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Design, Synthesis, and Biological Activity of Thioguanine-Modified Pleuromutilin Derivatives

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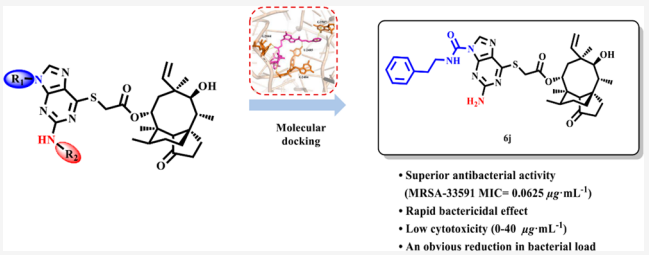
ABSTRACT: Antibiotic overuse has caused the increasingly serious problem of bacterial drug resistance, with numerous marketed antibiotics exhibiting significantly reduced activity against drug-resistant bacteria. Therefore, there is urgent demand for the development of novel antibiotics. Pleuromutilin is a tricyclic diterpene exhibiting antibacterial activity against Gram-positive bacteria and is currently considered the most promising natural antibiotic. In this study, novel pleuromutilin derivatives were designed and synthesized by introducing thioguanine units, and their antibacterial activities against drug-resistant strains were evaluated *in vitro* and *in vivo*. Compound **6j** was observed to have a rapid bactericidal effect, low cytotoxicity, and potent antibacterial activity. The *in vitro* results suggest that **6j** has a significant therapeutic effect on local infections, and its activity is equal to that of retapamulin, an anti-*Staphylococcus aureus* pleuromutilin derivative.



KEYWORDS: *Pleuromutilin analogues, Thioguanine, Synthesis, In vitro and in vivo assays, Structure-activity relationship studies*

Staphylococcus aureus (*S. aureus*) is a widespread and highly infectious Gram-positive bacterium that causes various diseases such as soft tissue infections, pneumonia,[1] septicemia,[2] osteomyelitis,[3] and mastitis.[4] Antibiotics are important for preventing and treating bacterial infections, but due to long-term antibiotic overuse, bacterial resistance has become even more prevalent. In 2019, the number of deaths from drug-resistant infections reached 920 000 globally, including more than 100 000 deaths from methicillin-resistant *S. aureus*.[5−8] These staggering numbers draw attention to the urgent need to develop new antibacterial drugs.

In 1951, pleuromutilin was isolated from *Pleurotus mutilus* and *P. passeckerianus*.[9] Pleuromutilin and its derivatives inhibit bacterial protein synthesis, primarily by binding to the central part of domain V of the 50s subunit at the bacterial ribosome.[10] Over 40 years have passed since a derivative of pleuromutilin, tiamulin,[11] was first marketed (Figure 1). Subsequently, valnemulin[12] was approved in 1999 for use in poultry as well, and in 2007, retapamulin[13] was developed as the first pleuromutilin-derived drug for human topical use. In 2019, lefamulin[14] was approved as a systemic drug for humans. According to the differences in the modification sites, semisynthetic pleuromutilin derivatives can be classified into parent ring modifications[15−17] and C-14 side chain modifications.[18−21] Possible locations for modifications of the parent ring include the tricyclic structure,[22] C-2 methylene,[15] C-3 carbonyl,[23] C-8 methylene,[17] C-13 methylene,[24] and C-19 double bond.[25] Disadvantages of parent ring modifications include the complexity of the reaction, difficulty of synthesis, and a decline in antibacterial activity. Due to these disadvantages, most researchers worldwide have shifted their attention to C-14 side chain modifications. Previous studies indicate that the introduction of thioether structures at the C-14 position is conducive to increasing the antimicrobial activity of compounds.[26,27] Additionally, all four approved pleuromutilin drugs use thioether structures as linkages; thus, this linkage structure was used in our compound design. Furthermore, purines are key functional groups that are frequently used in drug modification because of their high heteroatom content and good water solubility. Existing research suggests that the presence of purine groups in the C-14 side chain of pleuromutilin derivatives can increase their antibacterial activity.[28,29] Thus, a combination of thioether and purine (such as thioguanine units) can be introduced into the pleuromutilin skeleton to develop new drugs with potent antidrug-resistant bacterial activity. In this study, we synthesized plueromutilin derivatives by introducing thioguanine units and evaluated their antibacterial activities *in vitro* and *in vivo* against drug-resistant strains.

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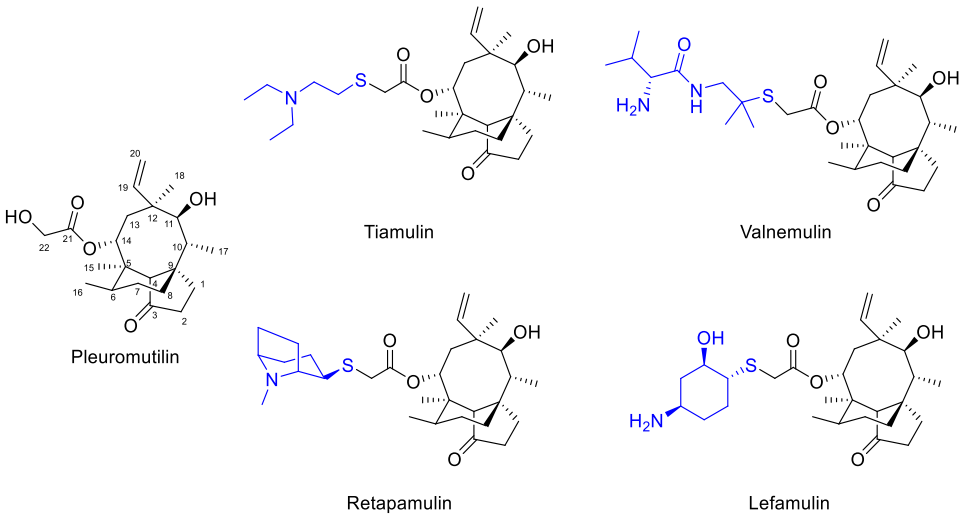
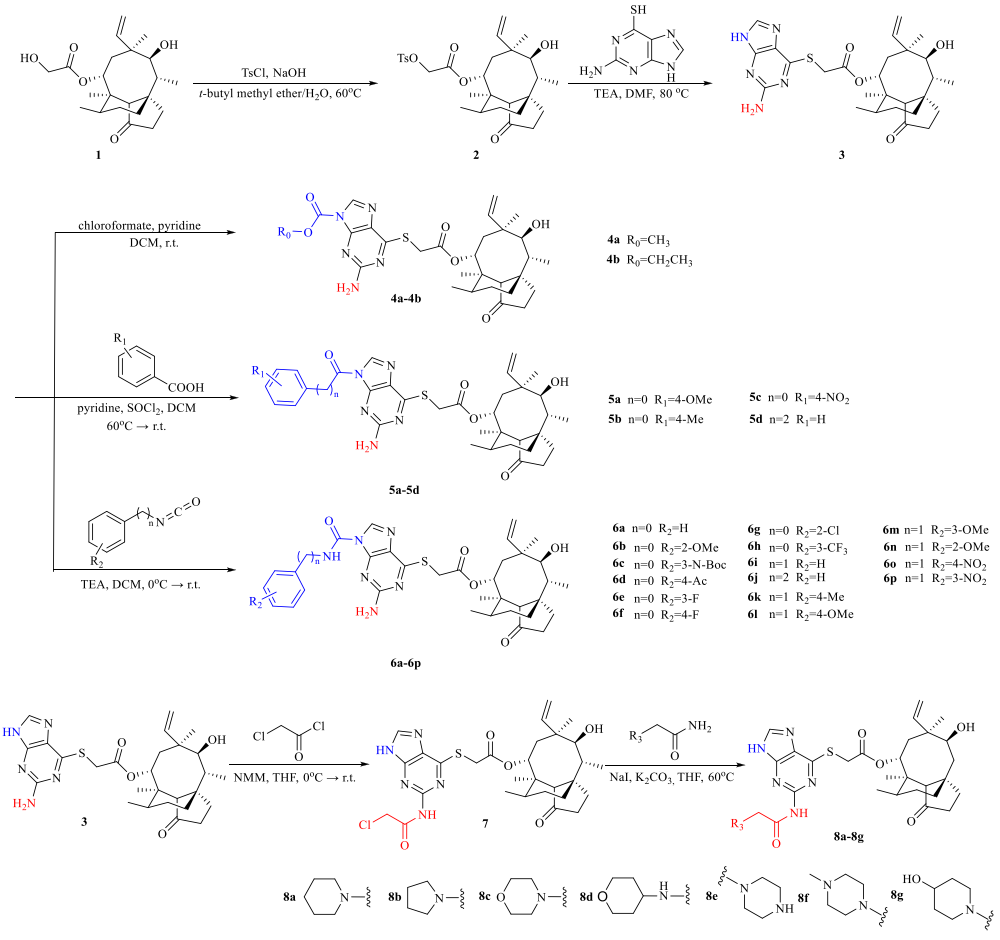


Figure 1. Approved drugs with pleuromutilin.

Scheme 1. Synthesis Route of Target Compounds



Thioguanine-modified pleuromutilin derivatives were synthesized via four strategies (Scheme 1). Hydroxyl at the C-22 position of pleuromutilin was activated with *p*-toluenesulfonyl chloride[30] to obtain intermediate **2** via nucleophilic substitution. Intermediate **2** was then reacted with 6-thioguanine and triethylamine (TEA) in dimethylformamide (DMF) to form intermediate **3** in an 85% yield, which was then reacted with chloroformates and pyridine in dichloromethane (DCM) to form carbamate derivatives **4a**−**4b** in 51−52% yields. Intermediate **3** was used to react with the benzoyl or acyl chloride derivatives (R = H, Me, OMe, and NO_2) to synthesize amide derivatives **5a**−**5d** in 45−60% yields. For the urea structure, intermediate **3** was reacted with the isocyanate derivatives (R = H, Me, and OMe) to synthesize urea derivatives **6a**−**6p** in 43−62% yields. The crystal structures of **6b** and **6i** were obtained from a solution of ethyl acetate/

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Table 1. MIC and MBC/MIC Values[a]

	MIC ($\mu g \cdot mL^{-1}$)/(MBC/MIC)					
Cpd	MRSA ATCC 33591	MRSA ATCC 43300	MRSE ATCC 51625	MSSA ATCC 29213	MSSE ATCC 12228	*S. aureus* ATCC 25923
3	0.125(4)	0.125(>4)	0.125(2)	0.125(>4)	0.125(>4)	0.25(>4)
4a	0.125(>4)	0.125(>4)	0.125(>4)	0.5(>4)	0.0625(>4)	0.125(>4)
4b	0.25(4)	0.125(>4)	0.25(>4)	0.125(>4)	<0.0078(>4)	0.25(4)
5a	0.5(>4)	0.125(>4)	0.25(4)	0.5(>4)	<0.0078(>4)	0.25(>4)
5b	0.125(>4)	0.25(>4)	0.125(>4)	0.125(>4)	0.0078(>4)	0.125(>4)
5c	0.125(>4)	**0.0625(>4)**	0.0625(>4)	0.0625(>4)	<0.0078(>4)	0.125(>4)
5d	0.125(4)	0.125(4)	0.125(>4)	0.125(>4)	0.0156(>4)	0.25(4)
6a	0.125(>4)	**0.0625(>4)**	0.125(>4)	0.125(4)	0.0156(>4)	0.125(>4)
6b	0.125(>4)	0.25(>4)	0.125(>4)	0.125(2)	0.0156(>4)	0.25(>4)
6c	0.25(4)	0.25(>4)	0.125(>4)	0.25(2)	0.0156(>4)	0.25(>4)
6d	**0.0625(>4)**	0.125(>4)	0.125(>4)	0.125(4)	0.0156(>4)	0.0625(>4)
6e	0.25(>4)	0.25(>4)	0.125(>4)	0.0625(>4)	0.0156(>4)	0.25(>4)
6f	0.125(>4)	0.125(>4)	0.125(4)	0.25(2)	0.0156(>4)	0.125(>4)
6g	0.125(>4)	0.125(>4)	0.0625(>4)	0.25(4)	0.0078(>4)	0.125(>4)
6h	0.25(>4)	0.25(>4)	0.125(4)	0.125(>4)	0.0156(>4)	0.25(>4)
6i	**0.03125(>4)**	**0.0625(>4)**	0.0625(>4)	0.0625(>4)	<0.0078(>4)	0.03125(>4)
6j	0.0625(4)	0.0625(4)	0.0625(>4)	0.125(>4)	<0.0078(>4)	0.0625(>4)
6k	**0.0625(>4)**	**0.0625(>4)**	0.0625(>4)	0.125(4)	<0.0312(>4)	0.125(>4)
6l	**0.0625(>4)**	**0.0625(>4)**	0.03125(>4)	0.0625(>4)	<0.0312(>4)	0.125(>4)
6m	0.125(4)	0.125(>4)	0.0625(2)	0.0625(4)	0.0156(>4)	0.125(>4)
6n	0.125(4)	**0.0625(>4)**	0.0625(>4)	0.0625(2)	0.0156(>4)	0.125(>4)
6o	0.25(>4)	0.25(>4)	0.0625(>4)	0.0625(>4)	<0.0078(>4)	0.125(>4)
6p	**0.03125(>4)**	**0.0625(>4)**	0.0625(>4)	0.0625(4)	<0.0078(>4)	0.0625(>4)
7	0.5(>4)	0.5(>4)	0.25(>4)	0.5(>4)	0.0156(>4)	0.5(>4)
8a	0.125(>4)	0.125(>4)	0.5(>4)	0.25(>4)	0.5(>4)	0.0625(>4)
8b	2(4)	1(>4)	1(4)	2(4)	2(>4)	2(>4)
8c	1(>4)	1(>4)	0.125(>4)	1(>4)	0.0156(>4)	0.5(Nd)
8d	0.5(>4)	16(>4)	1(>4)	8(>4)	2(>4)	0.5(>4)
8e	32(>4)	64(>4)	16(>4)	32(>4)	4(>4)	32(>4)
8f	16(>4)	32(>4)	1(>4)	8(4)	4(>4)	16(>4)
8g	16(>4)	32(>4)	2(>4)	16(>4)	2(>4)	16(>4)
T	1(4)	2(4)	1(>4)	1(>4)	0.5(4)	2(4)
V	0.25(1)	0.125(4)	0.125(>4)	0.125(>4)	2(>4)	0.25(1)
R	0.0625(2)	0.0625(4)	0.03125(4)	0.125(4)	0.01562(>4)	0.0625(4)

[a]T is tiamulin and V is valnemulin; Nd is not detected.

petroleum ether by using the slow evaporation method at ambient temperature (Figures S32 and S33).

Intermediate **3** was also reacted with chloroacetyl chloride and *N*-methylmorpholine in tetrahydrofuran (THF) via substitution to generate the amide intermediate **7**, followed by a substitution reaction with K_2CO_3 in the presence of NaI in THF to give the secondary amine derivatives (**8a**−**8g**) in 40−50% yields.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values are important indicators for determining antibacterial activity. In this study, the antibacterial activities of our synthesized compounds were evaluated against six *Staphylococcus* strains: *S. aureus* ATCC 25923, MSSA ATCC 29213, MSSE ATCC 12228, MRSA ATCC 33591, MRSA ATCC 43300, and MRSE ATCC 51625. Tiamulin, valnemulin, and retapamulin were used as control drugs. The results showed that most of the compounds exhibited potent antibacterial activity against the tested strains.

Among carbamate derivatives **4a**−**4b** and amide derivatives **5a**−**5d**, almost all compounds showed superior antibacterial activity to the approved drug tiamulin, with MIC values of 0.0625−1 $\mu g \cdot mL^{-1}$. Compound **5c**, with an electron-withdrawing group (NO_2), exhibited optimal antibacterial activity.

The antibacterial activities of urea derivatives **6a**−**6p** were significantly enhanced and similar to or higher than those of the carbamate and amide derivatives (**4a**−**4b** and **5a**−**5d**, respectively). This result confirms that the introduction of the urea group is conducive to increasing the antibacterial activity. Some compounds (e.g., **6d**, **6i**−**6l**, and **6p**) exhibited significant antibacterial activity, with MIC values as low as 0.0625 $\mu g \cdot mL^{-1}$. Compared with **6a**, **6i** and **6j** achieved lower MIC values, suggesting that the antibacterial activity of the compounds in which the urea group and benzene ring are linked by carbon atoms may be higher than that of the compounds in which they are directly linked. Thus, antibacterial activity can presumably be enhanced by introducing appropriate hydrophobic chains and extending the distance between the urea bond and the aryl group. A similar phenomenon was reported in a previous study.[31] Substituents on the benzene ring also affected the antibacterial activity. Using methoxy substitution as an example, the *para* substitution derivative (**6l**) exhibited higher activity than *ortho* and *meta* substitution derivatives (**6b** and **6m**, respectively),

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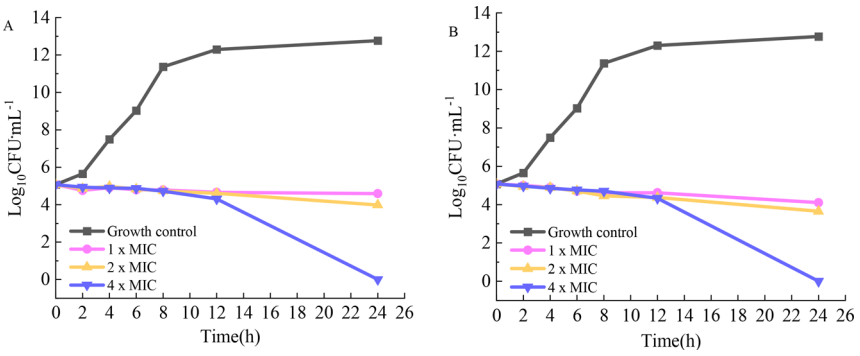

Figure 2. Time-kill curves of tiamulin (A) and **6j** (B) against MRSA ATCC 33591.

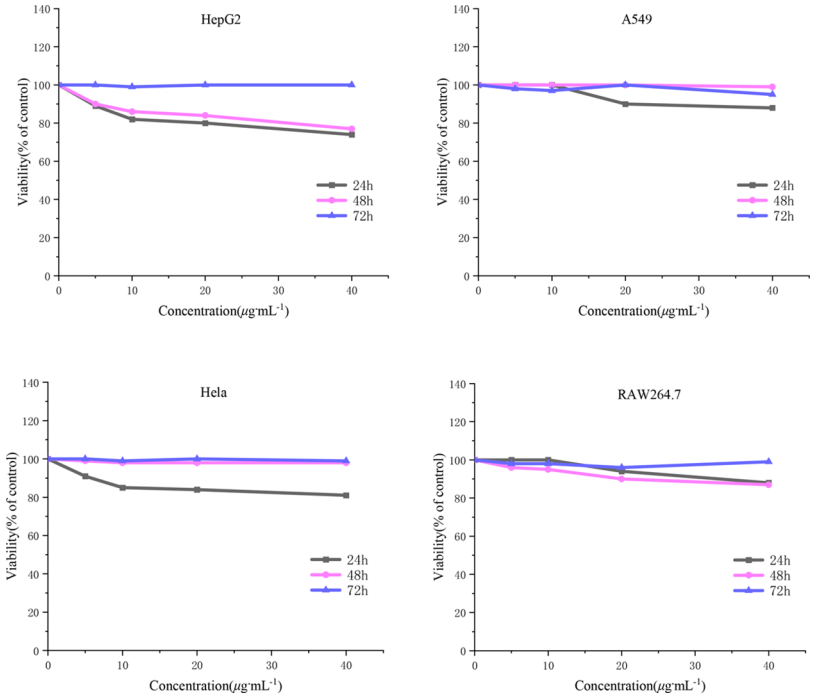

Figure 3. Toxicity of compound **6j** to the different cell lines.

suggesting that the sites of substituents on the benzene ring also affect the antibacterial activity.

Compared to the other compounds, pleuromutilin derivatives **8a**−**8g**, linked by a chloroacetyl group, exhibited reduced antibacterial activity. Among *N*5-substituted derivatives **8a**−**8g**, **8a** exhibited the optimal antibacterial activity with an MIC value of 0.125 $\mu g \cdot mL^{-1}$, whereas its activity was not significant compared with that of the *N*1-substituted derivatives. The activity of most of the *N*1-substituted derivatives was superior to that of the *N*5-substituted derivatives, suggesting that substitution at different sites on the purine ring significantly affects the activity of the derivatives.

The MBC values of the targets were examined to determine whether they were bacteriostatic or bactericidal. As shown in Table 1, most of the compounds exhibited MBC/MIC values higher than 4, indicating bacteriostatic effects, and individual compounds exhibited bactericidal effects at low concentrations. Notably, **6j** showed bactericidal effects against MRSA ATCC 33591 at a concentration of 1 × MIC.

Time-kill curves of tiamulin (Figure 2A) and **6j** (Figure 2B) against MRSA ATCC 33591 were obtained to study the correlation between the compound concentration and bactericidal rate. Three experimental concentrations (1×, 2×, and 4 × MIC) were assayed using tiamulin as the positive control. Figure 2 shows that the bacteria in the growth control group grew rapidly, particularly during 2−8 h, with a growth rate of 10^6. The number of colonies at 8 h was approximately 10^{12} $CFU \cdot mL^{-1}$. At concentrations of 1× and 2 × MIC, the results indicated that both **6j** and tiamulin inhibited bacterial growth and reduced the bacterial colony count to a certain extent but did not have any significant bactericidal effect. In the 4 × MIC group, **6j** exerted a significant bactericidal effect at 12 h, similar to that of tiamulin, both of which exhibited time dependency. Although **6j** showed bactericidal activity similar to that of tiamulin, the concentration of **6j** was 0.25 $\mu g \cdot mL^{-1}$, which is 16 times lower than the bactericidal concentration of tiamulin, suggesting that **6j** performs more effectively at a low concentration.

Cell survival and growth were examined at 24, 48, and 72 h through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The specific tetrazole salt is transformed into insoluble formazan via enzymatic activity in the mitochondria of living cells.[32] The cytotoxicity of **6j** was examined in HepG2, HeLa, and A549 cancer cells as well as

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RAW264.7 normal cells. The results indicated that the viability of the above cells was not significantly affected, and cell survival was more than 75% in the concentration range of 0−40 $\mu g \cdot mL^{-1}$ (Figure 3). These results suggest that **6j** exhibits low cytotoxicity against both tested cancer and normal cell lines. Moreover, at a prolonged incubation time of 72 h, **6j** was shown to slightly affected cell proliferation.

Thigh infection models were used to examine MRSA bacterial load and histopathological changes to determine the antimicrobial effect of **6j** *in vivo*. The results indicated that **6j** exhibited a better antibacterial effect than tiamulin and was comparable to that of retapamulin. As shown in Figure 4, the

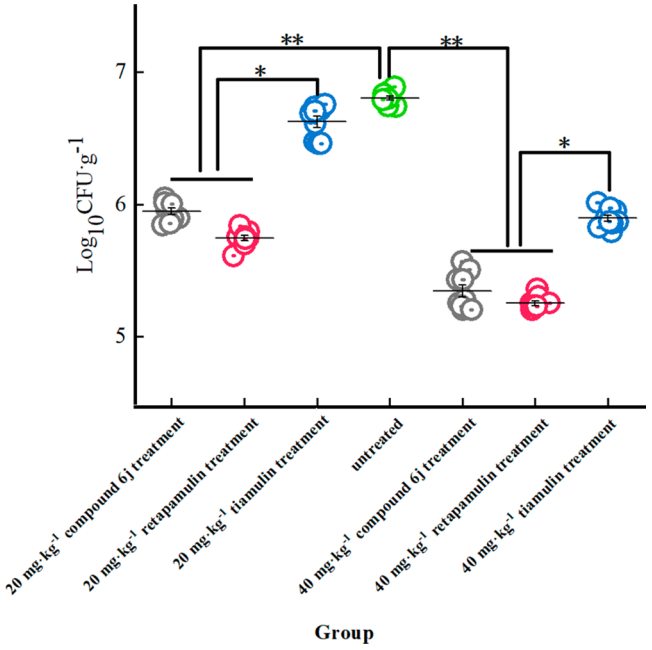

Figure 4. Bacterial load of different groups in thigh infection models of *MRSA*. * represents significant difference (vs the control group). ** represents extremely significant difference (vs the control group).

bacterial load in the model group is approximately 10^8 CFU·g^{-1}, which was reduced to approximately 10^7 CFU·g^{-1} after treatment with 40 mg·kg^{-1} of tiamulin. This is comparable to the effect of using **6j** or retapamulin at a lower dose of 20 mg·kg^{-1}, and the bacterial load can be reduced to as low as 10^5 CFU·g^{-1} at a dose of 40 mg·kg^{-1}.

Pathological changes were observed under a light microscope by hematoxylin and eosin (H&E) staining. Compared to the blank control group (Figure 5A), inflammatory cell infiltration and accumulation are observed in the MRSA-infected group (Figure 5B), whereas a significant reduction in inflammation is observed in the experimental group administered with **6j** or retapamulin Figure 5C−F). Notably, the inhibitory effect of **6j** on inflammation was dose-dependent. The thigh muscle in the group treated with 40 mg·kg^{-1} of **6j** (Figure 5D) was histologically normal, without a significant number of inflammatory cells in the blank control group and an effect comparable to that of retapamulin (Figure 5F).

The healing effect of **6j** on MRSA-infected mice was examined using a murine skin wound model. Compared to the uninfected group, the MRSA-infected control group exhibited skin deterioration, local skin blackening, and wound suppuration. Comparative analysis of the control group and the treatment group indicated that **6j** and retapamulin ointment facilitated wound repair. In particular, the groups that were administered **6j** and retapamulin ointment began to scab at the edge of the wound after 2−3 days and gradually healed. At 5−6 days, the wound healing status of the 1% and 2% **6j** ointment treatment groups was similar to that of the uninfected group, and the treatment effect of the 3% **6j** and 2% retapamulin ointment treatment groups was significant (Figure 6).

The expression of proinflammatory factors (for example, TNF-α and IL-6) is significantly correlated with wound healing. High expression of proinflammatory factors is a feature of systemic autoimmune and metabolic disorders, which causes abnormal inflammation of the wound, inhibits healing, and hinders wound repair.[33,34] MCP-1 induces the expression of CC chemokine receptor 2 (CCR2) in various cells during tissue injury or infection. CCR2 causes cells to move to the site of tissue injury or infection with high MCP-1 concentrations, facilitating the development of an inflammatory response.[35−38] In this study, the levels of TNF-α, IL-6, and MCP-1 in murine serum were measured using the ELISA method. Compared to the control group, the secretion levels of TNF-α, IL-6, and MCP-1 in the 1, 2, and 3% **6j** and 2% retapamulin ointment groups were significantly lower than those in the control group. This result suggests that **6j** and retapamulin can improve the inflammatory response to MRSA ATCC 43300. In particular,

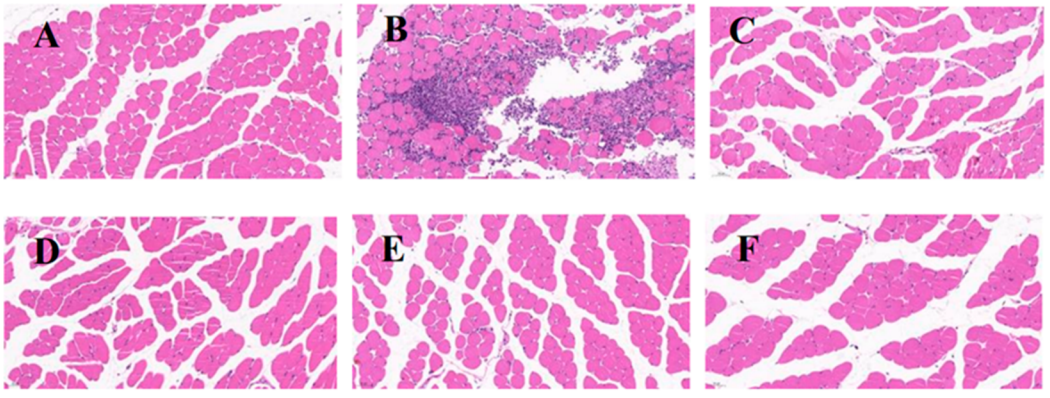

Figure 5. Effect of **6j** on pathological changes in MRSA-infected skin (HE: 200×). Blank control (A) group, untreated (B) group, 20 mg·kg^{-1} **6j** treatment (C) group, 40 mg·kg^{-1} **6j** treatment (D) group, 20 mg·kg^{-1} retapamulin treatment (E) group, 40 mg·kg^{-1} retapamulin treatment (F) group in thigh after challenge of *MRSA*.

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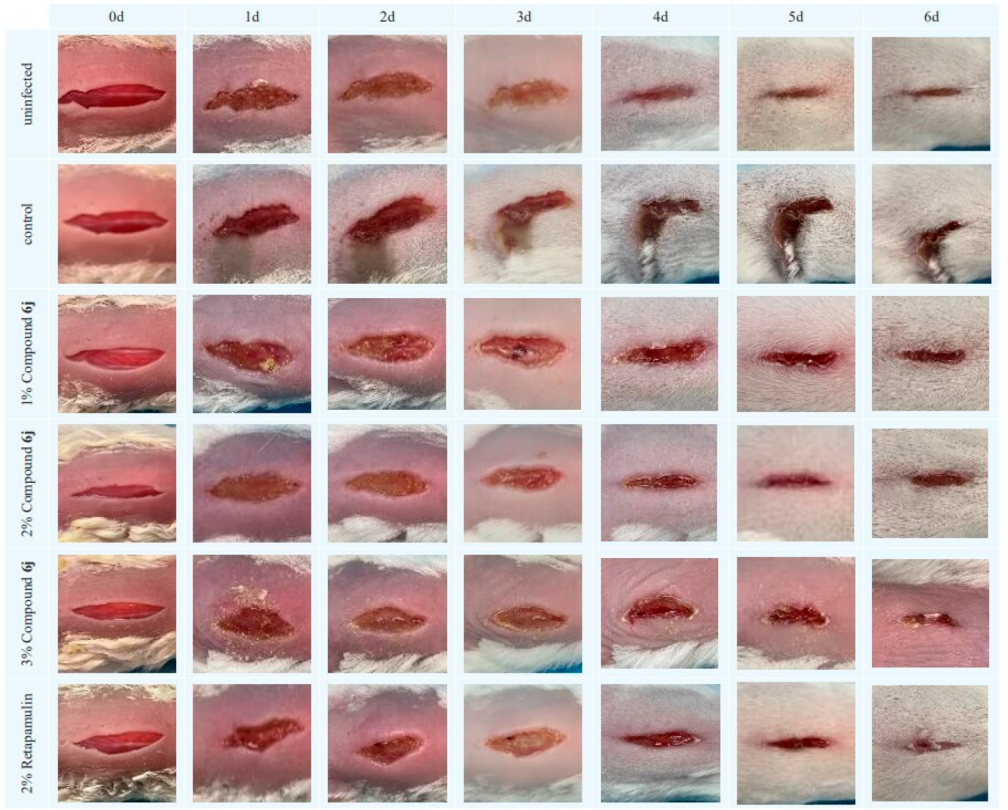

Figure 6. Observation of the wound healing process in each mice group at different times.

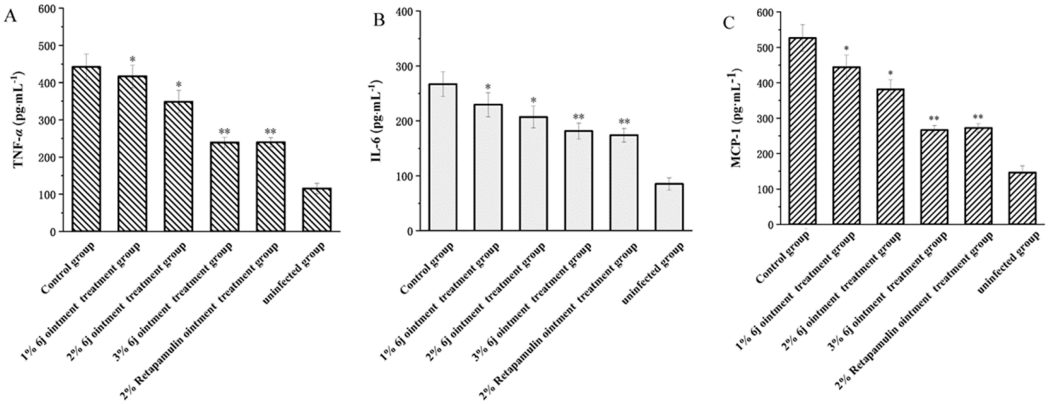

Figure 7. Effect of compounds on secretions of TNF-α (A), IL-6 (B), and MCP-1 (C).

both 3% **6j** and 2% retapamulin showed similar therapeutic effects (Figure 7). The effects on the downstream pathway signals will be explored in further detail.

The docking model was based on the crystal structures of *Deinococcus radiodurans* and tiamulin (PDB ID: 1XBP).[39] The binding mode of the target compound was studied by deleting the original ligand to obtain the receptor and using target compounds **5c**, **6j**, and **8a** as ligands.

As shown in Figure 8, the oxygen atom in the nitro group of **5c** formed a hydrogen bond with N−H on residue U2564, and the carbonyl group at position C-21 formed a hydrogen bond with residue G2044. Moreover, residue G2484 formed a hydrogen bond with the carbonyl group on the parent ring of pleuromutilin. Compound **6j** formed two hydrogen bonds with residue G2044 via the carbonyl group at the C-21 side chain, whereas residue G2484 formed a hydrogen bond with the carbonyl group at the C-3 position of the pleuromutilin parent ring. Residue U2046 also formed a hydrogen bond with NH_2 in the purine. The carbonyl groups at the C-3 and C-21 positions of **8a** formed hydrogen bonds with the residues G2044 and G2484, respectively. Furthermore, U2564 interacted with the purine ring via a hydrogen bond.

A series of thioguanine-modified pleuromutilin derivatives were prepared, and their antibacterial activities against Gram-positive bacterial strains were examined. Almost all the synthesized compounds showed satisfactory antibacterial activity, and **6j** exhibited significant antimicrobial activity against MRSA. The time-killing curve results indicated that **6j** exhibited bactericidal activity similar to that of tiamulin and had more advantages and prospects at low concentrations. In

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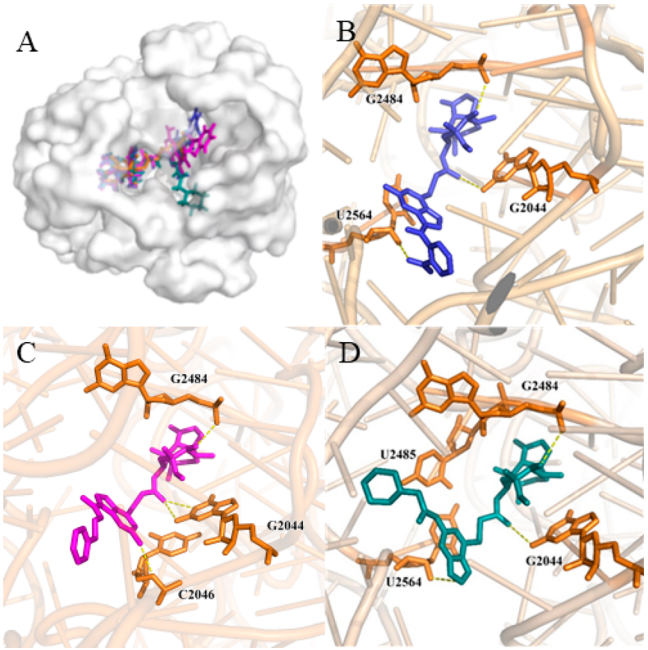

Figure 8. Interactions between ligands with PTC residues. Superimposed poses of selected compounds **5c** (purple), **6j** (magenta), and **8a** (cyan) in the PTC of the 50s ribosomal (A). Interactions between compound **5c** with residues (B). Interactions between compound **6j** with residues (C). Interactions between compound **8a** with residues (D).

addition, **6j** exhibited satisfactory antibacterial activity *in vivo*, and its effect was similar to that of the approved drug retapamulin, which can facilitate wound healing and significantly downregulate the expression of inflammatory factors in serum. These results revealed that **6j** is a potential drug candidate against MRSA infection, thus providing a reference for the development of anti-MRSA drugs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.3c00004.

Detailed experimental information: Synthetic experimental procedures, biology experimental methods, data of HPLC, ^{1}H NMR, ^{13}C NMR, and HRMS, molecular docking of some compounds, and the crystal data of some compounds (PDF)

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■ ABBREVIATIONS

K_2CO_3, potassium carbonate; MeCN, acetonitrile; DCM, dichloromethane; EDCI, N-(3-(dimethylamino)propyl)-N′-ethylcarbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; CDI, N,N-carbonyldiimidazole; THF, tetrahydrofuran; NMM, 4-methylmorpholine; NaI, sodium iodide; r.t., room temperature; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration

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