SEÇÃO VII.
BIOLOGIA

DOI 10.36074/09.10.2020.v3.03

IN VITRO ACTIVITY OF PRODIGIOSIN ISOLATED FROM *SERRATIA MARCESCENS* IN COMBINATION WITH POLYENES AND AZOLES AGAINST *ASPERGILLUS NIGER*

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Biopigment are natural compounds (secondary metabolite) produced by many organisms represent one of the important sources of potential lead compounds. Prodigiosin (5[(3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene) is a red, tripyrrole, water insoluble, bioactive pigment produced by number of different bacteria, actinomycetes and some fungi [1]. Notwithstanding the scarce knowledge of its mechanism of action, prodigiosin appears as a pluripotent molecule, with various health-related properties; the most important being: an anticancer agent [2,3], an immunosuppressant, an antiprotozoal and an antibacterial agent [2,4,5]. 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 [2,3,6].

The rate of emergence of fungicide resistance is greater than the pace of fungicide discovery, and the long registration process for new compounds adds further delays. This situation parallels the situation for antibiotics. Increased research activity is thus needed to develop new antifungal drugs [7]. Therefore, combination therapy is a new option for combating such pathogens. Combinations of antibiotics with non-antibiotic activity-enhancing compounds offer a productive strategy to address the widespread emergence of antibiotic-resistant strains [8].

Materials and methods. As a pigment producer, we used the species *S. marcescens*, namely the pigment-forming strain, which 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). *Aspergillus niger* ATCC 704 was evaluated against four antifungal agents that included 2 polyene antimycotics: amphotericin B (AMB) and nystatin (NYS), and 2 azole antifungals: intraconazole (INZ) and fluconazole (FNZ) in combination with prodigiosin pigment (PG), using the method of broth microdilution.

The MICs of AMB, ITZ, NYS and FNZ (HiMedia Laboratories, Mumbai, India) were determined following the recommendations of the EUCAST protocol for filamentous fungi [9]. All antifungal drugs (except for FNZ, which solubilized in sterile water) were solubilized in dimethyl sulfoxide (DMSO) and the working solutions were prepared in RPMI 1640 medium with 2% glucose, L-glutamine and a pH indicator but

without bicarbonate (Sigma-Aldrich, São Paulo, Brazil). Conidial suspensions were obtained from sporulated *Aspergillus* cultures and adjusted to contain 2-5×10^6 conidia/mL by counting in a hemocytometer. To obtain a final concentration of 2-5×10^5 conidia/mL, 1:10 dilutions were prepared in sterile distilled water. For the microplate preparation, in each well, 100 µL of the final conidial suspension was added to 100 µL of each of the antifungal drug concentrations. Growth and negative control are included in all tests. The incubation time is about 48 h at +35ºC and MIC is read visually. The MIC value is a no-growth visual endpoint.

To evaluate the effect of the combination, the fractional inhibitory concentration index (FICI) calculated for each antifungal combination [10] 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 (1). Briefly, FICI was calculated as follows:

$$x = \left[\frac{MIC_{A(+B)}}{MIC_A}\right] + \left[\frac{MIC_{B(+A)}}{MIC_B}\right] \quad (1)$$

Where, $MIC_{A(+B)}$ = MIC of A in the presence of drug B; MIC_A = MIC of drug A alone; $MIC_{B(+A)}$ = 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.

The purity of prodigiosin isolated from the pigmented strain was determined by high-performance liquid chromatography (HPLC-MS) on the Agilent 1200 device (Agilent Technologies, USA) with diode-matrix and mass-selective detectors. The quantitative determination of the red pigment was done by measuring the absorbance at 530 nm using double beam UV-Visible spectrophotometer.

Research results. Combinatorial compounds sensitivity assays showed that INZ, NYS, and FNZ with PG (in ratio 1:1) presented remarkably synergistic activities against *Asp. niger* (Table 1) with the FICIs from 0.374 to 0.499. These values reflect a 4-fold reduction in the MIC for INZ, NYS, and FNZ for both culture media and a >4-fold reduction in the MIC for PG (synergistic MIC) compared to the MIC of each compound alone.

Table 1

Value of MIC (range) of combined effect of antimycotics and prodigiosin relative to *Aspergillus niger*

Antifungal compounds	MIC (range), µg/ml				FICI	Interpretation
	MIC_A	$MIC_{A(+B)}$	MIC_B	$MIC_{B(+A)}$		
	Aspergillus niger ATCC 704				–	–
prodigiosin	22.5	–	–	–		
amphotericin B	–	22.5	1	2	3.000	indifference
intraconazole	–	2.8	12.5	3.12	0.374	synergy
nystatin	–	5.6	3.74	0.93	0.497	synergy
fluconazole	–	5.6	25	6.25	0.499	synergy

*Note: MIC_A – prodigiosin pigment; MIC_B – amphotericin B, intraconazole, nystatin, fluconazole; $MIC_{A(+B)}$ – prodigiosin (+ amphotericin B, intraconazole, nystatin, fluconazole); $MIC_{B(+A)}$ – amphotericin B, intraconazole, nystatin, fluconazole (+ prodigiosin); * – P < 0.05.*

Interestingly, when prodigiosin assayed in combination with a different fixed concentration of antimycotics, the MIC of compounds showed similar values of

reduction, suggesting that the fungistatical/fungicidal character of prodigiosin was no dominant in the combination.

Conclusions. In addition to screening for novel antifungal compounds, combination therapy is considered a potential alternative strategy for treating invasive fungal infections. The results of the synergistic effect confirm and supplement the literature on the potentiation of conventional antibiotics and antimicrobial agents from natural resources. The study has demonstrated that prodigiosin can enhance the activity of individual antimycotics providing lower FICI values, which is an important finding of our study. The study has indicated that synergistic combinations of antifungal agents being susceptible to pathogenic fungi had a great potency to prevent resistance. The resultant synergy in the combination of prodigiosin and polyenes or azoles is a novel concept, as such combinations will have different mechanisms of action, which may lead to new choices of therapeutic agents for the treatment, especially infections having no effective therapy available.

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