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ORIGINAL ARTICLE

Photodynamic enhancement of the activity of antibiotics used in urinary tract infections

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Abstract

Photodynamic therapy (PDT) has been proven to kill different microbial cells. However, to our knowledge, none of the available reports describes the modulatory effect of this therapy on the antibacterial activity of antibiotics against *Escherichia coli* rods being the main causative agent of urinary tract infections (UTIs). Therefore, the aim of our study was to verify if the PDT can enhance the antibacterial activity of antibiotics recommended in the treatment of UTIs. An attempt to determine the optimal conditions of PDT to enhance the bactericidal activity of ciprofloxacin, amikacin, and colistin has been made. In order to find the optimal antimicrobial conditions, the efficacy of four protocols associated with the use of different energy doses (70 and 120 J/cm^2) and chlorin e6 (Ce6) concentrations (50 and 100 μg/mL) has been verified. The antibacterial effect of combined PDT and antibiotics was assessed by the time-kill assay. The best results were achieved for Ce6 at a concentration of 100 μg/mL and the energy dose 120 J/cm^2 for bacterial suspensions treated with ciprofloxacin. Taken together, our results showed that PDT using Ce6 improves the antibacterial activity of antibiotics effectively inhibiting bacterial growth and being promising in the elimination of bacterial UTIs in humans.

Keywords Antimicrobial photodynamic therapy (aPDT) · Chlorin e6 · Ciprofloxacin · Colistin · Amikacin · Uropathogenic *Escherichia coli*

Introduction

Escherichia coli is the most prevalent gram-negative agent causing urinary tract infections (UTIs) [1]. The increasing number of recurrent and chronic UTIs [2, 3] and extensive use of antibiotics leading to the selection of multi-drug-resistant bacterial strains underlines the urgent need for the further discovery and improvement of alternative ways of microorganisms inactivation [4, 5]. For years, antimicrobial photodynamic therapy (aPDT) has been used effectively in the eradication of gram-positive and gram-negative bacteria [6, 7]. Various bacterial cells' structures and components are the targets for PDT in contrast to one major target in the case of antibiotics [8, 9]. Thus, PDT reduces the risk of developing resistance of microorganisms exposed to it [10].

Chlorin e6 (Ce6) is a second-generation photosensitizer used in PDT. It has been reported to possess noteworthy advantages, e.g., short photosensitizing period, selective accumulation in the target tissue and cell's parts, relatively good absorption of red light, and minimal side effects [11, 12]. The combination of the aPDT and conventionally used antibiotics to treat severe bacterial infections shows significant potential, being a chance for more effective therapies also in UTIs [7, 13, 14].

According to the European Association of Urology guidelines on urological infections, ciprofloxacin and amikacin are recommended for empirical antimicrobial therapy in pyelonephritis [15]. The increase of antibiotic resistance in gram-negative bacteria has resurrected the importance of polymyxin antibiotics. Colistin, being polymyxin E, is a last-resort antibiotic very often used against multi-drug-resistant Enterobacteriaceae strains causing multiple infections and, among others, UTIs [16–19]. All three, ciprofloxacin, amikacin, and colistin, are well-known antimicrobials belonging to different groups of antibiotics. Amikacin (AN) is an

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aminoglycoside antibiotic which acts by binding to the bacterial 30S ribosomal subunit and inhibiting in this way protein synthesis. Ciprofloxacin (CIP) belongs to fluoroquinolones. It inhibits the bacterial enzyme DNA gyrase and prevents replication of bacterial DNA during bacterial growth and reproduction. Colistin (CL) is an antibiotic belonging to cyclic cationic polypeptides. Thanks to its amphiphilic nature, CL can easily penetrate into the bacterial cell and integrate with the cell membrane phospholipids thus disrupting the cell's structure [9]. It is well-known that the use of antibiotics for a long time is undesirable in clinical practice due to their adverse effects on the bacteria inducing multi-drug resistance. For that reason, it is very important to find more effective therapeutic methods preventing this disquieting phenomenon. aPDT seems to be such a method.

For the reasons presented shortly above, the aim of our study was to evaluate if aPDT can enhance the activity of antibiotics recommended in UTI treatment.

Material and methods

Bacteria

Uropathogenic *Escherichia coli* strain (UPEC060) came from the collection of clinical bacterial isolates of the Department of Biology and Medical Parasitology, the Wrocław Medical University, Poland. The strain was maintained on slopes containing nutrient broth and glycerol in a final concentration of 40%, stored at − 20 °C.

Photosensitizer and light source

Chlorin e6 (Ce6) was purchased from Frontier Scientific (Porphyrin Products, Logan, USA) and dissolved in DMSO and sterile water (1:1) to obtain a stock concentration of 500 μg/mL. Finally, the Ce6 concentrations of 50 μg/mL and 100 μg/mL were then used to measure photodynamic efficiency against *E. coli* strain. As a light source, a diode laser LASER COUPLER 635 (Wroclaw, Poland) has been used to irradiate Ce6 at the wavelength 635 nm, at the power density of 0.29 W/cm^2, and at the total energy density of 70 J/cm^2 or 120 J/cm^2, with no thermal side effects.

Antibiotics

Three antimicrobial agents with different bacterial cell targets were used in this study: colistin sodium methanesulfonate (CL; Colistin®, Polfa Tarchomin S.A., Warsaw, Poland), ciprofloxacin lactate (CIP; Proxacin®, Polfa S.A., Warsaw, Poland), and amikacin disulfate salt (AN; Biodacyna®, BIOTON S.A., Warsaw, Poland).

MIC determination

Minimum inhibitory concentrations (MICs) of antibiotics were performed in Mueller-Hinton broth (MHB; Emapol, Gdansk, Poland) according to CLSI guidelines for broth microdilution susceptibility testing [20]. In the current study, the MICs of CL, CIP, and AN were 0.5 μg/mL, 0.0039 μg/mL, and 1.0 μg/mL, respectively. Subinhibitory concentration (0.5× MIC) of each antibiotic was then used in the experiments.

Bacterial culture conditions used in aPDT experiments

The bacteria were grown overnight at 37 °C in the presence of 0.5× MICs of each antibiotic. Next, bacteria were harvested by centrifugation (4000 rpm/20 min) and resuspended in PBS to reach a final concentration of 1–2 × 10^8 CFU/mL (0.5 McFarland). The control sample contained no antibiotic.

aPDT experimental conditions

The work steps of the experimental study are shown in Fig. 1. We used the experimental groups treated with (*i*) antibiotic (CL, AN, or CIP); (*ii*) Ce6 and red laser light (L); (*iii*) antibiotic and light (CL + L, AN + L, CIP + L) and the sample containing bacteria treated with no antibiotic nor light (control).

Cultured overnight bacterial suspensions (1–2 × 10^8 CFU/mL) were plated to the wells of a 96-well plate. Then, MHB and Ce6 stock solutions were also added to the wells to obtain the final concentrations of Ce6 (50 μg/mL or 100 μg/mL). The plate was incubated for 15 min at room temperature (in the dark) before exposure to the red light at 70 J/cm^2 or 120 J/cm^2. PDT parameters were used in four combination protocols: 50 μg/mL + 70 J/cm^2; 50 μg/mL + 120 J/cm^2; 100 μg/mL + 70 J/cm^2; 100 μg/mL + 120 J/cm^2.

Then, samples were diluted and cultured in triplicate on nutrient agar plates (Biomed, Poland) immediately (t_0), at 1 h (t_1), and 3 h (t_3) after irradiation. Agar plates were incubated at 37 °C for 24 h and the number of colony-forming units per milliliter was counted (Fig. 1).

Statistical analysis

The results are given as a mean value from three separate experiments. All values were expressed as a mean ± SD. The differences in the growth of bacteria exposed to different combinations of agents: antibiotics/Ce6/light and unexposed bacteria were analyzed by the parametric *t* test for independent samples. Statistical calculations were made using Statistica 13.1 (Stat Soft, Kraków, Poland). P values < 0.05 were considered to be statistically significant.

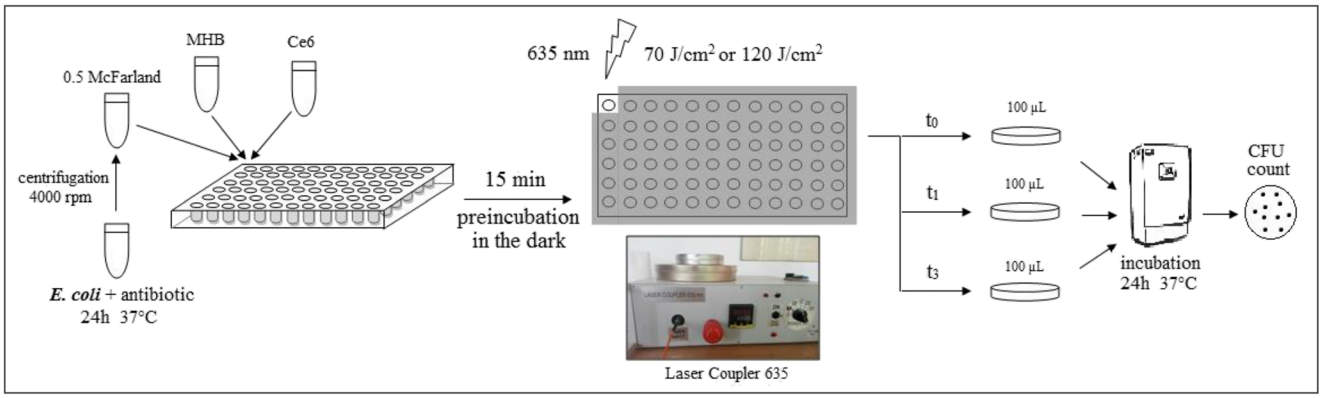

Fig. 1 Scheme demonstrating the flow of experiments

Results

The preliminary study (data not shown) showed no statistically significant changes in bacterial survival when UTI060 was treated with (1) Ce6 in the dark, (2) antibiotic and Ce6 in the dark, (3) light without Ce6, and (4) antibiotic and light without Ce6 in comparison to the control sample containing untreated bacteria.

In further experiments, the impact of aPDT on the bacteria incubated overnight in the presence of 0.5× MIC of antibiotics (CL, AN, CIP) and untreated bacteria (no antibiotics nor light) has been determined. In the experiments, Ce6 at concentrations of 50 μg/mL and 100 μg/mL, and a light dose of 70 J/cm^2 and 120 J/cm^2 in four different combinations as described in the "Material and methods" section was used to find optimal conditions of PDT modulating the activity of antibiotics.

The results of bacterial survival in the presence of Ce6 at a concentration of 50 μg/mL and light dose 70 J/cm^2 are shown visually in Fig. 2. The number of colonies (CFU/mL) of bacteria incubated overnight in antibiotics and then irradiated decreased in comparison to the number of colonies of bacteria unsubjected to PDT. However, the statistically significant decrease of viable bacteria was observed only at t_0 and t_1 in the case of rods treated with CL and CIP ($p < 0.05$). The greatest reduction of the colony number was noticed at t_0 for *E. coli* incubated with CIP, comparable to the nonirradiated bacteria. The colony-forming units per milliliter decreased 3.8 times, from 8.3×10^7 to 2.2×10^7, respectively.

The increase of Ce6 concentration from 50 to 100 μg/mL and illumination of the bacterial suspensions with a total energy dose of 70 J/cm^2 caused the statistically significant decrease of bacterial survival at t_0 in the case of all antibiotics and t_1 for bacteria treated with CL and CIP ($p < 0.05$) (Fig. 3). The greatest reduction of the colony number was also noticed at t_0 for rods incubated with CIP. The colony number decreased by 2.7 times, from 8.4×10^7 CFU/mL in the control to 3.1×10^7 CFU/mL in the illuminated sample (Fig. 3).

Figure 4 shows the results of bacterial survival in the presence of Ce6 at a concentration of 50 μg/mL irradiated with light of the total energy dose increased from 70 to 120 J/cm^2. Such experimental conditions caused a significant reduction of bacterial survival in all examined samples at t_0 and t_1 ($p < 0.05$). At t_0, the greatest reduction of colony-forming units per milliliter (4.2 times from 8.3×10^7 to 1.9×10^7) was noticed in the sample treated with CIP. While at time t_1, the concentration of viable bacteria decreased the most in the case of *E. coli* incubated with AN. The colony-forming units per milliliter was reduced from 1.6×10^8 in the control sample to 5.2×10^7 in the examined one.

Simultaneous use of the high-energy dose (120 J/cm^2) and Ce6 concentration (100 μg/mL) caused a significant reduction of bacterial viability at t_0, t_1, and t_3 ($p < 0.05$) (Fig.5). Although the most profound effect of synergy between all antibiotics and PDT was noticed at t_1. The number of colony-forming units per milliliter was reduced 3.3 times for CL, 4.0 times for AN, and 4.9 times in case of CIP.

Discussion

PDT is a method widely used in dermatology and periodontics, less frequently in ophthalmology, gastroenterology, or other medicine branches [21–24]. This method is also successfully used against gram-positive and gram-negative bacteria in in vitro studies [25–31]. However, it should be underlined that there are only a few reports describing the use of aPDT in urological infection [13, 14]. To our knowledge, current work is the first report focused on the modulatory effect of the aPDT on antibacterial activity of antibiotics possessing different mechanisms of action against UPEC rods. The UPEC060 strain used in our study was genetically characterized previously and classified to phylogenetic group B2 [32]. The Ce6 used in this study is structurally closely related to porphyrins but with a higher degree of saturation of the ring system [33]. It is worth noticing that the main advantages of Ce6 are low toxicity, fast and sufficiently selective accumulation in the target tissue, and higher photosensitizing efficacy than porphyrins [6, 33]. Since different protocols concerning Ce6

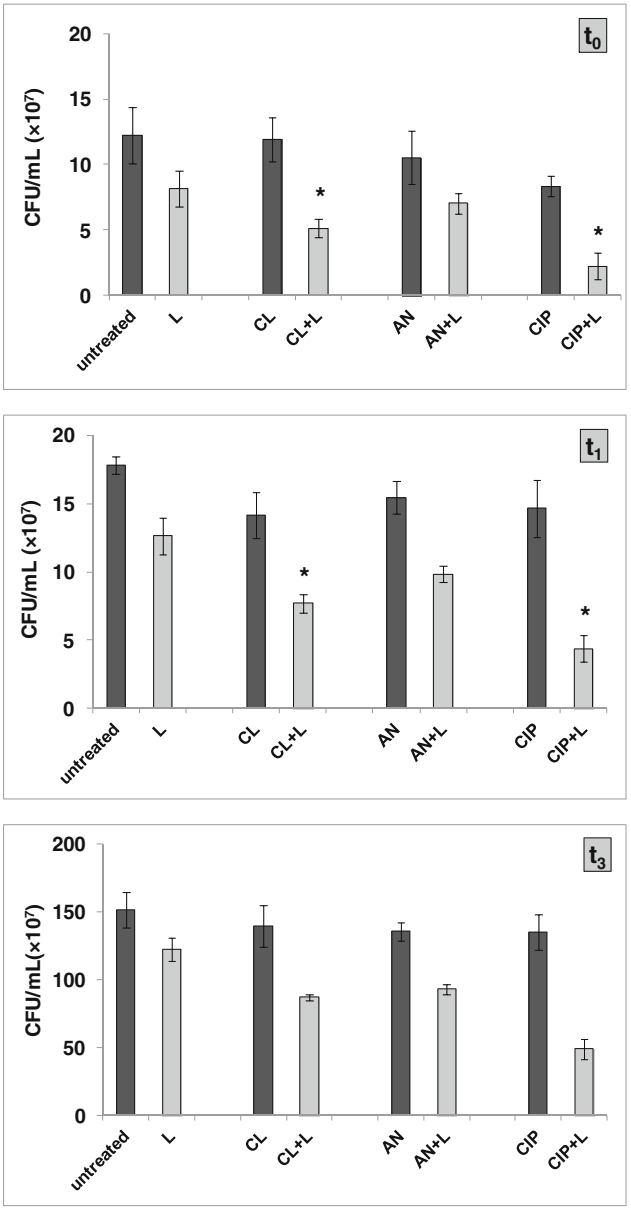

Fig. 2 Effect of PDT (Ce6 50 μg/mL; energy dose 70 J/cm^2) on the survival of *Escherichia coli* treated with antibiotics. Untreated, no antibiotic, no irradiation; L, light; CL, colistin; AN, amikacin; CIP, ciprofloxacin. Values represent the mean ± SD of three separate experiments. The asterisk indicates a statistically significant result ($p < 0.05$)

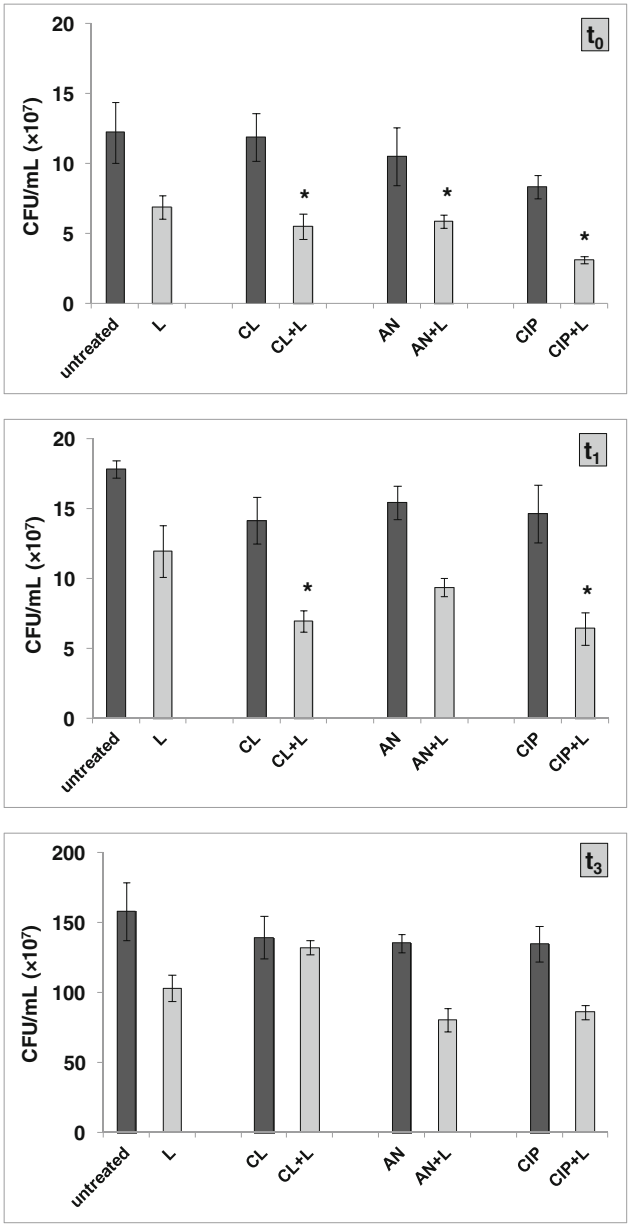

Fig. 3 Effect of PDT (Ce6 100 μg/mL; energy dose 70 J/cm^2) on the survival of *Escherichia coli* treated with antibiotics. Untreated, no antibiotic, no irradiation; L, light; CL, colistin; AN, amikacin; CIP, ciprofloxacin. Values represent the mean ± SD of three separate experiments. The asterisk indicates a statistically significant result ($p < 0.05$)

concentration and/or light energy were used in experiments performed by other research groups [34, 35], we examined few Ce6/light combinations to find the most effective one. As described in the "Material and methods" section, the efficacy of four protocols (combining the values given in [34, 35]) associated with the use of different energy doses (70 and 120 J/cm^2) and Ce_6 concentrations (50 and 100 μg/mL) has been verified.

In our study, the representative members (CIP, CL, AN) of the three major classes of antibiotics have been used. Despite the fact that the in vivo therapeutic result of treatment is the best when the antibiotic concentration between consecutive doses is above the MIC, we used the sub-lethal doses of the antimicrobials. Looking at the pharmacokinetic curves of antibiotics, it can be seen that their concentrations exceed the MIC values for only a certain period of time. Then, they become lower than the MIC especially in tissues, i.e., the sites of infections where antibiotic concentrations are frequently lower than those in the blood [36].

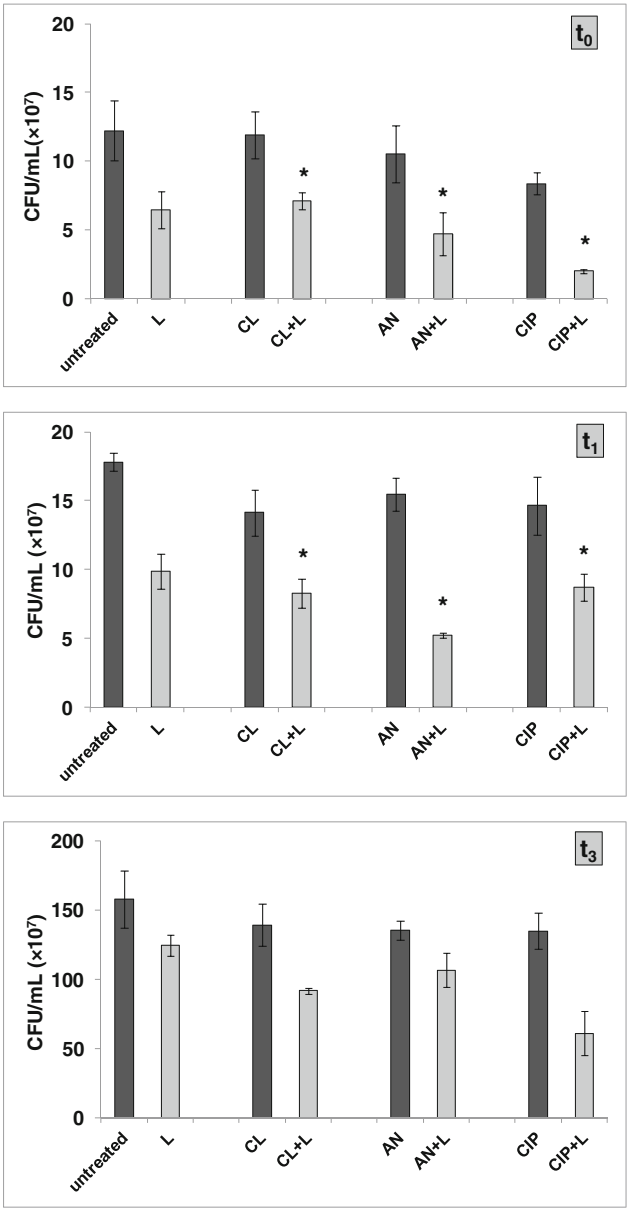

Fig. 4 Effect of PDT (Ce6 50 μg/mL; energy dose 120 J/cm^2) on the survival of *Escherichia coli* treated with antibiotics. Untreated, no antibiotic, no irradiation; L, light; CL, colistin; AN, amikacin; CIP, ciprofloxacin. Values represent the mean ± SD of three separate experiments. The asterisk indicates a statistically significant result ($p < 0.05$)

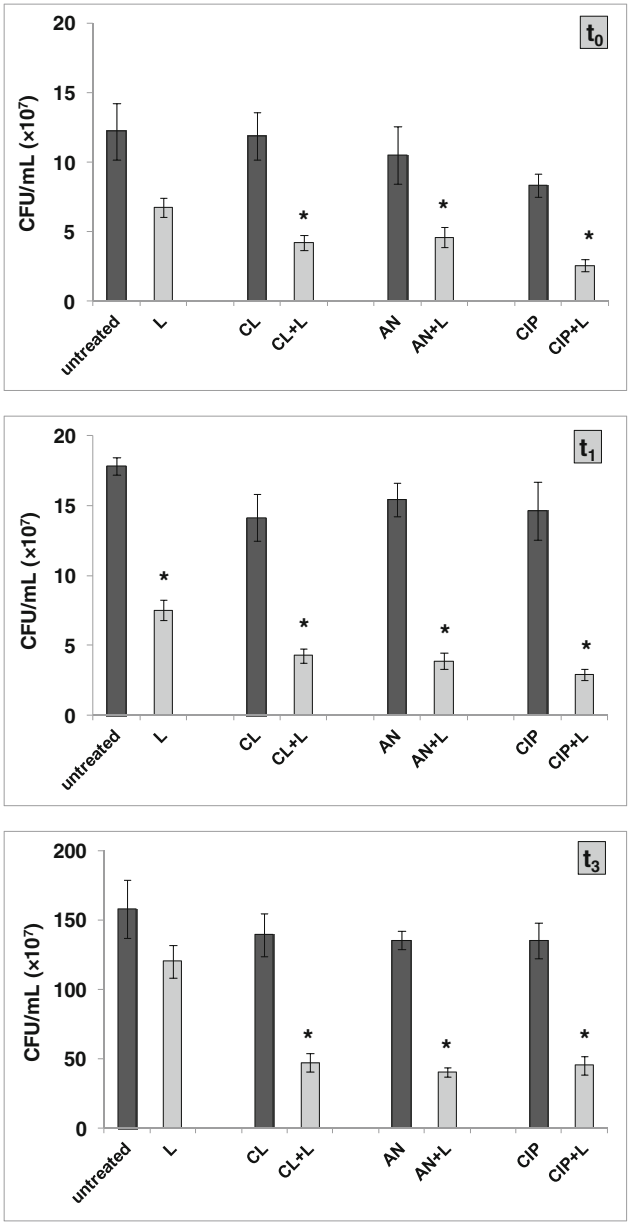

Fig. 5 Effect of PDT (Ce6 100 μg/mL; energy dose 120 J/cm^2) on the survival of *Escherichia coli* treated with antibiotics. Untreated, no antibiotic, no irradiation; L, light; CL, colistin; AN, amikacin; CIP, ciprofloxacin. Values represent the mean ± SD of three separate experiments. The asterisk indicates a statistically significant result ($p < 0.05$)

Ce6 is moderately hydrophobic; therefore, it shows the affinity to lipid-containing cell structures. It seems, however, that it was not able to penetrate the well-organized outer envelope of gram-negative bacteria, accumulate inside in a large enough amount, and exert a bactericidal effect when used alone. This can be deduced by comparing "untreated" and "L" bars in Figs. 2, 3, 4, and 5. Since antibiotics were used in sub-MIC concentrations, they also showed no bacteria-killing effect.

Analysis of the obtained results showed that when antibiotics and aPDT were used together, they showed remarkable antibacterial activities (Figs. 2, 3, 4, and 5). Moreover, their combined antimicrobial effect was dependent on the PDT conditions. The most profound effects were achieved for Ce6 at a concentration of 100 μg/mL and the energy dose 120 J/cm^2 (Fig. 5). It is worth noticing that under these experimental conditions, a significant reduction of bacterial survival was observed even after 3 h from irradiation (Fig. 5). Such prolongation of antibacterial effect was not observed in any

other experimental protocol used (Figs. 2, 3, and 4). Such a promising antibacterial effect was achieved due to the combined action of the antibiotic and the excited photosensitizer. Our experiments have shown, however, that the effects obtained for three antibiotic/Ce6 combinations were somehow different, depending on the kind of antibiotic used. The best antigrowth effect of aPDT was noticed for bacterial samples treated with CIP (Fig. 5). Presumably, this phenomenon results from the oxidative damage of DNA by PDT and the inhibition of bacterial gyrase by CIP, in both cases resulting in the impairment of the DNA replication [8, 9]. The enhancement of the antibacterial activity of antibiotic by PDT was also observed in *E. coli* suspensions treated with CL (Fig. 5). The mechanism of CL activity is connected with the interactions of its cationic polypeptide ring and the anionic phosphate groups present in the cell membrane of the gram-negative bacteria [9]. These interactions cause the displacement of Ca^{2+} and Mg^{2+} ions increasing cell membrane permeability leading to the leakage of bacterial cell contents. Reactive oxygen species generated during PDT are responsible for the bacterial cell membrane disruption and therefore can enhance the antimicrobial effect of CL [8]. The weakest, but also statistically significant, combined effect of PDT and antibiotics was noticed in the case of UPECs exhibited to AN (Fig. 5). AN impairs the process of protein synthesis by inhibition of small ribosomal subunits. It is not directly connected with the main targets (DNA, cell membranes) of reactive oxygen species generated during photodynamic reactions [8, 9]. It seems, therefore, that aPDT better enhances the activity of CIP and CL than AN. Recently, the effects and mechanisms of combined antibiotics and aPDT action have been described [25–30, 37, 38]. One of them is the work of Ronqui et al. [26], who described the synergistic effect of aPDT and CIP against *E. coli* and *Staphylococcus aureus* growing in planktonic and biofilm cultures. The results were significant when CIP was administered before the aPDT treatment as well as when the CIP administration was followed by an aPDT. In the case of both bacterial species, the number of colony-forming unites per milliliter was reduced; however, gram-positive bacteria were more susceptible to PDT than gram-negative ones, which is consistent with other literature [29, 31]. Another study of combining aPDT with two fluoroquinolones (CIP and norfloxacin) was presented by Pereira et al. [29]. This study demonstrated that irradiation of *E. coli* and *S. aureus* with blue or red light in the presence of CIP is more effective than antibiotic monotherapy. A similar result was also obtained with the combined use of aPDT and norfloxacin. Another interesting application of combining aPDT and antibiotic treatment was described by Boluki et al. [38]. They used aPDT and CL against pan-drug-resistant *Acinetobacter baumannii* strain. The obtained results showed that the application of aPDT resulted in increased bacterial drug susceptibility. The authors found that the expression of the *pmrA* and *pmrB* genes, which are responsible for the synthesis of lipid A—strictly linked with resistance to CL—was lower than in untreated cells. These results may suggest a mechanism underlying the synergy between antimicrobials and light therapy. One more interesting and valuable research was conducted by Pourhajibagher et al. [37]. They evaluated the efficacy of aPDT in combination treatment with CL against pan-drug-resistant *A. baumannii* and found that combined therapy eliminated bacteria in all tested CL concentrations.

Taken together, our results showed that aPDT using Ce6 improves the antibacterial activity of antibiotics effectively inhibiting bacterial growth and being promising in the elimination of bacterial urinary infections in humans.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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