Antibacterial Efficacy of Liposomal Formulations Containing Tobramycin and N-Acetylcysteine against Tobramycin-Resistant Escherichia coli, Klebsiella pneumoniae, and Acinetobacter baumannii

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Abstract: The antibacterial activity and biofilm reduction capability of liposome formulations encapsulating tobramycin (TL), and Tobramycin-N-acetylcysteine (TNL) were tested against tobramycin-resistant strains of E. coli, K. pneumoniae and A. baumannii in the presence of several resistant genes. All antibacterial activity were assessed against tobramycin-resistant bacterial clinical isolate strains, which were fully characterized by whole-genome sequencing (WGS). All isolates acquired one or more of AMEs genes, efflux pump genes, OMP genes, and biofilm formation genes. TL formulation reduced the MIC of the same isolates to 16 mg/L. TNL formulation was the only effective formulation against all A. baumannii strains compared with TL and conventional tobramycin (in the plektonic environment). Biofilm reduction was significantly observed when TL and TNL formulations were used against E. coli and K. pneumoniae strains. TNL formulation reduced biofilm formation at a low concentration of 16 mg/L compared with TL and conventional tobramycin. In conclusion, TL and TNL formulations particularly need to be tested on animal models, where they may pave the way to considering drug delivery for the treatment of serious infectious diseases.

Keywords: antimicrobial resistance; Escherichia coli; Klebsiella pneumoniae; Acinetobacter baumannii; liposomes; tobramycin; N-acetylcysteine; multidrug-resistant; resistant genes

1. Introduction

Antimicrobial resistance (AMR) has a substantial impact on human health and is becoming a major global health concern [1,2]. AMR has increased globally both in the community and hospital settings, but mainly in intensive-care units (ICUs) [3]. Among antimicrobial-resistant bacteria, Gram-negative bacteria (GNB) form the most serious threat due to the continuous emergence of resistance to almost every class of antibiotics [1]. Multidrug resistance (MDR) is the consequence of multiple bacterial-resistance mechanisms,
such as overexpression of efflux pumps and/or certain outer membrane proteins, as a result of gene mutations that alter the bacterial membrane permeability [4]. Aminoglycosides (AG) remain potent antimicrobial agents with a broad-spectrum activity, and are used to treat severe infections caused by aerobic Gram-negative rods [5,6]. They act by binding to the 30S or 50S ribosomal subunits, leading to mRNA miscoding and protein synthesis inhibition [7,8]. Nevertheless, several bacterial species including *E. coli*, *K. pneumoniae* and *A. baumannii* have developed resistance toward AG’s antibiotics, including tobramycin (Figure 1) [9,10].

**Figure 1.** (a) Chemical structure of tobramycin; (b) chemical structure of N-acetylcysteine.

Meanwhile, *N*-Acetylcysteine (NAC) (Figure 1), an acylated variant of l-cysteine amino acid, is a known antibiotic adjuvant for treating respiratory infections due to its mucolytic activity [11]. Moreover, several reports indicated that NAC has substantial activity against bacterial biofilms [12–15]. Moreover, NAC might protect against aminoglycoside toxicity as reported in several research studies [16–19]. It is well known that all AGs, including tobramycin, are associated with severe nephrotoxicity and ototoxicity [20–22].

Liposomes are nano-scale spherical membranous vesicles composed of lipids and/or phospholipids; the key characteristic of these structures is their naturally occurring single or multiple bilayer membranes, which confer protection of the loaded drug [23]. Since the 1970s, liposomes have gained attention for the use as a drug carrier, due to their low toxicity and ability to encapsulate both hydrophobic and hydrophilic compounds [24]. Currently, liposomes are versatile drug carriers in pharmaceutical industries. Using liposomes as delivery systems has many advantages, such as decreasing the side effects of the loaded drug, improving stability and activity, and enhancing the drug concentrations at the site of infection [25–30]. Moreover, using liposomes to deliver antibiotics at the site of infection decreases the total administered dosage, which limits the evolution of resistance bacteria [24]. The action of the liposomes is quick enough to kill the bacteria even before it can develop resistance [31–33]. As reported, the encapsulation of aminoglycosides into liposomes has improved the therapeutic index of these agents, by increasing the accumulation of the drug in the site of infection [34] and reducing the ototoxicity and nephrotoxicity of the drug [35]. In this study, we investigated the ability of liposomal-encapsulated tobramycin and tobramycin-N-Acetylcysteine to overcome resistance in *Escherichia coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*. This work is the first study that has examined the antibacterial activity of tobramycin and N-acetylcysteine entrapped in liposomes against antimicrobial-resistant Gram-negative bacteria with a known genomic background.

2. Materials and Methods

2.1. Sample Collection, Identification, and Susceptibility Tests

Antimicrobial-resistant *E. coli* (*n* = 7), *K. pneumoniae* (*n* = 9), *A. baumannii* (*n* = 5), and *Staphylococcus aureus* ATCC 29213 reference strains were obtained from the Clinical
Microbiology Laboratory at the Department of Pathology and Laboratory Medicine, King Abdulaziz Medical City (KAMC), Riyadh. Susceptibility testing was performed using the VITEK 2 compact automated system (Biomerieux, Lyon, France). The minimum inhibitory concentration (MIC) of tobramycin was determined using the micro broth dilution method following EUCAST guidelines [36].

2.2. Whole Genome Sequencing and Bioinformatic Analysis

Prior to the genome sequencing, the bacterial DNA was extracted using the MagnaPure compact system (Roche, Basel, Switzerland). DNA library was constructed using Nextera XT Library Prep Kit (Illumina, San Diego, CA, USA). Short-read sequences were generated using the Illumina MiSeq System (Illumina, San Diego, CA, USA) with the $2 \times 300$ bp paired-end protocol. The antimicrobial resistant genes and virulence factors were identified using ABRicate (version 0.9.8) (Seemann T, Github https://github.com/tseemann/abricate, accessed on 10 November 2021) [37] with the Megares [38], Resfinder [39] and virulence factors database (VFDB) [40].

2.3. Preparation and Characterization of the Tobramycin Liposomes (TL) and Tobramycin-N-Acetylcysteine Liposomes (TNL) Formulations

The liposome nanovesicles were prepared by the rehydration-rehydration vesicles (DRV) method of Alhariri et al. [41]. Basically, the liposomes were prepared by mixing 1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and cholesterol (UFC Biotechnology, Amherst, NY, USA) in the molar ratio 4:2:1 in chloroform. A Rotavapor® R-300 (BÜCHI, Flawil, Switzerland) was used to evaporate the chloroform until a lipid film formed, followed by a stream of nitrogen gas for 5 min, to flush any traces of chloroform. The liposomal vesicles were prepared by dissolving 1 mg tobramycin in PBS, pH 7.4, and 1 mg of tobramycin and 50 mM of N-acetylcysteine (NAC) in PBS to form the tobramycin liposome (TL), and the tobramycin-NAC liposome (TNL) formulations. The formulations were homogenized by sonication (Ultrasonic processor UPS 125, Maharashtra, India) for 3 min using cycles of a 10 s run and 2 s pause. The Zetasizer (Malvern, UK) was used to determine the size of the prepared liposomes. The liposomes were washed with PBS by centrifugation for 20 min at maximum speed, and then lyophilized for 48 h in the CHRIST lyophilizer (Osterode am Harz, Germany). The liposomes were reconstructed by gradually adding 10% of the original volume of PBS.

2.4. Tobramycin Encapsulation Efficiency (EE%) of the TL and TNL Formulations

A microbiological assay was used to measure the tobramycin encapsulated inside the liposomal formulations. Overnight bacterial cultures of S. aureus ATCC 29213 were first adjusted to 0.5 MacFarland and then diluted at $5 \times 10^5$ CFU/mL in 250 mL of Mueller-Hinton agar (HIMEDIA) and cooled down to 45 °C. The suspensions were later poured into square culture plates. Triton X-100 (UFC biotechnology, Amherst, NY, USA) (0.2%) was used to lyse the liposomes and release the encapsulated antimicrobial agents for 30 min at 37 °C. After solidification of the seeded plate, 6 mm diameter wells were made and filled with 20 µL of the liposome lysate. The plates were incubated at 37 °C for 24 h, and the average of the inhibition zones was measured. In addition, the quantification of the tobramycin was also performed for the confirmation of the results by using an ultra-high-performance liquid chromatography–tandem mass spectrometer (UHPLC-MS/MS). The UHPLC system consisted of an LPG-300RS quaternary rapid separation pump with an integrated degasser, WPS-300TRS autosampler, TCC-300RS Column compartment and Xcalibur™ 4.3 software (Revision A, Thermo Fisher Scientific, Waltham, MA, USA) [42]. All samples were centrifuged, filtered through 0.22 µm filters and, in some cases, diluted before analysis. Separation was done with a Thermo Scientific™ Syncronis™ C18 column (100 × 2.1 mm, 3 µm particle size). The oven temperature was maintained at 40 °C, and the mobile phase was LC/MS grade, water plus 0.1% formic acid (A) and methanol, plus 0.1% formic
acid (B). A linear gradient program was used at a flow rate of 0.300 mL/min: 0.0–2.0 min 2% (B), 2.0–5.0 min from 2% to 98% (B), 5.0–6.0 min from 98% (B), 6.5–9.0 min from 98% to 2% (B), and finally 7.0–10.0 min 2% (B). The identification and quantification of tobramycin was performed on a triple quadrupole mass spectrometer (TSQ Altis, Thermo Fisher Scientific). The mass spectrometer was equipped with an electrospray ionization (ESI) source, which was operated at the following conditions: gas temperature was 300 °C, sheath gas 50, aux gas 10, capillary voltage: 3500 V, and argon gas was used for the collision cell. Tobramycin was detected in an ESI positive mode at a retention time Rt = 0.59 min and quantified using selected reaction monitoring (SRM). The transition ions (m/z) associated with tobramycin were 468 → 163 (22 eV), 468 → 205 (21 eV) and 468 → 324 (14 eV). A standard calibration curve (R2 = 0.993) was created using eight different concentrations of tobramycin, ranging from 200 to 1000 ppb.

The following equation was used to calculate the encapsulation efficiency:

\[
EE\% = \frac{\text{Concentration of encapsulated drug}}{\text{Initial concentration of drug}} \times 100
\]  

2.5. The Stability of the TL and TNL Formulations in Biological and Storage Conditions

Anonymous patient samples of plasma and sputum were obtained after the routine work was done, and before discarding the samples from the Medical Laboratory, King Abdulaziz Medical City, National Guard Hospital, Riyadh, Saudi Arabia. The sputum samples were diluted 1:10 (w/v) in PBS before autoclaving. The retention of tobramycin in the prepared liposomes was tested at 37 °C in plasma and sputum, and at 4 °C and 37 °C in PBS. The samples were collected at the time intervals 0, 1, 6, 12, 18 and 24 h, and were harvested and centrifuged at 4 °C at 20,000 rpm. The concentration of tobramycin in the supernatants obtained was checked with the agar well-diffusion method, as described in the encapsulation efficiency (EE%) section.

The following equation was used to calculate the retention of the drugs:

\[
\text{Retention of encapsulated drug} = \frac{\text{Initial concentration} - \text{released concentration}}{\text{Initial concentration}} \times 100
\]

2.6. Antibacterial Activity of TL and TNL Formulations

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the free form of tobramycin, TL and TNL formulations were tested with the microbroth dilution method. Serial dilution (range 1024–16) of the free tobramycin, and the liposomal formulations prepared in the Mueller-Hinton broth were checked on overnight bacterial cultures that were diluted at 5 × 10^5 CFU/mL. All samples were seeded on Mueller-Hinton agar for the next day to determine the MBC.

\[
\text{Decrease}\% = \frac{\text{Decrease}}{\text{New number}} \times 100
\]

2.7. Biofilm Reduction of TL and TNL Formulations

The biofilm reduction assay was performed as described by Paula-Ramos et al. with minor modifications [43]. Fresh bacterial cultures, grown in Mueller-Hinton broth, were adjusted at the 0.5 MacFarland standard, diluted (1:100) into fresh media and then incubated in flat-bottom 96-well plates for 72 h at 37 °C in a shaking incubator at 75 rpm. Planktonic cells were removed by washing twice with sterile dH2O. After washing, 100 µL of Mueller-Hinton broth was added to each well and the plates were incubated again for 48 h.

After 72 h of incubation, the planktonic cells were removed by washing twice with water, and the treatments were added as follows. The biofilms were treated with the MIC concentration of each isolate and incubated for 24 h at 37 °C, and 50 mM of NAC and the MIC of tobramycin were tested as well.
The biofilms were stained with 0.1% crystal violet at room temperature for 15 min and washed with sterile distilled water. The plates were dried at room temperature and the biofilms were solubilized though incubation with 99% of ethanol for 15 min. The suspension was transferred to a new plate and the optical density (OD) was measured at 570 nm using a SpectraMax (Molecular Devices, San Jose, CA, USA) plate reader. The reduction percentage was calculated as follows:

\[
\text{Decrease} = \text{Original number (OD)} - \text{New number (OD)}
\] (4)

The original number was the positive biofilm control (no treatment) and the new number obtained after the treatment.

2.8. Statistical Analysis

The results were analyzed with XY tables with Graphpad Prism 9 software, (Version 9.3.0) [44]. ANOVA one-way analysis was used to compare the groups and measure the \(p\)-value. Note: no significant (ns) = \(p > 0.05\), significant = \(*p \leq 0.05\), very significant = \(**p \leq 0.01\), and highly significant = \(***p \leq 0.001\). All experiments were done in triplicate.

3. Results

3.1. Susceptibility Profiles of the E. coli, K. pneumoniae, and A. baumannii Clinical Isolates

A total of 21 tobramycin resistant clinical isolates were examined in this study. The isolates included E. coli \((n = 7)\), K. pneumoniae \((n = 9)\), and A. baumannii \((n = 5)\).

The results of the Antimicrobial Susceptibility Testing (AST) using the VITEK 2 compact automated system for E. coli, K. pneumoniae, and A. baumannii are provided in the Supplementary Materials. All the E. coli isolates were identified as multidrug-resistant organisms (MDRO) (Table S1 from Supplementary Materials). All the tested K. pneumoniae isolates were identified as pan-drug-resistant organisms (PDR), except for isolate KP_086 and KP_095, which were classified as extensively drug-resistant organisms (XDR) (Table S2). Regarding the A. baumannii isolates, all the isolates were completely resistant to all the tested agents belonging to the third and fourth cephalosporins, beta-lactamase inhibitors, carbapenems, aminoglycosides and fluoroquinolone classes. All of the A. baumannii isolates were identified as XDR organisms (Table S3) [45].

3.2. Whole Genome Sequencing and Bioinformatic Analysis

The whole genome sequencing analysis detected the presence of multiple outer membrane proteins, efflux pump genes, and genes involved in the formation and development of a biofilm in all the tested organisms. Among the detected OMPs, ompA in E. coli, A. baumannii, and K. pneumoniae isolates, and omp37 in K. pneumoniae. Furthermore, the efflux pump gene acrD was detected in all E. coli and K. pneumoniae isolates, with several other efflux pump genes. On the other hand, the majority of ade efflux pump genes were present in A. baumannii isolates. Biofilm formation genes were detected as well, including bap, csu, and PNAG, in A. baumannii, and csg genes in E. coli (Only the major genes are listed in Table 1, for more details please refer to Table S4).

Moreover, the presence of the aminoglycoside-modifying enzyme genes (AMEs) was detected in all the tested organisms. One or more of the AMEs, particularly the tobramycin-modifying enzymes, were detected in the isolates including N-acetyltransferases (ACC), O-adenyltransferases (ANT), O-phosphotransferases (APH), and methyltransferase armA and rmtF (for more details please refer to Table S5).
Table 1. Remarkable detected genes: outer membrane genes, efflux pump genes and biofilm formation genes in the tested organisms, (Table S4).

| Category               | *Escherichia coli* | *Acinetobacter baumannii* | *Klebsiella pneumoniae* |
|------------------------|-------------------|---------------------------|------------------------|
| Outer membrane proteins (OMPs) | *ompA*  | *ompA* | *ompA*, *omp37* |
| Efflux pumps           | *acrA*, *acrB*, *acrD*, *acrE*, *acrF*, *acrS*, *mdtA-C*, *mdtE-K*, *mdtM-P* | *adeA-C*, *adeL*, *adeK*, *adeR*, *adeT1*, *adeT2*, *abeM*, *abeS* | *acrA*, *acrB*, *acrD* |
| Biofilm formation      | *csgB*, *csgD*, *csgF*, *csgG* | *pgaA-D*, *csuA-D*, *bap*, *csgB*, *csgD*, *csgF*, *csgG* | - |

3.3. TL and TNL Formulation Characterization

In the current study, we succeeded in formulating liposomes with a size less than bacterium size; the size results illustrated that our prepared TL and TNL formulations were 347.33 ± 62.27 and 229.47 ± 47.57 nm, respectively (Table 2). The polydispersity index (PDI) of our formulations tended to be more heterogenic in size, as the PDI for TL and TNL were 0.85 and 0.68, respectively, representing the diverse particle sizes (Table 2).

Table 2. The average of triplicate reads of the particles size and PDI.

| Formula | Peak       | PDI   |
|---------|------------|-------|
| TL      | 347.33 ± 62.27 | 0.85  |
| TNL     | 229.47 ± 47.57 | 0.68  |

The encapsulation efficiency percentage (EE%) of tobramycin inside the TL and TNL formulations were 7.1 and 12.8, respectively. The TNL had a greater encapsulation efficiency of tobramycin and consequently, a higher released concentration, 127.6 mg/L compared with 71 mg/L for TL (Table 3).

Table 3. The Encapsulation efficiencies EE% and the released concentrations of the prepared formulations.

| Liposomal Formulations | Entrapped Concentration (mg/L) | EE% |
|------------------------|--------------------------------|-----|
| TL                     | 71                             | 7.1 |
| TNL                    | 127.6                          | 12.8|

TL: tobramycin liposomes. TNL: tobramycin-N-acetylcysteine liposomes.

3.4. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Tobramycin against the Clinical Bacterial Isolates

The *E. coli*, *K pneumoniae*, and *A. baumannii* isolates were highly resistant to conventional tobramycin with MICs and MBCs ranging between 32–2048 mg/L (Table 4). The MIC of the conventional tobramycin against the *E. coli* isolates of EC_077, EC_089, EC_162, and EC_219 was at 64 mg/L, and able to eradicate these isolates (MBC) at 128 mg/L. Moreover, the EC_057, EC_068, and EC_083 *E. coli* isolates were suppressed and eradicated by conventional tobramycin at 32 mg/L (MIC) and 64 mg/L (MBC).
Table 4. The MIC and MBC of tobramycin, TL, and TNL against *E. coli*, *K. pneumoniae*, and *A. baumannii*.

| Isolate ID | Tobramycin (mg/L) | TL (mg/L) | TNL (mg/L) |
|------------|------------------|-----------|------------|
|            | MIC | MBC | MIC | MBC | MIC | MBC |
| *E. coli* clinical isolates |     |     |     |     |     |     |
| EC_057     | 32  | 64  | 32  | 64  | 16  | 32  |
| EC_068     | 32  | 64  | 32  | 64  | 16  | 32  |
| EC_077     | 64  | 128 | 32  | 64  | 16  | 32  |
| EC_083     | 32  | 64  | 32  | 64  | 16  | 32  |
| EC_089     | 64  | 128 | 8   | 16  | 16  | 32  |
| EC_162     | 64  | 128 | 32  | 64  | 16  | 32  |
| EC_219     | 64  | 128 | 32  | 64  | 32  | 64  |
| *K. pneumoniae* clinical isolates |     |     |     |     |     |     |
| KP_002     | 1024 | 2048 | 8   | 16  | 16  | 32  |
| KP_017     | 512  | 1024 | 32  | 64  | 32  | 64  |
| KP_019     | 32  | 64  | 16  | 32  | 32  | 128 |
| KP_026     | 1024 | 2048 | 8   | 16  | 16  | 32  |
| KP_050     | 1024 | 2048 | 8   | 16  | 16  | 32  |
| KP_057     | 1024 | 2048 | 16  | 32  | 16  | 32  |
| KP_059     | 1024 | 2048 | 16  | 32  | 16  | 32  |
| KP_086     | 256  | 512  | 16  | 32  | 32  | 64  |
| KP_095     | 1024 | 2048 | 16  | 32  | 32  | 64  |
| *A. baumannii* clinical isolates |     |     |     |     |     |     |
| RAB_005    | 128  | 256  | 128 | 256 | 16  | 32  |
| RAB_009    | 128  | 256  | 128 | 256 | 16  | 32  |
| RAB_014    | 128  | 256  | 128 | 256 | 16  | 32  |
| RAB_030    | 128  | 256  | 128 | 256 | 16  | 32  |
| RAB_055    | 128  | 256  | 128 | 256 | 16  | 32  |
| *S. aureus* ATCC 29213 | 4   | 8   |     |     |     |     |

*This was done for validation purposes.

For the *K. pneumoniae* isolates, the majority of the isolates were extremely resistant to conventional tobramycin. For instance, the growth of isolates KP_002, KP_026, KP_050, KP_057 and KP_95 were inhibited (MIC) at 1024 mg/L and eradicated at 2048 mg/L (MBC). The MIC were 32, 256 and 512 mg/L, for the isolates KP_019, KP_086, and KP_017, the MBC were 64, 512, and 1024 mg/L, respectively. Lastly, all the *A. baumannii* isolates were resistant to the conventional tobramycin at 128 mg/L MIC and 256 mg/L MBC.

3.5. TL and TNL Formulations Stability within Biological and Storage Conditions

The TNL formulation was highly stable, and time seemed to have little or no effect on the drug release of the liposomes (Figure 2). The TL formulation had a lower stability than our TNL, but maintained a sustained drug release over the 24 h (Figure 3). Of the tested environments, plasma had a constant drug release starting at 79% at hour 1, and 80.7% at hour 24. The same phenomenon was observed with sputum, except for a slight decrease at hour 6 (77.8%). Regarding the stability of TL in PBS at 37 °C and 4 °C, the released drug varied. The highest retention percentage for TL at PBC 4 °C was 81.8% at hour 24, and the lowest was 77.1% at hour 18. For the PBS incubated at 37 °C, the highest retention percentage was 84.18% at hour 18, and the lowest was 77.8% at hour 12. No significant differences were detected in the stability of all the tested conditions for both TL and TNL formulations (p value > 0.05).
3.6. The Antibacterial Activity of the TL and TNL Formulations against the Genetically Resistant Clinical Bacterial Isolates

The TL and TNL formulations were tested for their minimum inhibitory and bactericidal activity against the multi-drug resistant *E. coli*, *K. pneumoniae*, and *A. baumannii*. The TL and TNL formulations reduced the MIC and MBC against the majority of the isolates (Table 4). The encapsulation of tobramycin inside the TL and TNL formulations improved its activity against seven isolates of *E. coli*. The MIC of these isolates decreased one-fold or two-fold, alternatively, in the cases of EC_057, EC_068, EC_083, EC_162 and EC_219. For EC_089, the MIC significantly decreased ~3-fold. The encapsulation of tobramycin and NAC decreased the MIC of *E. coli*, approximately one-fold against EC_057, EC_068 and SA0219, and two-fold against EC_089 and EC_162. The isolate EC_089 was positive for the detected genes, although it did not harbor any of the tobramycin-modifying enzymes. The MIC of the free tobramycin for this isolate was 64 mg/L, and the encapsulation of tobramycin in the liposomal formulation TL decreased the MIC three-fold (8 mg/L). The co-encapsulation of tobramycin and NAC in a liposomal formulation (TNL) decreased two-fold for this particular isolate (MIC = 16 mg/L).

Overall, the TL had a greater activity against *K. pneumoniae* than *E. coli*, which may be due to the different genetic profile of the two species. The decreased MIC of the TL against *K. pneumoniae* was remarkable. Six isolates were highly resistant to tobramycin (MIC 1024 mg/L) and the MIC decreased six-fold against KP_002, KP_026, KP_050, KP_057, KP_059 and KP_095. The activity was also observed for the TNL.

All the isolates of *A. baumannii* were highly resistant to tobramycin, with a MIC of 128 mg/L. The tested *A. baumannii* isolates were not affected by the TL formulation with no improvement, as the MIC results of the TL formulation were comparable to the conventional tobramycin. The TNL had a great activity against all the *A. baumannii*
isolates (MIC = 16 mg/L). This phenomenon could indicate the possible synergy between tobramycin and N-acetylcysteine, particularly against *A. baumannii*.

3.7. Biofilm Reduction Activity of TL and TNL Formulations against Clinical Bacterial Isolates

In this study, we tested the reduction activity of the liposomal formulations against mature biofilms formed by the selected Gram-negative clinical bacterial isolates with genetical mutations.

The most significant results were observed against biofilms formed by the *E. coli* isolates. The current study confirmed that treatment with 50 mM of the free form of NAC can reduce mature biofilms formed by EC_162 (*p* value = 0.0003) and EC_219 (*p* value = 0.0008) isolates by 48.35% and 33.82%, respectively (Figures 4 and 5). However, encapsulating NAC and tobramycin inside liposomes increased the reduction percentage to 77.18% and 72.04% against the same isolates. The same was observed for biofilms formed by *K. pneumoniae* (Figures 6 and 7). The encapsulation of tobramycin inside liposomal formulations improved its reduction percentage against all of the tested isolates. For *K. pneumoniae* biofilms, the reduction percentage of KP_050 and KP_059, when treated with free NAC, were 36.15% and 14.22%, which increased to 72.71% and 68.20% when treated with TNL, (Figures 6 and 7). The *A. baumannii* strains exhibited a higher sensitivity to the TNL formulation, though the strains remained resistant to conventional tobramycin or encapsulated tobramycin inside a liposomal formulation (TL). Interestingly, the liposomal formulations that contained the TNL were able to maintain the biofilms of *A. baumannii* strains at a very low concentration (16 mg/L), and the other drug forms did the same, but at higher concentration (128 mg/L) (Figures 8 and 9).

**Figure 4.** Biofilm reduction assay for isolate EC_162. (*p* value = 0.0003, *** highly significant; error bars reflect the standard error of the mean (SEM)).
Figure 5. Biofilm reduction assay for isolate EC_219. (p value = 0.0008, *** highly significant; error bars reflect the SEM).

Figure 6. Biofilm reduction assay of isolate KP_050. (p value = 0.0343, * significant; error bars reflect the SEM).
Figure 7. Biofilm reduction assay of isolate KP_059. ($p$ value = 0.0411, * significant; error bars reflect the SEM).

Figure 8. Biofilm reduction assay of isolate RAB_005. ($p$ value = 0.0027, ** very significant; error bars reflect the SEM).
Figure 9. Biofilm reduction assay of isolate RAB_009. (p value = 0.0027, ** very significant; error bars reflect the SEM).

4. Discussion

Escherichia coli, Klebsiella pneumoniae, and Acinetobacter baumannii are bacterial threats for public health, due to the raised concern of antibiotic resistance [46–49]. Tobramycin is a family member of aminoglycoside antibiotics, which have been used for treating infections caused by the abovementioned pathogens [50–52]. Scientifically, it has been proven that these bacteria are aggressively resistant to antibiotics in biofilm mode [53–55]. As documented, several research groups have reported the activity of NAC against bacterial biofilm formation, as well as its antibacterial activities [12–14], [56–59]. Furthermore, it has been reported by numerous research groups that NAC can be encapsulated inside liposomal formulations [60–65]. Therefore, we considered modifying the conventional tobramycin formula by using drug delivery systems; we are the first research group to have developed tobramycin with NAC inside the liposomal formulation. We have reported here the superior activity of our liposomal NAC formulation (TNL), as an antibacterial formula that has the mucolytic ability to reduce bacterial biofilm formations and reduce tobramycin resistance.

In this study, we used the multidrug-resistant organisms (MDRO) of E. coli isolates, the pan-drug resistant organisms (PDR) of K. pneumoniae isolates, and drug-resistant organisms (XDR) of A. baumannii isolates. The results shown in the Supplementary Materials sheets show that the TL and TNL formulations were able to reduce bacterial resistance phenomenally, as shown in Table 3. We investigated the genetic variations of these particular bacteria, in order to explain the antibacterial activity of our liposomal NAC formulations. We found that the outer membrane proteins (OMPs), including ompA and the efflux pump acrABD-tolC, were present in all seven E. coli isolates. Notably, the AcrAD-TolC and KDPE, and MTD efflux pumps were also present in all tested E. coli isolates. These pumps are responsible for the resistance to multiple aminoglycosides agents, including tobramycin. The deletion of the transporter gene (arcD) results in a reduction of MIC for the aminoglycoside agents, including tobramycin, gentamycin, amikacin, kanamycin, and neomycin [66,67]. The whole-genome data revealed that all the tested E. coli isolates were positive for the...
presence of acrD, and all were highly resistant to tobramycin. The operon genes, including csgA, csgB, csgD, csgF, and csgG, were also detected in all the E. coli clinical isolates, and were involved in the formation and development of a biofilm. Curli fimbriae (csgA) are surface protein genes that are essential for many functions, including adhesion, cell aggregation, and biofilm formation [68]. In addition, mutations of the csgA gene can cause a defect in the bacterial surface attachment ability and biofilm formation [69]. (Table S4). In addition, all K. pneumoniae clinical isolates expressed different outer membrane protein (OMP) genes, such as omp37 and ompA. Many multidrug efflux pump genes were also detected, including acrA, acrB, acrD, mdt, and emrD. (Table S4).

With regard to the A. baumannii isolates, genetic data revealed that they already contained all the genes listed above, the same as E. coli and K. pneumoniae, except for one efflux pump system. (Table S4). Interestingly, we observed that the outer membrane A genes (ompA), which have a major role in the adherence, invasion, and biofilm formation of A. baumannii, were present in all the isolates. Moreover, we observed the presence of two of the three efflux pump systems in all the isolates, namely adeABC and adeFGH. The AdeABC genes, adeA, adeB and adeC, were present in all the A. baumannii isolates, and all were associated with a high level of resistance to tobramycin. As mentioned in the Table S4, the overexpression of these pumps plays a role in biofilm formation in the clinical isolates of A. baumannii [70].

Furthermore, the presence of the aminoglycoside-modifying enzyme genes (AMEs) were also detected in the E. coli, K. pneumoniae, and A. baumannii. In the E. coli (Table S5), one or more of the AMEs, particularly the tobramycin-modifying enzymes, were detected in the isolates. However, in the isolate EC_089, none of the tobramycin modifying enzymes were detected, even though this particular strain displayed remarkable resistance to tobramycin, recorded at 64 and 128 mg/L for MIC and MBC, respectively. Notably, we detected other aminoglycoside-modifying enzymes for this isolate (EC_089), including N-acetyltransferases (ACC) aac(3)-Ila and aac(6')-Ib-cr, O-adenyltransferases (ANT), such as ant(2’)-Ia, and O-phosphotransferases (APH) which included aph(6)-Id and aph(3’)-Ib. Similarly, the same occurred in the K. pneumoniae isolates, and all the isolates were encoded with more than one of the AME genes. The isolates KP_002, KP_019, KP_045, KP_050, KP_057, KP_059, and KP_095 were encoded with the aminoglycoside resistant methyltransferase (armA) gene, except for one isolate, KP_017, which harbored the rmtF gene. (Table S5). Likewise, A. baumannii isolates were not different from the E. coli and K. pneumoniae isolates. All the isolates harbored the aph(6)-Id, aph(3’)-Ib, and armA (Table S5).

Finally, in terms of the biofilm formation genes, we detected several genes including the biofilm-associated protein (bap), Csu fimbriae (csuA, csuA/B, csuB, csuC, csuD, csuE), and PNAG (pgaA, pgaB, pgaC, pgaD). In addition, the bfmRS two-component system and abalR quorum-sensing system were present in all three bacterial isolates.

On the other hand, the sizes of the liposome formulations were expected to range from 25 nm to 2.5 µm, based on which they were classified as small (≤100 nm), intermediate (100–250 nm), large (≥250 nm) or giant (>1 µm) [71,72] The average size of bacterial cells is approximately 1 µm, and for liposomal formulations to fuse properly with the bacterial membrane and release their contents, they must be smaller than the bacterial cells. The size of the nanoparticle is an important factor in drug delivery to eukaryotic cells. It contributes to the tissue distribution, pharmacokinetics, and clearance of these delivery systems. The size of the nanocarriers differs based on the route of administration, for example, for intravenous administration, the particle size can be ranged between 200 nm and 2000 nm [73,74].

As reported by Messiaen et al., 2013, the size of the prepared tobramycin liposomes were 426.3 (±26.4) and 228.5 (±34.9), making the sizes of the TL and TNL average [71]. However, the size of the TNL liposomes were closer to those described by Hasanin and others, which had a mean diameter of 200 nm [72,75]. The polydispersity index (PDI), also known as the heterogeneity index, is a description of the size distribution in the tested sample. The PDI values range from 0.0 (homogeneity) and 1.0 (heterogeneity) [76,77].
Our encapsulation concentration was close to the previously reported DPPC/cholesterol-tobramycin liposomes by Messiaen et al., 2013. Their encapsulation was 141 (±35) ug/mL, and higher than the liposomes reported by Halwani et al., 2008 (0.2 mg/mL) [30,71]. The stability tests of the TL and TNL formulations supported a better understanding of the drug release in different biological and storage environments. Though the stability tests of our formulation gave us a better understanding of the drug release in different biological and storage environments, the stability of the TL formulation can be improved by implementing different preparation methods or changing the physical and chemical conditions of the current preparation method, such as heat and pH levels, to increase the entrapment of the drugs [78].

Overall, MIC and MBC of the TL and TNL were significantly decreased compared with the conventional tobramycin, which indicates that we succeeded in enhancing the antibiotic activity by using liposomes as a drug vehicle. Prior studies have also succeeded in improving the delivery of conventional tobramycin [28,76]; for instance, Marier et al., 2003, used liposomal tobramycin to treat pulmonary infections caused by *Pseudomonas aeruginosa* in rats [75]. Moreover, tobramycin liposomes exhibited strong bactericidal activity against a large range of resistant bacteria, including Gram negative bacteria [79].

This could be due to the presence of the *armA* gene (the aminoglycoside resistant methyltransferase), in all the *A. baumannii* isolates, which confers a high level of resistance to a wide range of aminoglycoside agents, including tobramycin [80–82]. This gene functions at the target site through methylation, which prevents the drug from recognizing its target site [80]. Biofilms are a community of bacteria embedded in a self-produced extracellular polymeric substance. The complex structure of biofilms prevents the entry of most antibiotics, and can mediate the adhesion of bacteria to various surfaces. Bacteria within biofilm can be up to 1000 times more resistant than its planktonic phenotypes [83]. It is known that NAC has antibiofilm activity. The activity includes several mechanisms, including the reduction of the biofilm formation process, the reduction of the matrix production or the disruption of the formed biofilms as in the current study [12,58,84,85]. Scientifically, the N-acetyl cysteine activity may have played a role as an antibiofilm and mucolytic agent [11,12,19]. The increased reduction activity of the encapsulated tobramycin compared with free tobramycin was reported by Sans-Serramitjana et al., (2017), who reported that the encapsulation resulted in a decreased minimal biofilm eradication concentration (MBEC) of the used drug for all tested *P. aeruginosa* isolates [86]. These results are compatible with our results; the encapsulation of tobramycin increased the reduction activity against the tested organisms. The activity of tobramycin in the liposome forms were also reported against *Burkholderia cepacia* biofilms [71]. A study by Marchese et al., was conducted to test the antibiofilm activity of NAC alone, or with antibiotics on biofilms formed by *E. coli* isolates. The results were similar to the current study [85]. In addition to the NAC inhibition of the biofilm matrix production in all the tested *E. coli* isolates, they found that NAC at concentrations between 2 and 8 mg/mL disrupted mature biofilms. The highest reduction % against *E. coli* was 60% [85].

5. Conclusions

The encapsulation of tobramycin and N-acetylcysteine successfully reduced the MIC of the resistant high-risk Gram-negative pathogens. In comparison with the conventional form of tobramycin, the encapsulated tobramycin in liposomal (TL) and N-acetylcysteine-liposomal (TNL) formulations increased the antibacterial activity against the tested pathogens. The TL and TNL formulations reduced the biomass of the biofilms. Using liposomes as delivery systems may enhance the treatment of infections caused by multidrug-resistant high-risk pathogens. In addition, the encapsulation efficiency and the stability of the prepared formulations can be improved by assessing other preparation methods.
Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pharmaceutics14010130/s1. Table S1. MIC of *E. coli* isolates by VITEK. Table S2. MIC of *K. pneumoniae* isolates by VITEK. Table S3. MIC of *A. baumannii* isolates by VITEK. Table S4. Detected efflux pumps, outer membrane, and biofilm formation genes in *E. coli, K. pneumoniae* and *A. baumannii*. Table S5. Detected Aminoglycosides modifying enzymes, 16S rRNA methyltransferases, and other genes in *E. coli, K. pneumoniae* and *A. baumannii*.

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16. Kranzer, K.; Elamin, W.; Cox, H.; Seddon, J.; Ford, N.; Drobniowski, F. A systematic review and meta-analysis of the efficacy and safety of N-acetylcysteine in preventing aminoglycoside-induced ototoxicity: Implications for the treatment of multidrug-resistant TB. *Thorax* 2015, 70, 1070–1077. [CrossRef]

17. Tepel, M. N-Acetylcysteine in the prevention of ototoxicity. *Kidney Int.* 2007, 72, 231–232. [CrossRef]

18. Somdas, M.A.; Korkmaz, F.; Gurgen, S.G.; Sagit, M.; Akcadag, A. N-acetylcysteine Prevents Gentamicin Ototoxicity in a Rat Model. *J. Int. Adv. Otol.* 2015, 11, 12–18. [PubMed]

19. Feldman, L.; Efрат, S.; Eviatar, E.; Abramsohn, R.; Yarovsky, I.; Gersch, E.; Averbukh, Z.; Weissgarten, J. Gentamicin-induced ototoxicity in hemodialysis patients is ameliorated by N-acetylcysteine. *Kidney Int.* 2007, 72, 359–363. [CrossRef] [PubMed]

20. Coca, A.; Martinez, A.; Soriano, E.; Blade, J.; Segura, F.; Ribas-Mundo, M. Tobramycin nephrotoxicity. A prospective clinical study. *Postgrad. Med.* J. 1979, 55, 791–796. [CrossRef] [PubMed]

21. Kahlmeter, G. Gentamicin and Tobramycin Clinical Pharmacokinetics and Nephrotoxicity Aspects on Assay Techniques. *Scand. J. Infect. Dis.* 1979, 11, 1–40. [CrossRef] [PubMed]

22. Wargo, K.A.; Edwards, J.D. Aminoglycoside-Induced Nephrotoxicity. *J. Pharm. Pract.* 2014, 27, 573–577. [CrossRef] [PubMed]

23. Kraft, J.C.; Freeling, J.P.; Wang, Z.; Ho, R.J. Emerging Research and Clinical Development Trends of Liposome and Lipid Nanoparticle Drug Delivery Systems. *J. Pharm. Sci.* 2014, 103, 29–52. [CrossRef]

24. Gomez, A.G.; Hosseimdoust, Z. Liposomes for Antibiotic Encapsulation and Delivery. *ACS Infect. Dis.* 2020, 6, 896–908. [CrossRef] [PubMed]

25. Mugabe, C.; Halwani, M.; Azghani, A.O.; Lafrenie, R.; Omri, A. Mechanism of Enhanced Activity of Liposome-Entrapped Aminoglycosides against Resistant Strains of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 2006, 50, 2016–2022. [CrossRef] [PubMed]

26. Drulis-Kawa, Z.; Dorotkiewicz-Jach, A. Liposomes as delivery systems for antibiotics. *Int. J. Pharm.* 2010, 387, 187–198. [CrossRef]

27. Fresta, M.; Spadaro, A.; Cerniglia, G.; Ropero, I.M.; Puglisi, G.; Furneri, P.M. Intracellular accumulation of ofloxacin-loaded liposomes in human synovial fibroblasts. *Antimicrob. Agents Chemother.* 1995, 39, 1372–1375. [CrossRef] [PubMed]

28. Sachetelli, S.; Khalil, H.; Chen, T.; Beaulac, C.; Sénéchal, S.; Lagacé, J. Demonstration of a fusion mechanism between a fluid bactericidal liposomal formulation and bacterial cells. *Biochim. Biophys. Acta Biomembr.* 2000, 1463, 254–266. [CrossRef]

29. Alexis, F.; Pridgen, E.M.; Langer, R.; Farokhzad, O.C. Nanoparticle Technologies for Cancer Therapy. In *Drug Delivery*; Springer: Berlin/Heidelberg, Germany, 2009; Volume 197, pp. 55–86.

30. Halwani, M.; Yeibo, B.; Suntres, Z.E.; Alipour, M.; Azghani, A.O.; Omri, A. Co-encapsulation of gallium with gentamicin in liposomes enhances antimicrobial activity of gentamicin against *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 2008, 62, 1291–1297. [CrossRef]

31. Huang, C.-M.; Chen, C.-H.; Pornpattananangkul, D.; Zhang, L.; Chan, M.; Hsieh, M.-F.; Zhang, L. Efficacy of neutral and negatively charged liposome-loaded gentamicin on planktonic bacteria and biofilm communities. *Curr. Med. Chem.* 2017, 24, 70–78. [CrossRef]

32. Pelgri, R.Y.; Friedman, A.J. Nanotechnology as a therapeutic tool to combat microbial resistance. *Adv. Drug Deliv. Rev.* 2013, 65, 1803–1815. [CrossRef]

33. Zhang, L.; Pornpattananangkul, D.; Hu, C.-M.; Huang, C.-M. Development of Nanoparticles for Antimicrobial Drug Delivery. *Curr. Med. Chem.* 2010, 17, 585–594. [CrossRef]

34. Xiong, Y.-Q.; Kupferwasser, L.I.; Zack, P.M.; Ebelk, K.; Noyes, N.R.; Morley, P.S. Comparative Efficacies of Liposomal Amikacin (MiKasome) plus Oxacillin versus Conventional Amikacin plus Oxacillin in Experimental Endocarditis Induced by *Staphylococcus aureus* by liposomal oleic acids. *Biomaterials* 2011, 32, 214–221. [CrossRef]

35. Schulze, M.; Kranzer, K.; Elamin, W.; Cox, H.; Seddon, J.; Ford, N.; Drobniowski, F. A systematic review and meta-analysis of the efficacy and safety of N-acetylcysteine in preventing aminoglycoside-induced ototoxicity: Implications for the treatment of multidrug-resistant TB. *Thorax* 2015, 70, 1070–1077. [CrossRef]

36. Seemann, T. Abricate, Github. Available online: https://github.com/tseemann/abricate (accessed on 10 November 2021).

37. Seemann, T. Abricate, Github. Available online: https://github.com/tseemann/abricate (accessed on 10 November 2021).

38. Doster, E.; Lakin, S.M.; Dean, C.J.; Wolfe, C.; Young, J.G.; Boucher, C.; E Belk, K.; Noyes, N.R.; Morley, P.S. MEGARes 2.0: A database for classification of antimicrobial drug, biocide and metal resistance determinants in metagenomic sequence data. *Antimicrob. Agents Chemother.* 2019, 63, 2640–2644. [CrossRef] [PubMed]

39. Zankari, E.; Hasman, H.; Cosentino, S.; Vestergaard, M.; Rasmussen, S.; Lund, O.; Aarestrup, F.M.; Larsen, M.V. Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 2012, 67, 2640–2644. [CrossRef]

40. Chen, L.; Zheng, D.; Liu, B.; Yang, J.; Jin, Q. VFDB 2016: Hierarchical and refined dataset for big data analysis—10 years on. *Nucleic Acids Res.* 2016, 44, D694–D697. [CrossRef] [PubMed]

41. Alhariri, M.; A Majarishi, M.; Bahkali, A.H.; Almajed, F.S.; O Azghani, A.; A Khiyami, M.; Alyamani, E.J.; Aljohani, S.M.; Halwani, M. Efficacy of neutral and negatively charged liposome-loaded gentamicin on planktonic bacteria and biofilm communities. *Int. J. Nanomed.* 2017, *ume12*, 6949–6961. [CrossRef] [PubMed]

42. XcaliburTM Software. Available online: https://www.thermofisher.com/order/catalog/product/OPTON-30965 (accessed on 10 November 2021).
43. Ramos, L.D.P.; Santos, C.E.D.R.; Mello, D.C.R.; Theodorou, L.N.; De Oliveira, F.E.; Brito, G.N.B.; Junqueira, J.C.; Jorge, A.O.C.; Oliveira, L. *Klebsiella pneumoniae* Planktonic and Biofilm Reduction by Different Plant Extracts: In Vitro Study. *Sci. World J.* 2016, 1–8. [CrossRef][PubMed]

44. Graphpad. Prism. Available online: https://www.graphpad.com (accessed on 10 November 2021).

45. Magiorakos, A.-P.; Srinivasan, A.; Carey, R.B.; Carmeli, Y.; Falagas, M.E.; Giske, C.G.; Harbarth, S.; Hindler, J.F.; Kahlmeter, G.; Olsson-Liljequist, B.; et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 2012, 18, 268–281. [CrossRef][PubMed]

46. MacKinnon, M.C.; Sargeant, J.M.; Pearl, D.L.; Reid-Smith, R.J.; Carson, C.A.; Parmley, E.J.; McEwen, S.A. Evaluation of the health and healthcare system burden due to antimicrobial-resistant *Escherichia coli* infections in humans: A systematic review and meta-analysis. *Antimicrob. Resist. Infect. Control.* 2020, 9, 200. [CrossRef]

47. Nji, E.; Kazibwe, J.; Hambridge, T.; Joko, C.A.; Larbi, A.A.; Dampney, L.A.O.; Nkansa-Gyamfi, N.A.; Lundborg, C.S.; Lien, I.T.Q. High prevalence of antibiotic resistance in commensal *Escherichia coli* from healthy human sources in community settings. *Sci. Rep.* 2021, 11, 3372. [CrossRef]

48. Dijkshoorn, L.; Nemec, A.; Seifert, H. An increasing threat in hospitals: Multidrug-resistant *Acinetobacter baumannii*. *Nat. Rev. Genet.* 2007, 5, 939–951. [CrossRef][PubMed]

49. Effah, C.Y.; Sun, T.; Liu, S.; Wu, Y. *Klebsiella pneumoniae*: An increasing threat to public health. *Ann. Clin. Microbiol. Antimicrob.* 2020, 19, 1. [CrossRef][PubMed]

50. Ruiz, J.; Bertran, S.; Saucà, G.; Julià, A.; Vila, X.; Gómez, E.; de Anta, M.T.J.; Vila, J. Isolation of an amikacin-resistant *Escherichia coli* strain after tobramycin treatment of previous recurrent episodes of respiratory tract infections caused by *Pseudomonas aeruginosa*. *Clin. Microbiol. Infect.* 2005, 11, 75–78. [CrossRef][PubMed]

51. Goudsen, R.; Bamford, C.; Van Zyl-Smit, R.; Cohen, K.; Maartens, G. Safety and effectiveness of colistin compared with tobramycin for multidrug-resistant *Acinetobacter baumannii* infections. *BMC Infect. Dis.* 2009, 9, 26. [CrossRef][PubMed]

52. Quah, S.Y.; Wu, S.; Lui, J.N.; Sum, C.P.; Tan, K.S. N-Acetylcysteine Inhibits Growth and Eradicates Biofilm of *Pseudomonas aeruginosa*. *In vitro activity of N-acetylcysteine against Burkholderia cepacia complex grown in planktonic and biofilm phase and biofilm. PLoS ONE 2018, 13, e0203941.* [CrossRef]

53. Asaad, A.M.; Ansari, S.; Aljan, S.E.; Awad, S.M. Epidemiology of Biofilm Producing *Acinetobacter baumannii* Nosocomial Isolates from a Tertiary Care Hospital in Egypt: A Cross-Sectional Study. *Infect. Drug Resist.* 2021, 14, 709–717. [CrossRef]

54. Zhao, T.; Liu, Y. N-acetylcysteine inhibit biofilms produced by *Pseudomonas aeruginosa*. *BMC Microbiol.* 2010, 10, 140. [CrossRef][PubMed]

55. Pollini, S.; Di Pilato, V.; Landini, G.; Di Maggio, T.; Cannatelli, A.; Sottotetti, S.; Cariani, L.; Aliberti, S.; Blasi, F.; Sergio, F.; et al. Interaction of N-acetylcysteine with DPPC liposomes at different pH: A physicochemical study. *New J. Chem.* 2020, 44, 14837–14848. [CrossRef]

56. Sun, J.; Liu, Y. Liposomal Antioxidants for Protection Against Oxidant-Induced Damage. *J. Toxicol.* 2011, 2011, 1–16. [CrossRef][PubMed]

57. Pollini, S.; Di Pilato, V.; Landini, G.; Di Maggio, T.; Cannatelli, A.; Sottotetti, S.; Cariani, L.; Aliberti, S.; Blasi, F.; Sergio, F.; et al. Interaction of N-acetylcysteine with *Stenotrophomonas maltophilia* and *Burkholderia cepacia* complex grown in planktonic phase and biofilm. *In vitro activity of N-acetylcysteine against Stenotrophomonas maltophilia and Burkholderia cepacia complex grown in planktonic phase and biofilm. PLoS ONE 2018, 13, e0203941.* [CrossRef]

58. Mitsopoulos, P.; Omri, A.; Alipour, M.; Vermeulen, N.; Smith, M.G.; Suntres, Z.E. Effectiveness of liposomal-N-acetylcysteine against LPS-induced lung injuries in rodents. *Int. J. Pharm.* 2008, 363, 106–111. [CrossRef][PubMed]

59. Arias, J.M.; Picot, R.A.C.; Tuttomondo, M.E.; Ben Altabez, A.; Diaz, S.B. Interaction of N-acetylcysteine with DPPC liposomes at different pH: A physicochemical study. *New J. Chem.* 2020, 44, 14837–14848. [CrossRef]

60. Sun, J.; Liu, Y. Liposomal Antioxidants for Protection Against Oxidant-Induced Damage. *J. Toxicol.* 2011, 2011, 1–16. [CrossRef][PubMed]

61. Alipour, M.; Buonocore, C.; Omri, A.; Szabo, M.; Pucay, K.; Suntres, Z.E. Therapeutic effect of liposomal-N-acetylcysteine against acaminophen-induced hepatotoxicity. *Drug Target. 2013, 21, 466–473.* [CrossRef]

62. Mitsopoulos, P.; Suntres, Z.E. Protective Effects of Liposomal N-Acetylcysteine against Paraquat-Induced Cytotoxicity and Gene Expression. *J. Toxicol.* 2011, 2011, 808967. [CrossRef][PubMed]

63. Mitsopoulos, P. The Role of Free-and Liposomal-N-Acetylcysteine in Parquat-Induced Cytotoxicity. Master’s Thesis, Lakehead University, Thunder Bay, ON, Canada, 2012.

64. Rosenberg, E.Y.; Ma, D.; Nikaido, H. AcrD of *Escherichia coli* Is an Aminoglycoside Efflux Pump. *J. Bacteriol.* 2000, 182, 1754–1756. [CrossRef]

65. Vidal, O.; Longin, R.; Prigent-Combaret, C.; Dorel, H.; Hooreman, M.; Lejeune, P. Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: Involvement of a new ompR allele that increases curli expression. *J. Bacteriol.* 1998, 180, 2442–2449. [CrossRef]

66. Swasthi, H.M.; Bhasne, K.; Mahapatra, S.; Mukhopadhyay, S. Human Fibrinogen Inhibits Amyloid Assembly of Biofilm-Forming *CsgA*. *Biochemistry 2018, 57, 6270–6273.* [CrossRef]
69. Uhlich, G.A.; Gunther, N.W.; Bayles, D.O.; Mosier, D.A. The CsgA and Lpp Proteins of an Escherichia coli O157:H7 Strain Affect HEp-2 Cell Invasion, Motility, and Biofilm Formation. *Infect. Immun.* 2009, 77, 1543–1552. [CrossRef]

70. Richmond, G.E.; Evans, L.P.; Anderson, M.J.; Wand, M.; Bonney, L.C.; Ivens, A.; Chua, K.L.; Webber, M.; Sutton, J.M.; Peterson, M.L.; et al. The Acinetobacter baumannii Two-Component System AdeRS Regulates Genes Required for Multidrug Efflux, Biofilm Formation, and Virulence in a Strain-Specific Manner. *mBio* 2016, 7, e00430-16. [CrossRef]

71. Messiaen, A.-S.; Forier, K.; Nelis, H.; Braeckmans, K.; Coenye, T. Transport of Nanoparticles and Tobramycin-loaded Liposomes in Burkholderia cepacia Complex Biofilms. *PLoS ONE* 2013, 8, e79220. [CrossRef] [PubMed]

72. Hasanin, A.; Omri, A. Liposomal N-acetylcysteine Modulates the Pathogenesis of *P. aeruginosa* Isolated from the Lungs of Cystic Fibrosis Patient. *J. Nanomed. Nanotechnol.* 2014, 5, 219–230. [CrossRef]

73. Chenthamara, D.; Subramaniam, S.; Ramakrishnan, S.G.; Krishnaswamy, S.; Essa, M.M.; Lin, F.H.; Qoronfleh, M.W. Therapeutic efficacy of nanoparticles and routes of administration. *Biomater. Res.* 2019, 23, 20. [CrossRef] [PubMed]

74. Halwani, M.; Hebert, S.; Suntres, Z.E.; Lefranc, R.; Azghani, A.O.; Omri, A. Bismuth–thiol incorporation enhances biological activities of liposomal tobramycin against bacterial biofilm and quorum sensing molecules production by *Pseudomonas aeruginosa*. *Int. J. Pharm.* 2009, 373, 141–146. [CrossRef] [PubMed]

75. Marier, J.F.; Brazier, J.L.; Lavigne, J.; Ducharme, M.P. Liposomal tobramycin against pulmonary infections of *Pseudomonas aeruginosa*: A pharmacokinetic and efficacy study following single and multiple intratracheal administrations in rats. *J. Antimicrob. Chemother.* 2003, 52, 247–252. [CrossRef] [PubMed]

76. Alexopoulou, E.; Georgopoulos, A.; Kagkadis, K.A.; Demetzos, C. Preparation and Characterization of Lyophilized Liposomes with Incorporated Quercetin. *J. Liposome Res.* 2006, 16, 17–25. [CrossRef] [PubMed]

77. Danaei, M.; Dehghankhold, M.; Ataei, S.; Hasanzadeh Davarani, F.; Javanmard, R.; Dokhani, A.; Khorasani, S.; Mozafari, M.R. Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems. *Pharmaceutics* 2018, 10, 57. [CrossRef]

78. Wei, X.-Q.; Zhu, J.-F.; Wang, X.-B.; Ba, K. Improving the Stability of Liposomal Curcumin by Adjusting the Inner Aqueous Chamber pH of Liposomes. *ACS Omega* 2020, 5, 1120–1126. [CrossRef]

79. Beaulac, C.; Sachetelli, S.; Lagace, J. In-vitro bactericidal efficacy of sub-MIC concentrations of liposome-encapsulated antibiotic against gram-negative and gram-positive bacteria. *J. Antimicrob. Chemother.* 1998, 41, 35–41. [CrossRef] [PubMed]

80. Galimand, M.; Sabtcheva, S.; Courvalin, P.; Lambert, T. Worldwide Disseminated armA Aminoglycoside Resistance Methylation Gene Is Borne by Composite Transposon Tn1548. *Antimicrob. Agents Chemother.* 2005, 49, 2949–2953. [CrossRef] [PubMed]

81. Bogaerts, P.; Galimand, M.; Bauraing, C.; Deplano, A.; Vanhoof, R.; De Mendonca, R.; Rodriguez-Villalobos, H.; Struelens, M.; Glupczynski, Y. Emergence of ArmA and RmtB aminoglycoside resistance 16S rRNA methylases in Belgium. *J. Antimicrob. Chemother.* 2007, 59, 459–464. [CrossRef]

82. Shrestha, S.; Tada, T.; Shrestha, B.; Kirikae, T.; Ohara, H.; Rijal, B.P.; Pokhrel, B.M.; Shergand, J.B. Emergence of Aminoglycoside Resistance Due to armA methylase in Multi-drug Resistant *Acinetobacter baumannii* Isolates in a University Hospital in Nepal. *J. Nepal Health Res. Counc.* 2016, 14, 72–76. [PubMed]

83. Olsen, I. Biofilm-specific antibiotic tolerance and resistance. *Eur. J. Clin. Microbiol.* 2015, 34, 877–886. [CrossRef] [PubMed]

84. Silveira, L.F.M.; Baca, P.; Arias-Moliz, M.T.; Rodriguez-Archilla, A.; Ferrer-Luque, C.M. Antimicrobial activity of alexidine alone and associated with N-acetylcysteine against Enterococcus faecalis biofilm. *Int. J. Oral. Sci.* 2013, 5, 146–149. [CrossRef] [PubMed]

85. Marchese, A.; Bozzolasco, M.; Gualco, L.; Debbia, E.A.; Schito, G.C.; Schito, A.M. Effect of fosfomycin alone and in combination with N-acetylcysteine on *E. coli* biofilms. *Int. J. Antimicrob. Agents* 2003, 22, 95–100. [CrossRef]

86. Serramitjana, E.S.; Jorba, M.; Fusté, E.; Pedraz, J.L.; Vinuesa, T.; Viñas, M. Free and Nanoencapsulated Tobramycin: Effects on Planktonic and Biofilm Forms of *Pseudomonas. Microorganisms* 2017, 5, 35. [CrossRef] [PubMed]