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Brief communication

6-(Propan-2-yl)-3-methyl-morpholine-2,5-dione, a novel cyclodidepsipeptide with modulatory effect on rat thymocytes

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ABSTRACT

A study has been carried out on the potential effect of a novel cyclodidepsipeptide, 6-(propan-2-yl)-3-methyl-morpholine-2,5-dione (PMMD), on rat thymocytes. Rat thymocytes were cultivated with increasing PMMD concentrations (0.1, 1, 10 μg/well), for 24 h, and evaluated for proliferative activity, viability, reactive oxygen species and mitochondrial membrane potential. The higher PMMD concentrations inhibited thymocytes proliferative activity mainly through induction of oxidative stress and resulting cytotoxicity, without any mitochondrial membrane potential alterations in thymocytes. The obtained results are correlated with previously published data on effects of 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones on rat thymocytes. The presence of methyl group in position 4 or/and the length of alkyl chain in position 3 of 6-(propan-2-yl)-morpholine-2,5-dione core plays a role for the obtained differences in the biological response between PMMD and two previously tested 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones.

1. Introduction

Cyclodidepsipeptides are the simplest members of cyclodepsipeptides family, which contain only one residue of amino acid and one residue of lactic, α-hydroxyisovaleric or other α-hydroxy acid. Biological activity of cyclodepsipeptides is well documented by numerous studies which were the subject of many review articles (Sarabia et al., 2004; Lemmens-Gruber et al., 2009; Bagavananthem Andavan and Lemmens-Gruber, 2010). There are some reports on cyclodidepsipeptides such as antimicrobial (Pavlovic et al., 2012; Yancheva et al., 2012), immunomodulating (Iijima et al., 1992), anticoagulant (Kagamizono et al., 1995), and inhibitory activity towards acyl-CoA:cholesterol acyltransferase (Hasumi et al., 1993) and α-glucosidase (Arcelli et al., 2004, 2005, 2007). As a part of our continuing study of identification (Smelcerovic et al., 2011), synthesis (Smelcerovic et al., 2011; Yancheva et al., 2012) and biological activities (Pavlovic et al., 2012; Yancheva et al., 2012) of cyclic didepsipeptides, recently we synthesized a novel didepsipeptide member containing an alanine moiety, 6-(propan-2-yl)-3-methyl-morpholine-2,5-dione (**PMMD**) (Scheme 1). Its structure was confirmed by IR, ^{1}H and ^{13}C NMR spectral data. DFT computational methods and experimental IR spectral techniques were employed to investigate the preferred conformations of different diastereomeric structures and the prototropic tautomerism of the compound. **PMMD** showed antimicrobial activity against four out of five tested bacterial strains, being the most effective against *Escherichia coli* (Yancheva et al., 2012). A reference survey shows that no other data concerning biological activity of **PMMD** has hitherto been published.

In our previous study (Pavlovic et al., 2012) we have shown that two cyclodidepsipeptides, 3,6-di(propan-2-yl)-4-methyl-morpholine-2,5-dione and 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione, do not induce the toxicity and mitochondrial membrane potential (MMP) decrease in rat thymocytes and, for the first time for this group of compounds, do not trigger the significant intracellular reactive oxygen species (ROS) production and exhibited antibacterial activity. On the other hand, higher concentrations of two studied 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones, which 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., were able to stimulate proliferative activity of thymocytes, with mechanisms not yet known, indicating potential stimulatory effect on the cells of the immune system (Pavlovic et al., 2012).

The aim of the present study was to investigate the potential effect of **PMMD** on rat thymocytes and to correlate with effects of two previously tested 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones. The results are discussed with respect to the differences in the chemical structure among those compounds.

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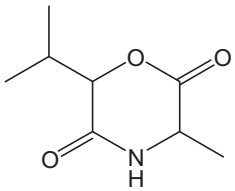

Scheme 1. Chemical structure of 6-(propan-2-yl)-3-methyl-morpholine-2,5-dione (**PMMD**).

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 **PMMD** was performed as described in our previous report (Yancheva et al., 2012).

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 **PMMD**, 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. 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 microliter 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 (Wallac Victor^{2}V, Turku, Finland). 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 (Pavlovic et al., 2012).

2.6. 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. 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.

2.7. Measurement of intracellular 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).

2.8. Determination of MMP

Changes in MMP of thymocytes, treated with **PMMD**, 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, 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. Statistical analysis

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

3. Results

Proliferative activity of rat thymocytes, triggered by optimal (5 μg/ml) concentration of ConA, was evaluated 24 h after incubation with increasing (0.1, 1, 10 μg) concentrations of **PMMD**. As shown in Fig. 1, cells incubation with 0.1 μg of **PMMD** induced slightly decreased proliferation activity but without ($p > 0.05$) statistically significance. On the other hand, thymocytes incubation with increased **PMMD** concentration resulted with significantly (1 μg, $p < 0.05$; 10 μg, $p < 0.05$) decreased proliferative rate (Fig. 1).

To test hypothesis that observed proliferative activity might be due to increased cell toxicity, we next evaluated the effect of tested compound (**PMMD**) on rat thymocytes viability, by using CCK-8 assay. Results, presented in Fig. 2, show that **PMMD** administration to cell culture, at concentration of 0.1 and 1 μg/well, resulted with no statistically significant increase ($p < 0.05$) of cytotoxicity. However, significantly increased cell toxicity was detected when thymocytes were cultivated with 10 μg of **PMMD** ($p < 0.01$), as compared to their respective controls (Fig. 2).

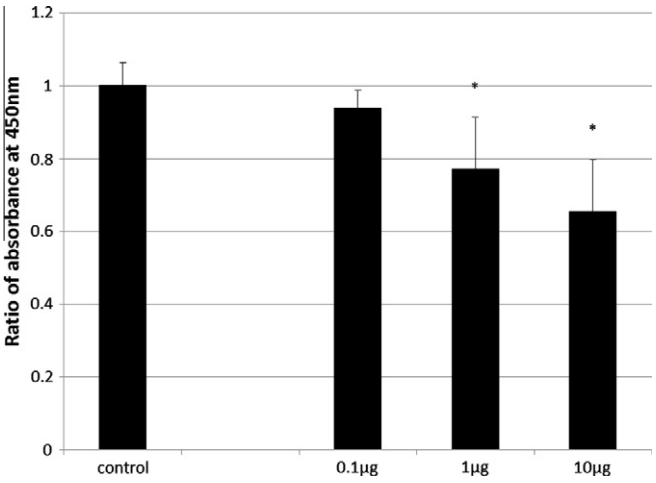

Fig. 1. The effect of **PMMF** on rat thymocytes proliferative activity. Rat thymocytes were cultivated with increasing concentrations of PMMD (0.1, 1, 10 μg/well) for 24 h and triggered with optimal concentration of ConA. The intensity of thymocytes proliferation was evaluated by using CCK-8 assay,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

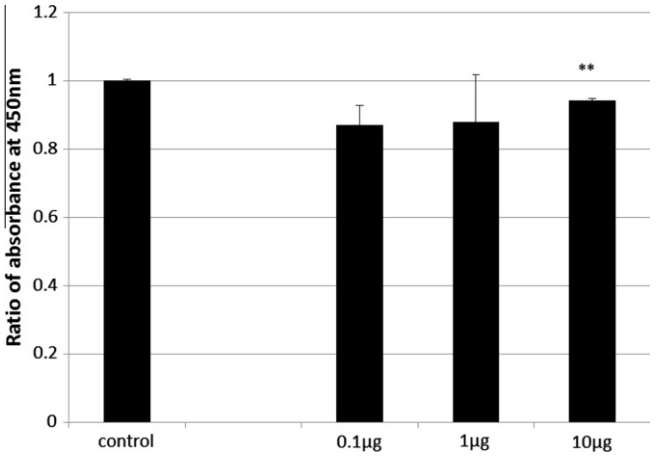

Fig. 2. The effect of **PMMD** on rat thymocytes toxicity Rat thymocytes (5×10^5 cells/well) were cultivated with increasing concentrations of **PMMD** (0.1, 1, 10 μg/well) for 24 h and cell toxicity was evaluated as described in Section 2. Results are presented as absorbance ratio ± SD for comparison with control samples. Abbreviations: $^{**}p < 0.01$.

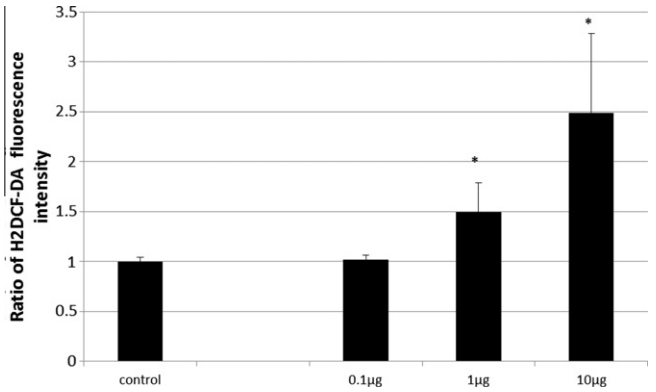

Fig. 3. The effect of **PMMD** on ROS production in rat thymocytes Rat thymocytes (5×10^5 cells/well) were cultivated with increasing concentrations of **PMMD** (0.1, 1, 10 μg/well) for 24 h and intracellular ROS production was evaluated by using redox-sensitive probe (H2DCF-DA), as described in Section 2. Results are presented as ratio of fluorescence intensity ± SD for comparison with control samples. Abbreviations: $^{*}p < 0.05$.

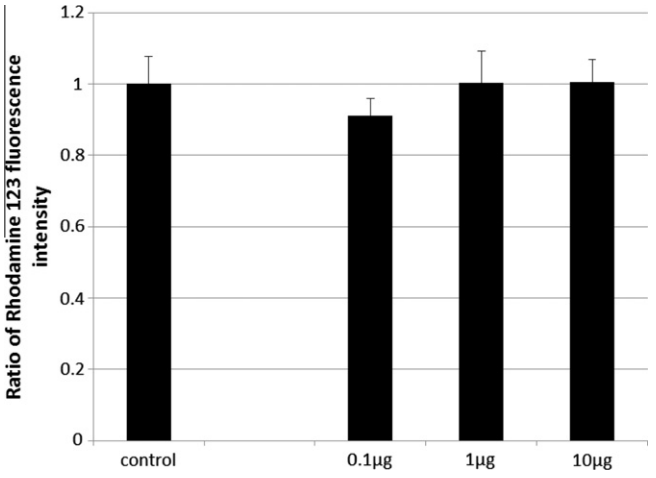

Fig. 4. The effect of **PMMD** on MMP of rat thymocytes Rat thymocytes (5×10^5 cells/well) were cultivated with increasing concentrations of **PMMD** (0.1, 1, 10 μg/well) for 24 h and MMP was evaluated by uptake of lipophilic cation Rhodamine 123 into mitochondria, as described in Section 2. Results are presented as ratio of fluorescence intensity ± SD for comparison with control samples.

Having in mind different molecular mechanisms involved in cell survival and proliferation, intracellular ROS production and MMP changes were analyzed. Obtained results demonstrate significantly increased H2DCF-DA fluorescence intensity, when cells were treated with **PMMD** (1 μg, $p < 0.05$; 10 μg, $p < 0.05$), indicating the increase of intracellular ROS production (Fig. 3). On the other hand, thymocytes exposure to increasing concentration of **PMMD** was not able to induce statistically significant alterations in MMP, as evaluated by intracellular Rhodamine 123 fluorescence intensity (Fig. 4).

4. Discussion

Cyclodepsipeptides are known to exhibit a broad spectrum of biological activities and present a great potential for pharmacological application. In the current study, we have shown that thymocytes exposure to 1 and 10 μg of **PMMD** significantly inhibited proliferative activity, in response to ConA, after 24 h of incubation. Simultaneously, application of 0.1 μg of **PMMD** in cell culture was not able to induce significant reduction of thymocytes proliferative rate, as evaluated by CCK-8 assay. Taking into account the previous findings, we next tested the possibility that decreased proliferative ability was mediated by increased thymocytes toxicity. Inhibited thymocytes proliferation was followed with significantly increased cytotoxicity when cells were cultivated with 10 μg of **PMMD**. However it should be noted that observed toxicity was followed with small standard deviation. On the other hand, thymocytes exposure to 0.1 and 1 μg of **PMMD** resulted with slightly increased cytotoxicity, but without any statistical significance. Immunosuppressive activity of different cyclodepsipeptides members have been documented earlier (Sarabia et al., 2004) and here we report, for the first time, an immunosuppressive activity of newly synthesized member of cyclodepsipeptides family. Similar immunosuppressive effect was reported in an earlier study showing that members of cyclodepsipeptides induced an enhancement of the gene activity of NFAT and NF-kB and blocked cell cycle progression of T cells at G1-S transition, which it is probably due to the inhibi-

tion of cyclin E-cyclin-dependent kinase (Zhang and Liu, 2001). On the contrary, our recent study showed that two 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones were not able to induce toxicity in rat thymocytes, after 24 h of incubation (Pavlovic et al., 2012). Because those compounds are tested under identical conditions the reason for this opposite effect is attributed to the differences in the chemical structure. Namely **PMMD** does not contain the methyl group in position 4 of 6-(propan-2-yl)-morpholine-2,5-dione core as two 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones. Also, **PMMD** contain a methyl group in position 3 of 6-(propan-2-yl)-morpholine-2,5-dione core unlike two previously investigated 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones containing longer alkyl chains (isopropyl or isobutyl) in this position.

Since it has been shown that free radicals and ROS generation may cause damage to DNA, lipids, proteins, and other biomolecules, with resulting anti-proliferative and cytotoxic effect (Pelicano et al., 2004), we next evaluated the potential mechanisms involved in these processes. Analysis of H2DCF-DA fluorescence revealed intensive ROS production when thymocytes were exposured to **PMMD** (1 and 10 μg), which corresponds with significantly inhibited proliferative activity and partially cell toxicity, indicating important role of ROS production in **PMMD**-induced immunosuppressive effect. On the other hand, application of increasing **PMMD** concentrations in cell culture was not able to alter the MMP in rat thymocytes, as evaluated by Rhodamine 123 fluorescence intensity. Immune cells are particularly sensitive to oxidative stress due to high content of polyunsaturated fatty acids in their plasma membranes (Victor et al., 2002). Changes in the ROS rate may significantly alter cellular homeostasis and consequently initiate different signaling processes, including cell death (Azad et al., 2010). Cell death depends, in part, upon mitochondrial dysfunction, which is often characterized by increased production of ROS, increased membrane permeability and eventual release of cell death mediators from mitochondria (Exline and Crouser, 2008). Taken together with our results, it seems that increased ROS production may represents an important factor in **PMMD**-induced immunosuppressive effect, with possible secondary immunological consequences. On the other hand, not detected MMP changes in thymocytes may not be the limitation factor in **PMMD**-induced immunosuppressive effect. These results are in line with a recent study (Pavlovic et al., 2012) showing that two 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones do not alter MMP in thymocytes. Further, it has been shown that members of cyclodepsipeptides inhibit an enzyme involved in the opening of the mitochondrial permeability transition pore, which is a critical event in some forms of necrotic and apoptotic cell death (Clarke et al., 2002). It has been postulated for long time that apoptosis and necrosis are functionally and morphologically distinct forms of cell death. However, this concept was changed due to observation that cells triggered to undergo apoptosis will die by necrosis when the intracellular ATP levels are depleted (Leist et al., 1997). Taking into account previous finding we can speculate that **PMMB** application to cell culture may not induce MMP alteration crucial for immunosuppressive effect and cell death, but in certain concentrations may lead to intracellular low energy levels with resulted impaired immune function, but this hypothesis requires further studies.

In summary, we have shown that higher **PMMD** concentrations inhibited thymocytes proliferative activity mainly through induction of oxidative stress and resulting cytotoxicity, without any MMP alterations in thymocytes. The presence of methyl group in position 4 or/and the length of alkyl chain in position 3 of 6-(propan-2-yl)-morpholine-2,5-dione core play a role for the obtained differences in the biological response between **PMMD** and two previously tested 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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