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Synergy between Synthetic Antimicrobial Polymer and Antibiotics: A Promising Platform to Combat Multidrug-resistant Bacteria

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The failure of many antibiotics in the treatment of chronic infections caused by multidrug- resistant (MDR) bacteria necessitates the development of effective strategies to combat this global healthcare issue. Here, we report an antimicrobial platform based on the synergistic action between commercially available antibiotics and a potent synthetic antimicrobial polymer that consists of three key functionalities – low-fouling oligoethylene glycol, hydrophobic ethylhexyl and cationic primary amine groups. Checkerboard assays with *Pseudomonas aeruginosa* and *Escherichia coli* demonstrated synergy between our synthetic antimicrobial polymer and two antibiotics, doxycycline and colistin. Co-administration of these compounds significantly improved the bacteriostatic efficacy especially against MDR *P. aeruginosa* strains PA32 and PA37, where the minimal inhibitory concentrations (MICs) of polymer and antibiotics were reduced by at least 4-fold. A synergistic killing activity was observed when the antimicrobial polymer was used in combination with doxycycline, killing > 99.999% of planktonic and biofilm *P. aeruginosa* PAO1 upon 20 min treatment at polymer concentration of 128 μg mL^{-1} (4.6 μM) and doxycycline concentration of 64 μg mL^{-1} (133.1 μM). In addition, this synergistic combination reduced the rate of resistance development in *P. aeruginosa* compared to individual compounds, and was also capable of reviving susceptibility to treatment in the resistant strains.

KEYWORDS: *combination therapy, antibiotic resistance, antimicrobial polymers, RAFT polymerization, biofilm*

The rising number of infections caused by multidrug-resistant (MDR) bacteria is a critical global healthcare concern.[1-5] Although resistance development is a natural phenomenon, the extensive overuse of antibiotics has accelerated the process in bacteria over the past few decades.[6-9] As a result, the efficiency of many antibiotics has diminished, causing longer hospital stays, higher medical costs and increased mortality.[10, 11] To address this global issue, more effective therapeutic approaches are required. These include improving the efficiency of current antibiotics through chemical modification or using adjuvants,[12-15] employing antimicrobial agents with novel cellular targets,[16-21] and applying combination therapy over monotherapy[22, 23].

In combination therapy, the co-administration of traditional antibiotics along with other antibiotics[24-26] or non-antibiotic drugs[27, 28] has been reported to improve treatment efficiency compared to using individual compounds. Furthermore, combination therapy not only hinders resistance development but also revives MDR bacteria's susceptibility to treatment.[11, 29] Additionally, synergistic combinations can effectively reduce the required treatment dose and possible side effects.[30] However, to achieve synergistic activity, compound pairings need to be selected rationally. A recent report on combination therapy shows that indifference or antagonism is more likely to occur than synergism.[31] Although many reports have been published on combination therapy using common antibiotics and drugs, only a few studies have focused upon using synthetic antimicrobial polymers in combination with antibiotics.[32-34]

By mimicking the fundamental composition of natural antimicrobial peptides, synthetic antimicrobial polymers have recently emerged as potential antimicrobial agents that target the bacteria cell membrane.[16, 17] Generally, electrostatic interactions between the cationic residues of antimicrobial polymers and the anionic components of bacteria cell walls or outer membranes is the first step in bactericidal activity, followed by the insertion of the polymer hydrophobic sub-units into the cytoplasmic membrane to induce membrane disruption/permeabilization leading to cell death. Such a bactericidal mechanism hinders resistance development in bacteria, thereby making synthetic antimicrobial polymers a promising co-agent in combination therapy to create more potent antimicrobial strategies.

On the basis of our experience in the development of antimicrobial polymers[35-37] and the potential benefits of combination therapy,[38-41] we herein investigate the efficacy of an antimicrobial platform based on our

lead synthetic antimicrobial polymer[35, 39] in combination with different classes of commercially available antibiotics (e.g., doxycycline from the tetracycline family, as depicted in **Figure 1**). The potency of these combinations is investigated via checkerboard assay and the synergistic combinations are further studied in terms of bactericidal activity and resistance development in bacteria.

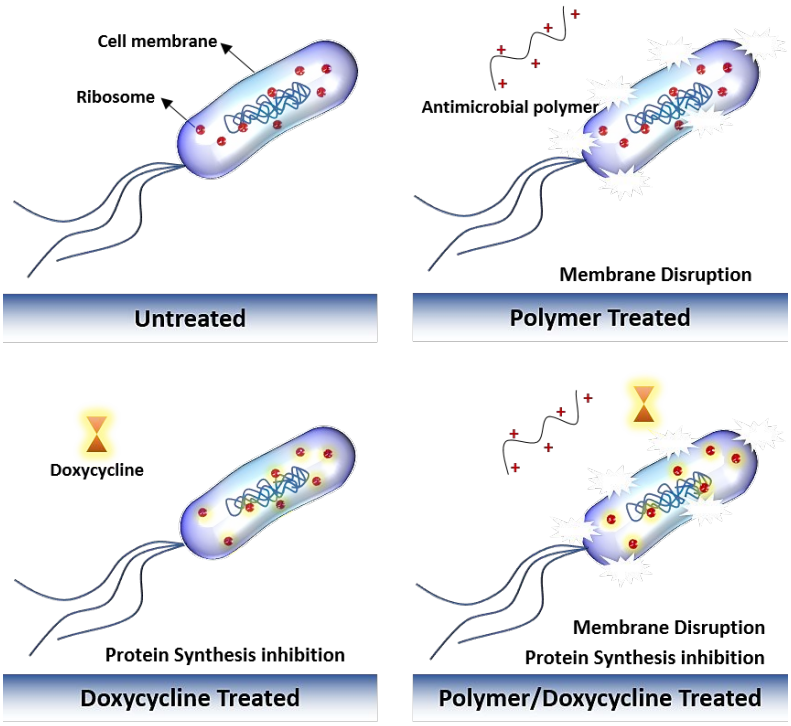


Figure 1. Synergistic antibacterial activity of synthetic antimicrobial polymer and doxycycline.

Materials and methods

Materials. Ethylenediamine (Sigma-Aldrich, ≥ 99%), di-*tert*-butyl dicarbonate (Aldrich, 99%), triethylamine (Scharlau, 99%), acryloyl chloride (Merck, ≥ 96%), oligoethylene glycol methyl ether acrylate (OEGA) M_n 480 g mol^{-1} (Aldrich), 2-ethylhexyl acrylate (Aldrich, 98%), trifluoroacetic acid (TFA) (Sigma-Aldrich, 99%), hexane (Merck), diethyl ether (Merck), 1,4-dioxane (Merck) and basic alumina (Al_2O_3) (LabChem) were used as received. 2,2'-azobis (2 methylpropionitrile) (AIBN) (Acros, 98%) was purified by recrystallization from methanol. Sodium sulfate (Na_2SO_4), magnesium sulfate ($MgSO_4$), sodium hydrogen carbonate ($NaHCO_3$), tetrahydrofuran, and acetone were obtained from Chem-Supply and used as received. Milli-Q water with a resistivity of > 18 MΩ·cm was obtained from an in-line Millipore RiOs/Origin water purification system. Antibiotics (amoxicillin, ampicillin, azithromycin dihydrate, ceftriaxone disodium salt hemi (hepatahydrate), ciprofloxacin hydrochloride, clarithromycin, colistin sodium methanesulfonate and doxycycline hydrochloride) were purchased from Sigma-Aldrich. Gentamicin sulfate was purchased from Enzo life sciences and tobramycin was purchased from Biogal.

Characterizations. ^{1}H Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker AC300F spectrometer. Deuterated solvents D_2O or $CDCl_3$ (obtained from Cambridge Isotope Laboratories) were used as reference solvents and samples with concentration of ca. 10-20 mg mL^{-1} were prepared. The monomer composition of the polymers was calculated using the following equation ∫a,b/6 : ∫c/9 : ∫d/2 where ∫a,b, ∫c, and ∫d correspond to the integrals of the characteristic protons of 2-ethylhexyl acrylate (methyl -C*H*$_3$- groups, δ_H 0.80-0.98 ppm), cationic monomer (*tert*-butyl -C*H*$_3$- groups, δ_H 1.38-1.52 ppm) and OEGA (ester -C*H*$_2$O- groups, 4.10-4.30 ppm), respectively (Figure S1, Supporting Information (SI)).

Gel permeation chromatography (GPC) analysis was performed using a Shimadzu liquid chromatography system equipped with a Shimadzu refractive index detector and two MIX C columns (Polymer Lab) operating at 40 ^{o}C. Tetrahydrofuran was used as the eluent at a flow rate of 1 mL min^{-1}. The system was calibrated with poly(methyl methacrylate) standards with molecular weights of 200 to 10^6 g mol^{-1}.

Zeta-potential measurements were conducted using a Malvern Zetasizer Nano ZS apparatus equipped with a He-Ne laser operated at λ = 633 nm and at a scattering angle of 173^o. Samples were prepared at a concentration of ca. 2 mg mL^{-1} where filtered Milli-Q water (using 0.45 μm pore size filter) was used as the solvent to solubilize the polymer.

Synthesis of cationic monomer. Cationic monomer *tert*-butyl (4-acrylamidobutyl) carbamate was prepared in the same manner as reported previously.[35] Briefly, ethylenediamine (0.3 mol) was dissolved in chloroform (400 mL), followed by the dropwise addition of di-*tert*-butyl dicarbonate (0.03 mol in 100 mL) over 2 h at 0−5 °C. The reaction mixture was stirred overnight at 25 °C. White precipitates were filtered, and the organic phase was washed exhaustively with water using a separation funnel to remove excess diamines. The organic layer was then dehydrated over $MgSO_4$, filtered, and dried using a rotary evaporation unit to yield a pale-yellow oil. Tetrahydrofuran (100 mL) was added to dissolve the intermediate product. Triethylamine (36 mmol) and acryloyl chloride (31.5 mmol) were added dropwise to the solution at 0−5 °C with N_2 bubbling. The contents were stirred at 25 °C for 1 h. The byproducts were filtered, and the solvent was removed in vacuo. The crude product was dissolved in chloroform (150 mL) and washed against brine (1 × 75 mL). The organic phase was stirred with $MgSO_4$ and basic Al_2O_3 for 10 min, filtered, and concentrated in vacuo. The product was further purified by repeated precipitation steps in hexane to yield the *tert*-butyloxycarbonyl (Boc)-protected monomer as a fine white powder, which was dried in vacuo.

tert-Butyl (2-acrylamidoethyl) carbamate: ^{1}H NMR (300MHz, $CDCl_3$,25 °C), δ_H 6.56 (br s, 1H, N*H*), 6.28 (dd, J = 17.1 Hz, 1.5 Hz, 1H, C*H*H=CH), 6.12 (dd, J = 17.1 Hz, 10.2Hz, 1H, CHH=C*H*), 5.65 (dd, J = 10.2 Hz, 1.5 Hz, 1H, CH*H*=CH), 5.05 (br s, 1H, N*H*), 3.49−3.41 (m, 2H, CH_2), 3.34−3.28 (m, 2H, CH_2), 1.45 (s, 9H, CH_3); ^{13}C NMR (300 MHz, $CDCl_3$,25 °C), δ_C 166.23, 157.50, 130.88, 126.30, 79.85, 41.05, 40.09, 28.35.

Synthesis of amphiphilic ternary random copolymer (P). The synthesis of amphiphilic ternary statistical copolymer proceeded in the same manner as reported previously.[35] Briefly, the RAFT agent benzyl dodecyl carbonotrithioate (11.6 μmol), AIBN (4.6 μmol), OEGA (350 μmol), *tert*-butyl (4-acrylamidobutyl) carbamate (580 μmol), and 2-ethylhexyl acrylate (230 μmol) were dissolved in 1,4-dioxane (such that the total monomer concentration in solvent is 1 M). The solution was purged with N_2 for 20 min in an ice bath. The polymerization was conducted for 20 h at 70 °C and then quenched in an ice bath with exposing to air. The polymer was purified by precipitation in a hexane/diethyl ether (7:3) mixture thrice and subsequently dried in vacuo. The Boc-protected polymer produced monomodal molecular weight

distributions with dispersity ($Đ$) value of 1.3 and the number-averaged molecular weight (M_n) of 17500 g mol^{-1} as evidenced by GPC analysis (Figure S2, SI)

The Boc protecting groups were removed using TFA in the same manner as reported previously.[35] In general, the polymer solution in dichloromethane (ca. 10 wt % polymer) was treated with TFA (20 mol equivalent with respect to the Boc group) for 3 h at 25 °C. Boc- deprotected polymer was subsequently precipitated into diethyl ether/hexane (4:1). The precipitate was isolated by centrifugation, dissolved in methanol, and reprecipitated two more times. The polymer was then dried in vacuo and further purified by dialysis against water (Cellu-Sep 3500 MWCO). The aqueous solution was lyophilized to yield the Boc-deprotected polymer **P**.

Minimum inhibitory concentration (MIC). The MIC of polymer and selected antibiotics were determined via broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Briefly, a single colony was cultured in 10 mL of Mueller-Hinton broth (MHB) at 37 ^oC with shaking at 200 rpm overnight. Subsequently, a subculture was prepared from the overnight culture by diluting 1:100 in 10 mL MHB and allowed to grow to mid-log phase, then diluted to the appropriate concentration for the MIC test. A twofold dilution series of 100 μL of polymers solution in MHB were added into 96-well microplates followed by the addition of 100 μL of the subculture suspension. The final concentration of bacteria in each well was ca. 5×10^5 cells mL^{-1}. The plates were incubated at 37 ^oC for 20 h, and the absorbance at 600 nm was measured with a microtiter plate reader (FLUOstar Omega, BMG Labtech). MIC values were defined as the lowest concentration of sample that showed no visible growth and inhibited cell growth by more than 90%. Positive controls without polymer and negative controls without bacteria were included. All assays included two replicates and were repeated in at least three independent experiments.

Checkerboard assay. The checkerboard assay was performed in 96-well cell culture plates (Costar, Corning®) containing polymer and antibiotic in MHB. Concentration gradients of polymer and selected antibiotics were prepared in the horizontal and vertical direction in a 10 × 8 layout, respectively. Bacterial suspensions were prepared in the same manner as abovementioned for MIC test and added to the plates. Positive and negative controls, without antimicrobial agent and bacteria respectively, were also included.

The plates were incubated at 37 °C for 20 h, and the absorbance at 600 nm was recorded subsequently. The MICs of individual polymer and antibiotics were computed from the corresponding column or row which contains only one component, while the remaining columns and rows were screened for fractional inhibitory concentration index (FICI). The FICI values were calculated as follows:

$$\text{FICI} = \frac{\text{MIC}_\text{A}\text{in combination}}{\text{MIC}_\text{A}} + \frac{\text{MIC}_\text{B}\text{ in combination}}{\text{MIC}_\text{B}} \qquad \text{Equation 1}$$

The FICI data was interpreted as follows: ≤ 0.5, synergistic effect; 0.5 < FICI < 1, indifference; and ≥ 1, antagonistic. All experiments were repeated in at least two independent experiments.

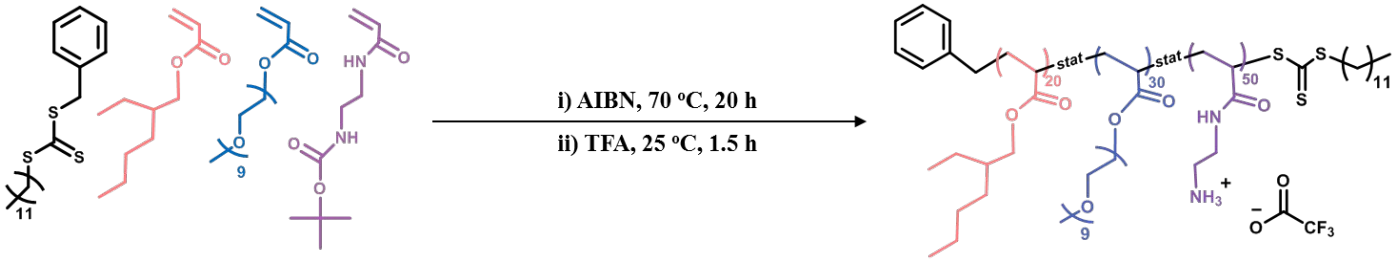
Killing studies. To evaluate the bactericidal activity of selected compounds and combinations, *P. aeruginosa* PAO1 and 6294 biofilms were grown. A single colony of PAO1 or PA6294 was cultured overnight in 10 mL of Luria Bertani medium (LB 10) at 37 ^{o}C with shaking at 200 rpm. The overnight culture was diluted 1:200 in freshly prepared M9 minimal medium containing 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, pH 7.0, supplemented with 2 mM $MgSO_4$, 100 μM $CaCl_2$ and 20 mM glucose. The bacterial suspension was then aliquoted (1 mL per well) into tissue-culture treated 24-well plates (Costar, Corning®). The plates were incubated at 37 ^{o}C with shaking at 180 rpm in an orbital shaker that does not stop agitation when the door is opened (model OM11, Ratek, Boronia, Australia) and the biofilm cultures were allowed to grow for 6.5 h without any disruption. The compounds were then added to the wells and the plates were incubated for 20 min. After treatment, the planktonic and biofilm viability analysis were determined by a drop plate method. For planktonic analysis, free-floating cells in the biofilm supernatant were serially diluted in sterile PBS and plated onto LB agar. For biofilm analysis, cells attached on the interior surfaces of the well (surface area 4.5 cm^2) were washed twice with sterile PBS to remove loosely attached bacteria, before being resuspended and homogenized in PBS by incubating in an ultrasonication bath (150 W, 40 kHz; Unisonics, Australia) for 20 min. Resuspended biofilm cells were then serially diluted and plated onto LB agar. Planktonic and biofilm colonies were counted and CFU was calculated after 24 h incubation at 37 ^{o}C. All assays included two replicates and were repeated in at least three independent experiments.

Antimicrobial Resistance Studies. To investigate the resistance development in *Pseudomonas aeruginosa* PAO1, bacterial suspensions at exponential phase (~ 10^7 cells mL^{-1}) were subjected to sequential passaging in the presence of selected compounds at sub-inhibitory concentrations (i.e., 1/4 × MIC, 1/2 × MIC, 1 × MIC and 2 × MIC) for 21 days. Cells were incubated at 37 °C and passaged at 24 h intervals. After incubation, the cultures were checked for growth. Cultures from the second highest concentrations that allow growth ($OD_{600} \geq 2.00$) were diluted to an OD_{600} of 0.01 per milliliter in fresh MHB containing 1/4 × MIC, 1/2 × MIC, 1 × MIC and 2 × MIC of selected compound. Assays were performed with two independent experiments.

Results and Discussion

The ability of synthetic antimicrobial polymers to combat MDR bacteria provides the key motivation for us to investigate their efficacy in combination therapy along with traditional antibiotics. For this, we used an antimicrobial polymer in the form of an amphiphilic random ternary copolymer (**P**) that was developed by our group.[35] Large quantities of polymer can be made in a facile manner via a controlled radical polymerization technique, termed reversible addition−fragmentation chain transfer (RAFT) polymerization (**Scheme 1**).[42, 43] This polymer was meticulously designed to have optimal antimicrobial activity and biocompatibility. It contains 30 repeat units of biocompatible oligoethylene glycol to impart low-fouling properties, 20 repeat units of hydrophobic ethylhexyl groups to induce membrane disruption, and 50 repeat units of primary amino groups to establish electrostatic interactions with bacterial membrane.[35, 36]

Scheme 1. Synthesis of antimicrobial polymer, **P**, which is an amphiphilic random ternary copolymer, via reversible addition−fragmentation chain transfer (RAFT) polymerization.



To assess the potential synergistic effect between **P** and commercially available antibiotics, their interactions were evaluated using checkerboard assay. **Figure 2** shows the chemical structure and mode of action of ten common antibiotics used in this study. These antibiotics were selected based on their ability to act on different mechanisms, such as targeting protein synthesis (doxycycline, clarithromycin, azithromycin, gentamycin and tobramycin), cell wall biosynthesis (ampicillin, amoxicillin, ceftriaxone), cell wall integrity (colistin) and DNA synthesis (ciprofloxacin) in bacteria.

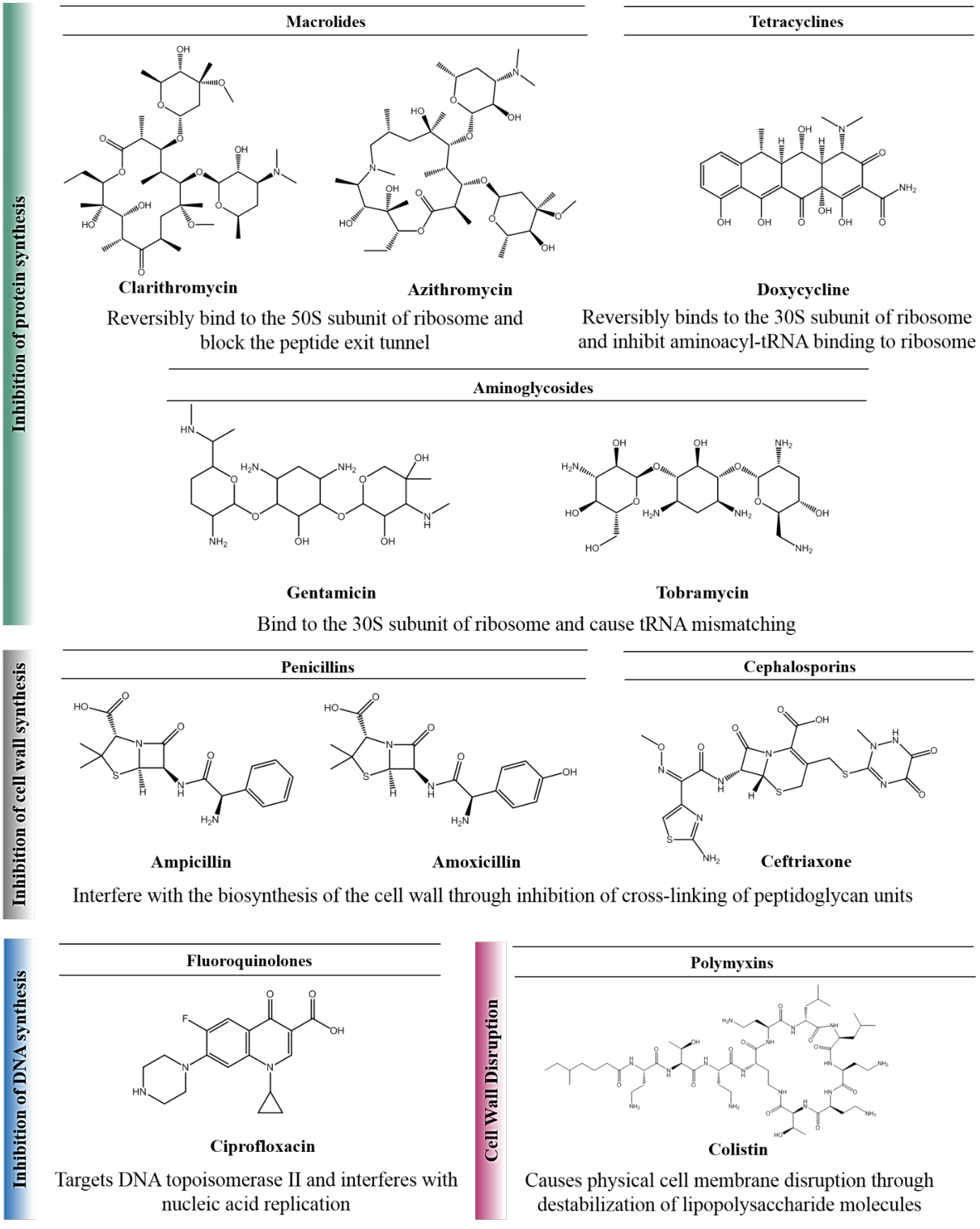


Figure 2. Chemical structure and reported mode of action of the antibiotics used in this work.[44, 45]

Checkerboard plots of tested combinations against two Gram-negative bacteria *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* K12 are represented in **Figures S3 and S4** (**SI**), Gram-positive bacteria were not included in this study as the antimicrobial activity of **P** is better against Gram-negative bacteria.[37] This assay is conducted to evaluate the bacteriostatic activity of proposed combinations. Minimum inhibitory concentrations (MICs) of the used antimicrobial agents alone and in combination, and fractional inhibitory concentration indexes (FICIs) of the tested combinations against *P. aeruginosa* PAO1 and *E. coli* K12 are shown in Tables S1 and S2 (SI). It is worthwhile noting that FICI is a parameter commonly used to determine the synergism/antagonism of compounds in combination therapy. Although both tested bacteria were susceptible to all selected antibiotics, only doxycycline (**D**) and colistin methanesulfonate (**C**) showed synergy (FICI $\leq$ 0.5) with **P**. Notably, indifference (0.5 < FICI <1) was the most common interaction between **P** and antibiotics, but none of the combinations demonstrated antagonism interaction (FICI $\geq$1) against the tested bacteria strains. It is noteworthy that clarithromycin showed synergy with **P** exclusively against *P. aeruginosa* PAO1.

The combination of **P** and **D** (**PD**) yielded synergistic activity against *P. aeruginosa* PAO1 and *E. coli* K12 with FICI values of 0.38–0.50 and 0.50, respectively. Co-administration of these antimicrobial agents resulted in at least a 4-fold decrease in MIC values of **P** and **D** against *P. aeruginosa* PAO1 (Table S1 and S2, SI). Meanwhile, **P** and **C** combination (**PC**) demonstrated stronger synergy against *P. aeruginosa* PAO1 (FICI = 0.38) compared to *E. coli* K12 (FICI = 0.50). Based on these results, both combinations demonstrated slight species-specific activity, where slightly greater synergy was observed against *P. aeruginosa* PAO1.

The mechanism behind the synergistic interactions is complicated; however, the mode of action of individual components might explain the observed synergy. When used in combination with other compounds that act on intracellular targets, membrane-targeting compounds are expected to modulate the intracellular drug concentration. Synergistic interaction will lead to increased drug influx while antagonistic interaction will do the opposite.[31] We postulate that in the synergistic combination **PD**, **P** can enhance **D** uptake through membrane wall disruption. In spite of this, we are unsure as to why the other antibiotics which act on intracellular targets mainly displayed neutral interactions. Synergistic interactions are also

probable when drugs that act on a similar mechanism are used in combination.[22, 31] Given that both **P** and **C** predominantly act on the same mechanism of membrane wall disruption, this might be the reason behind the observed synergistic activity in **PC.**

Following these results, the potency of the synergistic combinations (**PD** and **PC**) were evaluated against four more *P. aeruginosa* strains including PA ATCC 27853, an invasive strain isolated from microbial keratitis (PA 6294)[46] and two MDR strains (PA32 and PA37) also isolated from cases of microbial keratitis[47, 48] via the checkerboard method (Figure 3 and Table 1).

PD and **PC** exhibited synergism against all tested *P. aeruginosa* strains. The greatest synergistic effect was found against MDR strains PA32 and PA37 for both **PD** and **PC**. The strongest synergistic activities with FICI values of 0.28 and 0.38 against PA32, and 0.25 and 0.31 against PA37 were observed for **PD** and **PC** respectively (Table 1). As an example, although PA32 showed resistance toward both **P** and **D**, we observed a 4- to 8-fold and 4- to 32-fold decrease in the MIC values of **P** and **D** respectively, when **PD** was used. **P** at a sub-MIC level as low as 16 μg mL^{-1} (MIC = 128 μgmL^{-1}) was able to reduce the MIC of **D** from 128 μg mL^{-1} to 4 μg mL^{-1} (Table 1) when used in combination.

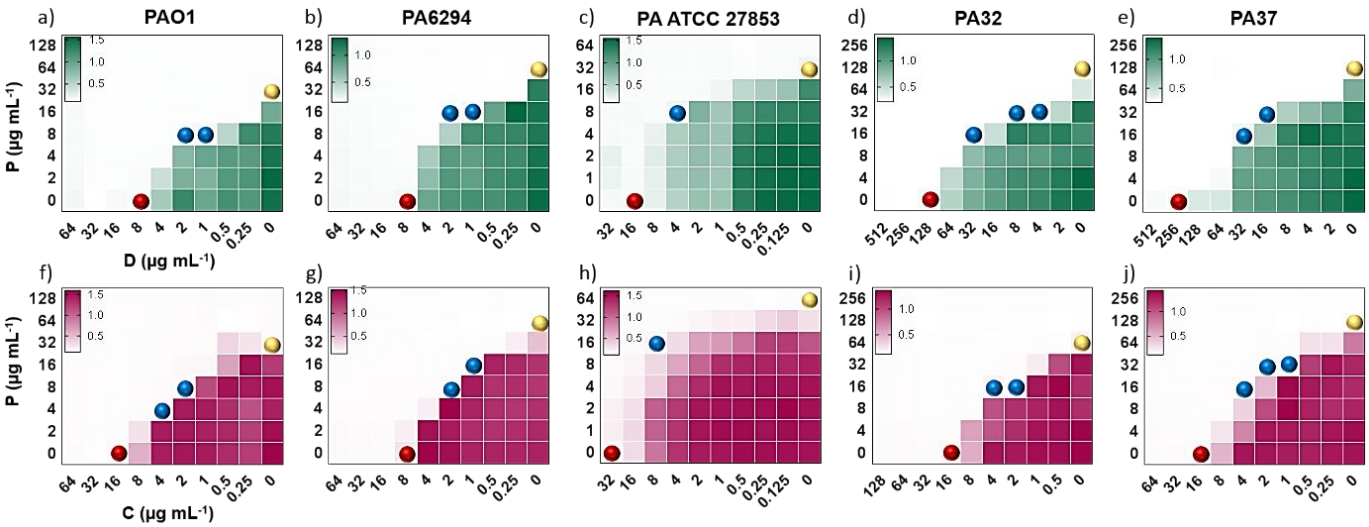


Figure 3. Checkerboard microdilution assay between **P** and **D** (a-e), and **P** and **C** (f-j) against *P. aeruginosa* PAO1, PA6294, PA ATCC 27853, PA32 and PA37. Bacterial growth, quantified by average OD_{600}, is illustrated as a linear gradient from white to green and burgundy where darker colors represent less growth inhibition. Yellow and red bullets represent MIC values for **P** and antibiotic, respectively. Blue bullets indicate concentrations exhibiting synergistic interaction. The data are representative of a minimum of two biological replicates.

Table 1. Checkerboard assay results indicating the synergistic activity of **P with D** and **C** and against five *P. aeruginosa* strains including PAO1, PA6294, PA ATCC 27853, PA32 and PA37. All the combinations showed moderate to high synergism (FICI ≤ 0.5)

	MIC (μg mL^{-1})					MIC (μg mL^{-1})				
	Alone		In Combination		FICI	Alone		In Combination		FICI
	P	D	P	D		P	C	P	C	
PAO1	32	8	8	1	0.38	32	16	4	4	0.38
			8	2	0.50			8	2	0.38
PA6294	64	8	16	1	0.38	64	8	8	2	0.38
			16	2	0.50			16	1	0.38
PA ATCC 27853	32	16	8	4	0.50	64	32	16	8	0.50
PA32	128	128	32	4	0.28	64	16	16	2	0.38
			32	8	0.31			16	4	0.50
			16	32	0.38			-	-	-
PA37	128	256	16	32	0.25	128	16	32	1	0.31
			32	16	0.31			32	2	0.38
			-	-	-			16	4	0.38

Subsequently, bactericidal and antibiofilm activity of **PD** and **PC** synergistic combinations were investigated. Colony-forming unit (CFU) analysis was used to assess the bactericidal activity of the combinations against both planktonic and biofilm bacteria. *P. aeruginosa* PAO1 biofilms were grown in cell culture media M9 for 6.5 h prior to incubation with selected compounds at different concentrations for 20 min.

First, the bactericidal activities of **PD** and **PC** at fixed **P** concentration of 128 μg mL^{-1} and three antibiotic concentrations of 16, 32 and 64 μg mL^{-1}, denoted as 128/16, 128/32 and 128/64 μg mL^{-1}, were examined against PAO1. For comparison, individual **P** and antibiotics (**D** and **C**) were also tested (Figure 4a-d).

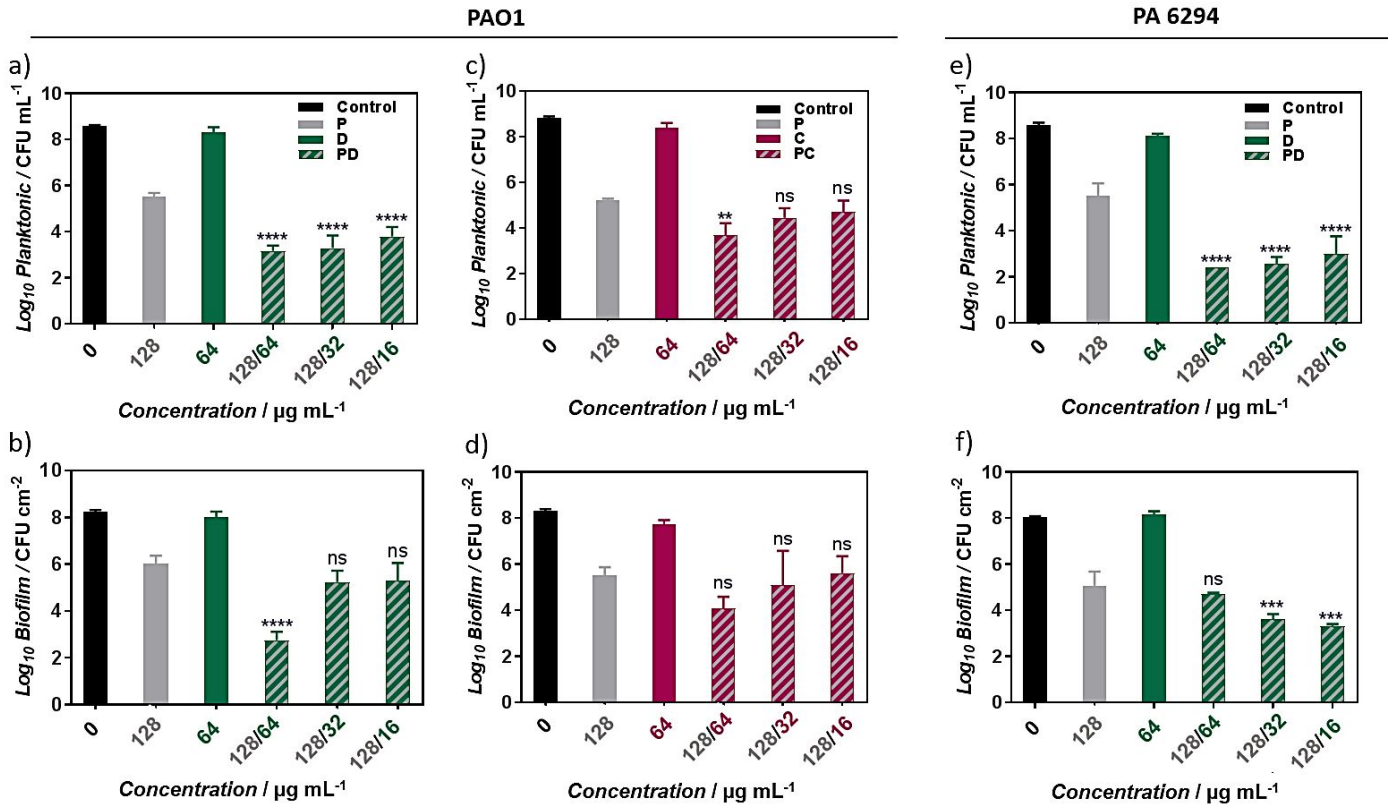


Figure 4. Bactericidal activity of individual components and combinations. Bactericidal activity of **P**, **D**, and **PD** on planktonic (a) and biofilm (b) *P. aeruginosa* PAO1. Bactericidal activity of **P**, **C**, and **PC** on planktonic (c) and biofilm (d) *P. aeruginosa* PAO1. Bactericidal activity of **P**, **D**, and **PD** on planktonic (e) and biofilm (f) *P. aeruginosa* 6294. All bactericidal activities were determined by colony-forming unit (CFU) analysis upon 20 min incubation. Data are representative of at least three independent experiments ± SD. Two-way ANOVA test − asterisks indicate statistically significant difference of **PD** and **PC** vs **P** treatment (**p < 0.01; ***p < 0.001; ****p < 0.0001; ns, non-significant (p > 0.01)).

For the planktonic cells, treatment with **P** at 128 µg mL^{-1} for 20 min resulted in an average of 3.3±0.24 log_{10} reduction in CFU compared to the untreated sample, while **D** and **C** did not cause any significant reduction in bacterial cell viability (Figure 4a, c and Table S3, SI). Both **PD** and **PC** exhibited bactericidal property against planktonic bacteria in a dose-dependent manner. Treatment with **PD** at concentrations of 128/64, 128/32 and 128/16 µg mL^{-1} led to 5.4±0.29, 5.3±0.61 and 4.8±0.48 log_{10} reductions in CFU, respectively, compared to the untreated sample. These results confirm the synergistic effect of **PD** in killing planktonic bacteria cells at the tested concentrations. **PC**, on the other hand, showed significant synergy only at 128/64 µg mL^{-1} causing 5.1±0.59 log_{10} reduction in CFU compared to the untreated sample.

Synergistic bactericidal activity was also observed against bacterial biofilm upon treatment with **PD** in the same dose-dependent manner (Figure 4b and Table S3, SI). We observed synergistic effect at the highest **D** concentration (128/64 µg mL^{-1}), whereas it was non-significant at lower **D** concentrations (128/32 and

128/16 μg mL^{-1}). Treatment with **PD** for 20 min at 128/64 μg mL^{-1} resulted in 5.5±0.43 $\log_{10}$ reductions in CFU, compared to the untreated biofilm. However, the differences between the bactericidal activities of **P** and **PC** at all tested concentrations were non-significant based on ANOVA analysis (Figure 4d and Table S3, SI).

We then tested the bactericidal activity of **PD** against *P. aeruginosa* 6294. We observed synergy against planktonic PA6294 with 6.2±0.10, 6.0±0.39 and 5.9±0.87 $\log_{10}$ reductions in CFU compared to the untreated sample at treatment concentrations of 128/64, 128/32 and 128/16 μg mL^{-1} respectively (Figure 4e and Table S3, SI). Interestingly, **PD** demonstrated a reverse dose-dependent trend against bacterial biofilm, where the lowest **D** concentration caused the highest reduction in CFU with a 4.7±0.14 $\log_{10}$ reduction in CFU at 128/16 μg mL^{-1} compared to the untreated sample (Figure 4f and Table S3, SI).

The observed synergy between **P** and **D** in the killing assays might be caused by the bacteriostatic characteristic of **D**, where it can potentiate the bactericidal activity of **P** by restricting bacterial growth.

Next, an antimicrobial resistance study was conducted to investigate if **PD** can induce resistance in *P. aeruginosa* PAO1. **P** and **D** were also included as controls. For this, 20 serial passages of bacterial cells were done over a period of 21 days in the presence of sub-MIC levels of **P**, **D,** and **PD** (Figure 5). It is worthwhile noting that based on our previous work *P. aeruginosa* PAO1 could not develop resistance toward **C** over a period of 22 days,[35] therefore **PC** and **C** were not included in the resistance study.

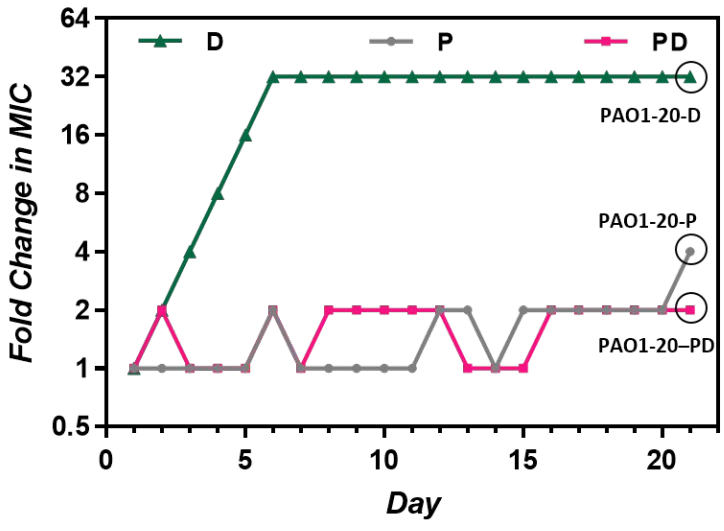


Figure 5. Resistance development monitoring in *P. aeruginosa* PAO1 in the presence of sub-MIC levels of **P**, **D** and **PD**. The y-axis indicates the changes in MICs of the compounds over a period of 21 days as compared

to the first day (0th passage). *Note that the bacteria cultures from 20th passage were used in supplementary MIC test.*

P. aeruginosa PAO1 developed resistance toward **D** rapidly, where the MIC increased 32 times the original value and plateaued after six days. **P**, on the other hand, triggered minor resistance in *P. aeruginosa* which is in accordance with our previous study.[35] The MIC value changed between 1 × MIC and 2 × MIC over the test period and finally reached 4 × MIC. This behaviour is attributed to the membrane disruption mechanism of **P** which minimizes the likelihood of resistance development in bacteria. The MIC for **PD** was less volatile, fluctuating between 1 × MIC and 2 × MIC but not exceeding 2 × MIC during the test period, which shows the potency of **PD** in suppressing resistance development in bacteria.

A supplementary antimicrobial activity test was subsequently performed against the obtained modified strains from the resistance study to evaluate their susceptibility to further treatments. For this, bacteria cells were derived from the 20th passage of all three treatments (**PAO1-20-D, PAO1-20-P** and **PAO1-20-PD**) and subjected to **P**, **D** and **PD**. Table 2 shows the MIC values of **P**, **D** and **PD** against unmodified and modified strains of *P. aeruginosa* PAO1.

Table 2. Supplementary antimicrobial activity test showing the susceptibility of modified strains of *P. aeruginosa* PAO1 (obtained from the 20th passage of resistant study, see Figure 5) to **P**, **D** and **PD.**

	MIC (μg mL^{-1})			
Treatment	**PAO1**	**PAO1-20-D**	**PAO1-20-P**	**PAO1-20-PD**
D	8	256	8-16	32
P	32	32	128	32
PD	8/2	16/4	32/8	16/4

For modified **D** strain (**PAO1-20-D**), we observed no change in the MIC value of **P**, while there was a two-fold increase in the MIC value of **PD** which might be the result of acquired resistance toward **D**. Although showing a minor resistance to **PD** treatment, **PD** is a potent treatment for **PAO1-20-D** by lowering the required **D** dose by 64 times compared to individual **D**. Not surprisingly, an increase in the MIC value for **PD** was observed against **PAO1-20-P**, where 4 × MIC was needed to effectively combat the modified **P**

strain. **D**, on the other hand, retained the same MIC value against **PAO1-20-P**. In the case of **PAO1-20-PD**, even after 20 passages in the presence of **PD**, no resistance was observed toward **P**, whereas 4 × MIC was required to inhibit the bacterial growth using **D**. However, this level of resistance to **D** is considered minimal compared to exclusively **D** treated PAO1(**PAO1-20-D**).

These observations suggest that: (i) the acquisition of resistance toward either **P** or **D** is hindered through co-administration, and (ii) when **PD** is used against modified strains (**PAO1-20-P** and **PAO1-20-D**), **P** is more efficient in reviving PAO1 susceptibility to **D**, rather than vice versa.

Conclusion

In summary, we investigated the efficacy of combination therapy involving our synthetic antimicrobial polymer and commercially available antibiotics. Polymer-drug interactions were studied via checkerboard assay where two synergistic combinations, containing doxycycline and colistin antibiotics, were detected. Synergistic combinations demonstrated bacteriostatic activity against Gram-negative bacteria such as *P. aeruginosa* and *E. coli*, where the greatest synergism was observed against MDR *P. aeruginosa* strains. A synergistic effect in planktonic and biofilm killing activity was also achieved using the combination of antimicrobial polymer and doxycycline, which suggests that doxycycline, even though as a bacteriostatic antibiotic, could potentiate the killing activity of polymer. In addition, serial passaging revealed that this synergistic combination can significantly hinder the generation of resistant mutant strains compared to individual compounds. This study shed valuable information on the potential use of synthetic antimicrobial polymers in combination with specific antibiotics in an effort to combat MDR bacteria.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Polymer characterization, including GPC and NMR, checkerboard microdilution assay, Figures S1-S4 and Tables S1-S3.

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