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RESEARCH ARTICLE

Metronidazole containing pyrazole derivatives potently inhibit tyrosyl-tRNA synthetase: design, synthesis, and biological evaluation

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As an important enzyme in bacterial protein biosynthesis, tyrosyl-tRNA synthetase (TyrRS) has been an absorbing therapeutic target for exploring novel antibacterial agents. A series of metronidazole-based antibacterial agents has been synthesized and identified as TyrRS inhibitors with low cytotoxicity and significant antibacterial activity, especially against Gram-negative organisms. Of the compounds obtained, **4f** is the most potent agent which inhibited the growth of *P*seudomonas aeruginosa ATCC 13525 (MIC = 0.98 μg/mL) and exhibited TryRS inhibitory activity (IC_{50} = 0.92 μM). Docking simulation was performed to further understand its potency. Membrane-mediated apoptosis in *P. aeruginosa* was verified by flow cytometry.

KEYWORDS
antibacterial agent, apoptosis, docking simulation, *Pseudomonas aeruginosa*, tyrosyl-tRNA synthetase

Bacterial infection has been a consistent and relentless threat to human health which has contributed innumerable financial burden to the healthcare system for a long time.[1] In addition, resistance to antibiotics, a widespread and thorny clinical problem, needs urgent attention due to a scarcity of new therapeutic agents.[2,3] Very recently, aminoacyl-tRNA synthetases (aaRSs) which exist in all creatures including the prokaryotes and eukaryotes have attracted impressive interest in antibacterial drug discovery.[4] Theoretically speaking, aaRSs are essential enzymes catalyzing a vital process in which amino acids are loaded to the cognate tRNAs.[5] It will lead to the failure of protein synthesis and cell growth arrest once they were inhibited in the cell. Moreover, because of the difference of significant evolutionary divergence between the human beings and microbes, aaRSs in the bacteria could be the targets selectively inhibited.[6,7] It is noteworthy that the tyrosyl-tRNA synthetase (TyrRS), a member of aaRSs family, has obtained increasing attention nowadays and may be a promising target for antibacterial agents.[7] Nevertheless, there is still no marketed drug targeting TyrRS[8] while our group had developed one kind of inhibitors with diaryl heterocycle scaffold (Fig. S1) and another series of inhibitors based on metronidazole skeleton (Fig. S2), both of which could selectively suppress it.[9,10]

Pyrazole, as a part of many natural and synthetic compounds and employed as the central building blocks for the synthesis of compound libraries,[11] exerts beneficial effects in the prevention of many diseases, including antimicrobial,[12] antitumor,[13] anti-inflammatory,[14] antidepressant,[15] and other activities. Nitroimidazole derivatives are provided with broad spectrum of biological activities,[16] especially antimicrobial activity. Among all the nitroimidazoles, metronidazole was used broadly in the treatment of infections induced by bacteria and other pathogenic protozoan parasites.[17] It is the truth

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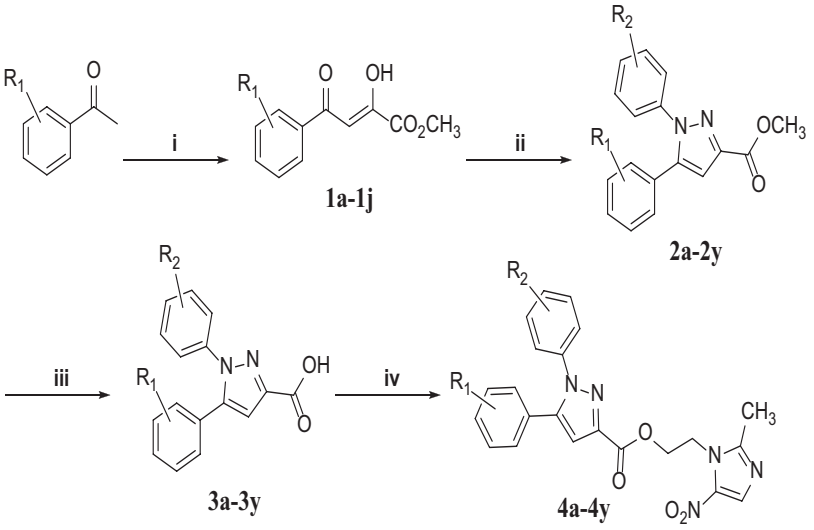

SCHEME 1 The synthetic routes of compounds **4a–y**. Reagents and conditions: (i) oxalic acid dimethyl ester, $NaOCH_3$, CH_3OH, reflux, 5 h; (ii) phenylhydrazine, diluted hydrochloric acid, CH_3OH, reflux, 6 h; (iii) KOH, H_2O, CH_3OH, reflux, 2 h; (iv) EDC, HOBT, DMAP, CH_2Cl_2, 45°C, overnight

that the metronidazole derivatives in organisms can produce electrophilic substances through biological reduction which can destroy proteins and nucleic acids; thus, the structure of metronidazole is widely applied in medicinal chemistry as an important pharmacophore.[18] In recent years, researchers increasingly tended to focus on the modification of the hydroxy group of metronidazole to screen more valuable drugs.[19] In our previous work, several pyrazole derivatives (Fig. S1) have been validated to inhibit aaRSs well. Encouraged by the findings, we have further confirmed that their efficacy mainly owes to the diaryl heterocycle containing pyrazole.[9] Furthermore, our group lately synthesized a class of compounds with metronidazole analogs targeting TyrRS (Fig. S2) and evaluated for their antibacterial activities.[10] Considering that multiple pharmacophores and complex scaffold are the common features of most antibacterial drugs,[20] we made the structural optimization of introducing the metronidazole on the pyrazole scaffold based on previous studies in this work.[19] The following docking simulation was carried out to predict the binding mode and affinity of the new compounds and enzyme. The results showed that the affinity of the new compounds was improved; thus, it was of interest to synthesize these compounds and evaluate their bioactivity.

In this research, we reported a sequence of metronidazole compounds bearing a pyrazole ring for the sake of finding new efficient antibacterial agents. We thereafter evaluated the antimicroorganism activity of them and also discussed the possible mechanism of their antibacterial action.

1 | RESULTS AND DISCUSSION

1.1 | Chemistry

All the novel metronidazole-pyrazole derivatives (**4a–y**) described herein were synthesized following the synthetic pathway depicted in Scheme 1, and the structures of the desired compounds are shown in Table S1. The starting chalcone derivatives, compounds **1** (**1a–j**), were synthesized from the reaction of acetophenone with dimethyl oxalate in the methanol mixed with sodium methoxide. Reaction of compound **1** with phenylhydrazine afforded the intermediates **2** (**2a–y**). Afterward, the intermediates **2** were hydrolyzed by KOH and drops of water in the methanol to give the last intermediate compounds **3** (**3a–y**), which contain a carboxyl group. Compounds **4a–y** hereafter were prepared from the reaction of compounds **3** with metronidazole in dichloromethane. The reactions were monitored by thin layer chromatography (TLC), and the crude products were purified by column chromatography and recrystallization with ethanol. All of the target compounds gave satisfactory analytical and spectroscopic data, that is, ^1H NMR, EI-MS, which are in accordance with their depicted structures. All of these compounds were reported for the first time. Furthermore, the crystal structure of compound **4l** was determined by single crystal X-ray diffraction analysis in Figure 1, and its crystal data, data collection, and refinement parameters for the compound **4l** were listed in Table S2.

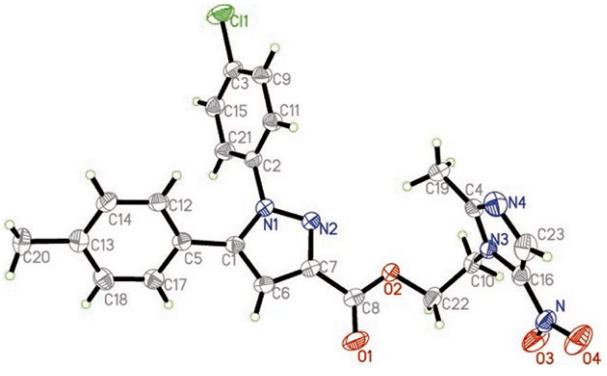

FIGURE 1 Crystal structure diagram of compound **4l**.

1.2 | Inhibitory activities of metronidazole-pyrazole derivatives against TyrRS

All the synthesized compounds (**4a**–**y**) were tested for inhibitory activity against TyrRS to obtain a further understanding of the structure–activity relationships (SAR). The IC_{50}s of these compounds are presented in Table S3. As we can see, the compound **4f** with 4-methoxy substituent in the benzene ring fragment (**A**) exerted the highest inhibitory activity against TyrRS with an IC_{50} of 0.92 μM and afforded over 60-fold improvement over the unsubstituted analog (**4r**). In comparison with **4r**, most substituted compounds were more potent, indicating that the modification on the aryl rings seems to be beneficial to enzyme inhibition.

Changing the position of the methyl group from the *para*-position (**4g**) to the *ortho*-position (**4p**) significantly enhanced the level of TyrRS inhibition. However, over 20-fold loss of enzyme inhibitory activity was observed when the 2-methyl group was replaced by fluoro atom (**4t**). On the other hand, the exchange of the fluoro atom from the *ortho*- to the *meta*-position resulted in an equally potent compound (**4x**), while the replacement of the methyl group to the *meta*-position (**4v**) led to a fourfold increase in potency. All the results suggest that compounds with electron-donating groups showed better inhibitory activities than those with electron-withdrawing groups.

In the case of compounds (**4q**, **4u**) with identical R_2 showed great difference of activity against TyrRS, while compounds (**4p**, **4q**) with same R_1 led to scarce alternation in potency. It

TABLE 1 Minimum inhibitory activity (MIC) of the synthetic compounds against microbes

Compounds	MIC[a] (μg/mL)			
	Gram-positive		Gram-negative	
	Staphylococcus aureus ATCC 6538	*Bacillus subtilis* ATCC 6633	*Escherichia coli* ATCC 35218	*Pseudomonas aeruginosa* ATCC 13525
4a	31.25	62.5	7.82	7.82
4b	31.25	62.5	31.25	31.25
4c	125	125	62.5	62.5
4d	31.25	62.5	15.63	15.63
4e	62.5	31.25	15.63	31.25
4f	3.91	3.91	1.96	0.98
4g	125	62.5	62.5	31.25
4h	62.5	62.5	62.5	31.25
4i	15.63	31.25	7.82	7.82
4j	15.63	15.63	15.63	7.82
4k	7.82	7.82	3.91	1.96
4l	15.63	31.25	3.91	3.91
4m	31.25	62.5	31.25	31.25
4n	62.5	62.5	31.25	15.63
4o	62.5	31.25	15.63	15.63
4p	15.63	15.63	3.91	3.91
4q	62.5	32.25	7.82	3.91
4r	62.5	31.25	31.25	31.25
4s	31.25	31.25	7.82	15.63
4t	125	125	125	62.5
4u	62.5	62.5	31.25	62.5
4v	15.63	15.63	15.63	7.82
4w	31.25	15.63	15.63	15.63
4x	62.5	62.5	62.5	31.25
4y	125	62.5	31.25	31.25
Penicillin G	1.96	0.98	7.82	7.82
Chloramphenicol	3.91	3.91	3.91	0.98
Metronidazole	62.5	62.5	31.25	62.5

[a]Values are the average of three independent experiments run in triplicate.

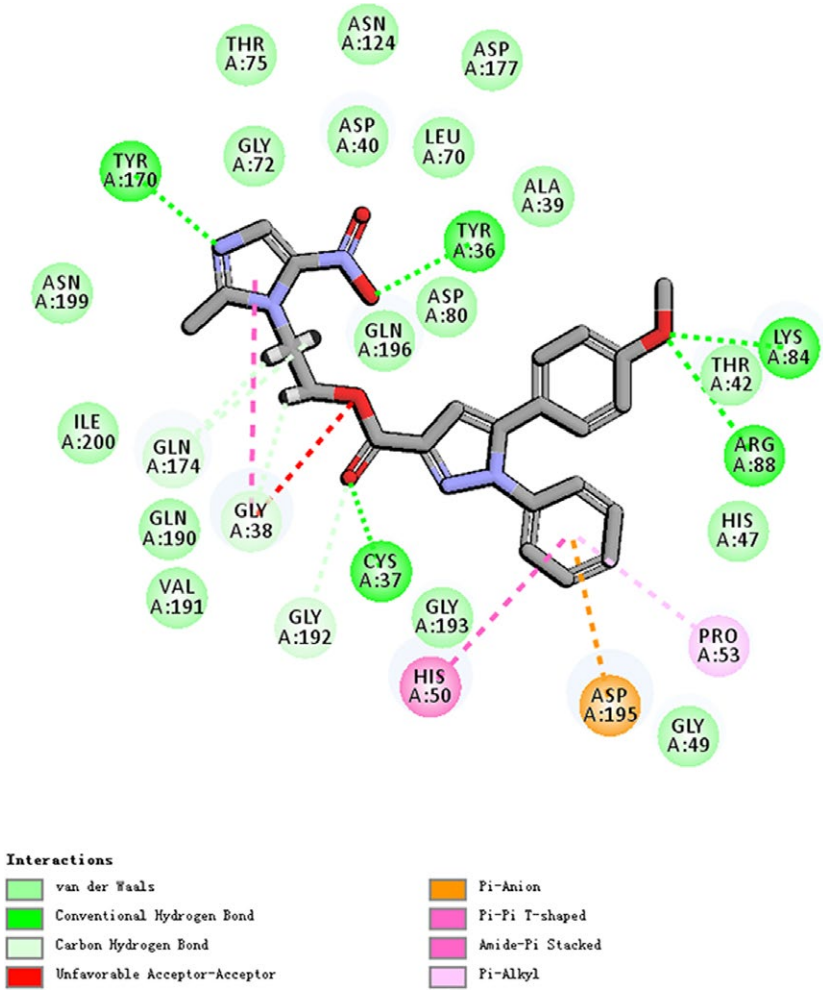

FIGURE 2 Ligand interaction diagram of compound **4f** with TyrRS using Discovery Studio program with the essential amino acid residues at the binding site are tagged in circles.

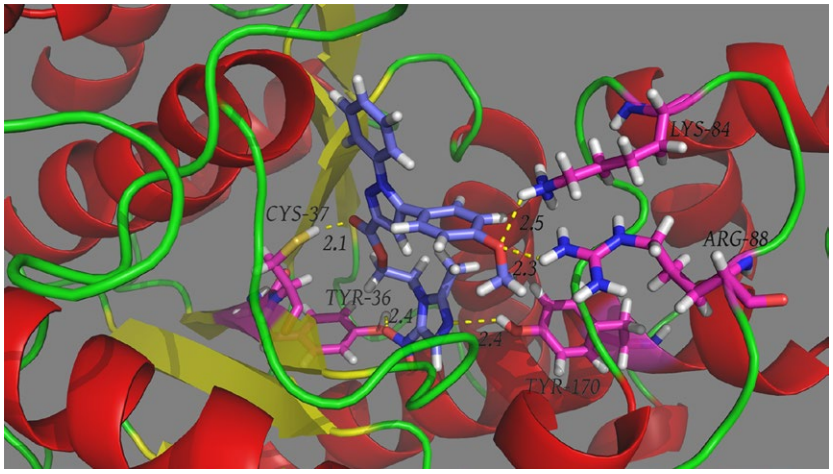

FIGURE 3 The 3D model structure of compound **4f** binding model with TyrRS.

illustrates that ring **A** seems to be more suitable for further modification with a substituent containing more diverse groups.

1.3 | Antibacterial activity

Encouraged by the results of TyrRS inhibitory assays, all the synthetic compounds were evaluated for their antimicrobial activities against two Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538) and Gram-negative bacteria (*Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 13525). The results are presented in Table 1. By and large, the obtained compounds showed more sensitivity to Gram-negative bacterial strains rather than Gram-positive bacterial strains, especially against *P. aeruginosa*. Compound **4f** and **4k** were found to exhibit impressive activity against both Gram-positive and

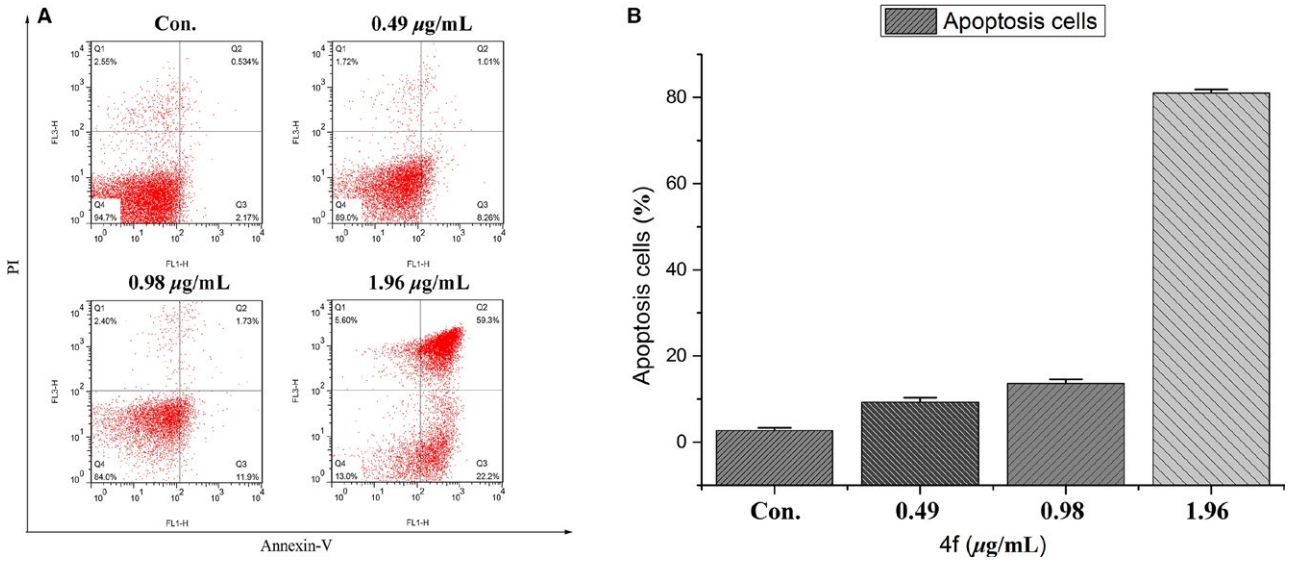

FIGURE 4 Annexin-V/PI dual-immuno-fluorescence staining after treatment with different concentrations of **4f** revealed significantly increased number of apoptotic cells (measured with Annexin-V^+/PI^+ cells). (A) Cells treated with 0, 0.49, 0.98 and 1.96 μg/mL **4f** for 14 h were collected and processed for analysis. (B) The percentage of apoptotic cells were calculated after the treatment of **4f**. Image is representative of three independent experiments.

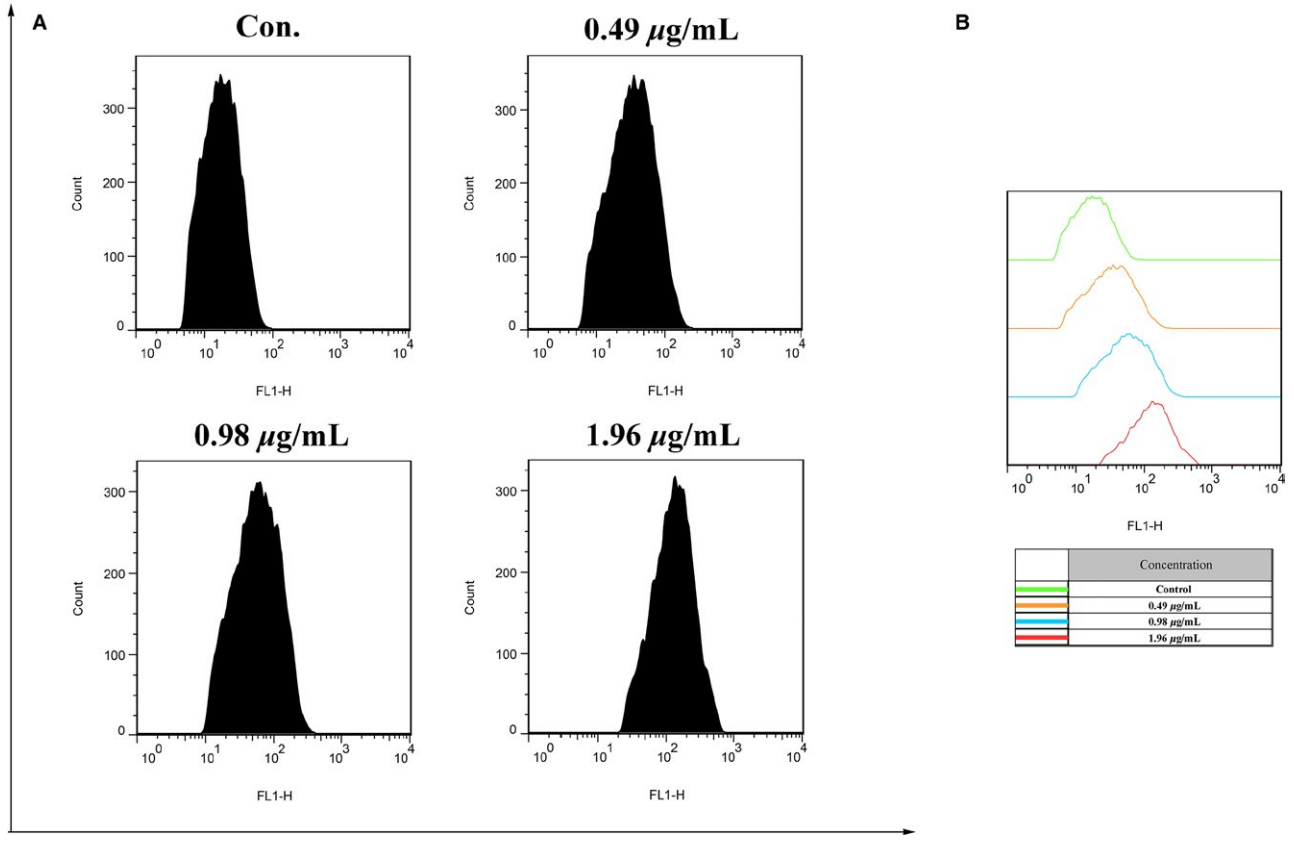

FIGURE 5 Effects of **4f** on membrane potential. Membrane potential ($\Delta\Psi$m) was analyzed using Rho123. The *P. aeruginosa* cells were treated with the designed concentration of **4f** (0, 0.49, 0.98, 1.96 μg/mL) at 37 °C for 14 h. *P. aeruginosa* cells were incubated with Rho123 (1 μM) for 30 min and then harvested for analysis by flow cytometry. (**A**) reflected the reduction of $\Delta\Psi$m. (**B**) presented the offset of the peaks.

Gram-negative bacterial strains and **4f** which presented the most active enzyme inhibition also showed the highest potency of antibacterial activity with MIC of 0.98 μg/mL against *P. aeruginosa* and 1.96 μg/mL against *E. coli*, which is close to marketed antibiotics. When it comes to the MIC values against Gram-negative bacteria (especially against

P. aeruginosa) and the corresponding IC_{50} values against TyrRS, a distinct correlation was found in almost all cases (Table 1 and Table S3), which indicates that the antibacterial activity may due to their inhibition of TyrRS.

1.4 | Molecular docking

For the purpose of giving an explanation and understanding of the excellent activity observed, we examined the interaction of the most potent inhibitor **4f** with TyrRS (1JIJ.pdb). All docking runs were applied CDOCKER Dock protocol of Discovery Studio 3.5. The docking model obtained in this study indicated that **4f** docks favorably into the active site of TyrRS (Figures 2 and 3) through five hydrogen bonds with Tyr36 (distance = 2.4 Å), Tyr170 (distance = 2.4 Å), Cys37 (distance = 2.1 Å), Lys84 (distance = 2.5 Å), and Arg88 (distance = 2.3 Å) residues. There were three important sections participating to form hydrogen bonds: nitrogen atom in nitroimidazole moiety and oxygen atom of nitro group were both responded to tyrosines; furthermore, methoxy group in the benzene ring contributed to form two hydrogen bonds with Lys84 and Arg88, respectively; finally, the ester bond connected the former two structures took part in the last hydrogen bond with cysteine. Besides, the higher binding affinity of **4f** might also be accounted for by some weak interactions, such as π bonds, van der Waals forces, and others. The above-mentioned interactions tightly anchoring **4f** to the active site of TyrRS probably explain its excellent inhibitory activity. As depicted in Figure S3, the CDOCK interaction energy of **4f** was −59.27 kcal/mol, which is the highest one of all. Our modeling results revealed that compound **4f** possessed an important influence on the interactions of the protein–ligand complex and was crucial to the potency of TyrRS inhibitory activity. Therefore, **4f** could be a potential inhibitor of TyrRS.

1.5 | 4f caused the apoptosis of *P. aeruginosa* in a dose-dependent manner

Earlier studies have showed that phosphatidylserine exposure, chromosome condensation, and DNA fragmentation, which are markers of apoptosis, could be observed in microbes treated with bactericidal antibiotic.[21–23] To further explore whether the apoptosis was involved in the antibacterial effect of target compound, *P. aeruginosa* cell apoptosis induced by different concentrations of compound **4f** (0.49, 0.98, 1.96 μg/mL) was performed. Annexin V and propidium iodide (PI) double staining by flow cytometry was conducted in this research. As shown in Figure 4, almost no apoptotic cells were found in the control group and a certain percentage of apoptotic cells were discovered with low concentrations of compound **4f**; however, the percentage of apoptotic cells significantly increased when concentration up to 1.96 μg/mL. The results consequently came to the conclusion that compound **4f** could induce bacterial cell apoptosis well.

1.6 | Effects of 4f on membrane-mediated apoptosis pathways

We measured the disruption of cell membrane potential (ΔΨm) using Rho123 stain to test whether compound **4f** could induce apoptosis of *P. aeruginosa* through destroying the cell membrane.[24] As depicted in Figure 5, compared with control group, *P. aeruginosa* cells which were treated with 0.49, 0.98, 1.96 μg/mL of compound **4f** for 14 h are all exposing the collapse of ΔΨm. The observations indicated that compound **4f** might induce irreversible programed cell death phenomenon through membrane-mediated apoptosis pathway.

1.7 | Cytotoxicity

To explore potential antibiotic, all the compounds were evaluated by the MTT assay for their toxicity against the human renal epithelial cells which was defined by the median cytotoxic concentration (CC_{50}) data. As shown in Table S3, most of the analogs possessed low cytotoxicity.

2 | CONCLUSION

Faced with the shortage of new therapeutic drugs to antibiotic-resistant bacteria, a battery of novel metronidazole-pyrazole derivatives targeted to TyrRS was synthesized and characterized. Meanwhile, their biological activities were evaluated. Antimicrobial activity and TyrRS inhibitory activity have identified that compound **4f** possessed the most potent inhibition activity against *B. subtilis*, *S. aureus*, *E. coli,* and *P. aeruginosa*, especially the Gram-negative bacteria (with MIC of 1.96 and 0.98 μg/mL) and inhibition of enzyme (with IC_{50} of 0.92 μM). The cytotoxicity test employing human renal epithelial cells indicated these derivatives were highly safe. A potential binding model with docking simulation of compound **4f** into TyrRS active site afforded a good understanding and explanation of its impressive performance. Analysis of the binding model showed that compound **4f** was stabilized by five hydrogen bonds. The following experiments including apoptosis and disruption of the membrane potential demonstrated **4f** exerts proapoptotic effects through a membrane-mediated apoptosis pathway. These findings bring the truth that compound **4f** would be a potential antibacterial agent for further research to light.

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