

Synergistic Activity of 3-Hydrazinoquinoxaline-2-Thiol in Combination with Penicillin Against MRSA

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Background: The growing resistance seen in various antibiotics, including those considered as last-resort options, underscores the pressing need for novel approaches and new substances to address MRSA infections. Combining antibiotics as a treatment approach can enhance effectiveness, expand the range of targeted bacteria, and minimize the likelihood of resistance emergence. This approach holds promise in addressing the escalating issue of antibiotic resistance.

Purpose: This study seeks to investigate the potential synergy between 3-hydrazinoquinoxaline-2-thiol and penicillin against a diverse array of MRSA isolates, thereby providing insights into their combined antimicrobial action.

Methods: Twenty-two clinical MRSA isolates subjected to broth microdilution to determine the Minimum Inhibitory Concentrations (MICs) of 3-hydrazinoquinoxaline-2-thiol and penicillin. Subsequently, a checkerboard assay was employed to evaluate the interaction between 3-hydrazinoquinoxaline-2-thiol and penicillin, focusing on the Fractional Inhibitory Concentration Index (FICI).

Results: The MICs of penicillin and 3-hydrazinoquinoxaline-2-thiol were determined for 22 clinical MRSA strains. Penicillin exhibited MICs within a range of 1024 to 128 $\mu\text{g/mL}$, while 3-hydrazinoquinoxaline-2-thiol displayed MICs varying from 64 to 8 $\mu\text{g/mL}$. Remarkably, the combination of 3-hydrazinoquinoxaline-2-thiol and penicillin yielded a synergistic effect, resulting in a significant reduction of MICs by up to 64-fold.

Conclusion: The potential of 3-hydrazinoquinoxaline-2-thiol in combination with penicillin as a viable solution against MRSA appears promising. However, to establish its practical utility, further extensive testing and experiments are essential.

Keywords: penicillin, antibiotic combination therapy, drug discovery, 3-hydrazinoquinoxaline-2-thiol

Introduction

Bacterial infections continue to pose a substantial global threat and remain a leading cause of mortality. This dire situation is exacerbated by the growing challenge of antibiotic resistance. This evolving resistance makes it increasingly difficult to effectively treat these infections, emphasizing the urgent need for novel therapeutic strategies and the development of innovative antibiotics.¹ Methicillin-resistant *Staphylococcus aureus* (MRSA) is accountable for a wide spectrum of diseases and stands out as a primary culprit behind hospital-acquired infections. These infections frequently lead to elevated levels of illness and death, prolonged hospital stays, and escalated treatment expenses. The formidable impact of MRSA on healthcare systems underscores the urgency of addressing this growing concern to mitigate its adverse effects on both patients and healthcare resources.²

β -lactam antibiotics exert their action by interfering with penicillin-binding proteins (PBPs), key players in the process of peptidoglycan crosslinking in bacterial cell walls.³ In the case of MRSA, resistance mechanisms are attributed to the presence of the *mecA* gene, responsible for encoding penicillin-binding protein 2A (PBP2A). PBP2A distinguishes itself by having a diminished affinity for the majority of β -lactam antibiotics, rendering them less effective against MRSA strains. This lowered susceptibility to these antibiotics underscores the significance of alternative treatment strategies to combat MRSA infections effectively.⁴ Handling MRSA infections has proven to be a formidable challenge,

primarily due to the limited therapeutic choices available. MRSA exhibits resistance to a range of antibiotics, including β -lactams, as well as last-resort antibiotic options. This resistance profile significantly narrows down the arsenal of effective treatments, amplifying the complexity of managing MRSA infections.⁵

At present, the arsenal of antibiotics suitable for MRSA treatment is quite limited, including options like vancomycin, linezolid, daptomycin and ceftaroline. These antibiotics have frequently been employed as the final resort for managing MRSA infections. However, it's important to note that they come with certain constraints in their clinical utility, and resistance to them has been frequently observed.^{3,6,7}

The process of developing novel antibiotics encounters a multitude of obstacles, encompassing regulatory hurdles, economic constraints, and substantial time investments. In light of these challenges, a pragmatic approach involves repurposing existing antibiotics and combining them synergistically in a well-thought-out manner. This strategy not only caters to the immediate and pressing clinical requirements but also benefits from the advantage that these agents have already gained approval for human use, streamlining the path to effective treatment solutions.^{8,9} Undoubtedly, the strategy of antibiotic combination therapy has proven to be efficacious in addressing the challenges posed by multidrug-resistant pathogens, exemplified by its successful application in treating organisms like *Mycobacterium tuberculosis* and *Helicobacter pylori*. This approach has enabled more comprehensive and effective treatment regimens, mitigating the impact of drug resistance and enhancing therapeutic outcomes in these cases.^{10,11}

Taking into account the utilization of antibiotic combinations offers a plethora of benefits, including the enhancement of treatment efficacy, the expansion of the range of pathogens targeted, a reduction in the likelihood of adverse side effects and toxicity, and a decreased risk of resistance development. This approach proves advantageous by concurrently addressing multiple aspects of bacterial infections, resulting in more effective and safer therapeutic strategies.¹¹

In a prior investigation, Elfadil et al demonstrated the notable efficacy of quinoxaline derivatives in combating various clinical strains of MRSA.¹² Quinoxalines, known for intercalating into DNA, exhibit preferential action against Gram-positive bacteria. Synthetic structures with the quinoxaline moiety possess antibacterial properties against a wide range of pathogens, acting through DNA interaction and inducing reactive oxygen species (ROS).¹³ Expanding on this, we propose that these quinoxaline derivatives have the capacity to restore the efficacy of penicillin when used in combination therapy against various MRSA clinical strains. This approach leverages the prior findings, presenting an innovative strategy to address the challenges posed by MRSA infections and potentially pave the way for more effective treatment options.

The aim of this study is to evaluate the in vitro antimicrobial activity of 3-hydrazinoquinoxaline-2-thiol when used in conjunction with penicillin against a range of clinical MRSA strains. This research endeavours to shed light on the potential synergy between these compounds and how it impacts the efficacy of treatment, further contributing to the understanding of innovative approaches to combat MRSA infections.

Materials and Methods

Sample Collection Growth Media and Condition

This study involved the assessment of 20 MRSA isolates, which were sourced from King Abdulaziz University hospital at King Abdulaziz University in Jeddah, Saudi Arabia. These isolates had been preserved in glycerol and stored at a temperature of -80°C . Prior to testing, all isolates were carefully thawed and cultured on Mannitol salt agar (provided by HiMedia, India) and incubated overnight at a temperature of 37°C within an aerobic environment.

Identification of the colonies was carried out using standard procedures, involving tests for catalase and tube coagulase. The process of sample collection was executed in strict adherence to the ethical guidelines and research protocols established by the Faculty of Applied Medical Sciences at King Abdulaziz University, under the reference number 38-712-456. The research was conducted in accordance with the principles outlined in the Declaration of Helsinki.

It's important to note that, as the clinical isolates utilized in this study were obtained as part of routine hospital laboratory procedures, the ethics committee granted an exemption from the requirement for informed consent.

Antibacterial Agents

The MRSA-targeting medications assessed in this study encompassed a 3-hydrazinoquinoxaline-2-thiol compound, sourced from Fluorophen Ltd in the United Kingdom and penicillin powder, acquired from Med ChemExpress.com

Susceptibility Testing

To assess antibiotic sensitivity, a broth microdilution test was conducted. The procedure involved creating a two-fold serial dilution of the antibiotics under examination in Mueller Hinton Broth obtained from Sigma-Aldrich in the United States. Subsequently, 100 μ L of the prepared antibiotic solution was dispensed into each well of 96-well plates from Corning, Italy.

The inoculum suspension density was meticulously adjusted to 0.5 McFarland using a suspension turbidity detector, specifically the Biosan Densitometers DEN-1B. Following this, 5 μ L of the prepared inoculum was introduced into each well containing varying concentrations of antibiotics. The plates were then incubated overnight at a temperature of 37°C. The antibiotic susceptibility testing was conducted in triplicate, and the resulting mean values were recorded for analysis.

The Minimum Inhibitory Concentration (MIC) refers to the lowest drug concentration capable of restraining the observable growth of a microorganism. The MIC results for the two antibacterial agents were assessed using the broth microdilution technique and were interpreted in accordance with the guidelines outlined by the Clinical and Laboratory Standards Institute (CLSI).^{14,15}

Checkerboard Assay

We employed the checkerboard broth assay to assess the interactions between antibiotics. To perform this, a two-fold serial dilution was prepared for each antibiotic using Muller-Hinton broth (MHB) Sigma, and 50 μ L of each dilution was dispensed into 96-well plates from Italy Inc.

The density of the inoculum suspension was carefully adjusted to 0.5 McFarland using a suspension turbidity detector, specifically the Biosan Densitometers DEN-1B. Subsequently, 5 μ L of the diluted bacteria were introduced into each well of the 96-well plates.

To evaluate the interactions, we calculated the fractional inhibitory concentration index (FICI) using the following equations: (MIC of drug A when used in combination) divided by (MIC of drug A when used alone) plus (MIC of drug B when used in combination) divided by (MIC of drug B when used alone). A FIC index of ≤ 0.5 was considered indicative of a synergistic effect.¹ The checkerboard assay was meticulously performed in triplicate to ensure robustness and reliability. The ensuing mean values obtained from these replicates were meticulously recorded, forming the basis for subsequent detailed analysis. This stringent approach to triplicate testing and subsequent mean calculation adds a layer of precision to the experimental process, ensuring a comprehensive and accurate assessment of the data.

Results

Mics of Penicillin and 3-Hydrazinoquinoxaline-2-Thiol Were Evaluated

It is essential to have determined the MICs of penicillin and 3-hydrazinoquinoxaline-2-thiol before proceeding with the checkerboard method. The MICs for penicillin range from 1024 to 128 μ g/mL (Table 1), high MIC values for penicillin are expected when dealing with MRSA strains. On the other hand, the MICs for 3-hydrazinoquinoxaline-2-thiol range from 64 to 8 μ g/mL (Table 2). With this information, we were able to design the checkerboard assay by combining various concentrations of penicillin and 3-hydrazinoquinoxaline-2-thiol to assess their potential synergistic or additive effects against the resistant strains. This assay will help you determine the interaction of these agents against different MRSA clinical strains.

Table 1 MICs of Penicillin in $\mu\text{g/mL}$ Against Different MRSA Clinical Strains

Strain Number	MIC of Penicillin
MRSA 2	128
MRSA 3	256
MRSA 1	512
MRSA 22	128
MRSA 20	256
MRSA 8	256
MRSA 7	256
MRSA 9	256
MRSA 10	256
MRSA 12	128
MRSA 14	256
MRSA 11	128
MRSA 15	256
MRSA 16	256
MRSA 23	128
MRSA 25	512
MRSA 27	1024
MRSA 28	512
MRSA 33	1024
MRSA 30	1024
MRSA 29	128
MRSA 34	512

Abbreviations: MIC, minimum inhibitory concentration; MRSA, Methicillin resistant *Staphylococcus aureus*.

Table 2 MICs of 3-Hydrazinoquinoxaline-2-Thiol in $\mu\text{g/mL}$ Against Different MRSA Clinical Strains

Strain Number	MIC of 3-Hydrazinoquinoxaline-2-Thiol
MRSA 2	16
MRSA 3	16
MRSA 1	32
MRSA 22	32

(Continued)

Table 2 (Continued).

Strain Number	MIC of 3-Hydrazinoquinoxaline- 2-Thiol
MRSA 20	16
MRSA 8	32
MRSA 7	8
MRSA 9	16
MRSA 10	16
MRSA 12	32
MRSA 14	32
MRSA 11	32
MRSA 15	32
MRSA 16	16
MRSA 23	16
MRSA 25	32
MRSA 27	8
MRSA 28	8
MRSA 33	64
MRSA 30	32
MRSA 29	32
MRSA 34	32

Abbreviations: MIC, minimum inhibitory concentration; MRSA, Methicillin resistant *Staphylococcus aureus*.

3-Hydrazinoquinoxaline-2-Thiol Synergises Penicillin Against Different MRSA Clinical Strains

A checkerboard assay was employed to evaluate the combined impact of 3-hydrazinoquinoxaline-2-thiol and penicillin on various clinical MRSA strains. When penicillin was administered in isolation, it failed to restrain the growth of MRSA. However, the intriguing observation was that when penicillin was used in conjunction with 3-hydrazinoquinoxaline-2-thiol, the MICs of penicillin decreased significantly, in some cases by up to 64-fold. Similarly, the MICs of 3-hydrazinoquinoxaline-2-thiol exhibited a noteworthy reduction when used in combination with penicillin, up to 8-fold lower (Figure 1).

What's even more remarkable is that this combination displayed a synergistic interaction against 22 distinct clinical MRSA strains, as evidenced by the FICI values consistently falling below 0.5 (Table 3). This strongly suggests that 3-hydrazinoquinoxaline-2-thiol complements penicillin effectively in combating MRSA strains, offering a potential avenue for improved treatment strategies (Figure 2).

Discussion

This study marks the pioneering demonstration of the synergistic potential between penicillin and 3-hydrazinoquinoxaline-2-thiol derivatives when confronting diverse MRSA strains. It's noteworthy that these MRSA strains exhibited

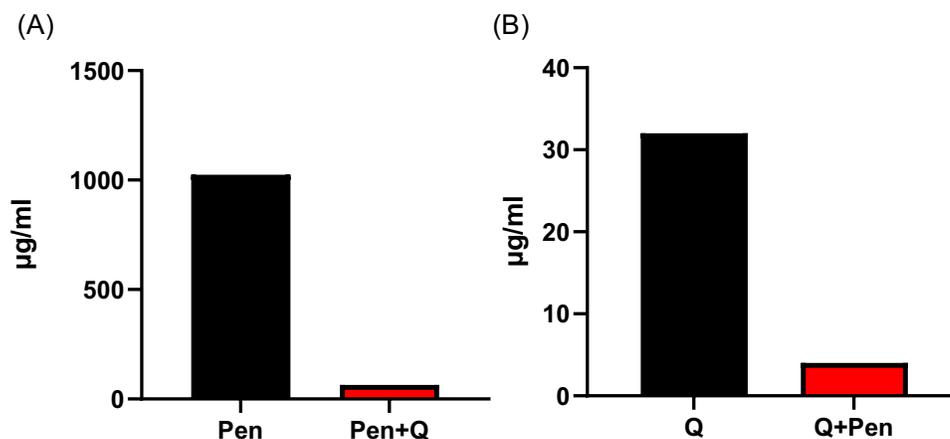


Figure 1 (A) Minimum inhibitory concentration (MIC) of penicillin was 1024 µg/mL and it was reduced to 64 µg/mL when it was combined with 3-hydrazinoquinoxaline-2-thiol against MRSA 27. (B) MIC of 3-hydrazinoquinoxaline-2-thiol was 32 µg/mL and it was reduced to 4 µg/mL when it was combined with penicillin against Methicillin resistant *Staphylococcus aureus* MRSA 33.

varying Minimum Inhibitory Concentration (MIC) values in response to penicillin, highlighting the diverse resistance profiles within the MRSA population. The revelation of this synergistic interaction not only expands our understanding of MRSA treatment but also offers a promising avenue for addressing antibiotic resistance in this context.

Table 3 The Interaction of Penicillin Against 3-Hydrazinoquinoxaline-2-Thiol 22 Clinical Different MRSA Strains

Strain Number	MIC of Penicillin	FIC of Penicillin	MIC of 3-Hydrazinoquinoxaline-2-thiol	FIC of 3-Hydrazinoquinoxaline-2-Thiol	FICI
MRSA 2	128	0.188	16	0.201	0.389
MRSA 3	256	0.135	16	0.25	0.385
MRSA 1	512	0.017	32	0.104	0.121
MRSA 22	128	0.063	32	0.198	0.261
MRSA 20	256	0.052	16	0.213	0.265
MRSA 8	256	0.073	32	0.125	0.198
MRSA 7	256	0.116	8	0.208	0.324
MRSA 9	256	0.135	16	0.25	0.385
MRSA 10	256	0.023	16	0.188	0.211
MRSA 12	128	0.146	32	0.213	0.359
MRSA 14	256	0.094	32	0.167	0.261
MRSA 11	128	0.105	32	0.104	0.209
MRSA 15	256	0.086	32	0.208	0.294
MRSA 16	256	0.026	16	0.213	0.239
MRSA 23	128	0.104	16	0.188	0.292
MRSA 25	512	0.051	32	0.213	0.264

(Continued)

Table 3 (Continued).

Strain Number	MIC of Penicillin	FIC of Penicillin	MIC of 3-Hydrazinoquinoxaline-2-thiol	FIC of 3-Hydrazinoquinoxaline-2-Thiol	FICI
MRSA 27	1024	0.065	8	0.333	0.398
MRSA 28	512	0.036	8	0.25	0.286
MRSA 33	1024	0.021	64	0.269	0.29
MRSA 30	1024	0.015	32	0.201	0.216
MRSA 29	128	0.167	32	0.208	0.375
MRSA 34	512	0.028	32	0.167	0.195

Abbreviations: MIC, minimum inhibitory concentration; MRSA, Methicillin resistant *Staphylococcus aureus*; FIC, Fractional inhibitory concentration; FICI, Fractional inhibitory concentration index.

Our research has unveiled a striking outcome: when used in combination with 3-hydrazinoquinoxaline-2-thiol, penicillin's Minimum Inhibitory Concentrations (MICs) saw a significant reduction, up to 64-fold. Likewise, a similar pronounced effect was observed when penicillin was paired with 3-hydrazinoquinoxaline-2-thiol, leading to a considerable decrease in the MICs of the 3-hydrazinoquinoxaline-2-thiol derivatives themselves. This potent cooperative action of 3-hydrazinoquinoxaline-2-thiol and penicillin was further substantiated in our experiments against a variety of clinical MRSA strains. These findings strongly indicate that the combined therapy of penicillin and 3-hydrazinoquinoxaline-2-thiol elicits a more robust response against MRSA strains compared to the efficacy of each individual drug.

Introducing a second antibiotic into the treatment regimen can potentially compensate for the limitations of the first antibiotic.¹⁶ Our results support the proposed concept: Penicillin alone could not halt MRSA growth, but in combination

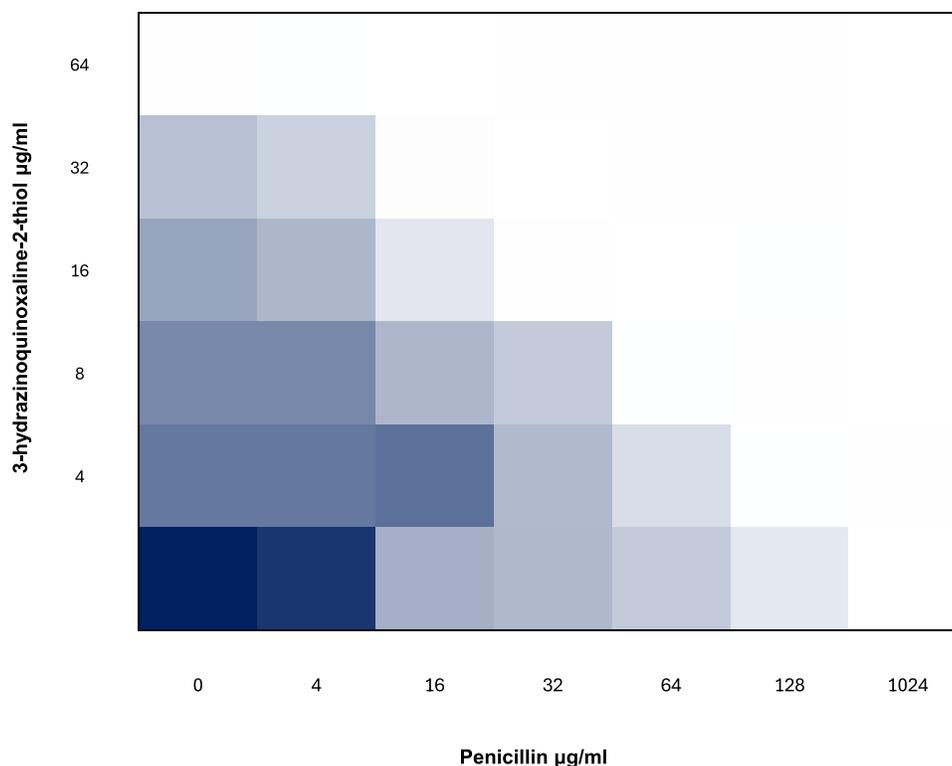


Figure 2 Checkerboard assay demonstrating the synergistic effect of penicillin with 3-hydrazinoquinoxaline-2-thiol against Methicillin resistant *Staphylococcus aureus* MRSA 33. The dark blue color represents 100% growth and the white color represents 0% growth.

with 3-hydrazinoquinoxaline-2-thiol, it demonstrated inhibitory efficacy. This synergy highlights the therapeutic potential of combined treatments against MRSA challenges.

Given the potential for toxic effects associated with high concentrations of 3-hydrazinoquinoxaline-2-thiol, penicillin, and other antibiotics, the utilization of reduced doses of each drug in a synergistic manner offers a promising strategy to mitigate potential toxicity.⁹ Our study supports the concept that penicillin, when combined with 3-hydrazinoquinoxaline-2-thiol, achieved MRSA growth inhibition at a significantly lower dose (32 µg/mL) compared to penicillin alone (512 µg/mL). This implies a potential for lower doses in combined therapy, potentially reducing adverse effects. Further research is needed to validate this promising result.

In our study, we made a noteworthy discovery regarding the efficacy of 3-hydrazinoquinoxaline-2-thiol in restoring the activity of penicillin against various clinical strains of MRSA. The synergistic effect observed between 3-hydrazinoquinoxaline-2-thiol and penicillin appears to be underpinned by a dual mechanism involving the inhibition of two distinct pathways. Firstly, penicillin hinders the action of penicillin-binding proteins (PBPs),¹⁷ and secondly, 3-hydrazinoquinoxaline-2-thiol impede DNA synthesis.¹⁸

The significance of this synergy lies in the fact that β -lactam antibiotics, like penicillin, primarily function by inhibiting PBPs. Importantly, each β -lactam antibiotic displays varying affinities for different bacterial PBPs. Consequently, the inhibition of PBPs disrupts the cross-linking of peptidoglycan in the bacterial cell wall.¹⁹ This disruption could potentially enhance the uptake and effectiveness of 3-hydrazinoquinoxaline-2-thiol.

It is worth noting that earlier research by Moellering and Weinberg indicated a substantial increase in the uptake of aminoglycoside antibiotics in the presence of penicillin or other antibiotics that interfere with the synthesis of bacterial cell walls.²⁰ Based on our findings, we hypothesize that the observed synergy between penicillin and 3-hydrazinoquinoxaline-2-thiol is driven by the activity of penicillin, which results in the impairment of the cross-linked cell wall. This impairment, in turn, facilitates the penetration and uptake of 3-hydrazinoquinoxaline-2-thiol, ultimately enhancing their bactericidal effects against MRSA.

Another plausible explanation for the enhanced effectiveness of the combination of penicillin and 3-hydrazinoquinoxaline-2-thiol against various MRSA strains could involve the increased production of reactive oxygen species (ROS). These ROS interfere with target-specific cellular processes and eventually lead to cell death. It is well-documented that bactericidal antibiotics, particularly β -lactams, can trigger the generation of ROS, which plays a significant role in bacterial cell killing.²¹ Interestingly, 3-hydrazinoquinoxaline-2-thiol are also known to have the capacity to generate reactive oxygen species.²²

Recent research has highlighted the phenomenon of β -lactam antibiotics inducing ROS production in *Enterococcus faecalis*.²³ Based on these findings, we speculate that the observed synergy between penicillin and 3-hydrazinoquinoxaline-2-thiol may be linked to the excessive production of ROS. However, further experiments are necessary to validate this hypothesis.

Additional investigations are imperative to gain a more comprehensive understanding of the synergistic interaction between penicillin and 3-hydrazinoquinoxaline-2-thiol. This entails conducting a series of crucial experiments.

Firstly, a Time Kill assay is warranted to meticulously evaluate the bactericidal effects of this combination. This assay will help us understand how effectively the combination eradicates bacteria over time, providing insights into its potential as a treatment strategy.

Furthermore, a resistance assay is essential to assess the likelihood of bacteria developing resistance to the penicillin and 3-hydrazinoquinoxaline-2-thiol combination. Understanding the resistance potential is crucial for the long-term effectiveness of this treatment approach.^{1,24} Incorporating an in vivo model is of paramount importance to assess the efficacy of the penicillin and 3-hydrazinoquinoxaline-2-thiol combination in a living organism. This model will not only allow us to gauge how well the treatment works in a complex biological environment but also delve into the pharmacokinetic and pharmacodynamic aspects of the combination, shedding light on its behaviour within the body and its interaction with bacteria at a molecular level.^{25,26}

Conclusion

In summary, for the first time we have demonstrated that the synergistic potential of 3-hydrazinoquinoxaline-2-thiol in combination with penicillin against a diverse spectrum of clinical MRSA strains. The findings from our study strongly indicate that this synergy could be advanced into clinical application. Nonetheless, there are several crucial steps that lie ahead to transition these combined antibiotics into clinical practice. Further research and clinical trials are imperative to fully explore the therapeutic potential, optimize dosing regimens, and ensure the safety and efficacy of this promising combination in real-world medical settings.

Abbreviations

MHB, Muller-Hinton broth; MRSA, Methicillin resistant *Staphylococcus aureus*; MIC, minimum inhibitory concentration; FIC, Fractional inhibitory concentration; FICI, Fractional inhibitory concentration index; ROS, Reactive oxygen species; PBP2A, penicillin-binding protein 2A.

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Disclosure

The authors declare no conflicts of interest in this work.

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