Tropical Journal of Pharmaceutical Research August 2020; 19 (8): 1731-1736
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i8.23

Original Research Article

A preliminary study of *in vitro* and *in vivo* synergistic effects of ciprofloxacin and D-tyrosine against *Pseudomonas aeruginosa* isolates

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Sent for review: 29 March 2020 *Revised accepted: 26 July 2020*

Abstract

Purpose: *To investigate the synergistic antimicrobial effects of ciprofloxacin and D-tyrosine against drug-resistant bacteria.*
Method: *The antimicrobial effects of ciprofloxacin and D-tyrosine on clinical isolates of multidrug-resistant (MDR) Pseudomonas aeruginosa (P. aeruginosa) no. 3556 were determined in vitro based on time-kill curve, and in vivo in P. aeruginosa-zebrafish infection model. Furthermore, 30 clinical isolates of multidrug-resistant P. aeruginosa were used in vitro to ascertain the synergistic effect of the two agents.*
Results: *Combined use of ciprofloxacin and D-tyrosine produced synergistic effects against the clinical isolate of P. aeruginosa no. 3556 in vitro and in vivo. Synergism occurred in 96.67 % (95 % CI, range 83.33 - 99.41 %) of the clinical isolates, and ciprofloxacin dose was reduced in 90 % (95 % CI, range 74.38 - 96.54 %) of the clinical isolates in vitro.*
Conclusion: *These preliminary results suggest that the combination of ciprofloxacin and D-tyrosine is a promising therapeutic strategy against MDR P. aeruginosa infections.*

Keywords: *Ciprofloxacin, D-tyrosine, Synergistic, P. aeruginosa, Zebrafish infection model, Time-killing curve*

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INTRODUCTION

Pseudomonas aeruginosa has become an increasingly notorious opportunistic pathogen in common bacterial infections, including community-acquired, healthcare-associated, and nosocomial infections [1]. The emergence of multidrug-resistant *P. aeruginosa*, including those resistant to broad-spectrum cephalosporins and carbapenems, has increased dramatically over the last two decades, and it is now a global health problem [1]. It is estimated that *P. aeruginosa* accounts for 5 to 10 % of infections in developing countries, 25 % of which are caused by multidrug-resistant *P. aeruginosa* [2]. Studies have shown that the mortality due to in-hospital infection caused by multidrug-resistant *P. aeruginosa* may be up to 31.1 % [2].

A major problem associated with antibiotic resistance is that pre-existing antibiotics are rapidly becoming ineffective against new and emerging antibiotic-resistant bacteria [3]. For these reasons, there is need to evolve novel and improved treatments against drug-resistant *P. aeruginosa* infections.

One promising solution is based on the use of combination of two or more conventional antimicrobial agents. This produces increased antimicrobial effects when compared with a single agent. Synergistic effect could offer a potential benefit for treatment of bacterial infections through drug combinations [4]. Many researchers have demonstrated that drug combinations produce synergistic effects. Examples are the use of vancomycin and β-lactams against *Staphylococcus aureus* highly resistant to vancomycin, and the use of ciprofloxacin-tobramycin combination against *P. aeruginosa* [5,6]. Moreover, the combination of a chemical compound and a conventional antimicrobial agent results in increased antibacterial effects, for example, silver nanoparticles in combination with antibiotics against gram-positive and gram-negative bacterial pathogens [7,8]. D-Amino acids have been found to affect biofilm formation. Studies have demonstrated that combination of D-amino acids and antimicrobial agents produces synergistic antimicrobial effects against pathogens [9-12]. However, studies on the effect of D-tyrosine as an antimicrobial enhancer against bacteria have resulted in controversial outcomes [12-14].

This study was aimed at determination of the synergistic antimicrobial effects of ciprofloxacin - D-tyrosine combination on clinically isolated *P. aeruginosa* No. 3556 *in vitro* based on time-kill curve, and *in vivo* in *P. aeruginosa*-zebrafish infection model.

EXPERIMENTAL

Bacterial strains, culture and preparation

Thirty-one clinical isolates of *P. aeruginosa* strains used in this study were kept in our laboratory. Multidrug-resistant *P. aeruginosa* strain No. 3556 selected from the above strains was used as reference strain *in vitro* based on time-kill curve, and *in vivo* in *P. aeruginosa*-zebrafish infection model. The strain was inoculated in Luria-Bertani (LB) agar plates, followed by overnight incubation at 37 °C. Then, a single colony on the plate was inoculated into 5-mL fresh LB broth and incubated, with shaking at 180 rpm, for 12 h at 37 °C. Bacterial suspension (500 µL) was inoculated into 5 mL fresh LB broth, followed by incubation at 37 °C, with shaking at 180 rpm, for 6 h. The bacterial cultures were subjected to centrifugation at 6,000 × g for 5 min. The harvested cell pellets were washed twice with phosphate buffered saline (PBS, pH 7.2) and serially diluted for use in this study.

Antibiotic and D-tyrosine preparation

Ciprofloxacin was purchased from Sigma–Aldrich (St. Louis, MO, United States). It was dissolved in sterile water to a concentration of 256 µg/mL. The drug susceptibility of *P. aeruginosa* strain No. 3556 was chosen according to the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute (CLSI), 2019) [15]. D-Tyrosine was purchased from Sigma–Aldrich (St. Louis, MO, United States), and was dissolved in PBS, followed by filtration through 0.22 µm membrane filters before use.

Optimization of D-tyrosine concentration

Pseudomonas aeruginosa No. 3556 was prepared in the same manner as described above. The concentration of D-tyrosine was optimized by co-culturing of 100 µL of bacterial suspension and 100 µL of serially diluted D-tyrosine (0 to 16 µM) in a 96-well plate. Eight hours later, the OD_{600} was read in a microplate reader. The experiment was done in 3 replicates. Bacteria culture without D-tyrosine was set as control.

In vitro time-kill curves for single and combination drugs

The effectiveness of ciprofloxacin and D-tyrosine, alone and in combination, against clinical isolate of multidrug-resistant *P. aeruginosa* No. 3556 was determined using the time-kill curve method. Time-kill assays were carried out according to the CLSI guidelines. In these assays, 5×10^5 CFU/mL inoculum was incubated in 96-well plate containing adjusted Mueller Hinton broth supplemented with ciprofloxacin and D-tyrosine, alone or in combination, in separate wells. In the entire experiment, the concentration of ciprofloxacin varied from 16 to 0.5 µg/mL, while the concentration of D-tyrosine was kept at 8 µM, based on the optimization result. Aliquots were removed from each tube and diluted serially (1:10) with PBS for use in determination of cell viability after 8 h of culture in Luria-Bertani (LB) agar plates. Time–kill experiment was performed in triplicate. According to a previous description [16], a combination of two antimicrobial agents was considered synergistic if it caused a ≥ 2 log

unit reduction in CFU/mL, relative to the sum of the reductions observed with the individual compounds at the end of the experiment.

Evaluation of the effectiveness of ciprofloxacin and D-tyrosine, in combination and singly, against clinical isolate of *P. aeruginosa*

The degrees of effectiveness of ciprofloxacin and D-tyrosine, alone and in combination, against 30 clinical isolates of *P. aeruginosa in vitro* were determined based on the procedure of time-kill curve for multiple-drug-resistant *P. aeruginosa* No. 3556. The concentration of ciprofloxacin varied from 32 µg/mL to 0.06 µg/mL.

In vivo assessment of the effectiveness of ciprofloxacin and D-tyrosine in combination and singly, on *P. aeruginosa*-zebrafish infection model

Each zebrafish was infected with 10 µL of a 5 × 10^5 CFU/ml bacterial suspension in the treatment group (n= 10 per group). The treatment groups were exposed to ciprofloxacin (8 µg/mL) and D-tyrosine (8 µM), singly and in combination. Zebrafish without bacteria served as the control group. The percentage survival in each experimental group was monitored from 0 h to 144 h post-infection.

Statistical analysis

Results from *in vitro* and *in vivo* experiments are presented as mean ± standard deviation (SD). GraphPad InStat version 6.01 (GraphPad Software, CA, USA) was used for statistical analysis of the differences amongst group means, based on one-way analysis of variance (ANOVA). Differences were assumed statistically significant at $p < 0.05$.

RESULTS

Optimized D-tyrosine concentration

The results showed that D-tyrosine interfered with the formation of biofilm, as shown in Figure 1 A. The formation of *P. aeruginosa* No. 3556 biofilm decreased with increase in the concentration of D-tyrosine from 0 to 8 µM, and subsequent microplate reader results showed that the bacterial concentrations were significantly decreased ($p = 0.01 < 0.05$). When D-tyrosine concentration was increased to 16 µM, there was no change in the formation of *P. aeruginosa* biofilm. Subsequent microplate reader results showed that there was no significant change in the bacterial concentration ($p = 0.23 > 0.05$). Thus, 8 µM D-tyrosine was selected as the optimum concentration used for investigating the effects of ciprofloxacin and D-tyrosine on clinical isolates of *P. aeruginosa* No. 3556 *in vitro* and *in vivo*.

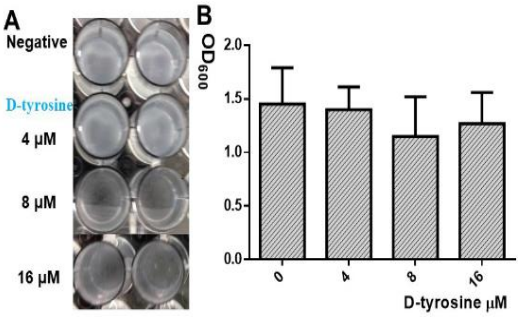

Figure 1: Optimization of D-tyrosine concentration. Effect of D-tyrosine on the formation of *P. aeruginosa* biofilm in 96-well plates (A), and comparison of bacteria growth in the presence or absence of D-tyrosine at various concentrations for 8 h (B).

Single and combined effects of ciprofloxacin and D-tyrosine *in vitro* based on time-kill curve test

The antimicrobial effects of ciprofloxacin and D-tyrosine, singly and in combination, were determined based on the growth properties of *P. aeruginosa* No. 3556. As shown in Figure 2, the bacteria growth when treated with ciprofloxacin or D-tyrosine alone was set as control group, and the results indicated that *P. aeruginosa* No. 3556 was resistant to both agents. In contrast, the combination of ciprofloxacin and D-tyrosine reduced the bacteria from 10 log CFU/mL to 6 log CFU/mL when the concentration of ciprofloxacin used ranged from 8 to 16 µM. The reduction margin in bacteria (4 log CFU/mL) indicated that the ciprofloxacin - D-tyrosine combination resulted in synergistic/additive effect *in vitro*. Obviously, the dose of ciprofloxacin could be decreased during combination of ciprofloxacin and D-tyrosine, indicating that the combination was better at killing drug-resistant bacteria than any of the agents when used alone.

Effectiveness of ciprofloxacin and D-tyrosine, alone and in combination, on clinical isolates of *P. aeruginosa in vitro*

As shown in Table 1, the degree of synergistic effect for combined use of ciprofloxacin and D-tyrosine was 96.67 % (95 % CI: 83.33 - 99.41). For ciprofloxacin-resistant strains, the degree of synergistic effect was 95.24 % (95 % CI: 77.33 - 99.15), while for ciprofloxacin-susceptible strain, the degree of synergistic effect was 100 % (95 % CI: 70.09 - 100). Moreover, the concentrations of

ciprofloxacin for 90 % (95 % CI: 74.38 - 96.54) of the strains were reduced, when compared with ciprofloxacin and D-tyrosine, in combination and singly, against clinical isolates of *P. aeruginosa in vitro*.

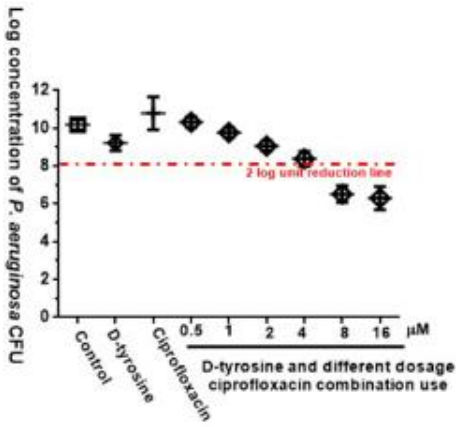

Figure 2: *In vitro* effect of ciprofloxacin and D-tyrosine on *P. aeruginosa*, singly and in combination, based on time-kill curve test

Effect of ciprofloxacin and D-tyrosine on *P. aeruginosa*-zebra fish infection model

As shown in Figure 3, the survival of untreated zebrafish was 100 % (from 0 to 144 h). This served as control. In the group injected with *P. aeruginosa* No. 3556, the survival of zebrafish was 10 % in 24 h, with no survival in 144 h. The percentage survival of zebrafish was 40 % in 24 h, 20 % in 48 h, and 20 % in 144 h for the group injected with *P. aeruginosa* No. 3556 and ciprofloxacin. In the group injected with *P. aeruginosa* No. 3556 and D-tyrosine, the survival of zebrafish was 20 % in 24 h, 10 % in 48 h, and 10 % in 144 h. In contrast, the survival of zebrafish was 70 % in 24 h, 50 % in 48 h and 40 % in 144 h in the group injected with *P. aeruginosa* No. 3556, ciprofloxacin and D-tyrosine. Obviously, ciprofloxacin and D-tyrosine improved the survival of zebrafish and produced good effect *in vivo*.

Table 1: Antimicrobial effectiveness of ciprofloxacin and D-tyrosine, alone and in combination, against clinical isolates of *P. aeruginosa*

Strain	MIC/CIP	R/S	Effective CIP concentration in combination use	Log unit reduction in CFU/mL	Synergism Yes or No
No.2241	8	R	4	4.50±0.14	Yes
No.2002	0.25	S	0.06	5.70±0.00	Yes
No.3130	16	R	4	3.92±0.41	Yes
No.3361	2	R	0.5	4.11±0.33	Yes
No.2781	2	R	2	2.94±0.27	Yes
No.2302	4	R	2	3.06±0.06	Yes
No.2987	32	R	8	4.95±0.23	Yes
No.3389	16	R	8	3.01±0.19	Yes
No.2904	0.5	S	0.12	2.75±0.09	Yes
No.3103	0.5	S	0.25	3.88±0.69	Yes
No.3867	16	R	4	3.42±0.52	Yes
No.3402	4	R	2	2.79±0.37	Yes
No.2725	2	R	0.25	5.00±0.45	Yes
No.2241	4	R	1	4.16±0.03	Yes
No.2321	8	R	0.5	3.00±0.14	Yes
No.3308	1	S	0.5	5.70±0.00	Yes
No.3352	0.25	S	0.25	2.41±0.10	Yes
No.3404	0.5	S	0.25	2.50±0.11	Yes
No.2988	2	R	0.5	4.20±0.53	Yes
No.3065	**32**	**R**	**32**	**1.07±0.27**	**No**
No.3321	8	R	4	5.13±0.14	Yes
No.2644	4	R	0.5	2.98±0.22	Yes
No.2241	8	R	1	4.44±0.48	Yes
No.2241	0.5	S	0.12	3.88±0.13	Yes
No.3901	4	R	1	4.61±0.21	Yes
No.3875	4	R	2	3.09±0.60	Yes
No.2802	16	R	4	4.65±0.07	Yes
No.3658	0.25	S	0.06	4.00±0.32	Yes
No.3716	0.5	S	0.25	3.78±0.41	Yes
No.2066	2	R	0.5	4.33±0.02	Yes

(MIC = minimum inhibitory concentration, CIP = ciprofloxacin, S = susceptible to ciprofloxacin, R= resistant to ciprofloxacin)

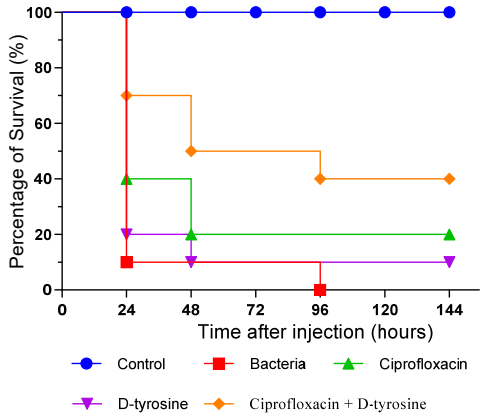

Figure 3: Effects of ciprofloxacin and D-tyrosine, singly and in combination, on zebrafish model of *P. aeruginosa* infection. Control = zebrafish alone, bacteria = zebrafish infected by bacteria, ciprofloxacin = zebrafish infected with bacteria and treated with ciprofloxacin alone, D-tyrosine = zebrafish infected with bacteria and treated with D-tyrosine alone, and ciprofloxacin + D-tyrosine = zebrafish infected with bacteria and treated with combination of ciprofloxacin and D-tyrosine

DISCUSSION

Bacterial resistance has always been a serious problem. Combined treatment with multiple drugs or chemical compounds may be a solution to the problem of drug resistance. In this preliminarily study, the effects of ciprofloxacin and D-tyrosine, singly and in combination, on multidrug-resistant *P. aeruginosa* were investigated *in vitro* and *in vivo*. D-Tyrosine is a bioactive substance. However, the report that D-amino acids may inhibit biofilms has remained controversial [13,14,17]. The results of the present study showed that D-tyrosine did not completely prevent the formation of biofilm, but inhibited the growth of bacteria by influencing the formation of biofilm to some extent. This might explain the synergistic effect of ciprofloxacin and D-tyrosine against the clinical isolates of *P. aeruginosa in vitro* and *in vivo*.

Time-kill curve results showed that combination of ciprofloxacin and D-tyrosine produced synergistic effect against *P. aeruginosa in vitro*. As explained above, it might be that D-tyrosine reduced the bacteria biofilm formation and accelerated the entry of ciprofloxacin into the cell, thereby increasing the efficacy of the drug. Furthermore, the use of D-tyrosine reduced the dose of ciprofloxacin in the time-kill curve. The decrease in drug dose during treatment could reduce the risk of drug-resistance. The combination of ciprofloxacin and D-tyrosine improved the survival time of zebrafish in *P. aeruginosa*-zebrafish infection model experiment. This result indicates that the synergistic effect seen *in vitro* was also produced *in vivo*.

Limitations of the study

This study has some limitations. Although it has demonstrated that combination of ciprofloxacin and D-tyrosine produced positive synergistic effect *in vitro* and *in vivo* in time-kill curve and *P. aeruginosa*-zebrafish infection model, respectively, single MDR *P. aeruginosa* is not sufficient as an index strain for a viable study. More MDR bacterial strains should be investigated in future in order to determine the precise mechanism involved in the observed synergism.

CONCLUSION

The findings of this study suggest that the ciprofloxacin/D-tyrosine combination produces synergistic effect *in vitro* and *in vivo*. This phenomenon offers a new strategy for tackling the current problem of drug-resistant bacteria.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by them. Huirong Li and Wei Jiang contributed equally to this work.

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