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Synergistic Effect of 14-Alpha-Lipoyl Andrographolide and Various Antibiotics on the Formation of Biofilms and Production of Exopolysaccharide and Pyocyanin by *Pseudomonas aeruginosa*▽

Xiangping Zeng,[1]† Xiangyang Liu,[2]† Jiang Bian,[2] Gang Pei,[2] Huanqin Dai,[2] Steven W. Polyak,[3] Fuhang Song,[2] Li Ma,[2] Yuqiang Wang,[1]* and Lixin Zhang[2]*

Institute of New Drug Research, Jinan University College of Pharmacy, Guangzhou 510632, China[1]; Chinese Academy of Sciences Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100190, China[2]; and School of Molecular and Biomedical Sciences, University of Adelaide, Australia 5005[3]

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Pseudomonas aeruginosa **produces a biofilm that provides the bacteria with an effective barrier against antibiotics. Here, we investigated the synergy of various antibiotics with 14-alpha-lipoyl andrographolide (AL-1), focusing upon synthesis of the biofilm. AL-1 also inhibited the production of the exopolysaccharide and pyocyanin components. We propose that AL-1 may potentially serve as a cotherapy to combat *P. aeruginosa*.**

Certain bacteria, such as *Pseudomonas aeruginosa*, can exist as biofilms on a variety of surfaces, including tissues and medical devices. Here, the bacteria cluster together with an extracellular matrix that often includes exopolysaccharide (EPS), proteins, and DNA (12), thereby providing an effective protection barrier against host immune responses and antibiotics (4). Hence, studying biofilm biology is necessary for better understanding the pathogenesis caused by *P. aeruginosa* in diseases such as chronic respiratory tract infections in cystic fibrosis patients and urinary tract infections (3, 24).

Biofilm synthesis of *P. aeruginosa* is controlled by a cell-to-cell signaling system known as quorum sensing (QS) (1, 2, 7, 8). Briefly, QS consists of three components: signal molecules, enzymes required for synthesis of signaling molecules, and receptors that function as ligand-dependent transcription factors. The QS system controls expression of approximately 11% of genes in the genome (21, 26, 27). The Las and Rhl systems produce and utilize acylated homoserine lactone (AHL) signaling molecules. In contrast, the *Pseudomonas* quinolone signal (PQS) system employs signaling molecules chemically related to quinolone (15). The *lasI* gene plays multiple roles in the establishment and maintenance of *P. aeruginosa* biofilms,

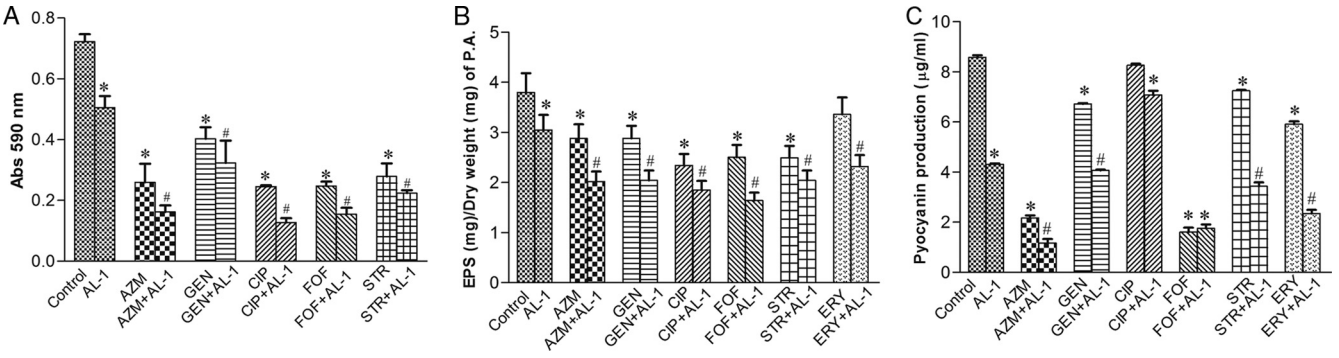

FIG. 1. Synergy effects of AL-1 on actions of antibiotics against biofilm formation (A) and EPS (B) and pyocyanin (C) production of *P. aeruginosa* PAO-1. *, significant difference ($P < 0.05$) compared to the control group; #, the combination of antibiotic and AL-1 showed a significant difference ($P < 0.05$) compared to the antibiotic alone. Each experimental value is expressed as the mean ± standard deviation (SD). Comparisons between groups were done using a one-way analysis of variance (ANOVA). The difference between each group and the control group was tested by using Bonferroni's comparison.

* Corresponding author. Mailing address for Lixin Zhang: Institute of Microbiology, Chinese Academy of Sciences, Beijing 100190, China. Phone: 86 10 6256 6511. Fax: 86 10 62538564. E-mail: lzhang03@gmail.com. Mailing address for Yuqiang Wang: Institute of New Drug Research, Jinan University College of Pharmacy, Guangzhou 510632, China. Phone: 86 20 8522 0850. Fax: 86 20 8522 4766. E-mail: yuqiangwang2001@yahoo.com.

† Both authors contributed equally to the work.

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TABLE 1. Effects of different antibiotics against *P. aeruginosa*

Antibiotic	MIC[a] (μg/ml)
FOF	32
STR	8–16
AZM	16
ERY	32–64
GEN	1–2
CIP	0.5–1.5
DAS-K	>1,140
AL-1	550

[a] MIC indicates a clinically resistant phenotype.

while RhlI and RhlR regulate the production of factors required for pathogenesis, such as pyocyanin, rhamnolipids, and elastase (7, 19). Both Las and Rhl QS systems regulate the *pel* biosynthetic operon that is responsible for the production of EPS (20), a component of the biofilm required for adherence to the substratum and maintenance of structure (9, 13). *P. aeruginosa* PAO-1 uses the type III secretion system (T3SS) to directly inject three virulence effectors (ExoS, ExoT, and ExoY), which also play roles in biofilm formation, into host cells. Since QS and T3SS are not directly involved in processes essential for bacterial growth, compounds that inhibit the above systems may offer a great advantage over conventional antimicrobial agents (10, 25, 28, 29).

Over the past 30 years, the natural product andrographolide, a diterpenoid lactone from the herb *Andrographis paniculata*, has been employed clinically in China to relieve symptoms of inflammation, fever, and pain due to bacterial and viral infections (11). We recently synthesized a novel derivative, 14-alpha-lipoyl andrographolide (AL-1), with antibacterial activity (5, 11). In this report, we demonstrated that AL-1 inhibited biofilm formation and sensitized the bacterium *P. aeruginosa* to a variety of antibiotics for distinct synergistic effects. Biofilm was initially investigated by staining adherent cells with crystal violet (18). Additionally, production of the two important biofilm components, i.e., EPS and pyocyanin, was measured. EPS was assayed by using the phenol solution-sulfuric acid detection method, as previously described (6, 14). Pyocyanin production was measured spectroscopically following extraction with chloroform (11). The *P. aeruginosa* strain PAO-1 was grown overnight at 37°C in L broth and compared against cells grown in the presence of 0.5 mM AL-1. In each case, significant decreases in biofilm production were observed upon treatment (Fig. 1A). There were similarly significant decreases in the amounts of EPS and pyocyanin (Fig. 1B and C). The cultures were also treated for synergistic activity alongside traditional antibiotics, such as potassium dehydroandrographolide succinate (DAS-K), fosfomycin (FOF), streptomycin (STR), azithromycin (AZM), erythromycin (ERY), gentamicin (GEN), and ciprofloxacin (CIP) (all purchased from Sigma-Aldrich). PAO-1 was grown in medium supplemented with

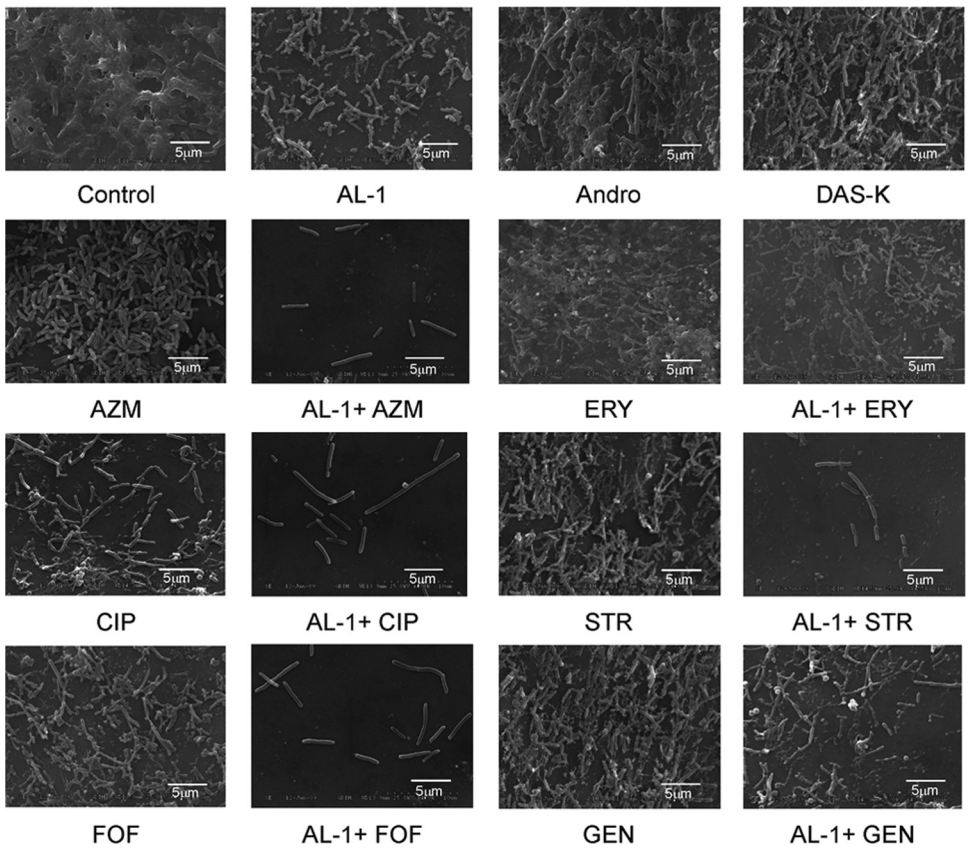

FIG. 2. Effects of AL-1 as a single agent and in combination with antibiotics against *P. aeruginosa* biofilm formation (magnification, ×4,000). The biofilm was grown on polyvinyl chloride catheter material for 7 days, during which the fresh culture medium containing antibiotics was changed every 2 days. The concentration of DAS-K and AL-1 was 0.5 mM, and antibiotics were 1/2 MIC.

AL-1 and antibiotics at 50% of their reported MICs. MICs were obtained by using the standard 2-fold serial dilution method, and the MIC determinations were repeated in triplicate. As expected, the antibiotics were more potent at inhibiting cell growth than AL-1 and DAS-K (Table 1). In all experiments, growth of the PAO-1 strain was not inhibited by treatment with AL-1 (data not shown). However, when these antibiotics were combined with AL-1, the inhibitory effect of AZM, GEN, CIP, and STR on biofilm formation was greatly increased (Fig. 1A). Furthermore, the production of both EPS and pyocyanin was significantly reduced in the AL-1 combination treatments in all cases compared to the effect of antibiotic alone (Fig. 1A, B, and C). Scanning electron microscopy (SEM) was also performed to visualize the *P. aeruginosa* biofilms. It showed that biofilm formation was almost completely inhibited when AL-1 was combined with AZM, CIP, and STR, while less synergistic effects were observed with ERY and GEN (Fig. 2).

The present study demonstrated that AL-1 has synergistic effects on antibiofilm and antivirulence factor activities when combined with AZM, CIP, FOF, STR, and GEN. AL-1 was identified as a potential inhibitor for the QS and T3SS systems (L. Ma, X. Zeng, X. Liu, H. Liang, J. Bian, G. Pei, H. Dai, F. Song, Y. Wang, and L. Zhang, unpublished data). Our data support the hypothesis that synergy arises due to inhibition of biofilm synthesis, specifically through decreased production of EPS and pyocyanin. The quinolone signaling system was not affected in this work. It is worth noting that previous toxicity study experiments in animal models have demonstrated that AL-1 has low toxicity (50% lethal dose [LD_{50}] of 1,243 mg/kg of body weight/day) (5) and therefore represents a chemotherapeutic worthy of further investigation. This synergistic mechanism of activity has been reported previously with AZM (17, 23), whereas CIP can inhibit QS-regulated virulence factor production (22). Although there is no evidence that FOF can target the QS-regulated genes, it has shown synergistic antipseudomonal activities with fluoroquinolones also by inhibition of biofilm formation (16). STR and GEN did not appear to interfere with QS. In conclusion, our data suggest that AL-1 is a potentially useful agent in combination therapies with conventional antibiotics to combat *P. aeruginosa* infections.

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