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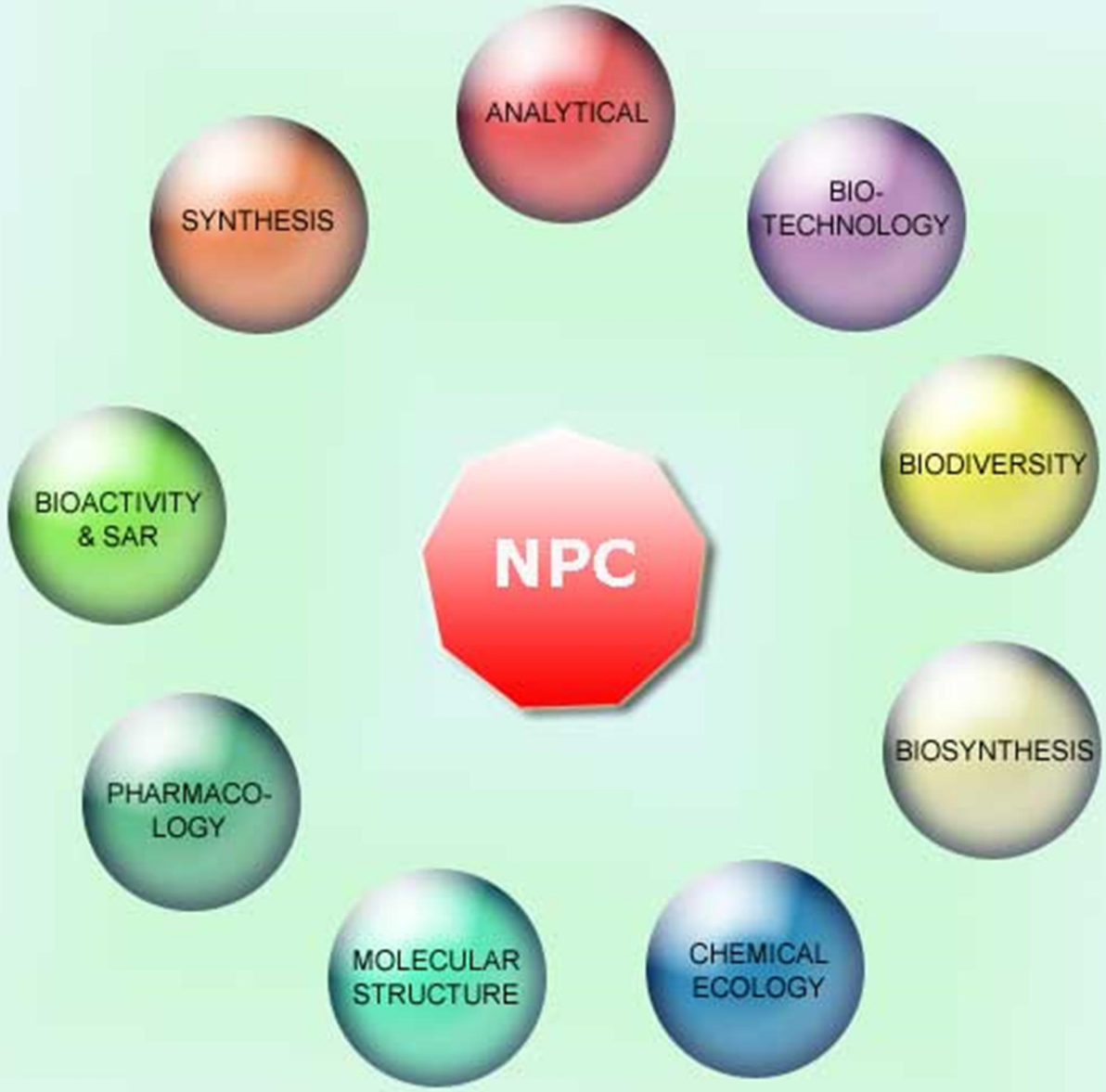

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Effects on MC3T3-E1 Cells and *In silico* Toxicological Study of Two 6-(Propan-2-yl)-4-methyl-morpholine-2,5-diones

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Recently, we found that two cyclodidepsipeptides, 3,6-di-(propan-2-yl)-4-methyl-morpholine-2,5-dione (**1**) and 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione (**2**), are excellent inhibitors of xanthine oxidase. In order to obtain more information about the toxicological potential of compounds **1** and **2** on bone cells, the current study was designed to evaluate the effect of these compounds on viability and proliferation of MC3T3-E1 cells. Compound **1** showed neither cytotoxic nor stimulatory effect on cell viability, while compound **2** showed a slight stimulatory effect on cell viability. Both studied compounds showed slight stimulatory effects on proliferation of MC3T3-E1 cells, in a dose dependent manner. Additionally, an *in silico* toxicological study of compounds **1** and **2** was performed, and the results indicate that they have a good probability of safe biological intake.

Keywords: Cyclodidepsipeptides, MC3T3-E1 cells, *In silico* toxicological study.

Behind the name "cyclodepsipeptides", we can unearth a rich world of compounds in terms of structural diversity and biological activity [1]. Cyclodepsipeptides are secondary metabolites of fungi and plants, or they originate from the marine environment [2]. Structurally, the diversity contained in this class of compounds arises from the type and number of amino acids, many of them completely unusual in nature, size of the macrocycle, and, usually, side chains that adorn the complex macrocycle [1]. Cyclodepsipeptides contain one or more amino acid(s) replaced by a hydroxy acid, resulting in at least one ester bond in the core ring structure [2]. Furthermore, it has been demonstrated that the ring form of depsipeptides is required for their biological activity [3,4]. The great interest that this class of natural products has elicited in the scientific community is explained by the diverse range of biological activities that they exhibit, including antitumor, antibiotic, antifungal, immunosuppressant, and antiinflammatory. This is supported by intriguing mechanisms of action and attractive molecular architecture [1].

Cyclodidepsipeptides are the simplest among the cyclodepsipeptides family containing one residue of amino acid and one residue of lactic, α-hydroxyisovaleric or other α-hydroxyacid [5]. They have an ester group and an amide group in the same 6-membered ring [6]. Two cyclodidepsipeptides, 3,6-di-(propan-2-yl)-4-methyl-morpholine-2,5-dione (**1**; $C_{11}H_{19}NO_3$, M = 213.27) and 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione (**2**; $C_{12}H_{21}NO_3$, M = 227.30), were found for the first time in natural products as potential precursors of enniatin B in the pathogenic fungus *Fusarium sporotrichioides* Sherb. This fungus was isolated from the stem of fresh *Hypericum barbatum* Jacq. [6]. The role of compounds **1** and **2** for the producing organism is questionable. For identification and confirmation, those compounds were synthesized and studied by density functional theory calculations and infrared spectroscopy [6]. Those two 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones were recently evaluated for antimicrobial, immunomodulatory [7], and antioxidant [8] activity, inhibitory activity toward xanthine oxidase (XO) *in vitro* and XO in rat liver homogenate, as well as for antiinflamatory response on human peripheral blood mononuclear cells (PBMCs) [9]. Our data indicate moderate antioxidant [8] and antimicrobial [7] potential of the studied compounds. Furthermore, they were excellent inhibitors of XO and significantly suppressed the activation of nuclear factor κB [9]. Both, **1** and **2** were confirmed as non-toxic in thymocites [7] and, therefore, may have promise for use in the treatment of gout and other excessive uric acid production or inflammatory conditions [9].

Hyperuricemia, a potentially harmful condition, favors precipitation of uric acid crystals in joints and tissues, leading to complications such as gout, nephrolithiasis and chronic nephropathy [10]. Gout is an inflammatory arthritis that is the result of the precipitation of serum urate into crystallized deposits of monosodium urate in and around the joints [11]. Also, changes in joints are frequently coupled with changes in surrounding bones such as new bone formation, sclerosis, osteophyte and spur. The finding that bone erosion is strongly associated with all features of new bone formation suggests that loss of bone and formation of new bone may be connected during the joint remodeling process in joints affected by gout. At present, it is unclear whether new bone formation in gout occurs before bone erosion and tophus formation, develops concurrently with bone erosion, or occurs as part of tissue remodeling in response to tophus and erosion [12]. MC3T3-E1 is a

nontransformed continuous osteoblast-like mouse calvarial cell line. Actively growing MC3T3-E1 cells are at the stage of osteoprogenitor cells. They show a typical fibroblastic morphology, possess the capacity to differentiate into osteoblasts [13,14] and frequently are used as model system for studies of cytotoxic and antiproliferative potential of different compounds on bone cells.

Compounds **1** and **2** possess inhibitory activity toward XO [9], a characteristic that recruits them as potential drugs in therapy of gout, clinical manifestation of which often includes changes on bones. In order to obtain more information about the toxicological potential of compounds **1** and **2** on bone cells, the current study was designed to evaluate the effect of these compounds on viability and proliferation of MC3T3-E1 cells. Additionally, an *in silico* toxicological study of two studied 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones was performed.

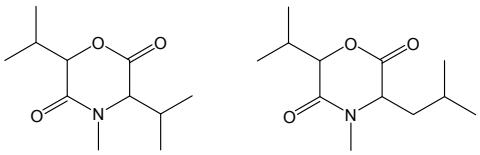

Figure 1: Chemical structures of the compounds under the study.

Effects of two 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones on viability and proliferation of MC3T3- E1 cells: MC3T3-E1 cells, osteoprogenitor cells derived from mouse calvaria, are a useful tool for examination of cytotoxic and antiproliferative activity of various compounds [14]. Registered change in MC3T3-E1 cell viability ranged from 103.6% to 96.8% after treatment with compound **1**, and from 105.1% to 118.7% (in relation to solvent control) after treatment with compound **2** (Figure 2). The change in MC3T3-E1 cell proliferation ranged from 117.7% to 126.2% and from 102.8% to 119.2% for compounds **1** and **2**, respectively (Figure 3). Treatment with compounds **1** and **2** for 24 h and 72 h in all effective concentrations had no visible effect on cell morphology.

MC3T3-E1 cells grow with a population doubling time of ~ 18 h [14] and it is expected that, under normal conditions, in a period from 24 h until 72 h the cells are divided at least once or more times, which multiply the number of cells. Compounds that reduce cell viability and/or suppress the cell division mechanism will lead to a reduction in the number of cells in culture that will manifest itself as a lower MTT reduction. MTT reduction is associated not only with mitochondria, but also with the cytoplasm and with non-mitochondrial membranes, including the endosome/lysosome compartment and the plasma membrane [17]. 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 [18]. We reported earlier [7] that cyclodidepsipeptides evaluated in this research do not decrease a mitochondrial membrane potential (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, the non-significant ROS production in thymocytes cultured with compounds **1** and **2**. Increasing concentrations of **1** and **2** were able to stimulate proliferation of rat thymocytes [7].

Extracts of dental materials were rated as severely, moderately, slightly or not cytotoxic or antiproliferative where activity relative to control was less than 30%, between 30% and 60%, between 60% and 90% or greater than 90%, respectively [19]. Reverse of the

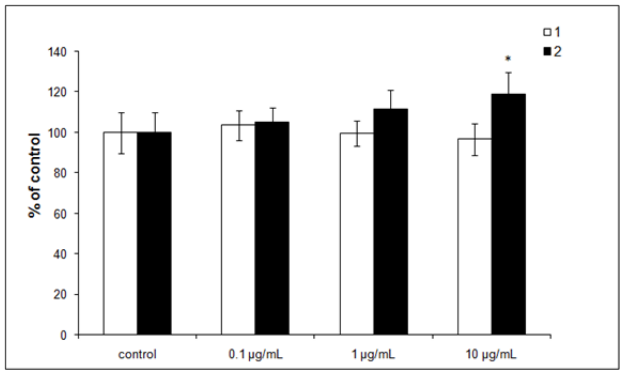

Figure 2: The effects of different concentrations of compounds **1** and **2** on viability of MC3T3-E1 cells. Results are presented as: mean percentage ± SD.
**P* < 0.05; ***P* < 0.01 compared with control

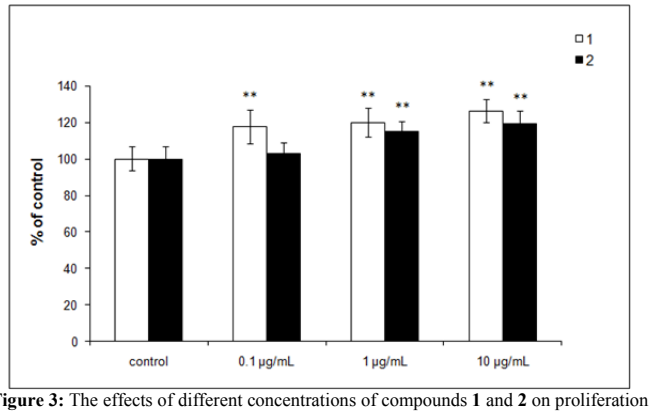

Figure 3: The effects of different concentrations of compounds **1** and **2** on proliferation of MC3T3-E1 cells. Results are presented as: mean percentage ± SD.
**P* < 0.05; ***P* < 0.01 compared with control

above mentioned classification, positive effect on cell viability or proliferation of tested compounds may be classified as not stimulatory, slightly, moderately and pronounced stimulatory where activity relative to control was between 100% and 110%, between 110% and 140%, between 140% and 170%, and more than 170%, respectively. Compound **1** showed neither cytotoxic nor stimulatory effect on cell viability. Compound **2** showed a slight stimulatory effect on cell viability in a dose dependent manner. Also, in a dose dependent manner compounds **1** and **2** showed a slight stimulatory effect on proliferation of MC3T3-E1 cells.

***In silico* toxicological study of two 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones:** In order to obtain a complete picture of the toxicity of the two studied 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones, we calculated absorption, metabolism and toxicity properties of compounds **1** and **2** using the OSIRIS Property Explorer [20], admetSAR [21] (Table 1) and ACD/I-Lab [22] (Tables 2 and 3).

The data obtained by OSIRIS Property Explorer indicate that the structures of compounds **1** and **2** are supposed to be non-mutagenic, non-tumorigenic, non-irritating and with no reproductive effects. The toxicological properties predicted by admetSAR (Table 1) suggest that two studied compounds might be able to pass through blood-brain barrier and penetrate into the CNS and, therefore, are considered as CNS active compounds. Compounds **1** and **2** were predicted to be capable of being absorbed by the intestine with high probability scores of 0.95 and 0.96, respectively. They are not likely to act as inhibitors of renal organic cation transporter and CYP450 enzymes, but are likely to act as substrates for CYP450 3A4. Additionally, the two studied compounds are supposed to be

weak/non-hERG inhibitors, non-AMES toxic, non-carcinogens, with low fish, *Tetrahymena pyriformis* and honey bee toxicity. While compound **1** is supposed to be ready biodegradable, compound **2** is not. Compounds **1** and **2** are predicted as Category III for risk for acute toxicity, including compounds with LD_{50} values greater than 500 mg/kg, but less than 5000 mg/kg; and "non-required" rat carcinogenicity class, including non-carcinogenic chemicals (Table 1).

Table 1: *In silico* predicted absorption, metabolism and toxicity properties of compounds **1** and **2** using admetSAR tool.

	Compound 1		Compound 2	
	Result	Probability	Result	Probability
Absorption				
BBB	+	0.8197	+	0.8311
HIA	+	0.9497	+	0.9604
ROC transporter	NI	0.9379	NI	0.8956
Metabolism				
CYP450 2C9 substrate	NS	0.8430	NS	0.8258
CYP450 2D6 substrate	NS	0.8522	NS	0.8158
CYP450 3A4 substrate	S	0.5844	S	0.6170
CYP450 1A2 inhibitor	NI	0.8453	NI	0.8578
CYP450 2C9 inhibitor	NI	0.9354	NI	0.9241
CYP450 2D6 inhibitor	NI	0.9577	NI	0.9461
CYP450 2C19 inhibitor	NI	0.9117	NI	0.8976
CYP450 3A4 inhibitor	NI	0.9845	NI	0.9828
CYP inhibitory promiscuity	Low	0.9699	Low	0.9726
Toxicity				
hERG inhibitor	Weak	0.9958	Weak	0.9939
	NI	0.9719	NI	0.9829
AMES toxicity	–	0.7156	–	0.7573
Carcinogens	–	0.9128	–	0.9053
Fish toxicity	Low	0.9656	Low	0.8434
Tetrahymena pyriformis toxicity	Low	0.9865	Low	0.9600
Honey bee toxicity	Low	0.6351	Low	0.6684
Biodegradation	Ready biodegradable	0.5259	Not ready biodegradable	0.6323
Acute oral toxicity	III	0.7319	III	0.7013
Carcinogenicity (Three-class)	Non-required	0.6836	Non-required	0.6797

*BBB – blood-brain barrier; HIA – human intestinal absorption; ROC – Renal organic cation; NS – non-substrate; S – substrate; NI – non-inhibitor; hERG – human ether-a-go-go-related gene.

Table 2: Health effects of compounds **1** and **2** calculated using ACD/I-Lab.

Health effects	Compound **1**	Compound **2**
Gastrointestinal system	0.48	0.59
Lungs	0.28	0.26
Cardiovascular system	0.53	0.52
Liver	0.1	0.09
Blood	0.11	0.12
Kidney	0.15	0.15

*Values around 0.00 indicate low, around 0.50 moderate, and around 1.00 high probabilities of health effects on certain organ systems.

According to the results obtained using ACD/I-Lab, compounds **1** and **2** have low probability of health effects on liver, blood, kidney and lungs, and moderate probability of health effects on cardiovascular and gastrointestinal system (Table 2). Using ACD/I-Lab software we predicted LD_{50} values in mg/kg after intraperitoneal, oral, intravenous and subcutaneous administration to mice, and after intraperitoneal and oral administration to rats (Table 3). The data indicate that compounds **1** and **2** are slightly toxic having oral LD_{50} values between 500 and 5000 mg/kg, and therefore are predicted as Category III for acute toxicity.

In conclusion, both tested compounds show a slightly dose dependent stimulatory effect on MC3T3-E1 cell proliferation. Summarizing the physico-chemical properties of cyclodidepsipeptides, we concluded in our recent review [5] that they obey the Lipinski "Rule of five" and meet all criteria for good solubility and permeability. The results of *in silico* studies shown in

Table 3: LD_{50} values of compounds **1** and **2** calculated using ACD/I-Lab.

Species/ Administration route	LD_{50} (mg/kg) Compound 1	Compound 2	Reliability (RI)
Mouse/Intraperitoneal	550	550	Borderline (0.47)
Mouse/Oral	2000	2100	Moderate (0.58)
Mouse/Intravenous	78	73	Not reliable (0.29)
Mouse/Subcutaneous	510	490	Borderline (0.42)
Rat/Intraperitoneal	860	850	Borderline (0.5)
Rat/Oral	2700	2800	Borderline (0.3)

Tables 1-3 indicate a good probability of compounds **1** and **2** being biologically safe for intake and make them promising leads for future development of safe and efficient agents. So, two studied 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones have promise to be used in the treatment of gout and other excessive uric acid production or inflammatory conditions.

Experimental

Cell culture: MC3T3-E1 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (PAA Laboratories GmbH), supplemented with 10% fetal calf serum (FCS) (Gibco, Great Britain), 100 IU/mL penicillin, 100 µg/mL streptomycin and 4 mM glutamine. Cells were incubated in a humidified chamber at 37ºC containing 5% CO_2.

Test compounds: The synthesis of the 2 cyclodidepsipeptides used in the study, **1** and **2**, was performed via *N*-(α-bromoacyl)-α-amino acids, as described in our previous study [6].

The effects of two 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones on the proliferation and viability of MC3T3-E1 cells were tested *in vitro* using MTT assay according to a slightly modified protocol [15].

Cell viability assay: Exponentially growing cells MC3T3-E1, (2×10^4 cells in 0.1 mL of medium/well) were seeded on 96-well plates in triplicate. Cells were incubated overnight in an atmosphere saturated with aqueous vapor, at 37°C with 5% CO_2. After 24 h, medium with various concentrations of ethanolic solutions of compounds **1** and **2** in a final volume of 0.1 mL was added to the wells. Effective concentrations of test compounds **1** and **2** were 0.1, 1, and 10 µg/mL.

At the same time, medium with an appropriate amount of 96% ethanol was added to control samples. Cells were incubated for 24 h. After the incubation the supernatant was removed and 0.1 mL of phosphate buffered saline (PBS) and 0.02 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) (Carl Roth, Germany), previously diluted in PBS, were added to each well and incubated for 2.5 h. After the estimated time, the reduced formazan product was dissolved in 0.1 mL of isopropanol per well. The absorbance was read at 540 nm on a multichanel spectrophotometer (Multiskan Ascent N°354, Thermo Labsystems, Finland).

Cell proliferation assay: Exponentially growing cells MC3T3-E1 (0.5×10^4 cells in 0.1 mL of medium/well) were seeded into 96-well plates in triplicate. Cells were incubated overnight in an atmosphere saturated with aqueous vapor, at 37°C with 5% CO_2. After 24 h, medium with various concentrations of ethanolic solutions of compounds **1** and **2** in a final volume of 0.1 mL was added to the wells. Effective concentrations of test compounds **1** and **2** were 0.1, 1, and 10 µg/mL. At the same time, medium with an appropriate amount of 96% ethanol was added to control samples. Cells were incubated for 72 h. After the estimated time, MTT assay was carried out in the same way as in the cell viability assay.

Data analysis: Absorbance of the control was regarded as 100% and values (%) of the different concentrations of test compounds were calculated as follows:

absorbance of the compound/absorbance of the control × 100% = percent of the compound in comparison with a control.

Results are presented as: mean ± SD. Statistical differences between different concentrations of the evaluated compounds in comparison with control were analyzed in SPSS 17.0 with Student's t-test and considered significant at $^{*}P < 0.05$ and $^{**}P < 0.01$.

Cell morphology: Cells that were used are adherent, and the possible toxic effect of the test compounds was evaluated through the possible change of their morphological characteristics. The normal morphology of MC3T3-E1 pre-osteoblast cells is polygonal [16]. Alteration of its phenotype was considered as a sign of a toxic effect. Cell morphological characteristics were observed after 24 h and 72 h of incubation using an invert microscope (AxioObserver.Z1 Carl Zeiss, Germany).

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