*J Antimicrob Chemother* 2022; **77**: 1635–1644
https://doi.org/10.1093/jac/dkac094 Advance Access publication 23 March 2022

Journal of Antimicrobial Chemotherapy

# Synergism inhibition and eradication activity of silver nitrate/potassium tellurite combination against *Pseudomonas aeruginosa* biofilm

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*Received 17 November 2021; accepted 28 February 2022*

**Objectives:** Antibiotic resistance, biofilm and persistent infection of *Pseudomonas aeruginosa* is a perilous challenge in the healthcare system. Hence, a vast number of novel antipseudomonas approaches are currently being pursued. Our group focuses on exploring the efficacy of metal(loid)-based antimicrobials (MBAs) towards novel infection control solutions.

**Methods:** Initially, nine MBAs were tested for biofilm prevention and eradication efficacy. Synergistic potentials were then screened systematically in a total of 1920 combinatorial MBA concentrations, in laboratory media [CAMHB and LB] and infection-related simulated wound fluid (SWF). The antibiofilm efficacy of the silver nitrate ($AgNO_3$; 'Ag') with potassium tellurite ($K_2TeO_3$; 'Te') combination was examined against clinical antibiotic-resistant isolates and compared with the most used antibiotics. The *in vitro* resistance acquisition test, for exploring the chance of getting future resistance, and meta-analysis, for estimating Ag/Te human cell cytotoxicity, were carried out.

**Results:** The Ag/Te combination was identified as the most effective agent against *P. aeruginosa* biofilm. The application of the Ag/Te combination was quite effective against all clinical isolates. Comparison of clinical isolates with indicator strains showed clinical isolates are gaining resistance against the antibiotics (especially gentamicin) and Ag, while they are susceptible to Te and particularly the Ag/Te combination. The chance of getting future resistance against Ag/Te as a mixture was remarkably lower than the individual application of each metal. Te has significantly lower human cell cytotoxicity in comparison with Ag.

**Conclusions:** Te could be an appropriate alternative against *P. aeruginosa* biofilms (existing or prevention thereof), especially in combination with Ag.

## Introduction

*Pseudomonas aeruginosa* is a common opportunistic bacterium that threatens hosts by developing persistent infections/contaminations in the respiratory system, wounds, urinary tract, blood and biofilms on medical devices such as catheters, stents, implants and artificial joints.[1] Consequently, *P. aeruginosa* biofilm infection is one of the most perilous challenges in healthcare and industry[2,3] and has led the WHO to list it as the first critical priority where new antibiotics are urgently needed.[4]

Dual antibiotic application is one of the most effective strategies for the eradication and prevention of biofilm infections.[5] Combining and mixing different antimicrobials is now the standard and recommended treatment and control method for *P. aeruginosa* biofilm infections.[6,7] Statistically, antimicrobial combination therapy decreases the chance of the pathogen developing antibiotic resistance. For instance, the probability of spontaneous resistance to the antibiotic X+Y combination is 1 in $10^{13}$, while independent chances of resistance to antibiotic X and antibiotic Y independently may be only 1 in $10^6$ or $10^7$.[5,8,9] Furthermore, lower antimicrobial dose requirements, limited side effects and the prevention of bacterial recovery after exposure are other advantages of dual application.[9–11]

Metal or metalloid [metal(loid)]-based antimicrobials (MBAs), especially silver (Ag), have been used since ancient times for infection control.[12,13] A comprehensive review by our research group demonstrated the history, potency and molecular mechanism of MBAs[12] as well as their efficacy against bacteria growing as a biofilm.[14] A recent study by our group showed some MBA combinations have strong synergistic activity against planktonic cultures of *P. aeruginosa*, *Escherichia coli* and/or *Staphylococcus aureus*.[11] Earlier work showed MBAs' antibiofilm synergistic activity in combination with quaternary ammonium compound antiseptics.[10]

Effective combinations of Ag with other antibiotics were reported by other research groups as well.[15]

This study began with an examination and screening of both biofilm prevention and eradication potency of nine MBAs, as well as synergistic potency of 1920 MBA concentration combinations in both laboratory media and simulated wound fluid (SWF). Silver nitrate ($AgNO_3$) and potassium tellurite ($K_2TeO_3$) were identified as the most effective antibiofilm combination. For reader ease, we will henceforth use 'Ag' and 'Te' to refer to $AgNO_3$ and $K_2TeO_3$, respectively. The Ag/Te combination was tested on antibiotic-resistant *P. aeruginosa* clinical isolates and compared with common antibiotics. We performed an *in vitro* resistance acquisition test to observe the chance of getting future resistance against Ag/Te, as a mixture and individually.

One of the most important criteria when developing a new antimicrobial is the side effects and host cell cytotoxicity of the compound. Cytotoxicity to host cells can damage local tissue and delay the wound-healing process.[16] With a range of research-specific methodologies being applied in previous publications, including cell line, treatment duration and endpoint, we conducted a systematic review and meta-analysis to evaluate eukaryotic cell cytotoxicity of our selected MBAs.

## Materials and methods

### Bacterial strains and culture media

Bacterial strains were stored at −70°C in Microbank vials as described by the manufacturer (Pro-Lab Diagnostics, Richmond Hill, ON, Canada). *P. aeruginosa* ATCC 27853, PAO1 and 39 clinical isolates (source: Foothills Hospital, Calgary, Alberta, Canada) were used in this study. Three different media were used as the growth and susceptibility testing media in this study: LB (VWR chemicals; Lot# 190756384), CAHMB (BD Bacto, Oxoid, Basingstoke, UK; Cat# X296B) and SWF [50% peptone water (0.85% NaCl, 0.5 g peptone per 500.0 mL):50% FCS (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA; Lot# 2212202RP)].[17,18]

### Stock and working MBA solutions

Similar to our previous study,[11] nine metal(loid) salts with antimicrobial features had been selected. The antibiofilm efficacy of the metal(loid) salts $AgNO_3$ (Ag), gallium (III) nitrate [$(Ga(NO_3)_3$; henceforth abbreviated to Ga], copper (II) sulphate ($CuSO_4$; Cu), $K_2TeO_3$ (Te), zinc sulphate ($ZnSO_4$; Zn), nickel sulphate ($NiSO_4$; Ni), tetrachloroaurate (III) ($AuCl_4$; Au), sodium selenite ($Na_2SeO_3$; Se) and aluminium sulphate [$Al_2(SO_4)_3$; Al], were explored. Further detailed information such as company source and stock solution preparation is presented in the Supplementary methods, available as Supplementary data at *JAC* Online.

### Prevention of biofilm formation by microtitre plate method

Briefly, bacteria stored at −70°C were subcultured at 37°C overnight to get a pure single colony, 75 μL of the desired concentration of metal(loid)s (provided in the media) were added to 96 wells, 75 μL of $1.0 \times 10^6$ cfu/mL bacteria were added into each well and incubated for 48 h at 37°C in a microplate shaker incubator at 150 rpm. The planktonic cells and the spent medium were discarded, and the adhered biomass was rinsed two times with distilled water. The minimum biofilm inhibitory (preventive) concentration (MBIC) was detected, and defined as the lowest concentration of an antimicrobial that resulted in an $OD_{650}$ difference of ≤10% (1 log difference in growth after 6 h of incubation) of the mean of three positive control well readings[19] (see Supplementary methods). Results from at least three separate biological replicates were averaged.[17,20]

### Biofilm cultivation

Biofilms were grown in a Calgary biofilm device [CBD; commercially available as the MBEC physiology and genetics assay (Innovotech Inc., Edmonton, Alberta, Canada)], as originally described by Ceri *et al.*[21] Starting from cryogenic stocks, *P. aeruginosa* ATCC 27853 was streaked out twice on tryptic soya agar (TSA). One hundred and fifty microlitres of $1.0 \times 10^7$ cfu/mL bacterial inoculum was transferred into each well of a 96-well microtitre plate, and the sterile peg lid of the CBD was inserted into the plate. The inoculated device was then placed on a microplate shaker at 150 rpm for 24 h of incubation at 37°C and 95% relative humidity.

### Eradication of established biofilms

After developing a biofilm on CBD, the pegs were rinsed twice with 0.9% saline to wash away planktonic bacteria, then placed into a 96-well microtitre plate containing two-fold serial dilutions of the MBAs in the 150 μL of each medium; a column was reserved for bacterial growth in the absence of the metal(loid) salts. The microtitre plate was then incubated for 24 h in a humidified incubator at 37°C on a gyrorotary shaker at 150 rpm. This approach was used to determine the minimum biofilm eradication concentration (MBEC) of each MBA.[22] The last well that had no bacterial biofilm and OD absorption was considered the MBEC.

### Synergism high-throughput susceptibility testing of microbial biofilm growth

'Chequerboard' arrangements of MBA combinations were made in 96-well microtitre plates as previously described.[10,23] When prepared, each chequerboard microtitre plate had one column of negative controls (just media without bacteria and MBAs) and one column of growth controls as a positive control (without MBA, with media and bacteria). It would also contain 10 different concentrations of MBAs alone, 8 different concentrations of Ag alone, and each MBA and Ag at 80 different combinations of concentrations leading to the binary metal(loid) concentration array in the chequerboard. For each chequerboard analysis, the same MBIC and MBEC steps (described in the Supplementary methods) were conducted for surveying biofilm eradication, prevention and synergism potency of MBA combinations.

### Determination of fractional biofilm inhibitory concentration (FBIC) and fractional biofilm eradication concentration (FBEC) for detection of synergism effects

The synergistic interactions rules suggested by the American Society for Microbiology for the testing of planktonic cells are used here for both the MBIC and MBEC synergism data obtained.[23] The FBIC and FBEC values for each combination of antimicrobial agents were calculated with the following formulae:

$$FBIC = \frac{\text{MBIC of antibiotic A in combination}}{\text{MBIC of antibiotic A alone}} + \frac{\text{MBIC of antibiotic B in combination}}{\text{MBIC antibiotic B alone}}$$

$$FBEC = \frac{\text{MBEC of antibiotic A in combination}}{\text{MBEC of antibiotic A alone}} + \frac{\text{MBEC of antibiotic B in combination}}{\text{MBEC antibiotic B alone}}$$

To evaluate antimicrobial interactions, we used the lowest FBIC/FBEC index method, as described by Bonapace *et al.*[24] and other studies.[24–27] The lowest FBIC/FBEC obtained for all inhibitory or eradication combinations on the chequerboard was considered to be the FBIC/FBEC for the pair. Finally, FBIC/FBEC values were interpreted as follows: FBIC/FBEC $<0.8$ = synergy; FBIC/FBEC $\geq 0.8$ and $\leq 1.2$ = partial synergy; and FBIC/FBEC $>1.2$ = antagonistic.

### *Antibiofilm potency of Ag/Te combination against clinical isolates in SWF*

The synergistic antibiofilm potency of the Ag/Te combination against selected clinical isolates was conducted to compare the resistance pattern of Ag, Te and the Ag/Te combination with ciprofloxacin and gentamicin.

### *In vitro development of resistance*

The methodology used was adapted from Gullberg *et al.*[28] Single colonies of *P. aeruginosa* PAO1 parental strains (susceptible) were incubated overnight at 37°C. The bacterial culture was serially passaged in SWF that contained a sublethal concentration ($^1/_8$ dilution from the MIC) to begin developing resistant strains: SWF with no MBA addition (control), SWF amended with 0.15 mM Ag, 0.015 mM Te or 0.15 mM Ag/0.015 mM Te. $OD_{600}$ was recorded for every strain before serial passage. Serial passage of 500-fold dilutions was performed every 24 h for 7 days. Every day, the development of resistant cells in each culture was monitored for their enrichment potency by cell counts (cfu) on Mueller–Hinton agar containing four different concentrations of metals. Cell counts were performed by back-calculating 10-fold dilutions in 0.9% saline, a 10 μL aliquot from each dilution was added to agar media (containing different concentrations of metals) and incubated for 20 h at 37°C, cfu was recorded, and a Boolean condition of 'growth' was recorded when colony counts exceeded 200 at a dilution.

### *Systematic review and meta-analysis for eukaryotic cell cytotoxicity of Ag and Te*

A systematic review and meta-analysis study was conducted to explore the human cell cytotoxicity of the most effective MBAs (Ag and Te) in this study. Detailed systematic review and meta-analysis methodologies are presented in the Supplementary methods.

### *Statistical tests and data analysis*

All data organization, analysis, mean, mode, standard deviation, calculation of FBIC and FBEC, and the 3D graphical representations were performed using Microsoft Excel 365 (Microsoft Corporation, Redmond, WA, USA). All experiments were repeated at least three times. For the meta-analysis, data cleaning and preparation was done in Microsoft Excel 365 and further analyses were carried out via Comprehensive Meta-Analysis Software Version 2.0 (Biostat, Englewood, NJ, USA).

## Results

### *Biofilm inhibition and eradication efficacy of MBAs*

Table S1 shows the MBIC and MBEC of nine MBAs (Ag, Ga, Cu, Te, Zn, Ni, Au, Se, Al) against *P. aeruginosa* grown as a biofilm produced in the CBD by shear force on a polystyrene surface.[21,29] For the standard ATCC 27853 indicator, Ag had the lowest mean (range) MBIC in CAMHB at 0.03 (0.03–0.125) mM, in LB at 0.125 (0.065–0.25) mM, and in SWF at 0.25 (0.125–1) mM, respectively. Like the MBIC, the MBEC values ranged by media type. Between the nine MBAs, Ag had the lowest mean (range) MBEC at 0.03 (0.015–0.5) mM in CAMHB, 0.065 (0.065–0.125) mM in LB and 0.5 (0.125–1) mM in SWF.

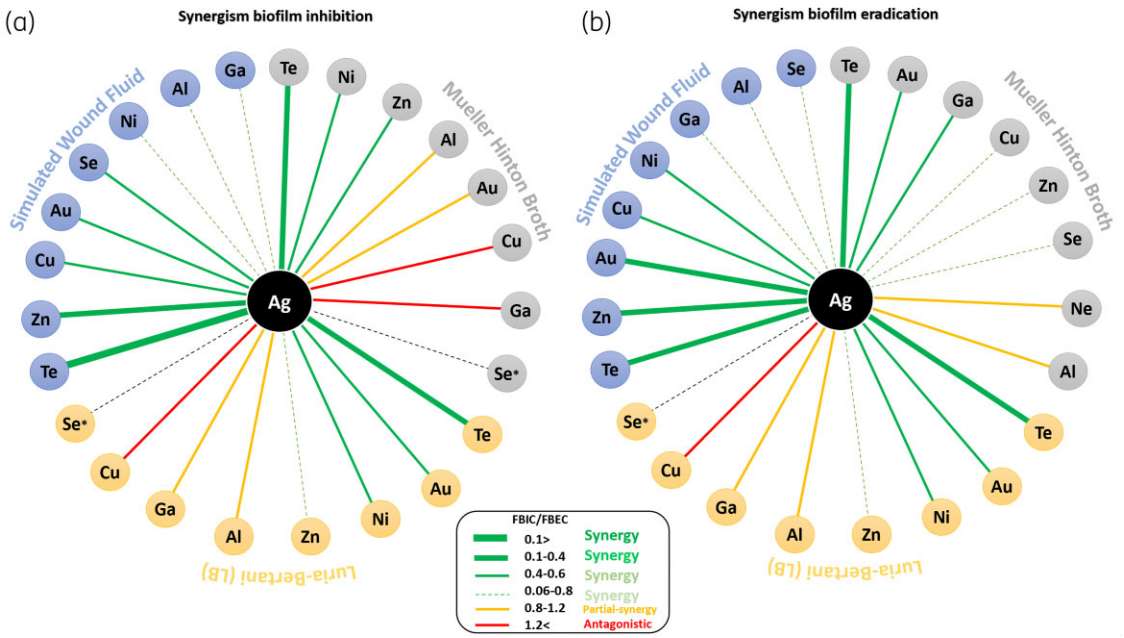


**Figure 1.** Synergism analysis against *P. aeruginosa* ATCC 27853 biofilm. (a) Estimated FBIC value for synergism biofilm inhibition efficacy. (b) Estimated FBEC values for synergism biofilm eradication efficacy of Ag with eight other MBAs in three different media. Detailed data and the most effective synergism concentrations are provided in Table S3. Data are presented as means of 2–5 (biological repeats) separate experiments over different days. *Results could not be determined from the concentration ranges examined experimentally, i.e. the agents did not effectively kill the biofilms. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

### *Synergistic biofilm inhibition and eradication efficacy of Ag in combinations with other MBAs*

The synergism potency of a total of 1920 combinations of MBA concentrations in a binary screening system was evaluated. The Ag was systematically paired with each of the other eight MBAs against *P. aeruginosa* ATCC 27853 biofilm (Figure 1). Also, Figures S1–S7 give the synergism patterns of both biofilm prevention and biofilm eradication as well as the FBIC/FBEC values for combinations of Ag with eight other MBAs against *P. aeruginosa*. For the convenience of the reader, the lowest FBIC/FBEC (highest synergism) combinations are ranked and presented in Table 1. The combination of Ag with Te had the lowest FBIC (0.093) (the highest level of synergism for biofilm inhibition) and the lowest FBEC (0.24) (the highest synergism for biofilm eradication) in SWF (0.015 mM Ag + 0.063 mM Te) or CAMHB (0.015 mM Ag + 0.008 mM Te), respectively (Figure 2).

The Ag/Te combination was highly synergistic and decreased the effective concentration of both agents. For biofilm inhibition, the Ag MBIC was 0.25 mM in SWF, while in combination with Te, the effective concentration of Ag decreased to 0.015 mM. The Te MBIC was 0.5 mM, while the Ag/Te combination decreased the effective concentration of Te to 0.063 mM. For the biofilm eradication, we observed a similar outcome. The MBEC for both Ag and Te was 0.5 mM, while the Ag/Te combination decreased the effective concentration of both agents to 0.065 mM.

### *Antibiofilm potency of Ag/Te combination against clinical antibiotic-resistant isolates*

After screening the antibiofilm efficacy of MBAs against the *P. aeruginosa* pathogen indicator strain, where Ag/Te was chosen as the most effective combination, the Ag/Te combination was then applied to clinical isolates to explore the efficacy profiles. This was also performed in SWF to get a clear idea of the best MBA candidate's potential efficacy for a clinical setting.

A total of 39 clinical isolates (20 cystic fibrosis and 19 burn wounds) of *P. aeruginosa* were also challenged with the two most common antibiotics for controlling biofilm-based infections (ciprofloxacin and gentamicin).[30] Not surprisingly, 17 (43%) of the clinical isolates displayed resistance to these antibiotics. Out of these 39, 15 (38%) isolates were resistant to gentamicin, while 2 (5%) isolates were resistant to ciprofloxacin. None of our isolates were resistant to both antibiotics.

**Table 1.** Synergism analysis against *P. aeruginosa* biofilm; estimated FBIC and estimated FBEC values for combinations of MBAs against *P. aeruginosa* ATCC 27853 biofilm

| | MBA | | Biofilm inhibition synergism activity (FBIC) | | | Biofilm eradication synergism activity (FBEC) | | |
|---|---|---|---|---|---|---|---|---|
| Medium | Agent A | Agent B | FIC | Interpretation | Concentrations (mM) | FEC | Interpretation | Concentrations (mM) |
| **SWF** | **Ag** | **Te** | **0.093** | **Synergy** | **0.015 Ag + 0.063 Te** | 0.27 | Synergy | 0.065 Ag + 0.063 Te |
| LB | Ag | Te | 0.36 | Synergy | 0.03 Ag + 0.06 Te | 0.37 | Synergy | 0.015 Ag + 0.125 Te |
| CAMHB | Ag | Te | 0.37 | Synergy | 0.015 Ag + 0.125 Te | **0.24** | **Synergy** | **0.015 Ag + 0.008 Te** |
| SWF | Ag | Zn | 0.38 | Synergy | 0.065 Ag + 4 Zn | 0.36 | Synergy | 0.125 Ag + 1.5 Zn |
| SWF | Ag | Cu | 0.5 | Synergy | 0.065 Ag + 2 Cu | 0.48 | Synergy | 0.007 Ag + 3 Cu |
| LB | Ag | Au | 0.5 | Synergy | 0.03 Ag + 0.125 Au | 0.48 | Synergy | 0.03 Ag + 0.065 Au |
| LB | Ag | Ni | 0.51 | Synergy | 0.065 Ag + 4 Ni<br>0.032 Ag + 8 Ni | 0.7 | Partial synergy | 0.015 Ag + 3 Ni |
| CAMHB | Ag | Ni | 0.53 | Synergy | 0.031 Ag + 0.5 Ni | 0.98 | Synergy | 0.25 Ag + 3 Ni |
| SWF | Ag | Au | 0.58 | Synergy | 0.065 Ag + 0.016 Au | 0.37 | Synergy | 0.03 Ag + 0.125 Au/0.015 Ag + 0.25 Au |
| CAMHB | Ag | Zn | 0.58 | Synergy | 0.065 Ag + 0.5 Zn | 0.72 | Synergy | 0.015 Ag + 3 Zn |
| SWF | Ag | Se | <0.62 | Synergy | 0.125 Ag + 12.5 Se | <0.77 | Synergy | 0.065 Ag + 25 Se |
| LB | Ag | Zn | 0.62 | Synergy | 0.015 Ag + 4 Zn | 0.96 | Partial synergy | 0.03 Ag + 1.5 Zn |
| SWF | Ag | Ni | <0.75 | Synergy | 0.25 Ag + 1 Ni | 0.49 | Synergy | 0.007 Ag + 3 Ni |
| SWF | Ag | Al | 0.75 | Synergy | 0.125 Ag + 12.5 Al | 0.75 | Synergy | 0.25 Ag + 25 Al |
| SWF | Ag | Ga | 0.75 | Synergy | 0.25 Ag + 12.5 Ga | 0.75 | Synergy | 0.25 Ag + 6.25 Ga |
| LB | Ag | Al | 0.9 | Partial synergy | 0.25 Ag + 25 Al | 1 | Partial synergy | 0.25 Ag + 50 Al |
| LB | Ag | Ga | 0.96 | Partial synergy | 0.031 Ag + 6.25 Ga | 0.58 | Synergy | 0.007 Ag + 1.56 Ga |
| CAMHB | Ag | Al | 1 | Partial synergy | 0.25 Ag + 50 Al | 1 | Partial synergy | 0.25 Ag + 50 Al |
| CAMHB | Ag | Au | 1 | Partial synergy | 0.065 Ag + 0.125 Au | 0.47 | Synergy | 0.007 Ag + 0.062 Au |
| LB | Ag | Cu | 2 | Antagonistic | 0.065 Ag + 8 Cu | 0.47 | Synergy | 0.015 Ag + 1.5 Cu |
| CAMHB | Ag | Ga | 2 | Antagonistic | 0.031 Ag + 2.5 Ga | 0.52 | Synergy | 0.007 Ag + 0.195 Ga |
| CAMHB | Ag | Cu | 2 | Antagonistic | 0.065 Ag + 8 Cu | 0.7 | Synergy | 0.007 Ag + 3 Cu |
| LB | Ag | Se | — | — | — | — | — | — |
| CAMHB | Ag | Se | — | — | — | <0.75 | Synergy | 0.015 Ag + 25 Se |

FBIC <0.8 = synergy; FBIC ≥0.8 and ≤1.2 = indifferent; FBIC >1.2 = antagonistic.
The lowest FBIC (the most effective biofilm inhibition synergism) and FBEC (the most effective biofilm eradication synergism) are highlighted in bold type. — indicates no FIC or FEC was obtained due to low antimicrobial efficacy.

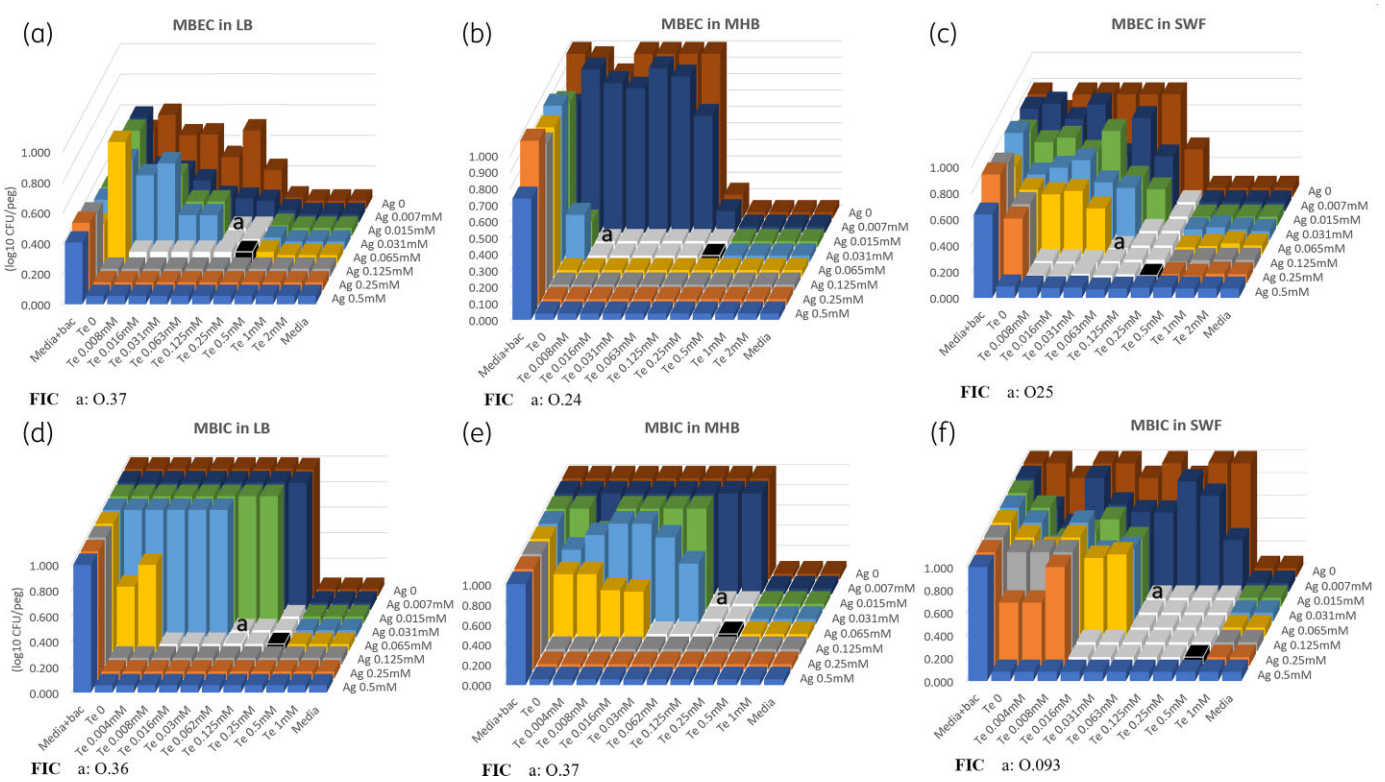


**Figure 2.** Synergism in *P. aeruginosa* ATCC 27853 biofilm inhibition and eradication activity of Ag/Te combinations upon culturing in three different media. (a) MBEC in LB; (b) MBEC in CAMHB; (c) MBEC in SWF; (d) MBIC in LB; (e) MBIC in CAMHB; and (f) MBIC in SWF ($n = 2$–5). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Ag, Te and the Ag/Te combination were applied to the 17 resistant clinical isolates of *P. aeruginosa* and their antibiofilm efficacy was compared with gentamicin and ciprofloxacin results. Figure 3 shows the MBIC results for clinical isolates and the standard ATCC 27853 indicator and PA01 strains. Unsurprisingly, due to the common application of Ag for various clinical approaches, the Ag and antibiotic (especially gentamicin) tolerances in clinical isolates were higher than in the indicator strain. However, clinical isolates were comparably susceptible to indicator strains when challenged with Te, which might be due to the limited application of the $TeO_3^{2-}$ oxyanion in healthcare and industry. Consequently, Te gave a lower MBIC in comparison with Ag, in most of the clinical isolates, while Ag had lower MBIC and MBEC for the ATCC 27853 strain in comparison with Te. All the clinical isolates were highly susceptible to our newly found Ag/Te combination.

### *In vitro assessment for the development of resistance*

For estimating the future chance of resistance against Ag and/or Te, an *in vitro* resistance acquisition test was performed. Serial passage of *P. aeruginosa* PAO1 was performed in SWF supplemented with sublethal ($^1/_8$ dilution from the MIC) concentrations of Ag, Te and the Ag/Te mixture. Culture growth ($OD_{600}$) was used as the first metric to determine the rate of resistance acquisition. Ag-supplemented broth culture growth was comparable with an unexposed growth control absorbance on all 6 recorded days of serial passage, suggesting Ag rapidly developed resistance. The Te-supplemented cultures took 4 days to match the control, whereas the Ag/Te combination-supplemented broth culture took 7 days for one of the replicates to approach the control's level of growth (Figure 4), indicating very slow rates of evolved tolerance.

The development of resistant cells and their enrichment potency was monitored daily through cfu on control and challenge media agar. Diluted samples of broth culture were applied to agar plates, incubated and observed for growth (Figure S8). Within the 7 days of passage, no growth on Ag/Te combination agar at the MIC was observed. The Ag-resistant strain saw resistance enrichment to all challenge conditions after 24 h of incubation in the sublethal medium. The Te-resistant strain produced consistent community growth on MIC agar after 4 and 5 days of sublethal incubation. Under Ag/Te exposure at 1.2 mM Ag/0.12 mM Te-supplemented agar, resistance never emerged. As a result, this experiment showed that singular usage of Ag and Te (especially Ag) shows strong potential for quickly gaining resistance compared with the Ag/Te mixture.

### *Systematic review and meta-analysis for eukaryotic cell cytotoxicity of Ag and Te*

The potential for clinical applications of MBAs is mostly as topical treatment to control wound infections and as part of coatings on indwelling medical devices. Therefore, human cell cytotoxicity of these components is critical and directly related to the wound-healing process. Consequently, we did a systematic review and

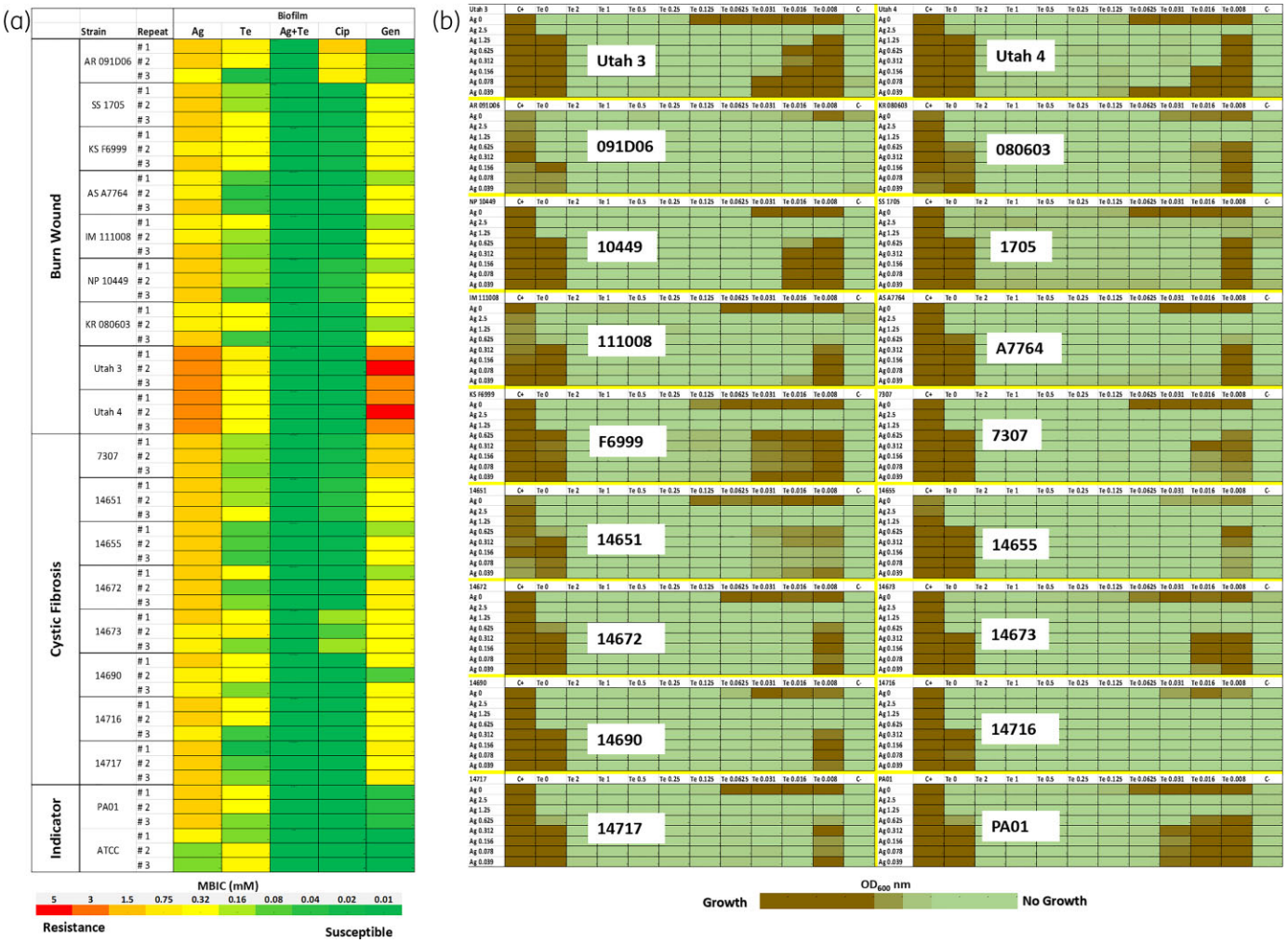


**Figure 3.** (a) MBIC of Ag, Te, Ag/Te combination, ciprofloxacin (Cip) and gentamicin (Gen) on clinical isolates and indicator strains of *P. aeruginosa* ($n = 3$). (b) Synergism chequerboard of synergism biofilm inhibitory of the Ag/Te combination on clinical isolates and indicator strains of *P. aeruginosa* ($n = 3$). Both the metal salts and antibiotic concentrations are in units of mM. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

meta-analysis to evaluate the cell cytotoxicity of selected MBAs. The literature search and study selection flow diagram for our meta-analysis is shown in Figure S9. Characterization of included studies is summarized in Table S2. The cell cytotoxicity of Te was remarkably lower than that of Ag (Table 2). In total, 50% cell viability after 24 h exposure with Ag was 7.5 μM while with Te it was 408 μM and the 75% cell viability was 5.2 μM and 103.3 μM for Ag and Te, respectively (Figure 5 and Figure S10). This helps validate the use of our Ag/Te combination, allowing for reduced levels of Ag to be used with a less cytotoxic partner. Unfortunately, to date, no toxicity data have been reported for the combination of Ag with Te.

## Discussion

Initially, nine MBAs were tested for biofilm prevention and eradication efficacy. This was followed by exploring for synergistic potentials of the eight with Ag for a total of 1920 combinatorial MBA concentrations. This was performed in media of varying richness of nutrients and relevance to the wound environment since nutrition and energy sources affect bacterial physiology and relative fitness towards stress.[31–33] Interestingly, some of the antagonistic combinations in the laboratory media, such as the Ag/Cu combination, produced synergistic results in the SWF or vice versa (Figure 1). This illustrates a problem in the literature that evaluating antimicrobial efficacy in laboratory media versus media more closely reflecting the application environment can result in the recommendation of inappropriate antimicrobial and antibiofilm formulations. Environmental factors appeared to influence not only synergism but disparities between biofilm prevention and eradication efficacy (Table S1). Our dataset showed that Ag has strong synergism in combination with some other MBAs. The lowest FBIC (the strongest biofilm inhibition) and FBEC (the strongest biofilm eradication) were displayed with the Ag/Te combination in both laboratory media and SWF. Furthermore, Te and Ag had the highest biofilm inhibition (MBIC) and eradication (MBEC)

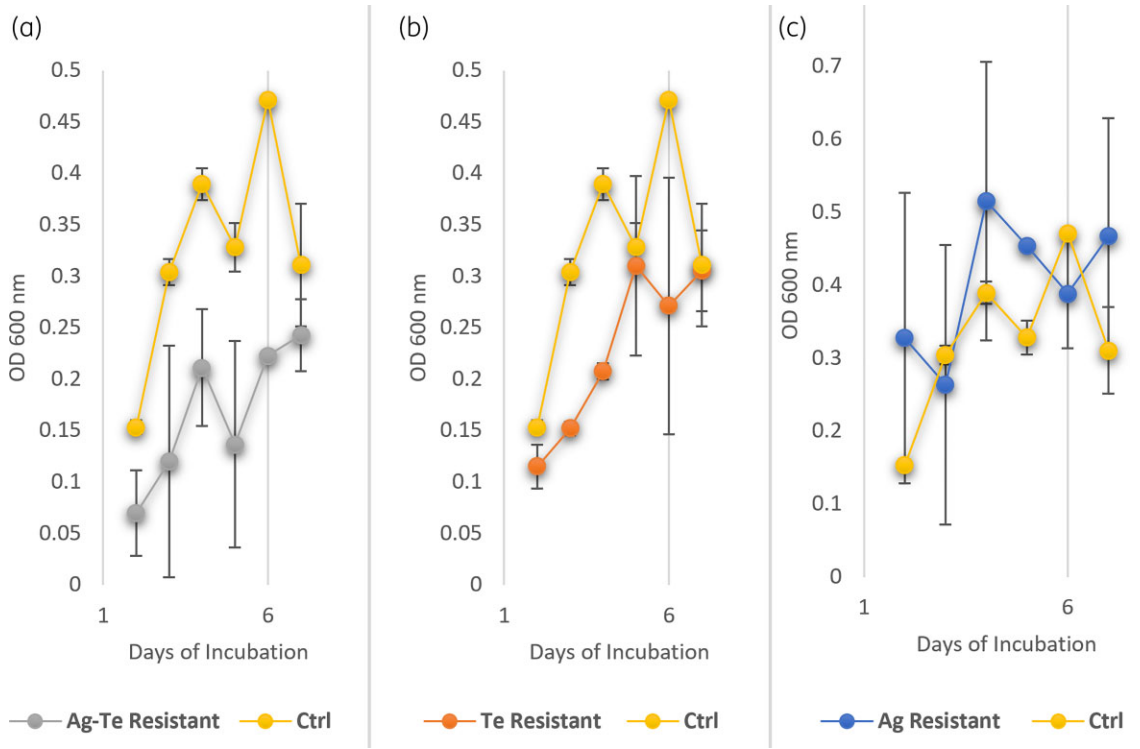


**Figure 4.** *In vitro* assessment for the development of resistance against Ag, Te and the Ag/Te combination. $OD_{600}$ of 7 days serially passaged *P. aeruginosa* PAO1 in 30% SWF supplemented with sublethal concentrations: 0.15 mM Ag and 0.015 mM Te (a), 0.015 mM Te (b) or 0.15 mM Ag (c) for the development of resistance ($n = 4$). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

**Table 2.** Meta-analysis on eukaryotic cell cytotoxicity of Ag and Te

| | | | Effect size | | | | 95% CI | | Test of null (2-tail) | | Heterogeneity | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MBA | Exposure time (h) | Toxicity | Number of studies | Mean (mM) | Standard error | Variance | Lower limit | Upper limit | *Z* value | *P* value | *P* value | $I^2$ |
| Te | 24 | 50% cell viability | 6 | 0.4079 | 0.111 | 12.3241 | 0.1903 | 0.6255 | 3.7 | <0.005 | <0.005 | 99.9 |
| | | 75% cell viability | 4 | 0.1033 | 0.0269 | 0.7260 | 0.0505 | 0.1561 | 3.8 | <0.005 | <0.005 | 95.9 |
| | | Stress oxidative index (0.5 mM) | 3 | 0.0013 (OR) | 0.0002 | 0.0 | 0.0009 | 0.0017 | 6.4 | <0.005 | 0.9 | 0.0 |
| | | Stress oxidative index (1 mM) | 3 | 0.0015 (OR) | 0.0002 | 0.0 | 0.0011 | 0.0018 | 7.3 | <0.005 | 0.8 | 0.0 |
| | 48 | 50% cell viability | 2 | 0.1184 | 0.0146 | 0.2136 | 0.0897 | 0.147 | 8.1 | <0.005 | <0.005 | 77.0 |
| | | 75% cell viability | 4 | 0.1029 | 0.0429 | 1.8396 | 0.0188 | 0.1869 | 2.4 | <0.005 | <0.005 | 100.0 |
| Ag | 24 | 50% cell viability | 21 | 0.0075 | 0.001 | 0.002 | 0.005 | 0.010 | 5.4 | <0.005 | <0.005 | 97.6 |
| | | 75% cell viability | 20 | 0.0052 | 0.0011 | 0.0012 | 0.0031 | 0.0073 | 4.8 | <0.005 | <0.005 | 95.7 |
| | | 50% intracellular ATP content | 3 | 0.0075 | 0.0012 | 0.0015 | 0.0051 | 0.0099 | 6.1 | <0.005 | 0.2 | 36.2 |
| | | 75% intracellular ATP content | 3 | 0.0058 | 0.0010 | 0.001 | 0.0039 | 0.0078 | 5.8 | <0.005 | 0.3 | 22.5 |

potency in comparison with other MBAs. Overall, the Ag/Te combination had the greatest antibiofilm synergy efficacy. Despite the similar characteristics to Te, Se antibiofilm activity was not observed in the conditions used here.

After selecting the most effective synergistic MBA combination (Ag/Te), its antibiofilm efficacy was examined against clinical isolates available to us and compared with the most commonly used antibiotics (ciprofloxacin and gentamicin) in

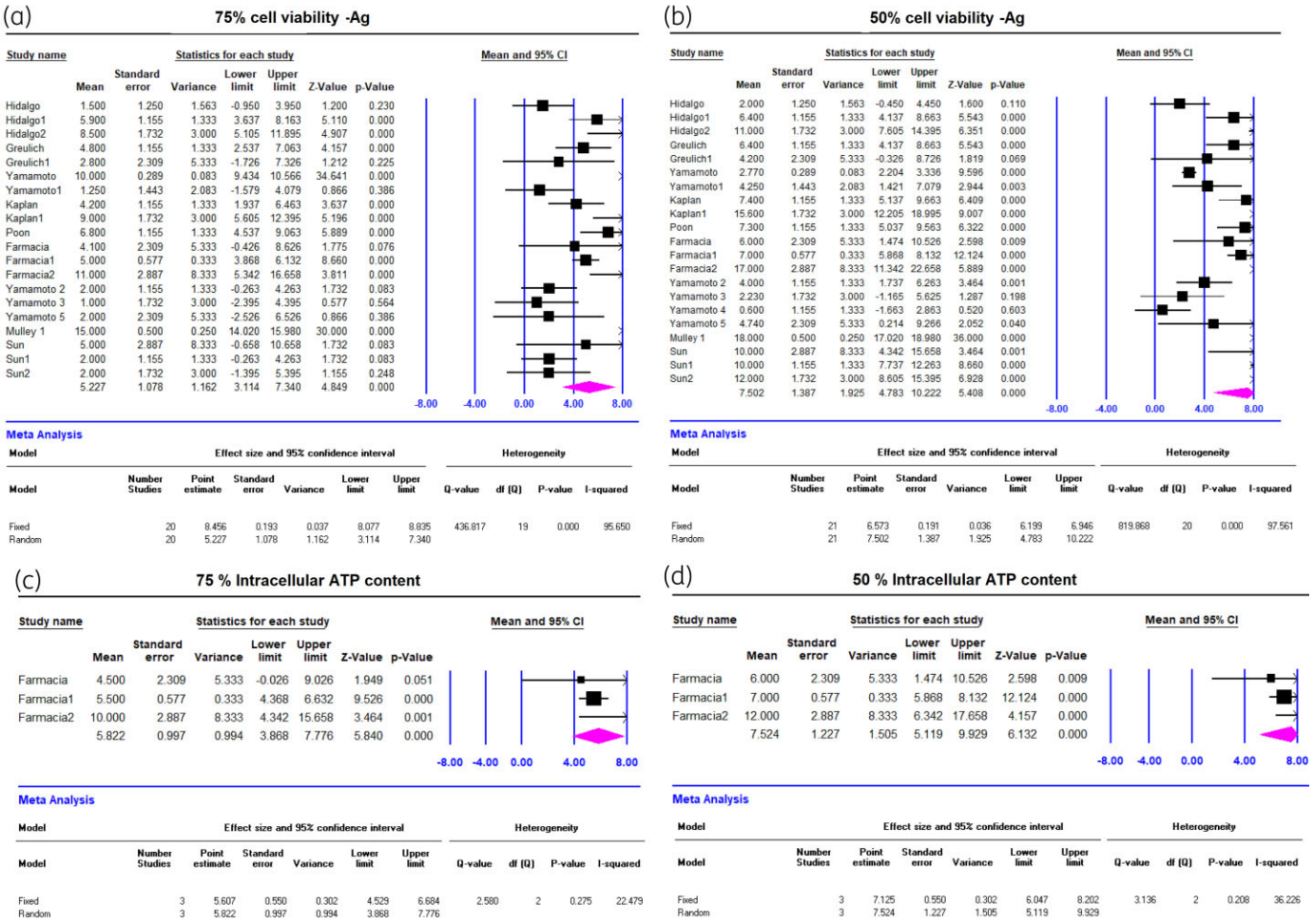


**Figure 5.** Meta-analysis Forest plots for eukaryotic cell cytotoxicity of Ag. (a) 75% cell viability of Ag; (b) 50% cell viability of Ag; (c) 75% intracellular ATP content; and (d) 50% intracellular ATP content. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

the control of *P. aeruginosa* biofilms (Figure 3). Most isolates were resistant to gentamicin and a few isolates were resistant to ciprofloxacin. The individual application of Ag was not effective, with most showing tolerance, and exposing strains to Te alone showed about half the isolates had tolerance. However, when Ag and Te were used in combination, all isolates were shown to be very susceptible. If we compared the susceptibility pattern of clinical isolates with the indicator strain (ATCC 27853) and PAO1, our data showed that clinical isolates are gaining resistance against the antibiotics, especially gentamicin, and Ag, while most are susceptible to Te. The most probable reason for accumulating resistance is the common application of Ag, ciprofloxacin and gentamicin for various approaches such as wound care, dentistry, veterinary, food industry etc.[34–40]

Despite introducing novel effective antimicrobials for MDR isolates, resistance can potentially evolve. Tepekule *et al.*,[9] in a mathematical model study, reported that antimicrobial combinations significantly delayed the emergence of resistance and rate of emergence of resistance in comparison with single antibiotic use. Therefore, we carried out an *in vitro* resistance acquisition test to estimate the future chance of resistance against the Ag/Te combination and compared the outcome if applied individually. Our results showed that resistance under the Ag/Te combination did not occur. Alternatively, Ag resistance occurred after one passage in sublethal Ag, and Te resistance was observed after 3 and 5 days in the different replicates. As a result, the chance of getting resistance against the Ag/Te combination was remarkably delayed when compared with Ag or Te alone. Comparison of $OD_{600}$ values confirmed these findings by showing the population density of resistant cultures approaching the control OD last in the combination application. Therefore, our results support the mathematical model of Tepekule *et al.*, in which combination applications significantly delay the onset of resistance and rate of emergence of resistance in comparison with single antibiotic use.

Many chemicals and materials have strong antibacterial activity, but they have human cell cytotoxicity as well.[41,42] The host cell cytotoxicity and side effects of antimicrobials are important factors in clinical approaches of antibacterials. Several studies have shown the cytotoxic properties of MBAs under different

experimental conditions, including the type of cell line, growth conditions, duration of exposure to the MBA, and the addition of growth supplements.[16,43] Therefore, a wide variety of reports are available for cell cytotoxicity of the MBAs Ag and Te. Thus, we did a systematic review and meta-analysis to summarize all available data to estimate the cytotoxic range of our selected MBAs (Ag and Te). The results showed that Te was less cytotoxic than Ag to eukaryotic cells. Generally, Ag 50% cell viability was 0.0075 mM while for Te it was 0.408 mM. On the other hand, Ag MBIC was 0.250 mM in SWF, while in combination with Te, the effective concentration of Ag decreased to 0.015 mM. Te MBIC was 0.500 mM, while the Ag/Te combination decreased the effective concentration of Te to 0.063 mM. For the biofilm eradication, we observed a similar outcome. Our data showed individual application with Ag or Te might be toxic for the host cells, but the combination application remarkably decreased the effective concentration with the same antibiofilm efficacy and with lower toxicity to the host.

### *Conclusions*

Considering our findings, the clinical application of the Ag/Te combination should be considered. The exploitation of combinations of antimicrobials can reduce working concentrations while synergistically maintaining antibacterial efficacy. Our experimental approach surveyed three different media formulations, including the clinically relevant highly complex SWF medium. Our approach here was to find formulations that could either prevent biofilms or eradicate existing biofilms of *P. aeruginosa*, two different clinical issues requiring different approaches. Additionally, we demonstrate that using combinations of MBAs would help decrease the acquisition of antimicrobial resistance in bacterial communities, an issue causing increasing concern, leading to reduction of efficacy of a variety of chemotherapeutics.[10,44,45]

## Acknowledgements

We thank members of C-Crest Laboratories Inc. for their suggestions on this work.

## Funding

Discovery Grant from the Natural Sciences Engineering Research Council of Canada (NSERC) to R.J.T. Mitacs Elevate Fellowship grant to A.P. from C-Crest Laboratories Inc, Montreal, Quebec, Canada.

## Transparency declarations

A patent has been filed on the resulting formulations; otherwise the authors declare no competing interests.

### *Author contributions*

Conceived and designed the study: R.J.T., A.P.; practical performance: R.J.T., A.P., D.G.; analysed the data: R.J.T., A.P., D.G.; wrote the paper: R.J.T., A.P., D.G.; participated in data analysis and manuscript editing: R.J.T., A.P., D.G. All authors have read and agreed to the published version of the manuscript.

## Supplementary data

Supplementary methods, Tables S1 to S3 and Figures S1 to S10 are available as Supplementary data at *JAC* Online.

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