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Stimulatory effect on rat thymocytes proliferation and antimicrobial activity of two 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones

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ABSTRACT

Recently we reported the identification and synthesis of cyclodidepsipeptides, 3,6-di(propan-2-yl)-4-methyl-morpholine-2,5-dione (**PPM**) and 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione (**BPM**), as potential precursors of enniatin B in *Fusarium sporotrichioides*. No data concerning biological activity of **PPM** and **BPM** have hitherto been published. The possible immunomodulatory effect and antimicrobial activity of **PPM** and **BPM** were investigated in this study, due to well known biological activities of enniatin B. The cytotoxicity effect of **PPM** and **BPM** on rat thymocytes demonstrated that increasing concentrations (0.1, 1, 10 μg/well) of **PPM** and **BPM** to cell culture, showed no significant effect on thymocytes toxicity. Simultaneously, incubation with studied cyclodidepsipeptides did not result with decreased mitochondrial membrane potential. Further, thymocytes exposure to increasing concentration of **PPM** and **BPM** was not able to induce significant reactive oxygen species (ROS) production in rat thymocytes. **PPM** and **BPM** administrations to cell culture in concentrations of 0.1 and 1 μg/well resulted with no significant increase of proliferative activity. However, significantly increased proliferative activity was detected with 10 μg of **PPM** ($p < 0.001$) and **BPM** ($p < 0.05$), as compared to their respective controls. The *in vitro* antimicrobial activity of **PPM** and **BPM** was tested against two Gram-positive and three Gram-negative bacteria. The results indicated that MIC values against tested strains ranged between 2.00 and 25.00 mg/ml. **PPM** showed much better activity against all tested bacteria in comparison with **BPM**. **PPM** was equally effective against both Gram-positive and Gram-negative bacteria, at the dose of 2.00 mg/ml.

1. Introduction

The family of cyclodepsipeptides comprises natural products and synthetic peptide lactones with at least one ester bond in their skeleton. The great interest that this class of compounds has elicited in scientific community is explained by their wide range of biological activities, intriguing mechanisms of actions and attractive molecular architecture. For example, they display a variety of biological effects, such as immunosuppressant, antibiotic, antifungal, antiinflammatory or antitumoral activities (Lemmens-Gruber et al., 2009; Sarabia et al., 2004; Bagavananthem Andavan and Lemmens-Gruber, 2010). In addition, many of these cyclic depsipeptides represent useful tools for the research of biological processes involved in cellular regulations. Either as potential new drugs or as biochemical tools, this class of compounds has offered fascinating disclosures in the area of pharmaceutical research together with extraordinary opportunities in organic synthesis for development and invention of new synthetic methodologies. In conjunction with this, the chemical synthesis of those naturally occurring compounds is of great importance, particularly when the isolation of natural product results in extremely laborious or ecologically unsuitable procedures (Sarabia et al., 2004).

Among the large family of cyclodepsipeptides, the simplest members are the cyclodidepsipeptides which have an ester group and an amide group in the same 6-membered ring. Two cyclo didepsipeptides, 3,6-di(propan-2-yl)-4-methyl-morpholine-2,5-dione (**PPM**; $C_{11}H_{19}NO_3$, $M = 213.27$) and 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione (**BPM**; $C_{12}H_{21}NO_3$, $M = 227.30$) (Scheme 1), were found for the first time in the natural products as potential precursors of enniatin B in the pathogenic fungi *Fusarium sporotrichioides*, isolated from the stem of fresh *Hypericum barbatum* Jacq. For identification and confirmation, those compounds were synthesized and studied by density functional theory calculations and infrared spectroscopy (Smelcerovic et al., 2011). No data concerning biological activity of **PPM** and

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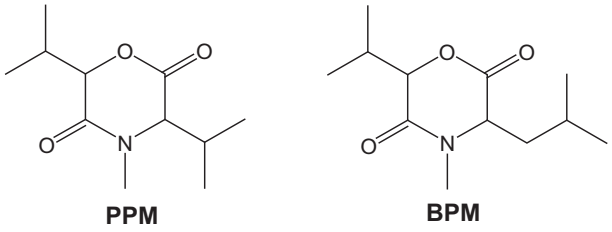

Scheme 1. Chemical structures of the compounds under study.

BPM have hitherto been published. Enniatins possess antibiotic (Fináková et al., 2007) and antiviral (McKee et al., 1997) activities. The current study was designed to evaluate the possible immunomodulatory effect and antimicrobial activity of two synthesized 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones, **PPM** and **BPM**, which are potential precursors enniatin B.

2. Experimental

2.1. Animals

Experiments were performed on adult male Wistar rats (150–180 g), 8–10 weeks old, bred at the Vivarium of the Institute of Biomedical Research, Medical Faculty, Nis, under conventional laboratory conditions and in accordance with national animal protection guidelines.

2.2. Materials

Culture medium (CM) was prepared using RPMI 1640 (Sigma, St. Louis, Mo., USA), according to the manufacturer's instructions. CM containing 25 mM HEPES, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal calf serum (FCS).

Concanavalin A (ConA) was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. ConA was dissolved in CM at concentration of 50 μg/ml.

2′,7′-Dichlorofluorescin diacetate (H2DCF-DA), Cell Counting Kit (CCK-8) and Rhodamine 123 were purchased from Sigma–Aldrich, St. Louis, Mo., USA.

The synthesis of the two cyclodidepsipeptides under study, **PPM** and **BPM**, was performed via *N*-(α-bromoacyl)-α-amino acids as noncyclic intermediate products, as described in our previous study (Smelcerovic et al., 2011).

2.3. Preparation of thymocytes

Rat thymocytes were isolated as described previously (Pavlovic et al., 2007; Cekic et al., 2011). The viability of the isolated cells, as determined by trypan blue dye exclusion test, was always over 95%. Isolated thymocytes were counted and adjusted to a density of 5×10^6 cells/ml of CM.

2.4. Cell culture

Isolated thymocytes were cultivated in 96-well round-bottom plates (NUNC, Aarhus, Denmark), containing a 100 μl of cell suspension (5×10^5 cells) in each well. Cells were treated with increasing concentrations (0.1, 1, 10 μg/well) of **PPM** and **BPM**, diluted in appropriate amounts of 96% ethanol. Control samples were cultured in CM, with appropriate amounts of 96% ethanol or with ConA alone. For further evaluation of the proliferative activity, thymocytes were treated with optimal concentration (5 μg/ml) of ConA (Pavlovic et al., 2006). All cell cultures were done in triplicates and cultivated for 24 h in an incubator (Galaxy, Wolf laboratories, USA) with 5% CO_2 at 37 °C.

2.5. Cell proliferation

The proliferation of rat thymocytes was estimated by CCK-8 assay, according to the manufacturer's guidelines. This assay is based on the cleavage of the tetrazolium salt WST-8 by mitochondrial dehydrogenase in viable cells. The absorbance at 450 nm of each well was measured with a Perkin-Elmer microplate reader (Wallac Victor^{2}V, Turku, Finland). For each sample, basal intensity values were subtracted from those obtained after different treatments and results were presented as ratio for comparison with control samples.

2.6. Measurement of intracellular reactive oxygen species (ROS) production

A redox-sensitive probe (H2DCF-DA) was used to determine changes in overall cellular ROS levels, as described previously (Das et al., 2005; Boldogh et al., 2003). The change in fluorescence (excitation 485 nm; emission 530 nm) was measured using a Perkin-Elmer fluorimeter (Wallac Victor^{2}V, Turku, Finland). For each sample, basal fluorescence intensity values were subtracted from those obtained after different treatments and results were presented as ratio of mean fluorescence intensity (MFI).

2.7. Analysis of cell viability

Cell viability of rat thymocytes, after cultivation period, was evaluated by CCK-8 assay as was previously described (Hori et al., 2002). Ten microliters of reaction mixture was added in each well. After 2 h of incubation, the solubilized formazan product was quantified spectrophotometrically, by using a microplate reader Perkin-Elmer. Absorbance was measured at 450 nm. For each sample, basal intensity values were subtracted from those obtained after different treatments. Absorbances were presented as ratio for comparison with control samples.

2.8. Determination of mitochondrial membrane potential

Changes in mitochondrial membrane potential (MMP) of thymocytes, treated with **PPM** and **BPM**, were evaluated by uptake of lipophilic cation Rhodamine 123 into mitochondria, as previously described (Wang et al., 2007; Pathak and Khandelwal, 2006). The fluorescence of intracellular Rhodamine 123 (excitation 485 nm; emission 530 nm) was measured by Perkin-Elmer fluorimeter (Wallac Victor^{2}V, Turku, Finland), as published earlier (Yang et al., 2008). For each sample, basal fluorescence intensity values were subtracted from those obtained after different treatments and results were presented as ratio of mean fluorescence intensity (MFI).

2.9. Assay for in vitro antibacterial activity

The *in vitro* antimicrobial activity of **PPM** and **BPM** was tested against a panel of laboratory control strains belonging to the American Type Culture Collection Maryland, USA (except one, belonging to National Collection of Type Cultures, see below). Antibacterial activity was evaluated against two Gram-positive and three Gram-negative bacteria. Gram-positive bacteria used were: *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538 while Gram-negative bacteria utilized in the assay were: *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella abony* NCTC 6017.

The minimal inhibitory concentration (MIC) of **PPM** and **BPM** against tested bacteria was determined by using a broth microdilution method in 96 multi-well microtitre plates (NCCLS, 2003). A stock concentration for **PPM** was 8 mg/ml and 100 mg/ml for

BPM. The inocula of the bacterial strains were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Dimethyl sulphoxide (10%, v/v aqueous solution) was used to dissolve and to dilute samples. A serial double dilution of the samples was prepared in a 96 well microtiter plate, using method of Sarker et al. (2007). Two columns in each plate were used as controls. One column was used as a positive control and contained a broad-spectrum antibiotic (doxycycline in a serial dilution of 200–0.05 μg/ml) to determine the sensitivity of Gram-negative and Gram-positive bacterial species. The other column contained the solvent as negative control. Tests were carried out in triplicate.

2.10. Statistical analysis

Results are presented as mean ± SD. Significant differences between the groups were analyzed with Student's *t*-test.

3. Results

The cytotoxicity effect of **PPM** and **BPM** on rat thymocytes, after 24 h of incubation, was evaluated by using CCK-8 assay. The obtained results, presented in Fig. 1, demonstrated that application of increasing concentrations (0.1, 1, 10 μg/well) of **PPM** and **BPM** to cell culture, showed no significant effect ($p > 0.05$) on thymocytes toxicity, during examination period. Simultaneously, MMP analysis resulted with no significant decrease ($p > 0.05$) of MMP in rat thymocytes during their cultivation with increasing concentrations of **PPM** and **BPM**. Furthermore, significant increase ($p < 0.05$) of MMP was detected only when cells were cultured with 10 μg of **BPM** compound (Fig. 2). In the next set of experiments we demonstrated that thymocytes exposure to increasing concentration of **PPM** and **BPM** was not able to induce statistically significant increase of ROS production in rat thymocytes, as determined by redox-sensitive H2DCF-DA probe (Fig. 3). Even the results given

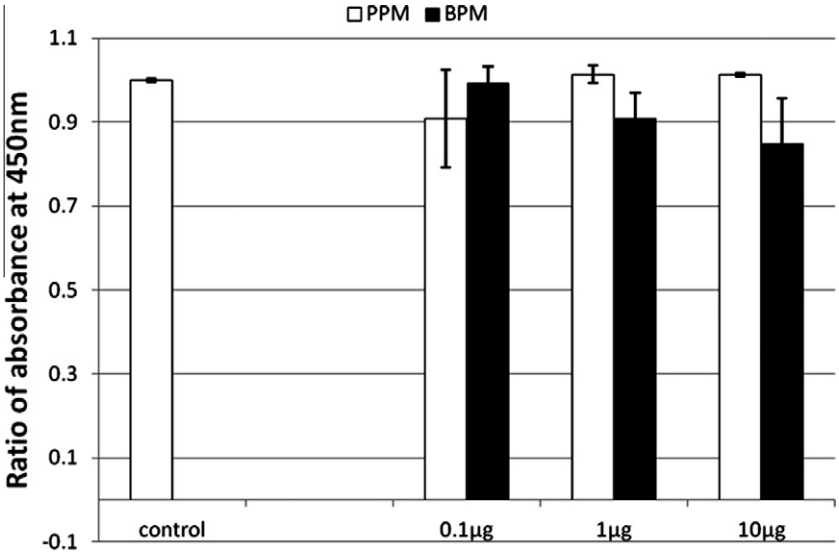

Fig. 1. The effect of **PPM** and **BPM** on cytotoxicity. Rat thymocytes (5×10^5 cells/well) were cultivated with increasing concentrations of **PPM** and **BPM** (0.1, 1, 10 μg/well) for 24 h and cell toxicity was evaluated as described in Section 2. Results are presented as mean percentage ± SD. Absorbances were presented as ratio for comparison with control samples.

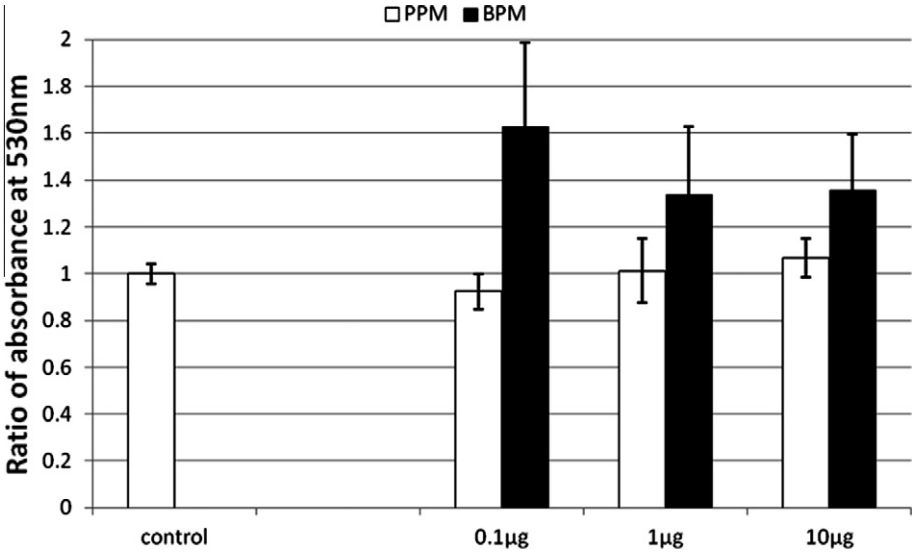

Fig. 2. The effect of **PPM** and **BPM** on ROS production in rat thymocytes. Cells (5×10^5 cells/well) were treated with increasing concentrations of **PPM** and **BPM** (0.1, 1, 10 μg/well) for 24 h and intracellular ROS production was evaluated by using redox-sensitive probe (H2DCF-DA), as described in Section 2. Results are presented as ratio of mean fluorescence intensity ± SD for comparison with control samples.

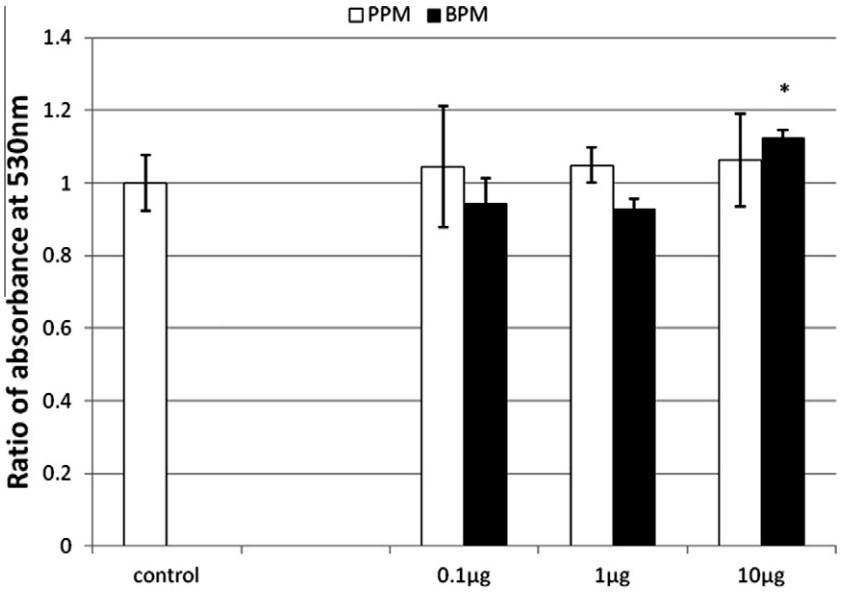

Fig. 3. The effect of **PPM** and **BPM** on mitochondrial membrane potential of rat thymocytes. Cells (5×10^5 cells/well) were treated with increasing concentrations of **PPM** and **BPM** (0.1, 1, 10 µg/well) for 24 h and mitochondrial membrane potential was evaluated by using Rhodamine 123, as described in Section 2. Results are presented as ratio of mean fluorescence intensity ± SD for comparison with control samples.

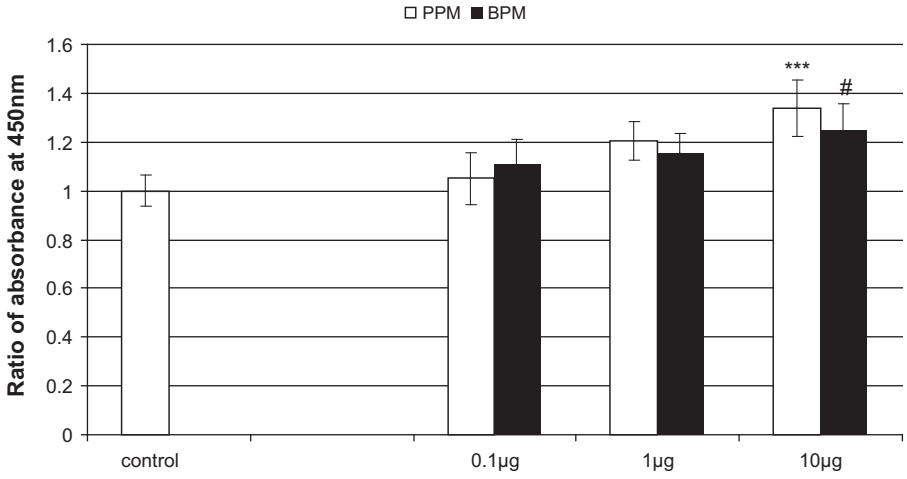

Fig. 4. The effect of **PPM** and **BPM** on rat thymocytes proliferative activity. Rat thymocytes were incubated with increasing concentrations of **PPM** and **BPM** (0.1, 1, 10 µg/well) for 24 h and triggered with optimal concentration of ConA. The intensity of thymocyte proliferation was evaluated by using CCK-8 assay, as described in Section 2. Results are presented as absorbance ratio ± SD for comparison with control samples. Abbreviations: $^{***}p < 0.001$, $^{\#}p < 0.05$ compared to control (non-treated) cells.

Table 1
Minimal inhibitory concentrations (MIC) of the samples **PPM** and **BPM**.

Microorganism	Tested samples		Positive control	Negative control
	PPM (mg/ml)	**BPM** (mg/ml)	Doxycycline (µg/ml)	DMSO (10%)
B. subtilis	2.00	25.00	25.00	n.a.
S. aureus	2.00	12.50	0.19	n.a.
P. aeruginosa	2.00	25.00	3.12	n.a.
S. abony	2.00	25.00	6.25	n.a.
E. coli	2.00	25.00	1.56	n.a.

n.a. – not active.

in Fig. 3 show the increase in ROS production in cell culture after **BPM** treatment, this increasing is not statistically higher ($p > 0.05$) than ROS production in control, non-treated, thymocytes.

Based on the previous findings, we next studied the possible effect of **PPM** and **BPM** on thymocytes proliferative activity. As shown in Fig. 4, **PPM** and **BPM** administrations to cell culture, in

concentrations of 0.1 and 1 μg/well, resulted with no statistically significant increase of proliferative activity ($p > 0.05$), by using CCK-8 assay. On the other hand, significantly increased proliferative activity was detected when thymocytes were incubated with 10 μg of **PPM** ($p < 0.001$) and **BPM** ($p < 0.05$), as compared to their respective controls (Fig. 4).

The results of antimicrobial activity of the samples **PPM** and **BPM** are listed in Table 1. The minimal inhibitory concentration (MIC) determinations were obtained by broth microdilution assay. The results indicated that MIC values against tested strains ranged between 2.00 and 25.00 mg/ml. **PPM** showed much better activity against all tested bacteria in comparison with **BPM**. **PPM** was equally effective against both Gram-positive and Gram-negative bacteria, at the dose of 2.00 mg/ml. **BPM** showed slightly better activity against Gram-positive bacteria (in comparison with Gram-negative bacteria), being the most susceptible against *S. aureus* (MIC = 12.50 mg/ml). The assayed samples were less effective than the antibiotic used as a referent standard (Table 1).

4. Discussion

Cyclodepsipeptidic secondary metabolites produced by marine and terrestrial organisms have unique structures comprised of unusual amino acids and non-amino acid moieties. The novel structural features and the wide spectrum of biological activities of these peptidic metabolites have generated considerable interest. However, it is rather difficult to isolate sufficient quantities of these metabolites for pharmacological and toxicological testing (Bagavananthem Andavan and Lemmens-Gruber, 2010). Recently a pathogenic fungus, *F. sporotrichioides* Sherb., was isolated from *H. barbatum* Jacq. (Smelcerovic et al., 2011). The volatile compounds of broth and mycelium were analyzed using gas chromatography-mass chromatography–mass spectroscopy and cyclodidepsipeptides, **PPM** and **BPM**, were found for the first time in the natural products. The role of **PPM** and **BPM** for the producing organism is questionable. The structures of **PPM** and **BPM** were confirmed by comparison of the analytical data for the natural products with samples obtained via synthetic methods (Smelcerovic et al., 2011). In the present study, we have shown that increasing concentrations of **PPM** and **BPM** were not able to induce toxicity or to decrease MMP in rat thymocytes, after 24 h of incubation. Simultaneously, thymocytes exposure to **PPM** did not result with statistically significant increase of intracellular ROS concentration. Rat thymocytes incubation with increasing concentrations of **BPM** resulted with slightly intracellular ROS production, without any statistical significance, representing a minor cytotoxic effect. Similar results were obtained in earlier study, showing that metacytofilin ((3R,6R)-3-benzyl-3-hydroxy-6-(methylamino)-6-(2-methylpropyl) morpholine-2,5-dione), another member of cyclodidepsipeptides family, was not able to induce toxicity in different cell lines and *in vivo* (Iijima et al., 1992). On the other hand, to our knowledge, this is first study reporting that members of cyclodidepsipeptides were not able to induce statistically significant intracellular ROS production. Immune cells are particularly sensitive to oxidative stress because of the high content of polyunsaturated fatty acids in their plasma membranes and a high production of ROS, which is part of their normal function (Victor et al., 2002). Also, intensive ROS production sensitize T cells to apoptosis (Hildeman et al., 2003), by decreasing the expression of Bcl-2 protein and resulted mitochondrial dysfunction (Tatton and Chalmers, 1998). Cell death depends, in part, upon mitochondrial dysfunction, which if often characterized by increased production of ROS, increased membrane permeability and eventual release of cell death mediators from mitochondria (Exline and Crouser, 2008). Extensive mitochondrial damage leads to loss of cellular ATP pools and to further inflammatory responses, resulting with wide range of human pathologies as a consequence of mitochondrial dysfunction (Crouser, 2004). Here, we report for the first time that two cyclodidepsipeptides do not decrease a MMP in rat thymocytes, after 24 h of incubation. These results correspond with data obtained from intracellular ROS analysis and cytotoxicity and may explain, in part, not significant ROS production in thymocytes cultured with **PPM** and **BPM**. On the other hand, significant MMP increase in cell treated with maximal **BPM** concentrations may represent one of the potential mechanisms involved in thymocytes proliferation. Taken together with our results it appears that **PPM**, after 24 h of incubation, was not able to induce oxidative stress and toxicity in rat thymocytes, while incubation with **BPM** resulted with slightly ROS increase but without statistically significant values.

Based on the previous findings, we hypothesized that **PPM** and **BPM** could have stimulatory effect on immune cell function. By using CCK-8 assay, our results show that maximal concentrations of **PPM** and **BPM**, used in our study, markedly stimulated the proliferative activity of rat thymocytes, indicating the immunostimulating effect, with potential secondary immunological consequences. On the other hand, lower **PPM** and **BPM** concentration did not have any significant effect. Immunosuppressive effect of metacytofilin has been documented earlier in mices (Iijima et al., 1992). However, it should be noted that immunosuppressive effect was documented *in vivo* and by using higher concentrations of metacytofilin, by measuring the antibody production and without any evaluation of ROS production, compared to the members of cyclodidepsipeptides used in our study. The reason for this opposite effect may be attributed to the differences in the chemical structure (differenent substituents in the positions 3 and 6 of the morpholine-2,5-dione moiety) between the metacytofilin and **PPM** and **BPM**. It has been shown that intracellular calcium increase, as well as production of interleukin-2, activates cascade of reactions that play a pivotal role in cell growth, differentiation and proliferation (Pacheco et al., 2004; Bootman et al., 2001). We can speculate that **PPM** and **BPM** could modulate the calcium influx and cytokine production, with resulting increased proliferative activity, but this hypothesis requires further studies. On the other hand, stimulatory effect on rat thymocytes proliferation may have important role, of these natural compounds, in boosting the immune system and, together with their antimicrobial activity, in successful microbe's eradication.

PPM and **BPM** showed antibacterial activity (see Table 1), while metacytofilin at 100 μg/ml had no antimicrobial activity against bacteria and fungi (Iijima et al., 1992). **PPM** may be used as potential antibacterial agent. To the best of our knowledge there are no data about the antimicrobial activity of other cyclodidepsipeptides.

In summary, we have shown that two cyclodidepsipeptides do not induce the toxicity and MMP decrease in rat thymocytes and, for the first time for this group of compounds, do not trigger the significant intracellular ROS production and exhibited antibacterial activity. On the other hand, higher **PPM** and **BPM** concentrations were able to stimulate proliferative activity of thymocytes, with mechanisms not yet known, indicating potential stimulatory effect on the cells of the immune system.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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