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Original Article

Development of a novel ultrashort antimicrobial peptide-levofloxacin conjugate with enhanced synergistic activity against multidrug and levofloxacin-resistant bacterial isolates

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Abstract

Background and purpose: Antimicrobial resistance poses a significant global health threat. A previously developed penta-amino acid ultrashort antimicrobial peptide (UP5), with alternating arginine and biphenylalanine units, showed strong antimicrobial activity, particularly in combination with levofloxacin. However, differences in pharmacokinetics between UP5 and levofloxacin may hinder their optimal clinical use. This study aimed to develop a covalent UP5-levofloxacin conjugate that retains the synergistic antimicrobial properties of both UP5 and levofloxacin.
Experimental approach: The UP5-levofloxacin conjugate was synthesized and tested for antimicrobial activity against multidrug-resistant gram-positive (*Enterococcus faecium*, *Staphylococcus aureus*, *Staphylococcus epidermidis*) and gram-negative bacteria (*Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*). Hemolytic activity was assessed on human erythrocytes, and selectivity against MDCK cells was determined. Comparative analysis was performed with individual components.
Findings/Results: The conjugate exhibited synergistic antimicrobial activity with minimum inhibitory concentration (MIC) values between 2.5 and 20 µM, overcoming levofloxacin resistance. It showed minimal hemolytic activity < 1% and a favorable selectivity index of 4.4-35.2. Cytotoxicity studies indicated selective toxicity towards MDCK cells, with an IC_{50} of 88.34 µM, significantly higher than its MIC values.
Conclusion and implications: The UP5-levofloxacin conjugate demonstrated enhanced antimicrobial efficacy against resistant bacteria, with minimal hemolytic activity and favorable selectivity toward normal cells. This conjugate presents a promising approach to combating antimicrobial resistance, offering potential for improved therapeutic strategies.

Keywords: Antimicrobial peptide; Antimicrobial resistance; Conjugation; Drug design; Levofloxacin; Synergism.

INTRODUCTION

Antimicrobial resistance and the rise of multidrug-resistant (MDR) bacteria continue to pose significant challenges to human health (1,2). In recent decades, a widespread emergence of resistance to nearly all available antibiotics has occurred, with some bacterial strains reported to exhibit pan-resistance, rendering them unresponsive to all classes of antibiotics (3). This trend raises concerns about humanity entering what is feared as the post-antibiotic era, where no antibiotics would be available for the treatment of infections caused by MDR bacteria (4,5). This issue is causing alarm within international and governmental health-oriented organizations, which are actively working to raise awareness about the threat of bacterial resistance and formulate strategies to mitigate its potential impact on human health (6,7).

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Antimicrobial resistance is exacerbated by a significant decline in the development of new antimicrobial agents. This situation places considerable strain on medical health practitioners in managing bacterial infections within the clinic (8,9). Institutions and healthcare providers are concentrating on implementing regulations for antibiotic prescriptions and increasing awareness among populations (10,11). Simultaneously, scientists in the field of drug research are committed to developing novel antibacterial agents capable of overcoming the new mechanisms of bacterial resistance (12,13).

Antimicrobial peptides (AMPs) represent one of the innovative strategies to combat microbial resistance that has gained considerable interest in the past two decades due to their antimicrobial potential (14,15). AMPs constitute a diverse category of naturally occurring molecules that serve as a primary defense mechanism in various living organisms (16). These proteins exhibit broad-spectrum activity, capable of directly targeting and eliminating bacteria, yeasts, fungi, and viruses (17,18). Characterized by their small size (< 10 kDa) and variable lengths and sequences, AMPs possess unique and attractive features (19). Notably, they demonstrate a low propensity to induce bacterial resistance, offering rapid and potent wide-spectrum antimicrobial activities against multidrug-resistant gram-negative and gram-positive bacteria (20,21). Additionally, AMPs have demonstrated synergistic effects when used in conjunction with conventional antibiotics (22). Regrettably, despite the initial enthusiasm surrounding AMPs as a potential alternative to antibiotics, numerous barriers hinder their widespread clinical application. This limitation can be attributed to their high systemic toxicity and increased cost of production (23,24). Additionally, the synergistic outcome that results from combining AMPs with antibiotics would be expected to generate significant challenges *in vivo,* even due to differing pharmacokinetics, posing obstacles to the development of effective combination therapies (25).

Recently, we successfully designed UP5, an ultrashort AMP (USAMP) composed of only five amino acids, featuring alternating sequences of arginine and biphenylalanine. UP5 exhibited potent antimicrobial properties against broad-spectrum representatives of both gram-positive and gram-negative bacteria. Notably, it demonstrated minimal toxicity towards mammalian cells and maintained excellent stability in serum plasma. Furthermore, UP5 displayed significant synergistic effects when combined with antibiotics, particularly demonstrating potent synergy when combined with levofloxacin, with fractional inhibitory concentration (FIC) indices reported as low as 0.1 (26,27). The design rationale behind UP5 aimed to develop an ultrashort peptide addressing the cost challenges associated with peptide synthesis and minimizing inherent toxicity to mammalian cells, common in AMPs.

This study aims to assess the feasibility of addressing the *in vivo* pharmacokinetic variations challenge associated with separately administering UP5 and levofloxacin by employing covalent conjugation and creating a hybrid molecule of both agents. This involves linking the carboxyl group of levofloxacin with the N-terminal side chain of UP5 through solid-phase peptide synthesis (SPPS). The conjugation strategy seeks to create a novel antimicrobial agent that preserves the synergistic mode of action between levofloxacin and UP5 but also maintains a unified pharmacokinetic profile. This approach is expected to facilitate ease of synthesis and administration for the combined therapeutic agent. Furthermore, the assessment of the conjugate will include levofloxacin-resistant clinical isolates of both gram-positive and gram-negative bacteria, listed in the World Health Organization's (WHO) as critical and high-priority pathogens. The goal is to determine whether the UP5 levofloxacin conjugate exhibits the capability to reverse quinolone resistance in these strains. Finally, the study will assess the *in vitro* toxicity of the conjugate in comparison with its individual components.

MATERIALS AND METHODS

Peptide synthesis

The synthesis of the UP5-levofloxacin conjugate followed standard fluorenylmethyloxycarbonyl (Fmoc) SPPS chemistry. Initially, a Rinker amide 4-methylbenzhydrylamine (MBHA) resin was

employed for peptide chain synthesis, involving consecutive coupling reactions, washings, and deprotections until the peptide achieved full elongation. Hydrazine hydrate in dimethylformamide (DMF) was used for deprotection, and the peptide MBHA resin was directly coupled with levofloxacin. The final global decoupling was conducted with trifluoroacetic acid/triisopropylsilane/water (TFA/TIS/H_2O). The resulting UP5 levofloxacin conjugate underwent purification through preparative high-performance liquid chromatography (HPLC). Mass confirmation and identification were performed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Bacterial strains

This study utilized several levofloxacin and MDR clinical isolates, encompassing both gram-positive and gram-negative bacteria. The selection of isolates was based on their specific resistance profile against levofloxacin, aiming to evaluate the peptide's potential to reverse bacterial resistance to levofloxacin. Additionally, the clinical isolates were chosen to mirror the WHO's list of critical and high-priority MDR pathogens, replicating the current antimicrobial resistance profile observed in clinical settings. All clinical isolates were obtained from the American Tissue Culture Collection (ATCC), and their characteristics, including their antimicrobial resistance profile, are detailed in Table 1.

Table 1. American tissue culture (ATCC) bacterial clinical isolates utilized in this study, including their antibiotic resistance profile and infection source.

Gram-positive MDR clinical isolates				
ATCC Number	**Name**	**Resistance profile**		**Source**
BAA 2316	*Enterococcus faecium*	Ampicillin	Benzyl penicillin	Human feces
		Ciprofloxacin	Levofloxacin	
		Erythromycin	Nitrofurantoin	
		Tetracycline	Vancomycin	
BAA 44	*Staphylococcus aureus*	Azithromycin	Clindamycin	Hospital
		Ciprofloxacin	Levofloxacin	
		Erythromycin	Gentamicin	
		Cefoxitin	Rifampin	
700565	*Staphylococcus epidermidis*	Amoxicillin-Clavulanic	Ampicillin	Human blood
		Cefazolin	Cefotaxime	
		Ciprofloxacin	Levofloxacin	
		Imipenem	Erythromycin	
		Piperacillin-tazobactam	Trimethoprim-sulfamethoxazole	
Gram-negative MDR clinical isolates				
BAA 1799	*Acinetobacter baumannii*	Cephalosporins	Ciprofloxacin	Human sputum
		Levofloxacin	Imipenem	
		Gentamicin	Ampicillin/Sulbactam	
		Ticarcillin	Trimethoprim-sulfamethoxazole	
2814	*Klebsiella pneumoniae*	Levofloxacin	Ciprofloxacin	Human with leukemia/lymphoma
		Gemifloxacin	Moxifloxacin	
		Amikacin	Tobramycin	
BAA 3197	*Pseudomonas aeruginosa*	Cephalosporins	Ciprofloxacin	Human with pneumonia
		Levofloxacin	Gentamicin	
		Imipenem	Piperacillin/Tazobactam	

MIC and MBC assays

The determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for UP5, levofloxacin, and UP5-levofloxacin conjugate followed the broth microdilution method outlined in the Clinical and Laboratory Standards Institute (CLSI) guidelines. Briefly, bacteria were grown in sterile 96-well polypropylene microtiter plates, and Muller-Hinton broth (MHB) served as the growth medium for the various bacterial strains that were recovered from frozen glycerol. After overnight growth in MHB, bacterial cells were diluted to 10^6 colony-forming units per milliliter (CFU/mL) in the same medium. Antimicrobial agents were prepared in various dilutions and in separate 96-well microtiter plates. Fifty microliters of each antimicrobial agent and 50 µL of diluted bacterial suspension were added to each well, with six replicates for each antimicrobial agent concentration distributed across six wells. The plates were incubated for 18 h at 37 °C, and bacterial growth was assessed by measuring OD at λ = 570 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. MIC, defined as the lowest concentration inhibiting bacterial growth, was determined. Each plate included a positive control column (50 µL bacterial suspension + 50 µL MHB) and a negative control column (100 µL MHB) to ensure bacterial growth and the sterility of MHB, respectively. MBC was determined by streaking 10 µL from clear negative wells and turbid positive control wells onto labeled nutrient media agar, followed by incubation for 24 h at 37 °C. The lowest concentration resulting in < 0.1% viable cells (achieving 99.9% killing) was identified as the MBC value. All experiments were conducted in triplicate.

Synergistic studies

Using the broth microdilution checkerboard technique (28), MICs of combinations involving UP5 and levofloxacin against levofloxacin-resistant and MDR clinical isolates were determined as described in the MIC/MBC section. Each well contained 25 µL of UP5 and 25 µL of levofloxacin at varying concentrations, along with a 50 µL inoculum (final volume 100 µL). MICs were determined in triplicate. The fractional inhibitory concentration (FIC) was calculated as follows:

$$FIC=\frac{MIC\ UP5\ (in\ combination)}{MIC\ UP5\ (alone)}+\frac{MIC\ levofloxacin\ \ (in\ combination)}{MIC\ levofloxacin\ \ \ (alone)} \quad \textbf{(1)}$$

In a combination well, one OD readout yields inhibition for a pair of concentrations; hence**,** we report two MICs in combination (one per agent) from the first no-growth well. When a solo MIC was "> 100 µM", the denominator 100 µM was used**,** and the FIC is reported as an upper bound (≤). FIC indices were interpreted as: ≤ 0.5 synergistic, > 0.5-1 additive, > 1-4 indifferent, and > 4 antagonistic.

Hemolytic assay

The evaluation of UP5 and UP5-levofloxacin toxicity on human erythrocytes was conducted through hemolytic assays to measure the extent of hemolysis induced by the novel conjugate, as previously outlined (30). Briefly, a 4% suspension of human erythrocytes (Zen-Bio Inc., Research Triangle Park, NC, USA) in 0.9% sodium chloride (NaCl) was exposed to various concentrations of UP5, levofloxacin, and UP5-levofloxacin conjugate at 37 °C for 1 h. Triton X-100 served as a positive control for inducing 100% hemolysis, while erythrocytes without the peptide served as negative controls. The percentage of hemolysis was determined using equation (2):

$$\text{Hemolysis} = \frac{(A-A0)}{AX-A0)} \times 100 \quad \textbf{(2)}$$

where A represents OD 570 nm with the peptide solution, A0 represents OD 570 nm in NaCl, and AX represents OD 570 nm with 0.1% Triton X-100.

Mammalian cell cytotoxicity

For the mammalian cytotoxicity assay, assessing the impact of UP5 and UP5-levofloxacin conjugate on mammalian cell proliferation was carried out using Madin-Darby canine kidney (MDCK) cells, obtained from the ATCC and used as an *in vitro* model for evaluating the peptides' cytotoxicity against mammalian cells. MDCK cells were seeded at a density of 5×10^3 cells per well in a 96-well plate. The cells were exposed to varying

concentrations of UP5 and UP5-levofloxacin, with the medium alone as a negative control. Triton X-100 (0.1%) was included as a positive control to induce complete cytotoxicity and validate the assay. Following a 24-h incubation period, 20 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well, and the plates were incubated for an additional 3 h. Subsequently, the growth medium was removed, and 200 µL of dimethyl sulfoxide (DMSO) was added to each well, vigorously mixed to dissolve the developed formazan crystals. The absorbance was measured using an absorbance microplate reader at 550 nm. Based on the half maximal inhibitory concentration (IC_{50}) data acquired for both UP5 and the UP5-levofloxacin conjugate, the selectivity index (SI) was calculated to measure their relative specificity in targeting microbial cells over host cells according to equation (3):

$$SI = \frac{\text{IC50 against host cells}}{\text{MIC against bacteria}} \quad \textbf{(3)}$$

Statistical analysis

The method of statistical analysis was performed using GraphPad Prism software (La Jolla, CA, USA). Data are expressed as mean ± SD unless otherwise specified.

RESULTS

UP5-levofloxacin design and synthesis

The UP5-levofloxacin conjugate was designed to be synthesized continuously through Fmoc SPPS methodology. This was based on the fact that levofloxacin contains only one active carboxylic moiety and, accordingly, could be treated as an amino acid during peptide coupling and synthesis. The complete structure and synthetic steps employed in the synthesis and the UP5-levofloxacin conjugate are displayed in Fig. 1. To confirm the success of the conjugate synthesis, purification, and characterization of the conjugate were performed using reverse phase-HPLC and mass spectrometry, which displayed that UP5-levofloxacin's purity exceeded 98%. The conjugate identity was confirmed by electrospray ionization mass spectrometry (ESI-MS) with the synthetic peptide displaying major peaks in the +2 and +3 charge states of 638.5 and 426.1 Da, which correspond to the molecular weight of the conjugate, which is 1275.1 Da (Fig. 2A and B).

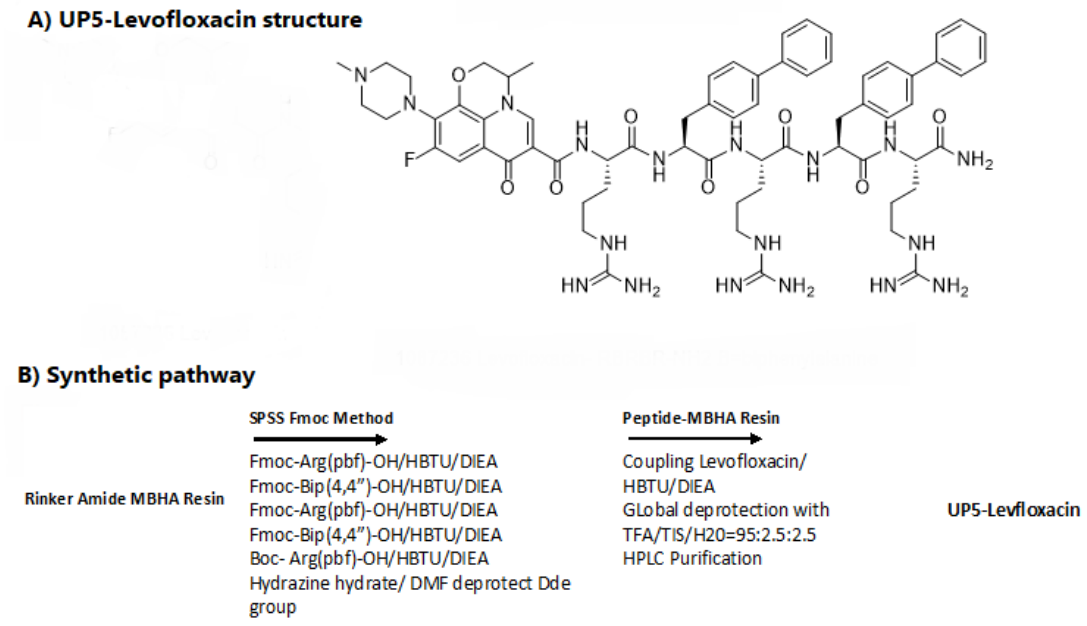

Fig. 1. (A) Chemical structure of UP5-levofloxacin. (B) Synthetic pathway for UP5-levofloxacin using Fmoc solid-phase peptide synthesis.

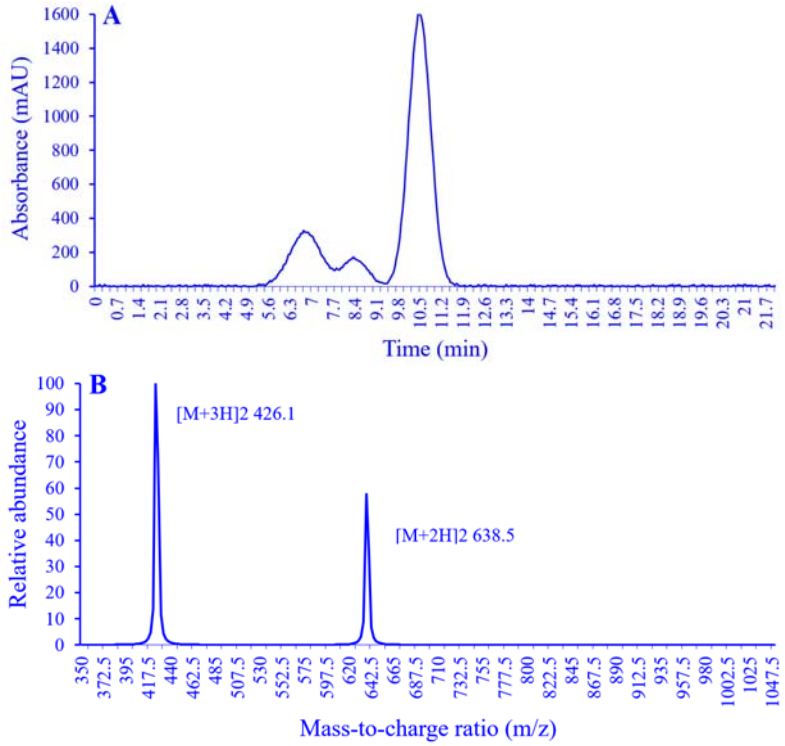

Fig. 2. (A) The HPLC profile of UP5-levofloxacin from 80/20 A/B to 55/45 A/B in 30 min; A: 0.1% TFA in water; B: 0.1% TFA in acetonitrile, indicating 99% purity of the synthesized peptide. The absorbance was recorded at λ of 214 nm. (B) Mass spectrometry of UP5-levofloxacin displays major peaks in the +2 and +3 charge states of 638.5 and 426.1 Da, which correspond to the molecular weight of the conjugate, which is 1275.1 Da. HPLC, High-performance liquid chromatography; TFA, trifluoroacetic acid.

Table 2. MIC and MBC values of the UP5-levofloxacin conjugate against the Gram-positive and Gram-negative clinical isolates employed in the study.

Gram-negative clinical isolates	MIC (µM)	MBC (µM)	Gram-positive clinical isolates	MIC (µM)	MBC (µM)
Acinetobacter baumannii ATCC 1799	2.5	2.5	*Enterococcus faecium* ATCC 2316	5	5
Klebsiella pneumoniae ATCC 2814	20	20	*Staphylococcus aureus* ATCC BAA-44	20	20
Pseudomonas aeruginosa ATCC 3197	20	20	*Staphylococcus epidermidis* ATCC 700565	10	10

MIC, Minimum inhibitory concentration; MBC, minimum bactericidal concentration.

Antimicrobial activity of UP5-levofloxacin conjugate

The antimicrobial efficacy of UP5-levofloxacin was assessed against a spectrum of levofloxacin and multidrug-resistant high-priority clinical isolates, representing both gram-positive and gram-negative bacteria. As displayed in Table 2, UP5-levofloxacin demonstrated potent antimicrobial activity against all clinical isolates used in this study. The highest activity against gram-positive bacteria was observed against *Enterococcus faecium* (*E. faecium,* ATCC 2316), exhibiting an MIC of 5 µM. The MIC values for *Staphylococcus aureus* (*S. aureus,* ATCC BAA-44) and *S. epidermidis* (ATCC 700565) were 10 µM and 20 µM, respectively. Against gram-negative bacteria, UP5-levofloxacin exhibited superior antimicrobial activity, particularly against *Acinetobacter baumannii* (*A. baumannii,* ATCC 1799), showing an MIC value of 2.5 µM. In the case of the other gram-negative isolates, namely *Klebsiella pneumoniae* (*K. pneumoniae,* ATCC 2814) and *Pseudomonas aeruginosa* (*P. aeruginosa,* ATCC 3197), MIC values were reported at 20 µM. The MBC values mirrored the MIC values across all six bacterial types, indicating that the peptide is exerting a bactericidal effect.

The UP5-levofloxacin conjugate successfully reversed levofloxacin resistance in all clinical isolates included in this study.

Synergistic and antimicrobial comparative studies

To assess and compare the antimicrobial activity of the UP5-levofloxacin conjugate and determine its capability in exhibiting superior efficacy compared to its individual components, as well as to verify its comparative synergistic activity consistent with its intended design rationale for conjugation, synergistic studies were conducted using the checkerboard technique. Specifically, UP5, levofloxacin, and their combinations were tested against all clinical isolates. For each isolate, the MIC values within the combination were recorded, and the FIC index was calculated. The evaluation of antibacterial potency resulting from synergism was assessed by interpreting the FIC indices, categorizing them as synergistic (FIC $\leq$ 0.5), additive (0.5 < FIC $\leq$ 1), indifferent (1 < FIC $\leq$ 4), or antagonist (FIC > 4) effect. These analyses were crucial for understanding the conjugate's performance relative to its individual components and confirming its intended synergistic activity as detailed in Table 3. The UP5-levofloxacin conjugate managed to surpass the antimicrobial activity of the individual ultrashort antimicrobial peptide UP5 and levofloxacin, with MIC values ranging from 2.5 to 20 µM. The UP5-levofloxacin conjugate exhibited the most significant enhancement in antimicrobial effectiveness against gram-negative bacteria, particularly A. baumannii, demonstrating a remarkable (4-40)-fold increase in activity compared to its individual components. For K. pneumonia and P. aeruginosa, the reported increase in activity was 3.75 to 5-fold and 1.25 to 2.5-fold, respectively. For gram-positive bacteria, the most significant improvement in activity was reported against E. faecium with a 10-fold increase in activity.

Table 3. MICs of UP5, levofloxacin individually, and in combination, including their FIC indices in comparison with the UP5-levofloxacin conjugate and the synergism threshold against both gram-negative and gram-positive clinical isolates.

Gram-negative Clinical isolates	Individual MIC (µM)		MIC in combination (µM)		FIC Index	Synergism threshold (µM)	Individual MIC
	Levofloxacin	UP5	Levofloxacin	UP5			UP5-levofloxacin
Acinetobacter baumannii ATCC 1799	> 100	10	12.5	5	≤ 0.625	5	2.5
Klebsiella pneumoniae ATCC 2814	> 100	75	25	9.375	≤ 0.375	37.5	20
Pseudomonas aeruginosa ATCC 3197	25	50	12.5	12.5	0.75	12.5	20
Gram-positive clinical isolates							
Enterococcus faecium ATCC 2316	50	50	12.5	3.125	0.3125	25	5
Staphylococcus aureus ATCC BAA-44	50	65	12.5	8.125	0.375	25	20
Staphylococcus epidermidis ATCC 700565	25	25	6.25	3.125	0.375	12.5	10

MIC, Minimum inhibitory concentration; FIC, Fractional inhibitory concentration.

Meanwhile, the conjugate's increased activity against both S. aureus and S. epidermidis was reported to be in the range of 2.5-3.25-fold and 2.5-fold, respectively. Furthermore, the UP5-levofloxacin conjugate exhibited antimicrobial concentrations below the synergistic threshold against all clinical isolates as calculated by the FIC index formula. It presented a comparable synergistic profile to the individual components of the conjugate, maintaining synergy and, in some instances, surpassing it, such as in its activity against A. baumannii (ATCC 1799). The only clinical isolate that exhibited an additive effect rather than a synergistic one when exposed to the conjugate was P. aeruginosa (ATCC 3197), a trend similar to the effect of its individual components UP5 and levofloxacin. Similar to previous synergistic studies, the UP5-levofloxacin conjugate demonstrated a comparable profile to its components., These results substantiate the success of the conjugation strategy in replicating the antimicrobial and synergistic profile of the combined individual constituents of UP5 and levofloxacin and even surpassing them.

Hemolytic assay

The hemolytic impact of both UP5 and UP5-levofloxacin on human erythrocytes was assessed, and the findings are summarized in Table 4. The hemolytic effects of UP5 and UP5-levofloxacin were investigated across concentrations ranging from 3.125 to 100 µM. Both peptides demonstrated comparable and minimal hemolytic activity, with the highest reported percentage of hemolysis being 3% for UP5 and 2.7% for UP5-levofloxacin at the highest concentration of 100 µM. Moreover, at the effective antimicrobial concentrations for the conjugate, which ranged between 2.5 and 20 µM, the reported percentage of hemolysis did not exceed 1%.

Mammalian cell cytotoxicity

The cytotoxicity of UP5 and UP5-levofloxacin was evaluated against mammalian cells, specifically MDCK cells, chosen as a representative of normal mammalian cell lines. UP5-levofloxacin displayed inhibitory effects on MDCK cell proliferation, with an average IC_{50} value of 88.34 µM (Fig. 3).

Table 4. Hemolytic effect of UP5 and UP5-levofloxacin conjugate on human red blood cells across a concentration range.

Concentration (µM)	Hemolysis% (mean ± SD)	
	UP5	UP5-levofloxacin
3.125	0	0.00
6.25	0.0	0.00
12.5	0.05 ± 0.003	0.00
25	0.09 ± 0.003	0.97 ± 0.008
50	1.04 ± 0.005	1.27 ± 0.014
100	3.04 ± 0.007	2.7 ± 0.004

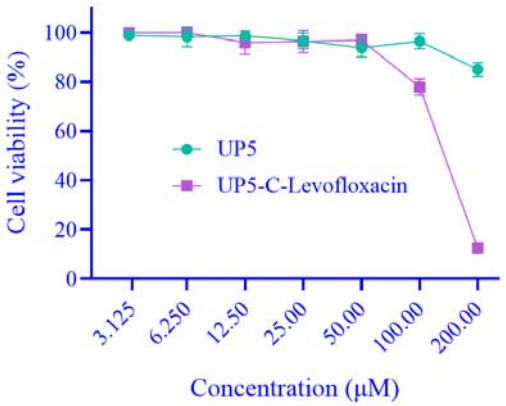

Fig. 3. Survival curves of normal MDCK cells were measured through MTT assay for UP5 and UP5-levofloxacin. Cells were exposed to various concentrations of the UP5 and the conjugate for 24 h at 37 °C. Control cells, indicating 100% proliferation, were used as a reference, and the growth sensitivity of treated cells was estimated by comparing mean absorption to control values. The data are expressed as mean ± SD, n = 3

Notably, this IC_{50} value was significantly higher than the average (MIC values obtained for the conjugate in antimicrobial susceptibility studies conducted against a broad spectrum of clinical isolates, including both gram-positive and gram-negative bacteria. The IC_{50} value for UP5 alone was not determined within the concentration range employed in the study (3,125-200), as the highest concentration tested (200 µM) resulted in only a 15% decrease in cell proliferation. Importantly, the conjugation of UP5 with levofloxacin led to an escalation in cytotoxicity, starting at concentrations of 88 µM and above. This heightened cytotoxic effect could be attributed to the enhanced membrane disruption efficacy resulting from the synergistic activity of both UP5 and levofloxacin, as observed in previous reports involving levofloxacin-conjugated compounds (31).

Table 5. The SI value of the UP5-levofloxacin conjugate was calculated to measure its relative specificity in targeting microbial cells over host cells.

Gram-negative clinical isolates	**MIC**	**SI Value**
Acinetobacter baumannii	2.5	35.2
Klebsiella pneumoniae	20	4.4
Pseudomonas aeruginosa	20	4.4
Gram-positive clinical isolates		
Enterococcus faecium	5	17.6
Staphylococcus aureus	20	4.4
Staphylococcus epidermidis	10	8.8

MIC, Minimum inhibitory concentration; SI, selectivity index.

The SI index measures the relative specificity of the antimicrobial agents for targeting microbial cells over host cells and helps assess the therapeutic potential of antimicrobial peptides by indicating their ability to kill or inhibit the growth of pathogens while minimizing harm to host cells. The SI index (Table 5) revealed that the UP5-levofloxacin conjugate had a favorable toxicity profile as the indices were all above 1, ranging from 4.4 to 35.2, indicating that the conjugate can effectively combat pathogens while minimizing harm to host cells.

DISCUSSION

Antimicrobial resistance remains a significant challenge facing humanity. The predominant factors contributing to this challenge are the proliferation of MDR bacteria and the absence of novel antimicrobial agents. The escalating misuse of antibiotics in clinical and agricultural domains further compounds the issue. Projections indicate a concerning trend, with the total annual global deaths estimated to soar to 10 million by 2050, potentially surpassing other leading causes of mortality, including cancer (32). This highlights the urgent need for comprehensive strategies to address and mitigate the impact of antimicrobial resistance on public health.

We previously designed an ultrashort antimicrobial peptide called UP5. This peptide is composed of five alternating amino acids of arginine and biphenylalanine (26). UP5 demonstrated significant antimicrobial activity against both gram-positive and gram-negative bacteria, while exhibiting minimal toxicity towards mammalian cells. A key finding from our previous research was UP5's ability to synergize effectively with levofloxacin. However, the development of antimicrobial peptide antibiotic combinations and advancing them towards the clinic faces the challenge of differences in pharmacokinetics inherent with the administration of the two agents, posing obstacles to the establishment of successful combination therapies. The current study successfully addressed this challenge by conjugating the antibiotic levofloxacin with UP5 through a cleavable amide linkage. The resultant conjugate antimicrobial activity was evaluated against levofloxacin and MDR clinical isolates, mimicking the current antimicrobial resistance profile observed in clinical settings. The synthesis of the conjugate was, in principle, based on the fact that levofloxacin possesses only one carboxylic reactive moiety, allowing its incorporation into the SPPS reaction as an individual amino acid. This amino acid could be linked through the N-terminal end of UP5. The synthetic approach proved successful, yielding a UP5-levofloxacin conjugate with a purity of up to 98%, accompanied by negligible synthesis-related impurities. To evaluate the effectiveness of the UP5-levofloxacin conjugate in achieving its design purpose, we determined the MIC values against a range of MDR bacteria recognized as critical and high-priority pathogens by the WHO. The conjugate demonstrated significant inhibitory and bactericidal activity against all clinical isolates of both gram-positive and gram-negative bacteria. Notably, it reversed levofloxacin resistance in strains such as *A. baumannii* and *E. faecium*, with MIC values as low as 2.5 μM and 5 μM, respectively. Additionally, the MIC value was 10 μM against *S. epidermidis*. Lower activity was observed against *S. aureus*, *K. pneumoniae*, and *P. aeruginosa*, with an MIC value of 20 μM. Furthermore, we conducted a comparative analysis of the antimicrobial efficacy of the UP5-levofloxacin conjugate against its individual components. This study aimed to investigate whether the peptide exhibited superior performance in comparison with its components and to confirm the consistency of its synergistic activity, which was the basis and intended rationale for the conjugation design. We employed the checkerboard technique for

synergistic studies, which has revealed that UP5-levofloxacin demonstrated significantly higher antimicrobial activity against all bacteria tested when compared to its individual components, UP5 and levofloxacin. The most notable enhancement in activity was observed against *A. baumannii*, where the conjugate exhibited a remarkable 4-40-fold increase in effectiveness compared to its components. In gram-positive bacteria, particularly *E. faecium*, a significant 10-fold improvement in activity was reported. In general, the UP5-levfloxacin conjugate performed better than its individual components against all clinical isolates, as detailed in the results section. Additionally, the UP5-levofloxacin conjugate maintained antimicrobial concentrations below the synergistic threshold, as calculated by the FIC index equation, against all clinical isolates that were affected by the synergistic combination of UP5 and levofloxacin. The conjugate displayed a synergistic profile comparable to its components, consistently maintaining or surpassing synergy. The only exception was *P. aeruginosa*, which exhibited an additive effect rather than a synergistic one. The mechanism underlying this enhanced activity is attributed to the conjugate's ability to undergo cleavage by various proteases within the bacterial membrane milieu. This cleavage releases both components, allowing them to exert their targeted antibacterial activities with superior outcomes compared to separate administration. Similar conjugate designs reported in the literature, such as the levofloxacin-indolicidin combination, the cyclic peptide [R4W4]-levofloxacin combination, and the [R4W4K]-levofloxacin conjugates, have demonstrated enhanced antimicrobial activity through peptide-antibiotic conjugation strategies, reinforcing the potential of our conjugate design (33-35).

This mechanism aligns with findings from other studies, where conjugated antimicrobial peptides have demonstrated enhanced efficacy against resistant strains of gram-positive and gram-negative bacteria (36). The molecular mechanism of the synergistic cleavage and mode of action of the conjugate needs to be elucidated in further mechanistic studies. In terms of implementation, the development of peptide-antibiotic conjugates offers a promising strategy to combat MDR bacteria. By combining the broad-spectrum activity of antibiotics with the unique mechanisms of action of antimicrobial peptides, these conjugates can overcome existing resistance mechanisms. For instance, conjugates have been shown to potentiate the activity of antibiotics like levofloxacin against MDR clinical isolates of *P. aeruginosa*, *Escherichia coli*, and *K. pneumoniae* (37). The hemolytic effects of UP5 and UP5-levofloxacin activity on human erythrocytes were evaluated across concentrations ranging from 3.125 to 100 µM, revealing comparable minimal hemolytic activity. At the highest concentration employed in the hemolytic study of 100 µM, UP5 and UP5-levofloxacin induced only 3% and 2.7% hemolysis, respectively. Additionally, at the effective antimicrobial concentrations for the conjugate (2.5-20 µM), the reported hemolysis did not exceed 1%. Mammalian cytotoxicity assessments have revealed an increase in the cytotoxic capacity of the conjugate compared to UP5 at concentrations of 100 µM and above. This elevation in cytotoxicity correlates with the higher efficacy of the conjugate against clinical isolates, while the selectivity index values remain favorable, compensating for the cytotoxic increase. Further *in vivo* studies, such as repeat dose toxicity evaluations and the calculation of *in vivo* animal lethal doses and infection models, could provide additional insights into the peptide's cytotoxicity.

In summary, the results of the current study revealed the success of the conjugation strategy in augmenting the antimicrobial efficacy of both levofloxacin and UP5 and demonstrating the potential benefit for designing a hybrid conjugated antibiotic-peptide molecule with a unified pharmacokinetic profile. These results provide evidence that the conjugate can be advanced for further advanced antimicrobial preclinical studies, including comprehensive *in vivo* animal efficacy and toxicity studies. Our research presented a novel approach to the escalating problem of antimicrobial resistance by introducing an ultrashort antimicrobial peptide, UP5, and successfully conjugating it with the antibiotic levofloxacin. The resultant UP5-levofloxacin conjugate exhibited remarkable antimicrobial activity against both gram-positive and gram-negative bacteria, including MDR strains. This conjugation strategy overcame the challenges associated with combining antimicrobial peptides and conventional antibiotics, providing a significant advancement in the development of effective

combination therapies. The study not only demonstrated the successful synthesis of the UP5-levofloxacin conjugate but also highlighted its superior efficacy compared to individual components. The conjugate effectively reversed antibiotic resistance, maintained antimicrobial concentrations below the synergistic threshold, and exhibited a synergistic profile comparable to its individual components.

CONCLUSION

Our results highlight the potential of UP5-levofloxacin conjugate to pave the way for further antimicrobial development. This research represents a significant step forward in the quest for effective antimicrobial therapies, offering a promising solution to combat multidrug-resistant bacterial infections. UP5-levfloxacin could prove to be of high value and a significant candidate for further antimicrobial development against MDR bacteria.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

A. Almaaytah and A. Alrashdan conceptualized the study; A. Almaaytah supervised the study; A. Alrashdan performed the experimental parts of the study; A. Almaaytah and A. Alrashdan analyzed the data; A. Almaaytah, A. Alrashdan and S. Sabi wrote the manuscript. All authors have read and approved the finalized article. Each author has fulfilled the authorship criteria and affirmed that this article represents honest and original work.

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