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In vitro ACTIVITY OF PRODIGIOSIN ISOLATED FROM *Serratia marcescens* IN COMBINATION WITH TWO GROUPS OF ANTIBIOTICS AGAINST GRAM-POSITIVE MICROORGANISMS

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The work was aimed to study the synergy of the antimicrobial activity of the prodigiosin pigment with antibiotics against bacteria of the genera *Bacillus*, *Staphylococcus* and *Streptococcus*. The serial dilution method was used to evaluate antimicrobial compositions, which included inhibitors of cell wall synthesis: ampicillin, benzylpenicillin, vancomycin, cefazolin, and metronidazole (nitroimidazole derivatives) in combination with the pigment prodigiosin isolated from *Serratia marcescens*. Each combination was tested against the studied strains. The fractional inhibitory concentration index (FICI) for each combination was calculated to determine synergy, and the results were interpreted as follows: FICI ≤0.5 — synergism; FICI> 4.0 — antagonism; and FICI> 0.5–4 — neutralism.

It was shown that the ethanol extract of prodigiosin in combination with benzylpenicillin, vancomycin, cefazolin, and metronidazole interacted differently synergistically depending on the type of microorganism. The combinations of prodigiosin and metronidazole showed a synergistic effect against *Bacillus subtilis*, vancomycin and cefazolin against *Staphylococcus aureus* and benzylpenicillin against *Streptococcus pyogenes*. Other combinations of prodigiosin and antibiotics showed a neutral effect, and in the case of cefazolin against *Str. pyogenes*, even an antagonistic effect.

Thus, the study showed the synergism of prodigiosin with antibiotics depending on the type of microorganism, contributed to a several-fold decrease in the minimum inhibitory and bactericidal concentrations of each component separately, and the results indicated that prodigiosin acted separately more efficiently against gram-positive non-spore-forming bacteria. This synergistic combination of antimicrobial agents had great potency to prevent bacterial resistance.

Key words: prodigiosin, antimicrobial compounds, antimicrobial synergy.

Prodigiosins seem to be ubiquitous secondary metabolites with a great variety of producers and even greater variation in production yields reported so far. In addition to the best known and studied prodigiosin-producing strains (*Serratia* and *Streptomyces*), representatives of *Pseudomonas*, *Vibrio*, *Alteromonas*, *Actinomadura*, *Saccharopolyspora*, and *Streptoverticillium* were also identified as producing one or a mixture of prodigiosins [1, 2]. A boom in marine microbiology and the search of the extreme habitats showed a considerable number of marine microorganisms and extremophyles, especially from family *Hahellacea* and *Pseudoalteromonas*, to be producers of prodigiosins [1, 3]. Notwithstanding the scarce knowledge of its mechanism of action, prodigiosin appeared as a pluripotent molecule with various health-related properties. The most important being: an anticancer agent [1, 4], an immunosuppressant, an antiprotozoal and an antibacterial agent, while also offering protection against UV [1, 3, 5], as well as inhibition of the growth of a wide range of gram-positive (*Staphylococcus* spp., *Bacillus* spp., etc.) and gram-negative (*Escherichia coli*, *Salmonella enterica*, etc.) bacteria [6].

Nevertheless, the antimicrobial properties of prodigiosin have often been questioned, particularly because of the high concentrations required for it to be effective, as these exceed the levels causing toxicity in mammalian cells. For this reason, it has been studied in

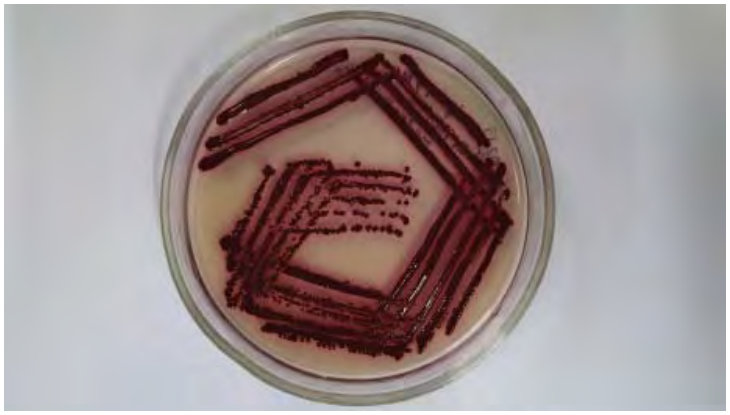
greater depth for its use in anticancer and immunosuppressive therapy, than as an agent to fight infectious agents [1, 4, 7], but antimicrobial resistance threatens a resurgence of life-threatening bacterial infections and the potential demise of many aspects of modern medicine. Despite intensive drug discovery efforts, no new classes of antibiotics have been developed into new medicines for decades, in large part owing to the stringent chemical, biological and pharmacological requisites for effective antibiotic drugs. A new option for combating such pathogens is combination therapy. Combinations of antibiotics and antibiotics with non-antibiotic activity-enhancing compounds offer a productive strategy to address the widespread emergence of antibiotic-resistant strains [8, 9]. The purpose of the study was to investigate the synergism of antimicrobial activity of the prodigiosin pigment in combination with antibiotics against gram-positive test strains of bacteria.

Materials and Methods

Isolation and identification of pigment-producing strains of bacteria. As a pigment producer, we used the species *S. marcescens*, namely the pigment-forming strain, which were isolated in the laboratories of the Department of Microbiology, virology, and immunology of Bogomolets National Medical University from the bentonite clays of Kurtsivskyi deposit (Crimea, Ukraine). Red color pigment-producing bacteria with different morphology and individual colonies were picked up separately and purified by quadrant streaking in nutrient agar plates for the isolation of bacterium *S. marcescens*. The pigmented colonies of bacteria were selectively isolated and transferred by the method of loop inoculum on nutrient agar surface of the following composition: peptone — 10 (g/l), glycerol — 10 (ml/l), K_2SO_4 — 10 (g/l), yeast extract — 2 (g/l), $MgCl_2$ — 1.4 (g/l), agar 15 (g/l), pH 6.5–7.0 (Fig.). Then Petri dishes with inoculated strains of *S. marcescens* were incubated in a thermostat at +28 °C for 24–72 h in an inverted position for the screening of pigment-producing strains. These obtained isolates were taken and identified by morphological and biochemical characterization using Bergey's manual of systematic bacteriology [10, 11].

The method of obtaining purified prodigiosin. The extraction of prodigiosin pigment from biomass of bacteria was carried out by double processing of biomass with 96% ethanol. The resulting preparation dried in air and reextracted. The procedure was repeated several times before the release of insoluble admixtures. The resulting homogeneous solution was designated as a crude pigment complex or ethanol extract. The ethanol extract was evaporated dry in a drying oven at a temperature of +45–50 °C and the residue dissolved in chloroform (10 ml/l of precipitate). The resulting solution was mixed with an equal volume of a water-ethanol mixture (4:1) and emulsified on a magnetic stirrer for 1 hour at room temperature. A water-ethanol mixture containing water-soluble admixture separated by a separating funnel. The procedure was repeated by increasing the volume content of ethanol by half. The drug was then redried in a vacuum oven and redissolved in ethanol (10 ml/g precipitate) [3].

Quantification of prodigiosin. The purity of prodigiosin isolated from the pigmented strain was determined by high-performance



***Serratia marcescens* isolated from the bentonite clays on nutrient agar surface**

liquid chromatography (HPLC-MS) on the Agilent 1200 device (Agilent Technologies, USA) with diode-matrix and mass-selective detectors. Detection was performed using a diode-matrix detector with 315 and 535 nm signal recording. The molecular weight of the compounds determined on a massive detector with ionization in positive and negative APCI mode. Determination of the absorption spectrals of the isolated pigment determined by UV/VIS spectrophotometry method. Absorption spectra of the extract were tested by Portlab 512 spectrophotometer in the range 400-700 nm. Absorption of bacterial cells before extraction noted at each stage. The concentration of pigment was calculated using the following equation [12]:

$$\text{Concentration of prodigiosin} = \frac{[OD_{534} - (1.381 \times OD_{620})] \times 1000}{OD_{620}}$$

where OD — optical density; OD_{534} — represent pigment absorption; OD_{620} — represent bacterial cells absorption; 1.381 — constant.

Evaluation of antibacterial activity. To evaluate the antimicrobial properties of prodigiosin pigment in combination with antibiotics, we used the next reference strains of microorganisms: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923 and *Streptococcus pyogenes* ATCC 21059. The strains obtained from Gromashevsky Institute of Epidemiology and Infectious Diseases of the National Academy of Medical Sciences of Ukraine and Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine.

Antimicrobial activities and synergistic interaction with antibiotics of the obtained purified prodigiosin pigment were determined at the Department of Microbiology, Virology, and Immunology of Bogomolets National Medical University. The minimum inhibitory and minimum bactericidal concentrations (MICs and MBCs) were determined by the micro broth dilution method. The broth microdilution format is traditionally set up as 2-fold dilutions of the compounds in sterile polystyrene plates at a lower volume of the drugs and nutrient medium used in the study. Each tube of a 96-well plate (except control) in a volume of 150 µl was injected with a suspension of microorganism cells in a liquid nutrient medium in the amount of 1×10^6 CFU/ml. The compounds were added in the same amount to the first tube, followed by 2-fold dilutions, and then incubated at + 37 °C for 24 h. The MIC and MBC were determined after 24 hours. The MBC was determined by adding 50 µl of the suspensions from the wells, which did not show any growth after incubation during MIC assays, to 150 µl of fresh broth. These suspensions were reincubated at +37 °C from 72 to 96 h. The MBC was determined as the lowest concentration of extract which inhibited 100% growth of microorganisms [13].

To evaluate the effect of the combination, the fractional inhibitory concentration index (FICI) calculated for each antibiotic combination [14] by computing the ratio of the MIC of the combination divided by the MIC of the antimicrobial alone for each agent and then adding those two ratios together (Equation A). Briefly, FICI was calculated as follows:

$$\text{Equation A: FICI} = \left[\frac{\text{MIC}_{A(+B)}}{\text{MIC}_A}\right] + \left[\frac{\text{MIC}_{B(+A)}}{\text{MIC}_B}\right]$$

where $\text{MIC}_{A(+B)}$ = MIC of A in the presence of drug B; MIC_A = MIC of drug A alone; $\text{MIC}_{B(+A)}$ = MIC of B in the presence of drug A; MIC_B = MIC of drug B alone. The FICI data were interpreted using the following criteria: Synergy defined as a FICI of ≤ 0.5; No interaction: > 0.5 to 4.0 (additive: > 0.5 to ≤ 1.0; indifference: FICI > 1.0 to ≤ 4.0) and antagonism by FICI of > 4.0. Discrepant MIC results and those combinations with FICI ≤ 1 were confirmed by performing an additional duplicate synergy test.

All studies performed in triplicate and the statistical processing of the obtained results carried out by using the specialized software Statistica 9.0 (StatSoft Inc., USA). A value of $P < 0.05$ was considered as statistically significant [15].

Results and Discussion

Combinatorial compounds sensitivity assays showed that metronidazole, vancomycin/cefazolin, and benzylpenicillin with prodigiosin (in ratio 1:1) presented remarkably synergistic activities against *B. subtilis*, *St. aureus*, and *Str. pyogenes*, which were selected for the study based on differences in the structure of their cell walls and differents sensitivity to antibiotics, and FICI values were ranging from 0.279 to 0.498. The MICs of five antimicrobials and FICI combinations of prodigiosin against test strains of microorganisms are shown in Table 1.

Table 1. **Value of MIC (range) of combined effect of antibiotics and prodigiosin relative to test strains of microorganisms**

Antimicrobial compounds	MIC (range), μg/ml				FICI	Interpretation
	MIC_A	$MIC_{A(+B)}$	MIC_B	MIC		
	Bacillus subtilis ATCC 6633					
Prodigiosin	11.2	–	–	–	–	–
Ampicillin	–	22.5^	250^	250^	–	–
Benzylpenicillin	–	11.2	250^	15	1.006	Indifference
Vancomycin	–	11.2	125^	62.5	1.500	Indifference
Cephazolin	–	5.6–11.2	250^	62.5–125	0.499–0.999	Indifference
Metronidazole	–	2.8	62.5	15.62	0.498	Synergy
Staphylococcus aureus ATCC 25923						
Prodigiosin	1.4	–	–	–	–	–
Ampicillin	–	0.7	0.78	0.78	1.500	Indifference
Benzylpenicillin	–	2.8	250^	1.87	2.014	Indifference
Vancomycin	–	0.05	0.05	0.01	0.279	Synergy
Cephazolin	–	0.35	1.95	0.24	0.373	Synergy
Streptococcus pyogenes ATCC 21059						
Prodigiosin	2.8	–	–	–	–	–
Ampicillin	–	2.8–5.6	6.25	6.25–12.5	3.000	Indifference
Benzylpenicillin	–	0.35–0.7	250^	0.46–0.93	0.126–0.252	Synergy
Vancomycin	–	0.35–0.7	0.19–0.39	0.19–0.39	0.611–1.249	Indifference
Cephazolin	–	1.4–2.8	1.95–3.90	15.62–31.25	4.503–9.012	Antagonism

Hereinafter: MIC_A — prodigiosin pigment; MIC_B — ampicillin, benzylpenicillin, vancomycin, cephazolin, metronidazole; $MIC_{A(+B)}$ — prodigiosin (+ ampicillin, benzylpenicillin, vancomycin, cephazolin, metronidazole); $MIC_{B(+A)}$ — ampicillin, benzylpenicillin, vancomycin, cephazolin, metronidazole (+ prodigiosin); ^— the absence of inhibitory effect when making the maximum test concentration; * — $P < 0.05$.

These results reflect a > 4-fold decrease in MIC for metronidazole and vancomycin, > 8-fold decrease for cefazolin and more than 250-fold for benzylpenicillin, and a greater than 4-fold decrease in prodigiosin (synergistic MIC) compared to the MIC of each compound. Other combinations of antibiotics with prodigiosin did not show synergistic activity.

Ampicillin, benzylpenicillin, vancomycin, cefazolin, and metronidazole in combination with prodigiosin were tested *in vitro* to determine whether they were bacteriostatic or bactericidal against the test strains of microorganisms. The MBC of five antimicrobial compounds and FICI combinations of them with prodigiosin against test strains of microorganisms are shown in Table 2.

In determining the MBC/MIC ratio for individual compounds, four different combinations of antibiotics with prodigiosin had a bactericidal action of 0.05 to 31.25 μg/ml. Interestingly, when prodigiosin was assayed in a combination with a different fixed concentration of antibiotics, the MBC of prodigiosin was in the range 0.1 to 5.62 μg/ml, probably because the bactericidal nature of prodigiosin was not dominant in the combination, due to the selective effect on the target structure different in the bacterial cell.

The first area of research is the combined use of prodigiosin with antibacterial drugs, presented in this publication, reproduced by the simultaneous effect of the pigment of *S. marcescens* and inhibitors of cell wall

Table 2. **Value of MBC (range) of combined effect of antibiotics and prodigiosin against test strains of microorganisms**

Antimicrobial compounds	MBC (range), µg/ml				FICI	Interpretation
	MBC_A	$MBC_{A(+B)}$	MBC_B	$MBC_{B(+A)}$		
	Bacillus subtilis ATCC 6633					
Prodigiosin	22.5	–	–	–	–	–
Ampicillin	–	22.5	250	250	–	–
Benzylpenicillin	–	22.5	250	30	1.120	Indifference
Vancomycin	–	22.5	125	250	–	–
Cephazolin	–	22.5	250	250	2.000	Indifference
Metronidazole	–	5.6	125	31.25	0.499	Synergy
Staphylococcus aureus ATCC 25923						
Prodigiosin	2.8	–	–	–	–	–
Ampicillin	–	1.4	0.78	1.56	1.500	Indifference
Benzylpenicillin	–	5.6	250	3.74	2.021	Indifference
Vancomycin	–	0.1	0.19	0.05	0.298	Synergy
Cephazolin	–	0.7	3.90	0.48	0.373	Synergy
Streptococcus pyogenes ATCC 21059						
Prodigiosin	5.6	–	–	–	–	–
Ampicillin	–	11.2	250	25	2.101	Indifference
Benzylpenicillin	–	1.4	250	1.87	0.256	Synergy
Vancomycin	–	1.4	0.78	0.78	1.249	Indifference
Cephazolin	–	5.6	7.80	62.5	9.002	Antagonism

synthesis on gram-positive non-spore-forming bacteria. The results of the synergistic effect confirm and supplement the literature on the potentiation of conventional antibiotics and antimicrobial agents from natural resources. The combinations of prodigiosin and cephazolin or vancomycin had synergistic effects on *St. aureus*. The same result for *Str. pyogenes*, but in the combination of prodigiosin with benzylpenicillin was obtained. On the opposite side, the combinations of prodigiosin and inhibitors of cell wall synthesis had no shown synergistic effect on gram-positive spore-forming bacteria.

The second area of research, presented in the paper, was on evaluate in the susceptibility of spore-forming bacteria to antimicrobial preparations, due to the impact of prodigiosin and nitroimidazole derivatives on *B. subtilis*. The combinations of prodigiosin and metronidazole had synergistic effects on *B. subtilis*, and the mechanism of action combination can associate with inhibiting nucleic acid synthesis by disrupting the DNA of microbial cells.

The study of the interactions of the compounds showed that prodigiosin may be competing for the same cellular target as antibiotics, leading to a neutral or antagonistic effect. Thus, the results of studies indicate that prodigiosin by peptidoglycan hydrolysis, which is predominant in the cell walls of gram-positive bacteria and/or accumulation inside the bacterial cell by mediating redox reactions, leads to impaired membrane permeability and/or disruption of DNA structure. We have demonstrated that prodigiosin can enhance the activity of individual antibiotics depending on the type of microorganism and possibly other non-clinically effective antibiotics against pathogenic bacteria while providing lower FICI values, which is an important finding of our study. The results indicate that prodigiosin acts more effectively against gram-positive non-spore-forming bacteria, and synergistic combinations of antimicrobial agents have

great potential for preventing resistance.

Summarizing, a set of five antibiotics with different structures was analyzed in the presence of prodigiosin and found that in total only four combinations had synergistic activity against test strains of gram-positive bacteria. The study has indicated that synergistic combinations of antimicrobial agents being susceptible to pathogenic bacteria had a great potency to prevent resistance. The resultant synergy in the combination of prodigiosin and inhibitors cell wall synthesis is a novel concept, as such combinations will have identical or different mechanisms of action, which may lead to new choices of therapeutic agents for the treatment, especially infections caused by multidrug-resistant microorganisms having no effective therapy available. Combinations of inhibitors cell wall synthesis or nitroimidazole derivatives with prodigiosin may warrant further clinical investigation for treating the diseases associated with pathogenic gram-positive microorganisms.

Conclusions

The work was performed at the Department of Microbiology, virology, and immunology of Bogomolets National Medical University within the framework of the initiative-search topic "Biological activity of prodigiosin pigment isolated from *Serratia marcescens*".

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АКТИВНІСТЬ *in vitro* ПРОДИГІОЗИНУ, ВИДІЛЕНОГО ІЗ *Serratia marcescens*, У КОМБІНАЦІЇ З ДВОМА ГРУПАМИ АНТИБІОТИКІВ ПРОТИ ГРАМПОЗИТИВНИХ МІКРООРГАНІЗМІВ

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Метою роботи було вивчити синергізм антимікробної активності пігменту продигіозину з антибіотиками щодо бактерій родів *Bacillus*, *Staphylococcus* та *Streptococcus*. Використовували метод серійних розведень для оцінювання антимікробних комбінацій, які включали інгібітори синтезу клітинної стінки: ампіцилін, бензилпеніцилін, ванкоміцин, цефазолін та метронідазол (похідні нітроімідазолу) у поєднанні з пігментом продигіозином, виділеним із *Serratia marcescens*. Кожну комбінацію тестували проти досліджуваних штамів. Індекс фракційної інгібувальної концентрації (FICI) для кожної комбінації обчислювали для визначення синергії, а отримані результати інтерпретували так: FICI ≤ 0.5 — синергізм; FICI > 4.0 — антагонізм; FICI > 0.5–4 — нейтралізм.

Показано, що етаноловий екстракт продигіозину в комбінації з бензилпеніциліном, ванкоміцином, цефазоліном та метранідазолом по-різному синергічно взаємодіє залежно від виду мікроорганізму. Комбінації продигіозину та метронідазолу проявляли синергічний ефект — проти *Bacillus subtilis*, ванкоміцин і цефазолін — проти *Staphylococcus aureus* і бензилпеніцилін — проти *Streptococcus pyogenes*. Інші комбінації продигіозину та антибіотиків виявляли нейтральний ефект, а у випадку цефазоліну проти *Str. pyogenes* — навіть антагоністичний ефект.

Таким чином, дослідження показало синергізм продигіозину з іншими антибіотиками залежно від виду мікроорганізму, що сприяє зменшенню у кілька разів мінімальної інгібувальної та бактерицидної концентрації кожного з компонентів окремо. Отримані результати вказують на те, що продигіозин окремо діє більш ефективно щодо грампозитивних неспороутворювальних бактерій, при цьому синергічні комбінації антимікробних агентів мають велику потенцію для запобігання резистентності бактерій.

Ключові слова: продигіозин, антимікробні сполуки, синергізм антимікробної дії.

АКТИВНОСТЬ *in vitro* ПРОДИГИОЗИНА, ВЫДЕЛЕННОГО ИЗ *Serratia marcescens*, В КОМБИНАЦИИ С ДВУМЯ ГРУППАМИ АНТИБИОТИКОВ В ОТНОШЕНИИ ГРАМПОЛОЖИТЕЛЬНЫХ МИКРООРГАНИЗМОВ

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Целью работы было изучение синергизма антимикробной активности пигмента продигиозина с антибиотиками в отношении бактерий родов *Bacillus*, *Staphylococcus* и *Streptococcus*. Использовали метод серийных разведений для оценки антимикробных композиций, которые включали ингибиторы синтеза клеточной стенки: ампициллин, бензилпенициллин, ванкомицин, цефазолин и метронидазол (производные нитроимидазола) в сочетании с пигментом продигиозином, выделенным из *Serratia marcescens*. Каждую комбинацию тестировали против исследуемых штаммов. Индекс фракционной ингибиторной концентрации (FICI) для каждой комбинации вычисляли для определения синергии, а полученные результаты интерпретировали так: FICI ≤ 0.5 = синергизм; FICI > 4.0 = антагонизм; FICI > 0.5–4 = нейтрализм.

Показано, что этаноловый экстракт продигиозина в комбинации с бензилпенициллином, ванкомицином, цефазолином и метронидазолом по-разному синергически взаимодействует в зависимости от вида микроорганизма. Комбинации продигиозина и метронидазола проявляли синергический эффект против *Bacillus subtilis*, ванкомицин и цефазолин — против *Staphylococcus aureus* и бензилпенициллин — против *Streptococcus pyogenes*. Другие комбинации продигиозина и антибиотиков проявляли нейтральный эффект, а в случае цефазолина против *Str. pyogenes* — даже антагонистический эффект.

Таким образом, исследование показало синергизм продигиозина с антибиотиками в зависимости от вида микроорганизма, что способствует уменьшению в несколько раз минимальной ингибиторной и бактерицидной концентрации каждого из компонентов в отдельности. Полученные результаты указывают на то, что продигиозин отдельно действует более эффективно в отношении грамположительных неспорообразующих бактерий, при этом синергические комбинации антимикробных агентов имеют большую потенцию для предотвращения резистентности бактерий.

Ключевые слова: продигиозин, антимикробные соединения, синергизм антимикробного действия.