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Effect of Photoactivated Hypericin on Growth and Antibiotic Susceptibility of Hospital-Related *Staphylococcus aureus* and *Enterococcus sp.* Clinical Strains

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Abstract

Resistance against commonly used antibiotics is a serious clinical problem in recent medical practice. There exist several bacterial strains in which the possibilities of their inhibition are very limited due to multidrug resistance. Antimicrobial photodynamic therapy (aPDT) represents an option how to effectively suppress the growth of resistant pathogens. In this work we have studied interactions of potent photosensitizer hypericin (Hyp) with hospital-related gram positive (Gram+) and gram negative (Gram−) bacterial strains and the effects of photodynamic activated Hyp on bacterial susceptibility and/or resistance of these strains to antibiotics. We demonstrated a significant influence of photoactivated Hyp on growth of *Staphylococcus aureus* and *Enterococcus sp*. We have also shown that it is extremely important to use the effective concentrations of Hyp for aPDT, which completely inhibit the growth of microorganisms. Otherwise, there appears an increase in resistance, probably due to the activation of efflux mechanisms, which are involved in the efflux of Hyp and antibiotics as well.

Keywords

Antimicrobial Photodynamic Therapy, Hypericin, Antibiotic Resistance, Methicillin-Resistant *Staphylococcus aureus*, Vancomycin-Resistant *Enterococcus sp.*

1. Introduction

The introduction of antibiotics in the treatment of infectious diseases in the first

half of the 20th century significantly reduced the number of serious and fatal bacterial infections. In the golden age of antibiotics, different classes of antibiotics have been discovered such as aminogycosides, tetracyclines, chloramphenicols, macrolides, glycopeptides, oxazolines (oxazolidinones), quinolones and streptogramines. An intensive pressure on microorganisms together with their short lifecycle leads over the time to the adaptation of microorganisms resulting in the development of resistance against many of commonly used antibiotics [1] [2]. Pathogens present on devices and hospital facilities attack patients through the respiratory and gastrointestinal tract shortly after hospitalization. The length of hospitalization directly increases the risk of transmission of resistant hospital strains between patients, resulting in contamination of the entire environment with repeated cycles of colonization of patients with multidrug-resistant ESKAPE microorganisms [3] [4] [5].

Staphylococcus sp. represents a significant group of Gram+ bacteria among which an important resistance against antibiotics is rapidly spreading. Except the resistance to beta-lactam antibiotics, caused by the production of beta-lactamases and/or through changes in binding proteins, resistance to other classes of antibiotics also occurs. Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) are a serious problem in hospitals, where MRSA spread rapidly, especially among older and seriously ill patients [6]. *Enterococcus sp.* represents the second major Gram+ nosocomial pathogens among which vancomycin resistance spreads. The spread of vancomycin resistance among Gram+ bacteria, including MRSA limits gradually our current options to treat serious infections.

It was reported that the number of deaths associated with multi-resistant ESKAPE strains will increase to 10 million per year until 2050 [2]. There is an urgent need for antimicrobial approaches inactivating the growth of microorganisms and effectively kill especially multiresistant nosocomial bacteria in the group ESKAPE [7]. From this point of view, antimicrobial photodynamic therapy (aPDT) seems to be a suitable and promising approach [8] [9] [10] [11].

aPDT is a simple, non-invasive, and effective method to suppress the growth of microorganisms in the environment. In the period of antibiotic failure, aPDT represents a suitable strategy how to work with this phenomenon. aPDT is a procedure utilizing combination of photosensitizers (pts), visible light of the wavelength equal to the wavelength of pts excitation and molecular oxygen. Upon illumination at the appropriate wavelength (depends on pts) the pts molecules are excited from their ground state S_0 to an electronically excited singlet state S_x. The excited state energy can be dissipated via several competing relaxation pathways and is required for the generation of cytotoxic reactive oxygen species (ROS), including singlet oxygen ($^1O_2$) which may destroy pathogenic microorganisms. Cycle of photon absorption by pts, generation of ROS and return of pts to energetic ground state (**Figure 1(a)**) can be repeated, so one pts molecule can generate thousands of ROS (including $^1O_2$) molecules [9] [10] [11]. Because the diffusion of $^1O_2$ is not longer than 0.3 μm (depending on surrounding environment), an oxidative damage takes place in close vicinity to the

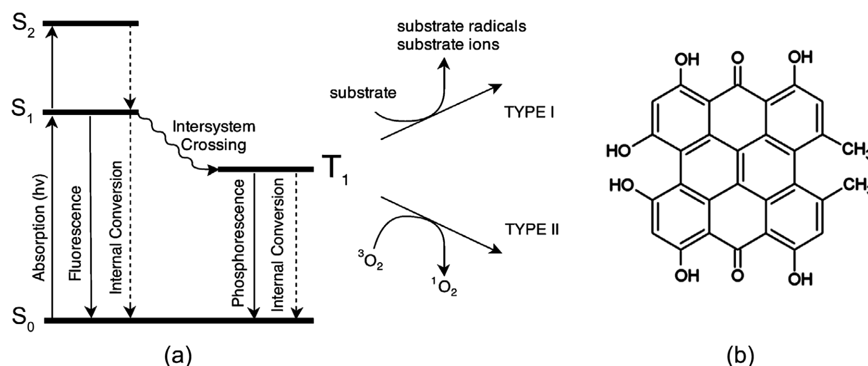

Figure 1. (a) Mechanism of Hyp photodynamic action—Jablonski diagram; (b) chemical structure of hypericin.

photoactive molecules. The main molecular targets of aPDT in cells are proteins, lipids and nucleic acids, whose oxidative damage is responsible for aPDT-mediating inactivation of bacteria. Because aPDT is a physical method of microorganisms killing, the emergence of resistance development to aPDT itself is unlikely. The question is how the presence of pts and the application of aPTD will affect the mechanisms by which bacteria protect themselves against pts and/or presence of the other antimicrobial agents.

Hypericin (Hyp) (**Figure 1(b)**) is a natural photosensitive molecule (7,14-dione-1,3,4,6,8,13-hexahydroxy 10,11-dimethyl-phenanthrol [1,10,9,8-opqra] perylene), one of the most popular herbal pigments displaying anti-depressant [12], on light dependent anti-tumor [13] [14] and anti-viral activity [14]. Several articles reported an application of Hyp in inactivation of Gram+ and Gram− microorganisms in the field of food safety [15]. Its photoactivity is characterized by high ROS generation, predominantly $^1O_2$ production through the type II mechanism of the photodynamic action [9] [14] (**Figure 1(a)**).

The aim of this study is to present a potential of aPDT application, however, also point out possible risks of Hyp induced aPDT in the inactivation of hospital-related clinical isolates. First, we investigated the Hyp uptake into Gram+ and Gram− bacteria and effect of photoactivated Hyp on growth and the susceptibility of irradiated bacterial strains against commonly used antibiotics. We demonstrated that photoactivated Hyp strongly affects growth of Gram+ bacteria immediately after irradiation. Because of Hyp activation, the minimum inhibitory concentrations (MICs) significantly decreased and survived bacteria have become more susceptible to antibiotics therapy in general. On the other hand, we obtained results suggesting that probably an activation of defense mechanisms in bacterial cells due to aPDT leads to manifestation of more resistant population, especially in *Staphylococcus aureus* isolates.

2. Material and Methods

2.1. Photosensitizer

Hypericin was purchased from Sigma. A stock solution of 1×10^{-3} M Hyp was

prepared in DMSO and stored in the dark at 4˚C. The final concentration of Hyp varied between 0.5×10^{-6} M and 5×10^{-6} M. The final concentration of DMSO in the incubation medium was less than 0.5% in all experiments [16].

2.2. Bacterial Cultures and Growth Conditions

Staphylococcus aureus, *Enterococcus sp.*, *Klebsiella pneumoniae* and *Escherichia coli* were isolated in the University Hospital in Kosice. Samples were inoculated on the blood agar plates and incubated at 37˚C overnight. Cells were then collected and resuspended in 1.5 ml of 0.75% physiologic solution. This suspension was accordingly diluted to 1×10^{7} CFU ml-1 (0.5 McFarland suspension) and use in all subsequent experiments. As a control both *Staphylococcus aureus CCM* 4626 and *Enterococcus faecalis CCM* 4224 were used.

2.3. Fluorescence Spectroscopic Analysis of Hyp Uptake

To study of Hyp uptake into tested bacterial isolates, cells were incubated with Hyp for 15 - 180 min in the dark at 37˚C. Then the cells were harvested and washed twice with PBS. The lysis of the cells and Hyp monomerization was realized by the adding of DMSO. Fluorescence emission spectra of Hyp in samples were measured by Fluoro-Max-2 ISA spectrofluorimeter (Jobin YVON-SPEX Instruments S.A., Inc., Longjumeau, France) using excitation wavelength 575 nm. All measurements were carried out at room temperature and were repeated for every Hyp concentration minimally three times. Obtained fluorescence spectra were treated using Origin Program (Microcal Software, Inc., Northampton, MA).

2.4. Hyp Photo-Activation—aPDT Protocol

Harvested bacterial cells was resuspended in 1.5 ml PBS. Hyp was added and samples were incubated in dark condition for 5 min, 10 min, 20 min, 60 min, 90 min and 120 min. Samples were irradiated by monochromatic homemade diode illuminator at 590 nm (close to maximum of Hyp absorption) and the light dose was in the range 1 - 10 J/cm^{2} [17]. After irradiation, bacterial suspension was inoculated on blood agar and Mueller-Hinton agar as well. Samples were incubated overnight at 37˚C.

2.5. Fluorescence Spectroscopic Analysis of ROS Production

To study of ROS production, after incubation with Hyp, the bacterial cells were incubated with DCFH-DA for 15 min in the dark at 37˚C. After incubation, the cells were harvested and washed twice with PBS. Fluorescence emission spectra of DCFH-DA in samples were measured by Fluoro-Max-2 ISA spectrofluorimeter (Jobin YVON-SPEX Instruments S.A., Inc., Longjumeau, France) using excitation wavelength 575 nm. All measurements were carried out at room temperature and were repeated three times. Obtained fluorescence spectra were treated using Origin Program (Microcal Software, Inc., Northamp-

ton, MA).

2.6. Bacterial Growth and Survival

1.5 ml of bacterial suspensions were prepared (optical density 0.5 McFarland). Samples were incubated with Hyp for 2 hours at 37˚C at the dark. Then cells were irradiated for 10 min or 20 (corresponding 5 and 10 J/cm^2) min. 1 µl, 10 µl and 100 µl of irradiated bacterial suspension was inoculated on blood agar and incubated at 37˚C overnight. Number of CFU was evaluated 24 hours after irradiation.

2.7. Antibiotic Susceptibility Testing

Antibiotic kits for *Staphylococcus aureus* and *Enterococcus sp.* were selected based on EUCAST and CLS rules. Following the antibiotic dilution disks were used for antibiotic susceptibility testing: cefoxitim (FOX-30), clindamycin (CLI-2), erythromycin (ERY-15), sulfam (TRIM), tetracycline (TE-30), ciprofloxacin (CIP), linezolid (LZD), tigecycline (TIG), teicoplanin (TEC), vancomycin (VA), gentamycin (CHL-30) for Staphylococcus aureus testing. Ampicilin (AMP), ampicillin-sulbactam (SAM), doxycycline (DOX), linezolid (LZD), ciprofloxacin (CIP), tigecycline (TIG), teicoplanin (TEI), vancomycin (VA), chloramphenicol (CHL-30) for *Enterococcus sp.* All disks were purchased from Oxoid, Ltd., UK.

2.8. Determination of Minimum Inhibitory Concentrations (MICs)

The minimum inhibitory concentrations (MICs) of all tested antibiotics were determined by standard Disc Dilution Tests (DDT). Bacterial suspension was inoculated on Mueller-Hinton agar, antibiotic disks were placed on plates and samples were incubated at 37˚C overnight. Inhibition zones were documented by using BACMED 4i system and MICs were calculated with the BEES expert system [18].

3. Results

3.1. Hyp Uptake in Gram+ and Gram− Clinical Isolates

Figure 2 presents time-dependence of Hyp uptake into *Staphylococcus aureus*, *Enterococcus sp*., *Klebsiella pneumoniae* and *Escherichia coli* clinical isolates. We observed a significant difference between Hyp uptake in Gram+ and Gram− bacterial strains. The intensity of Hyp fluorescence, corresponding to the Hyp monomers concentration, was approximately three-times higher in Gram+ strains when compared Hyp fluorescence in Gram− isolates. Intracellular fluorescence intensity increased rapidly within the first 30 min of the incubation (**Figure 2(a)**). After this time, the increase in fluorescence intensity was slower, but still detectable. The highest fluorescence was observed at about 60 min in Gram− bacterial species, while in Gram+ bacteria the highest intensity was observed 120 min after incubation. Incubation longer than 120 min did not lead to further increase of the Hyp fluorescence intensity.

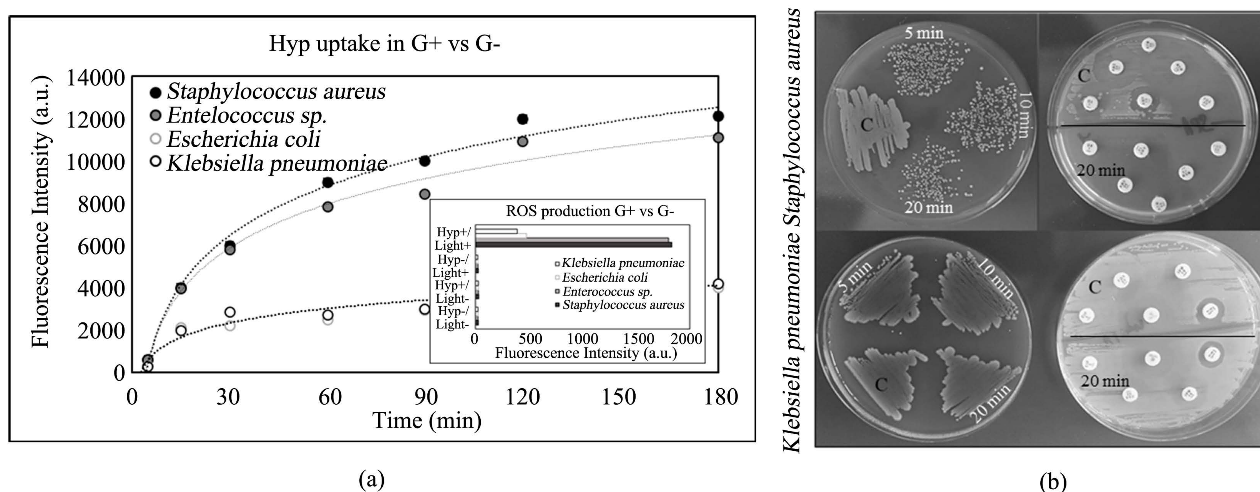

Figure 2. (a) Hyp fluorescence as a function of incubation time in G+ and G− clinical isolates; (b) representative images of growth (left) and susceptibility against selected antibiotics (right) in *Staphylococcus aureus* (upper) and *Klebsiella pneumoniae* (bottom) in the absence of Hyp, C and 24 hours after Hyp-photodynamic action.

3.2. ROS Production after Hyp Photoactivation (Hyp-aPDT) in Gram+ and Gram− Clinical Isolates

ROS production was assessed by using fluorescence spectroscopy after DCFH-DA (Thermo Fisher Inc., UK) staining of bacterial cells. Application of Hyp−/Light+ or Hyp+/Light− did not affect amount of ROS produced by bacterial cells. Intensity of DCFH-DA fluorescence was only slightly increased, and results were comparable with Hyp−/Light− controls. Our results show a huge production of ROS immediately after Hyp photoactivation (Hyp+/Light+). The intensity of ROS generation was directly dependent on the amount of Hyp loaded in bacterial cells. Similarly, to the Hyp uptake, we obtained significant differences in ROS production in Gram+ and Gram− bacterial strains (Figure 2(a), inserted graph), which is directly related to the different intracellular concentration of hyp in Gram+ and Gram− strains (Figure 2).

3.3. Effect of Hyp-aPDT on Gram+ and Gram− Clinical Isolates

Based on the data obtained in the previous experiments, we compared the effect of Hyp induced aPDT on the members of Gram+ (*Staphylococcus aureus*) and Gram− (*Klebsiella pneumoniae*) bacterial strains. To evaluate the effect of photoactivated Hyp on bacterial growth and survival, two sets of experiments were realized. First, we focused on assessment of the optimal concentration of Hyp and effective light dose needed for significant reduction of bacterial growth. The growth of Staphylococcus aureus was significantly suppressed, and the level of inhibition was dependent on Hyp concentration used (Figure 2(b), left panel). 3×10^{-6} M was assessed as effective Hyp concentration with inhibition potential on Gram+ bacteria. Mentioned Hyp concentration was used in all following experiments. At the same experimental conditions, no effect was observed in the case of *Klebsiella pneumoniae*. We also studied the effect of Hyp-aPDT on

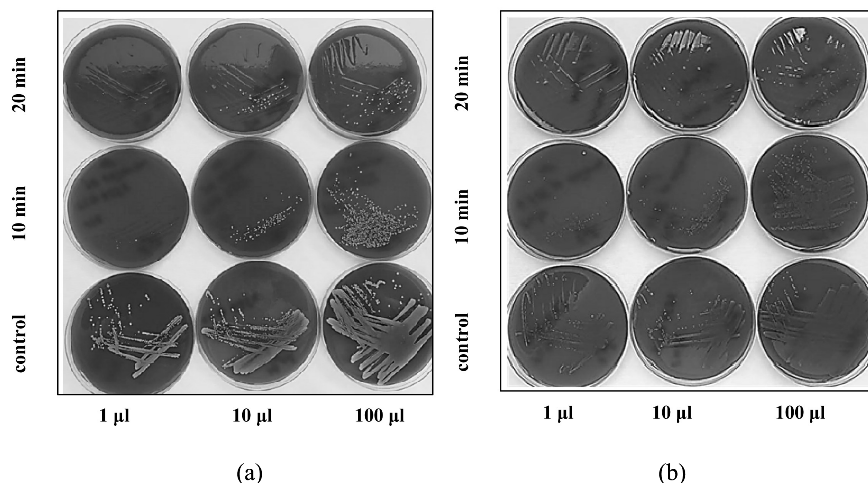

Figure 3. Representative images of colony morphology of (a) *Staphylococcus aureus* (MRSA) and (b) *Enterococcus sp.* (VRE) in the absence of Hyp and 24 hours after Hyp-photodynamic action (5 J/cm^2 vs 10 J/cm^2).

susceptibility to antibiotics typically used against Staphylococcus aureus and *Klebsiella pneumoniae*, as well. A significant difference between Gram+ and Gram− bacteria was observed again. *Staphylococcus aureus* was completely sensitive to the used antibiotics while in the case of *Klebsiella pneumonia*, Hyp induced aPDT had no effect on the antibiotic profile (**Figure 2(b)**, right panel).

Due to the weak response of Gram− bacteria to aPDT, we focused further experiments on representatives of Gram+ bacteria, *Staphylococcus aureus* and *Enterecoccus sp.*, as well. A working concentration of Hyp 3.0×10^{-6} M was determined as the optimal Hyp concentration for aPDT and this Hyp concentration was used in all experiments focused on MICs determination. **Figure 3** shows that Hyp activation (5 J/cm^{-2} vs 10 J/cm^{-2} irradiation dose) significantly decreased amount of CFU within 24 hours after Hyp-aPDT in *Staphylococcus aureus* and *Enterococcus sp*. The results demonstrated that the effectiveness of Hyp-aPDT strongly depends on the volume of the innoculated sample. If 1 µl of a bacterial suspension (0.5 McFarland) was used for inoculation, aPDT application resulted in complete suppression of bacterial growth. With increasing volumes of inoculated suspension (10 µl vs 100 µl), bacterial growth was less affected and effectiveness of Hyp-aPDT was less in general.

3.4. Minimum Inhibitory Concentrations Profiles

Control strain *Staphylococcus aureus CCM* 4626 was susceptible to all tested antibiotics with MIC ranging 0.077 - 1.11 mg/l. Photodynamic activation of Hyp led to the spreading of inhibition zones around the discs and a decrease of MIC immediately after irradiation. When aliquots were seeded 24 hours after irradiation results shown significant changes in the antibiotic profile. The irradiated samples showed resistance to CLI-2, TE-30, CIP-5, LZD, TIG and GEN, while they showed intermediate sensitivity to FOX-30 and ERY-30 (**Figure 4**).

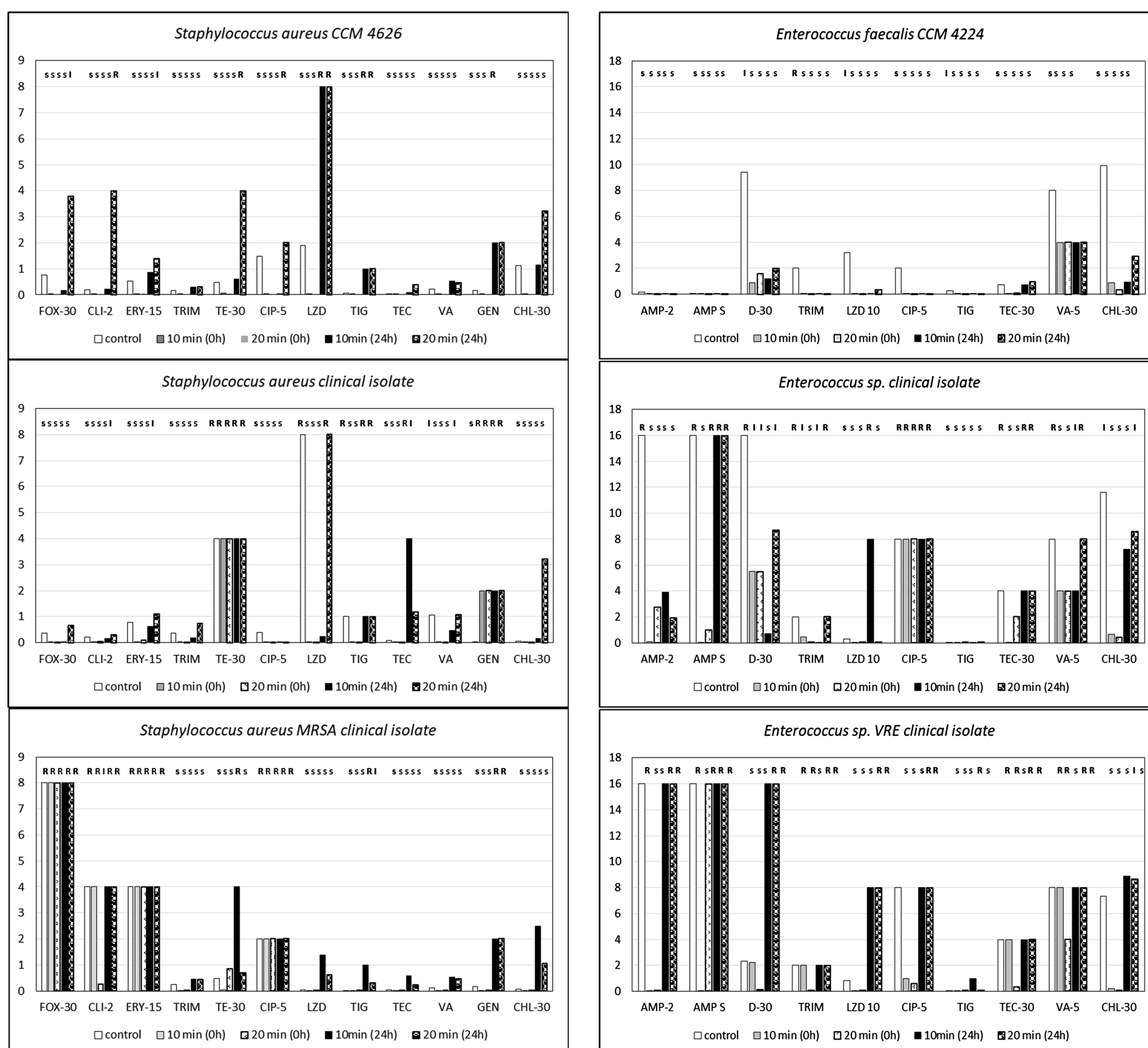

Figure 4. MICs of selected antibiotics in the absence of Hyp (control) or after hypericin-photodynamic action (5 J/cm^2 vs 10 J/cm^2) in *Staphylococcus aureus* and *Enterococcus sp.* clinical isolates. (S) susceptible, (I) intermediate, (R) resistant.

The clinical strain *Staphylococcus aureus CCM* 4399 was sensitive to FOX-30, CLI-2, ERY-15, TRIM, CIP-2, GEN and CHL-30 and resistant to TE and LZD under control conditions. When inoculating the culture immediately after irradiation, similarly to the control strain, there was a decrease in the MIC of all monitored antibiotics, except of TE-30 and GEN. After 24 h, the samples showed intermediate sensitivity or resistance to CLI-2, ERY-15, TE-30, LZD, TIG, TEC, VA and GEN which correspond to M efflux MLSB resistance phenotype.

Clinical strain *Staphylococcus aureus CCM 4356* was sensitive to TRIM, TE-30, LZD, TIG, TEC, VA, GEN and CHL-30 under control conditions. Because of resistance to FOX-30, CLI-2, ERY-15 and CIP-2 strain was classified as an isolate with the MRSA phenotype (c-MLSB MRSA Qnr). Hyp photoactivation had no significant effect on sensitivity immediately after irradiation. 24 hours after ir-

radiation, we observed a decrease in inhibition zones and an MICs increase from 0.483 mg/l to 4 mg/l ml for TE-30 and an increase from 0.172 mg/l to 2 mg/l for GEN similarly as in control.

The control strain *Enterococcus faecalis CCM 4224* was sensitive to all tested antibiotics under control condition, except for D-30 and TRIM. Incubation with Hyp and subsequent irradiation led to an increase of sensitivity, regardless of the time elapsed since the irradiation.

The clinical strain of *Enterococcus sp. CCM 4525* was sensitive to LZD-10 and TIG, of intermediate sensitivity to CHL-30 and resistant to AMP-2, AMP S, D-30, TRIM, CIP-5, TEC-30 and VA-5 under control conditions corresponding to VRE phenotype. Irradiated samples inoculated immediately after irradiation showed larger inhibition zones compared to control and reduced MICs for all antibiotics monitored except CIP-2. An aliquot of the samples inoculated 24 h after irradiation showed increase in MIC value and VRE phenotype again.

The clinical strain *Enterococcus sp. CCM 4585* was sensitive to D-30 and LZD 10, of intermediate sensitivity to TIG and CHL-30 and resistant to ANP-2, AMP S, TRIM, TEC and VA-5 under control conditions corresponding to VRE phenotype. Irradiation and Hyp activation increased the sensitivity against all tested antibiotics. We observed increase of resistance to the tested antibiotics and the VRE phenotype 24 hours after irradiation.

4. Discussion

aPDT is easy to apply, cheap and effective method to suppress the growth of pathogenic micro-organisms. This approach should be used in hospitals, the food industry or at the purification of bacterial contaminated waters [7] [8] [9]. Considering that aPDT is a multi-target process, it can be used also for treatment of bacterial infections caused by microorganisms expressing a high resistance against commonly used antibiotics. Hyp is a photoactive molecule showing anti-viral, anti-bacterial and anti-fungal properties, which effectively suppresses the growth of microorganisms [12] [13] [14]. Compared to other photosensitive molecules, such as curcumin and hypocrelin A, Hyp seems to be more effective, because some authors reported, that Hyp suppressed E. coli growth to 99.9% in Hyp concentration of 30 μM and a light dose of 5.9 J/cm^2. Curcumin (75 μM) and hypocrelin A (25 μM) achieved inhibition of growth of microorganisms with 90% efficacy after using light dose of 12 J/cm^2 [19] [20] [21] [22].

In this work, we have used Hyp to inactivate different bacterial strains. Our results show that an increase of Hyp concentration in culture medium leads to a higher cellular uptake of Hyp, and a high light dose used for Hyp activation increases an efficacy of aPDT resulting in significant reduction of cell survival mainly in Gram+ bacteria. The inhibitory effect of photoactivated Hyp on *Staphylococcus aureus* has been studied in several works. Yow *et al.* (2012) reported that the combination of Hyp (0 - 40 μM) and light irradiation (5 - 30 J/cm^2) induce significant killing of *Staphylococcus aureus* but is not effective in case of *E.*

coli. Complete inactivation of microorganisms was achieved by using Hyp at concentration 8 μM and light dose 30 J/cm² [23]. In general, the effective Hyp concentrations and light doses reported by different authors vary quite significantly [24]. The reason for this fact is that the efficacy of aPDT at similar concentrations of Hyp depends significantly on the wavelength of the used light. de Mello *et al.* (2013) used yellow light and light doses 10, 20 and 40 J/cm², Malacrida *et al.* (2020) used light at a wavelength of 570 - 610 nm and other authors irradiated Hyp with red light at a wavelength 665 nm [25] [26] [27]. In our work we activated Hyp with an orange light (590 nm), which corresponds to Hyp absorption maximum, and this allowed us to use effective concentration of Hyp to achieve the desired effect in Gram+ clinical strains of *Staphylococcus aureus* and *Enterococcus sp* [27]. In general, the change in the survival of microorganisms during and/or after aPDT is due to an increase in ROS concentration inside the cells. Overproduction of ROS beyond the control of antioxidant mechanisms leads to oxidative damage of the plasma membrane and biomacromolecules, resulting in the destruction of pathogenic microorganisms. Differences in susceptibility to aPDT among different microorganisms (Gram+ vs Gram−) strongly depend on their cell wall composition. A better sensitivity of Gram+ bacteria to aPDT is explained by the fact that the cell wall of Gram+ bacteria is formed by a thick layer of relatively permeable peptidoglycan and lipoteichoic acid, through which photosensitive molecules reach the cytoplasmic membrane more easily. The complicated cell wall of Gram− bacteria, on the other hand, slows down permeability for pts and thus negatively affects the effectiveness of aPDT [25]. Our results confirm that Gram+ strains have a significantly higher uptake of Hyp into cells compared to Gram−. The potency of aPDT also differs due to the different intracellular concentrations of captured Hyp. According to our results, Gram+ bacteria were more susceptible to generated oxidative stress, because intensity of oxidative stress depends on intracellular Hyp concentration which was significantly higher when compared to Gram− strains. In contrast, we did not observe a significant decrease in survival after aPDT in Gram− bacteria (*E. coli* and *Klebsiella pneumoniae*). It is probable that at the concentrations we used, insufficient amount of Hyp diffused through the cell wall and its photoactivation caused ROS increase only to the extent that microorganisms can control and eliminate. Another possibility is that the Hyp molecules are trapped on the outside of the cell wall and there are no suitable targets in their vicinity that could be significantly damaged by ROS. In any case, the amount of ROS produced in Gram− strains was lower due to the lower uptake and lower intracellular concentration of monomeric Hyp. Various approaches have been reported in the literature to achieve a more efficient passage of pts through the Gram− cell wall. One of them is to use polymyxin B or TRIS-EDTA pre-treatment [28], but we did not address this in our study.

In the 1980s, it was believed that antibiotics are the way to defeat infectious diseases caused by bacteria. However, due to over-prescriptions and overuse of

antibiotics, multi-resistant bacterial strains have been spreading. This fact is forcing scientific groups around the world to find more effective approaches to overcome an existing resistance. The mechanism of action of aPDT is based on the production of highly reactive singlet oxygen and/or other ROS. *E. coli* and *Klebsiella pneumoniae* survival was not significantly reduced at selected Hyp concentrations and light doses, and the results showed that their sensitivity to antibiotics did not change significantly. We have found that photoactivation of Hyp affects susceptibility of *Staphylococcus aureus* and *Enterococcus sp*. to commonly used antibiotics. *Staphylococcus aureus* and *Enterococcus sp.* were significantly more sensitive to the presence of antibiotics in short time-period after irradiation. The post-irradiation sensitivity to antibiotics was the result of intensive oxidative stress, which affects intracellular macromolecules non-specifically, including antibiotic deactivating enzymes and efflux systems, when are located quite near emerging ROS. Moreover, ROS can also affect the transcription of genes whose products are involved in the development of resistance against antibiotics. One of the intracellular targets for Hyp can be SarA—an important transcriptional regulator which indirectly controls many virulence factor genes in Staphylococcus aureus. The results of Wang *et al.* (2019) show that Hyp had an inhibition effect on SarA expression and increased *β*-lactam efficiency in MRSA [29]. We observed a similar increase in the effectiveness of *β*-lactam antibiotics (FOX-30, AMP and AMP-S) after 10 min of irradiation in the examined G+ strains, although in our case the sensitivity of MRSA to FOX-30 was not affected. In general, our results suggest that light-activated hypericin significantly decreased the minimum inhibitor concentrations of used antibiotics, especially at lower light doses and in the shorter post-irradiation period. On the contrary, we surprisingly observed an increase in MICs and a significant increase in the resistance of originally sensitive Gram+ clinical strains, especially after a higher light dose and in a longer time interval after irradiation. Painter *et al*. (2015) demonstrated that exposure of Staphylococcus aureus to sublethal oxidative stress leads to gentamicin resistant variants which have a greater catalase activity than wild-type bacteria [30]. We detected gentamycin resistance after Hyp activation in all tested *Staphylococcus aureus* strain. From our point of view ROS-stressed bacterial cells try to eliminate generated ROS by anti-oxidative cellular mechanisms and try to export Hyp out of the cell. At the same time, it is possible that the efflux systems which are used for the export of Hyp from cells are also involved in the efflux of antibiotics. Another possibility is that Hyp increases the pressure to expression a larger number of efflux pumps and thus indirectly contributes to the increase of resistance against antibiotics. Changes in efflux mechanisms are also indicated by the phenotypic manifestation of resistance acquired after Hyp-aPDT photoactivation, when resistance to macrolides and lincosamides, increased significantly. Nevertheless, aPDT is a promising method capable to eliminate pathogenic micro-organisms. However, it is still necessary to look for alternative approaches so that they are not left without relevant treatment of common infections. Other strategies still include the development

of new antimicrobial substances and their targeted delivery to the site of intervention, as well as the development of new methods promoting immune system thereby will further reduce the over-use of antibiotics [31].

5. Conclusion

ROS are attractive small molecules able to kill pathogenic microorganisms. We demonstrated that photoactivated Hyp (Hyp-aPDT) had an inhibition effect on growth of Gram+ clinical strains isolated from hospitalized patients. Number of survived cells strongly depends on intracellular concentration of Hyp and light dose used for Hyp activation. It is desirable to use effective concentrations of Hyp (pts) and light doses to achieve complete eradication of microorganisms. Otherwise, at sub-optimal concentrations, microorganisms actively fight against pts presence, which ultimately leads to the activation of mechanisms responsible for the increase of antibiotic resistance. The bacteria can activate the efflux mechanisms by which they attempt to remove Hyp from the cells, which in the second instance leads to an increase of antibiotic resistance especially against aminoglycozides, macrolides and lincosamides.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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