥98%	0.1M PBS，pH6.0	5120
	CEFS	98%	Water	5120
	CZ	99%	0.1M PBS，pH6.0	5120
	CH	89.5%	pH 6.0 of 0.1 mol/L phosphoric acid slow salt flush for dissolution and volume determination (water)	5120
Proanthocyanidins	PROs	>98%	Water	5120

Note: Penicillin G potassium salt PG, Amoxicillin AMX, Ampicillin AM, Ceftriaxone CEF, Ceftazidime CAZ, Ceftiofur Sodium CETS, Cephalexin monohydrate CN, Cefquinome sulfate CEFS, Cefazolin CZ, Ceftiofur hydrochloride CH, Proanthocyanidins PROs .

Table 2: Basic information of 12 clinical strains of MRSA

Strain name	Source
ATCC 29213	Laboratory Preservation
ATCC 33591	Laboratory Preservation
MRSA 2AY54	Shandong Pig Source
MRSA DY22	Shandong Pig Source
MRSA DY67-1	Shandong Pig Source
MRSA DY32	Shandong Pig Source
MRSA 2AY16-1	Shandong Pig Source
MRSA 2AY56-1	Shandong Pig Source
MRSA 2AY67-2	Shandong Pig Source
MRSA AY61	Shandong Pig Source
MRSA 2AY27	Shandong Pig Source
MRSA 2AY68-1	Shandong Pig Source

Table 3: 10 kinds of commonly used clinical antibacterial drugs MIC results determination range

Name	Quality control strain ATCC29213			Clinical strain range		
	S	I	R	S	I	R
PG		0.25-2		≤0.12	-	≥0.25
AM	≤0.5	0.5-2	≥2	≤0.25	0.25-0.5	≥0.5
AMX	0.125	0.125-0.5	0.5	≤4	4-8	≥8
CEF		1-8		≤8	16-32	≥64
CAZ		4-16		≤8	16	≥32
CETS	0.25	0.25-1	1	≤4	4-16	≥16
CN		0.12-0.5		≤8	16	≥32
CEFS		0.25-1		≤2	4	≥8
CZ		0.12-0.5		≤8	16	≥32
CH	0.25	0.25-1	1	≤4	4-16	≥16

Note: Cefazolin and cefadroxil belong to the first generation of cephalosporin antibiotics, with reference to the critical range of cefothiophene MIC.

Table 4: Statistics on MIC results of 10 clinically used antimicrobial drugs (%)

Name	PG	AM	AMX	CEF	CAZ	CETS	CN	CEFS	CZ	CH
Drug resistance rate (R)	100.00 ±0.00	100.00 ±0.00	81.82 ±0.00	27.27 ±0.00	100.00 ±0.00	27.27 ±0.00	36.36 ±0.00	72.73 ±0.00	18.18 ±0.00	18.18 ±0.00
Intermediation rate (I)	0.00 ±0.00	0.00 ±0.00	9.09 ±0.00	36.36 ±0.00	0.00 ±0.00	27.27 ±0.00	9.09 ±0.00	27.27 ±0.00	18.18 ±0.00	18.18 ±0.00
Sensitivity rate (S)	0.00 ±0.00	0.00 ±0.00	9.09 ±0.00	36.36 ±0.00	0.00 ±0.00	45.46 ±0.00	45.45 ±0.00	0.00 ±0.00	63.64 ±0.00	63.64 ±0.00

Table 5: Statistics of drug -resistant spectral results

Name	Spectral type	Number of species
ATCC33591	PG+AM+AMX+CAZ+CEF+CETS+CN+CEFS+CZ+CH	10
MRSA 2AY54	PG+AM+AMX +CETS	4
MRSA DY22	PG+AM+AMX +CETS	4
MRSA DY67-1	PG+AM+AMX +CETS+CZ+CEFS	6
MRSA DY32	PG+AM+AMX +CETS+CZ	5
MRSA 2AY16-1	PG+AM+AMX +CETS	4
MRSA 2AY56-1	PG+AM+AMX+CETS+CZ	5
MRSA 2AY67-2	PG+AM+AMX +CAZ+CETS+CN+CEFS+CZ	8
MRSA AY61	PG+AM+CETS+CZ	4
MRSA 2AY27	PG+AM+CETS+CEFS+CZ+PG	6
MRSA 2AY68-1	PG+AM+AMX +CETS+CN+CH	6

Table 6: FIC results of PRO with 10 antibacterial drugs (μg/mL)

Name	Solid MIC		Combined MIC		FIC index determination	Ways of role
	PRO	CEFS	PRO	CEFS		
PG	200.00±0.00	512.00±0.00	50.00±0.00	256.00±0.00	0.75±0.00	additive
AM	200.00±0.00	512.00±0.00	50.00±0.00	128.00±0.00	0.50±0.00	additive
AMX	100.00±0.00	512.00±0.00	50.00±0.00	128.00±0.00	0.75±0.00	additive
CEF	256.00±0.00	64.00±0.00	128.00±0.00	2.00±0.00	0.53±0.00	additive
CAZ	128.00±0.00	512.00±0.00	64.00±0.00	64.00±0.00	0.63±0.00	additive
CETS	100.00±0.00	128.00±0.00	100.00±0.00	64.00±0.00	1.00±0.00	irrelevant
CN	200.00±0.00	256.00±0.00	50.00±0.00	128.00±0.00	0.75±0.00	additive
CEFS	100.00±0.00	8.00±0.00	25.00±0.00	1.00±0.00	0.38±0.00	synergistic
CZ	200.00±0.00	32.00±0.00	50.00±0.00	16.00±0.00	0.75±0.00	additive
CH	100.00±0.00	32.00±0.00	50.00±0.00	8.00±0.00	0.75±0.00	additive

Table 7: FIC results of 10 clinical strains of PRO and CEFS (μg/mL)

Name	Solid MIC PRO	Solid MIC CEFS	Combined MIC PRO	Combined MIC CEFS	FIC index	Ways of role
MRSA 2AY54	200.00±0.00	4.00±0.00	50.00±0.00	1.00±0.00	0.50±0.00	synergistic
MRSA DY22	256.00±0.00	8.00±0.00	1.00±0.00	4.00±0.00	0.50±0.00	additive
MRSA DY67-1	256.00±0.00	8.00±0.00	16.00±0.00	2.00±0.00	0.38±0.00	synergistic
MRSA DY32	256.00±0.00	8.00±0.00	8.00±0.00	4.00±0.00	0.50±0.00	additive
MRSA 2AY16-1	256.00±0.00	4.00±0.00	32.00±0.00	4.00±0.00	1.13±0.00	irrelevant
MRSA 2AY56-1	256.00±0.00	8.00±0.00	32.00±0.00	4.00±0.00	0.63±0.00	additive
MRSA 2AY67-2	256.00±0.00	8.00±0.00	16.00±0.00	4.00±0.00	0.51±0.00	additive
MRSA AY61	256.00±0.00	8.00±0.00	16.00±0.00	4.00±0.00	0.51±0.00	additive
MRSA 2AY27	512.00±0.00	8.00±0.00	32.00±0.00	4.00±0.00	0.63±0.00	additive
MRSA 2AY68-1	290.40±88.08	6.80±1.83	30.30±26.88	3.20±1.25	0.58±0.19	synergistic

Table 8: Statistics of MIC and MBC results of 3 MRSA strains (μg/mL)

Name	MIC Mean ± standard deviation	MBC Mean ± standard deviation
PRO	123.00±14.00	400.00±0.00
CEFS	6.00±2.00	16.00±0.00

Analysis of the synergistic effect of procyanidin and CEFS

In this paper, we again found that PRO also has a synergistic effect with CEFS. PROs are polyphenolic compounds formed by the combination of flavan-3-ol monomers. They appear as reddish-brown powder, soluble in hot water and most organic solvents, and possess antioxidant (Ferreira *et al.*, 2021), anti-inflammatory (Zhang *et al*., 2018), antiviral and anticancer properties (Li *et al*., 2022). They have the potential to become candidates for anticancer drugs. Additionally, PROs belong to the class of flavonoid active monomers, while cefquinome sulfate belongs to the fourth-generation cephalosporins.

Studies have identified hibifolin as an inhibitor of SrtA and determined that it can reduce the virulence of methicillin-resistant MRSA. The FICI of CEF combined with hibifolin is 0.312 (FICI <0.5 indicates synergy), similar to the results in this study (Song *et al*., 2022).

Scholars have sought additional inhibitors by identifying structurally similar active monomers from traditional Chinese medicine. For example, studies on the synergistic effects of AMA derivatives and AMA derivatives bound to NDM-1-expressing MEM have shown that AMA stereoisomers perform better than AMA analogs (Koteva *et al.,* 2016; Zhang *et al*., 2017). Our research team, based on structural features such as NDM-1 stereoisomers and structural analogs, has preliminarily screened out four NDM-1 inhibitors (unpublished data).

CONCLUSION

In recent years, natural medicines have shown tremendous potential in the international pharmaceutical market. In China, they have developed into traditional Chinese medicine (TCM), which is unique to the country. Active components in TCM not only play a crucial role in clinical efficacy but also serve as a source for modern drug innovation, holding significant research value in the global pharmaceutical field. Initial studies have found that 4μg/mL and 8μg/mL of PROs reduce the MIC of CEFS to 1μg/mL and 2μg/mL, respectively, restoring the sensitivity of methicillin-resistant MRSA. This lays a theoretical foundation for further research on its mechanisms.

PROs have been shown to treat childhood diarrhea, diabetes, enhance cancer immunity, and alleviate neurological dysfunction. Commercially available low-molecular-weight proanthocyanidin-malvidin-3-O-glucoside conjugates from red wine have already been developed. This indicates the significant potential of PROs in clinical applications. Further research should focus on monitoring the safety of the drug, assessing potential risks and adverse reactions, and establishing a safety evaluation system, which are core aspects of new drug development. This study can continue to investigate whether different concentrations of PROs combined with CEFS exhibit obvious toxicity in mammalian red blood cells and cultured cell lines. Oral administration of PROs can be observed for any adverse reactions or toxic effects, thereby providing a more comprehensive understanding of the mechanisms of PROs.

Exploring and validating the safety and effectiveness of clinical drugs is a complex and challenging process in new drug development and repurposing existing drugs. This study can continue by administering PROs and CEFS together in mice to see if it improves their survival rate and effectively reduces bacterial loads and pathological damage in target organs. Given the limitations of oral gavage administration, such as lower bioavailability, which restricts the optimal synergistic therapeutic effect *in vivo*, future research should focus on developing novel drug formulations based on the pharmacological characteristics of active inhibitors. For example, using biotin, integrins, targeting peptides, or constructing nanolipid carriers to encapsulate small molecules can enhance the stability and bioavailability of the combination therapy, optimizing the best combination dosing regimen to achieve the best therapeutic outcome.

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