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Brief communication

Two 6-(propan-2-yl)-4-methyl-morpholine-2,5-diones as new non-purine xanthine oxidase inhibitors and anti-inflammatory agents

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ABSTRACT

Two cyclodidepsipeptides, 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione (**1**) and 3,6-di(propan-2-yl)-4-methyl-morpholine-2,5-dione (**2**), were evaluated for inhibitory activity against commercial enzyme xanthine oxidase (XO) *in vitro* and XO in rat liver homogenate as well as for anti-inflammatory response on human peripheral blood mononuclear cells (PBMCs). Both of cyclodidepsipeptides were excellent inhibitors of XO and significantly suppressed the nuclear factor of κB (NF-κB) activation. Allopurinol, a widely used XO inhibitor and drug to treat gout, relevated stronger inhibitory effect on rat liver XO activity than those of compounds **1** and **2**. Molecular docking studies were performed to gain an insight into their binding modes with XO. The studied morpholine-diones derivatives exerting XO inhibition and anti-inflammatory effect may give a promise to be used in the treatment of gout and other excessive uric acid production or inflammatory conditions.

1. Introduction

Cyclodepsipeptides are known to exhibit a broad spectrum of biological activities, such as immunosuppressant, antibiotic, antifungal, anti-inflamatory or antitumoral activities (Sarabia et al., 2004). Two cyclodidepsipeptides, 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione (**1**) and 3,6-di(propan-2-yl)-4-methyl-morpholine-2,5-dione (**2**) (Fig. 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). Recently, we investigated the possible immunomodulatory effect and antimicrobial activity of **1** and **2**, due to well known biological activities of enniatin B (Pavlovic et al., 2012).

The prototypical xanthine oxidase (XO) inhibitor allopurinol, has been the cornerstone of the clinical management of gout and conditions associated with hyperuricemia for several decades. More recent data indicate that XO also plays an important role in various forms of ischemic and other types of tissue and vascular injuries, inflammatory diseases, and chronic heart failure. Allopurinol and its active metabolite oxypurinol showed considerable promise in the treatment of these conditions both in experimental animals and in small-scale human clinical trials (Pacher et al., 2006). Recently, Burns and Wortmann discussed febuxostat and several other drugs that target various steps in the pathogenesis of gout (Burns and Wortmann, 2011).

Nuclear factor of κB (NF-κB) is a sequence-specific transcription factor activated in response to inflammatory stimuli, known to be involved in the inflammatory and innate immune responses. NF-κB, which was discovered by Sen and Baltimore in 1986 as a factor in the nucleus of B cells that binds to the enhancer of the kappa light chain of immunoglobulin (Sen and Baltimore, 1986), has since been shown to be expressed ubiquitously in the cytoplasm of all cell types, conserved from Drosophila to man. It translocates to the nucleus only when activated, where it regulates the

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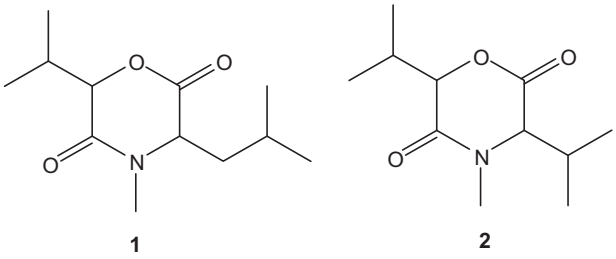

Fig. 1. Chemical structures of the compounds under study.

expression of over 200 genes that control the immune system, growth and inflammation (Pahl, 1999). NF-κB may function as the cellular checkpoint of metabolic stress conditions, such as hyperglycemia, oxidative, nitrosative stress and hyperuricemia, which may reflect disease processes associated with progression of autoimmune or autoinflammatory conditions (Kocic et al., 2011).

In the present study, two synthesized cyclodidepsipeptides **1** and **2** were evaluated for inhibitory activity against commercial enzyme XO *in vitro* and XO in rat liver homogenate as well as for anti-inflammatory response on human peripheral blood mononuclear cells (PBMCs). Molecular docking studies were performed in order to examine the binding mode of **1** and **2** with XO.

2. Materials and methods

2.1. Synthesis

The synthesis of the compounds **1** and **2** were performed via *N*-(α-bromoacyl)-α-amino acids as noncyclic intermediate products, as described in our previous study (Smelcerovic et al. 2011).

2.2. Evaluation of xanthine oxidase inhibition

2.2.1. Inhibition of commercial xanthine oxidase

Commercial bovine milk XO, purchased from Sigma–Aldrich, was employed for *in vitro* evaluation of enzyme inhibition, by measuring uric acid formation spectrophotometrically at 293 nm (Kizaki and Sakurada, 1977).

The inhibition was studied in a series of test-tubes with the reaction mixture (total volumen 350 μl), prepared in a following order: (i) *Test samples* contained 0.02 units of XO, 150 ng or 15 ng of test inhibitor **1** or test inhibitor **2** (both diluted in ethanol), 0.017 μmol of xanthine (Serva), and 20 μmol TRIS–HCl buffer (pH 7.8); (ii) *Solvent control samples* contained the same amount of XO, appropriate amount of ethanol, xanthine and TRIS–HCl buffer; (iii) *Control samples* contained the same amount of XO, xanthine and TRIS–HCl buffer adjusted to the same volume; and (iv) *Test substrate samples* were group of samples which contained in reaction mixture only XO, the compound **1** or **2** and TRIS–HCl buffer, in order to test if the compound **1** or **2** may be possible XO substrates. The tubes were allowed to incubate at 37 °C for 60 min, together with the corresponding duplicate blank aliquots, where the enzyme was omitted. After incubation, the reaction was stopped by adding perchloric acid, afterwards the XO was added in corresponding duplicate blank samples. The degree of enzyme inhibition was determined by measuring the difference in absorbance, associated with uric acid formation. It was calculated as a percent of change of the solvent control samples which involves only the effect of solvent ethanol. All experiments were conducted with six repetitions and averaged.

2.2.2. Inhibition of rat liver xanthine oxidase

The study was conducted in 6 weeks old female albino Wistar rats, weighing from 150 to 200 g. Rats were purchased from the farm of Military Medical Academy (Belgrade, Serbia) and bred in the vivarium of the Faculty of Medicine, University of Nis, under standard conditions. Animals used for the procedure were treated in accordance with the NIH Guide for Care and Use of Laboratory Animals (1985).

All experiments on animals were performed according to guidelines and had been approved by the Animal Ethics Board at the University of Nis Medical School beforehand.

Animals were anesthetized with ketamin (60 mg/kg) given intraperitoneally, and then sacrificed. Their livers were removed and cut in small pieces and homogenized in ice cold water, using homogenizer (IKA® Works de Brasil Ltda Taquara, RJ 22713-00). The homogenates (10% w/v) were centrifuged at 1500×*g* for 10 min at 4 °C. The resulting supernatant was separated and used for the biochemical analysis.

Inhibition of rat liver XO was monitored spectrophotometrically by a procedure similar to that described in Section 2.2.1. and procedure used in our recent report (Veljkovic et al., 2012).

The reaction mixture (total volume 2150 μl) was prepared in a following order: (i) *Test samples* contained 100 μl of 10% rat liver homogenate, 100 μg or 10 μg of **1** or **2** (diluted in ethanol), 1 μmol of xanthine (Serva), and 200 μmol TRIS–HCl buffer (pH 7.8); (ii) *Solvent control samples* contained the same amount of rat liver homogenate, appropriate amount of ethanol, xanthine and TRIS–HCl buffer; and (iii) *Control samples* contained the same amount of rat liver homogenate, xanthine and TRIS–HCl buffer adjusted to the same volume. Duplicate blank samples were prepared for each group of samples in the same way as the test solutions (i–iii). The obtained inhibition was calculated as a percent change of the control which involves the effect of appropriate amount of ethanol. Allopurinol was used as positive control. All experiments were performed in triplicate and averaged.

2.3. In vitro experiments on PBMC

Peripheral venous blood (350 ml) from 24 year old male healthy volunteer was drawn between 8 and 9 h into sterile heparinized tubes and was processed within 2 h. PBMC were isolated under sterile conditions by centrifugation in Ficoll Histopaque 1077 (Lymphoprep, Nycomed Pharma) according to the manufacturer instructions. Cell viability was above 90% using Trypan blue stain exclusion. After washing in PBS, obtained PBMC were resuspended in RPMI 1640 medium and 10% FCS.

2.4. Detection of NF-κB

All chemical were purchased from Sigma while the antibodies were purchased from Santa Cruz Biotechnology. Anti-inflamatory activity of **1** or **2**, diluted in appropriate amounts of ethanol (see Section 2.2.), was examined via determination of NF-κB, as described in our previous paper (Kocic et al., 2011). Washed PBMCs (each aliquot of 100 μl contained 10^5 cells) were distributed in 12-well plates and cultured for 4 h at 37 °C in 5% CO_2. The 50-μl aliquots of each sample were plated in 12 U-bottom 96-well culture plates. The cells were fixed by using 70% methanol and permeabilized with 0.1% Triton PBS. They were incubated with anti–NF-κB primary antibody (p65 C-20: sc-372 epitope mapping at the C-terminus of NF-κB p65), washed three times, and incubated with the FITC-conjugated secondary antibody. The mean fluorescence intensity (MFI; logarithmic scale) of cell populations was determined and analyzed on a Victor™ multiplate reader (Perkin Elmer-Wallace, Wellesley, MA). The results presented were obtained following subtraction of blank values treated with secondary antibodies only. The fluorescence intensity of cells indicated for both up or down regulating populations for each stimulus used, compared to intact PBMCs as well as to control samples with appropriate amounts of ethanol, and calculated as a percent change of the control PBMCs which involves the effect of appropriate amount of ethanol. All experiments were conducted with six repetitions and averaged.

2.5. Molecular docking

Molecular docking was carried out into the salicylic acid active site of XO (PDB entry code 1FIQ) using MOE software (Molecular Operating Environment, 2011). Water molecules from initial pdb were removed. Conformational search for preparation of the ligands was carried out by LowModelMD method which performs molecular dynamics perturbations along with low frequency vibrational modes with energy window 7 kCal/mol, and conformational limits of 1000. Placement of conformers was prepared according to alpha-triangle method on selected pharmacophores. Scoring of docking poses was performed by affinity dG, calculated with MMFF94x force field.

3. Results and discussion

3.1. Xanthine oxidase inhibition

1 or **2** were not substrates for XO when used instead of xanthine in reaction mixture. A significant inhibitory activity of two synthesized cyclodidepsipeptides on XO was observed to occur in a dose-dependent manner (Fig. 2).

2 exhibits more potent effects than **1**, and inhibits commercial bovine milk XO with an IC_{50} of about 0.2 μM (see dose of 42.9 ng/ml in Fig. 2), which is in the range of the allopurinol inhibitory activity towards XO (IC_{50} values of 0.2–50 μM) (Pacher et al., 2006).

The inhibitory activity of **1** and **2** on XO were further tested in rat liver homogenate and compared with allopurinol. **2** were found to be more active against rat liver XO than **1** (Fig. 3), which is in accordance with the inhibitory effect on activity of commercial bovine milk XO (Fig. 2). Allopurinol relevated stronger inhibitory effect than **1** and **2** in both tested concentrations (Fig. 3). However, allopurinol does have a number of serious side effects, and the cellular and molecular mechanisms of these side effects are incompletely understood. Some data indicate that the renal toxicity of allopurinol is related to impairment of pyrimidine metabolism (Horiuchi et al., 2000). There are no reliable or rapid screening tools that would predict the safety profile of novel XO inhibitors in terms of hypersensitivity reactions or organ toxicity; contact hypersensitivity mouse ear models and toxicity studies in rodents are being used to predict such side effects (Horiuchi et al., 1999). Intuitively, one would predict that novel XO inhibitors that would move away from the purine-based inhibitor structure may have fewer of the allopurinol-like side effects (of course, they may introduce new types of side effects or toxicities) (Pacher et al., 2006). Recently we have shown that **1** and **2** do not induce the toxicity and mitochondrial membrane potential decrease in rat thymocytes. The studied compounds do not trigger the significant intracellular reactive oxygen species production and exhibited antibacterial activity. The higher concentrations of **1** and **2** 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). Therefore, **1** and **2** have potential to be used as novel XO inhibitors.

3.2. Rational design of xanthine oxidase inhibitors and molecular docking study

Recent studies on novel non-purine isosters of allopurinol (Okamoto et al., 2004; Nepali et al., 2011a,b) showed that consideration of certain structural features leads successfully to the development of new potent XO inhibitors. Those features should be designed to provide: (i) hydrophobic and hydrogen bonding interactions with enzyme; (ii) site for hydroxylation near the molybdenum metal; and (iii) non-purine skeleton to avoid unnatural nucleotide metabolites. It is also known that small molecules as salicylic acid could inhibit the enzyme without binding to the metal complex itself, only by blocking the approach of potential substrates toward the molybdenum (Enroth et al., 2000). For this reason it was thought worthwhile to test two synthesized cyclodidepsipeptides **1** and **2** for inhibitory activity against commercial enzyme XO *in vitro* as well as for anti-inflammatory response on PBMCs. Both compounds possess non-purine skeleton, alkyl and

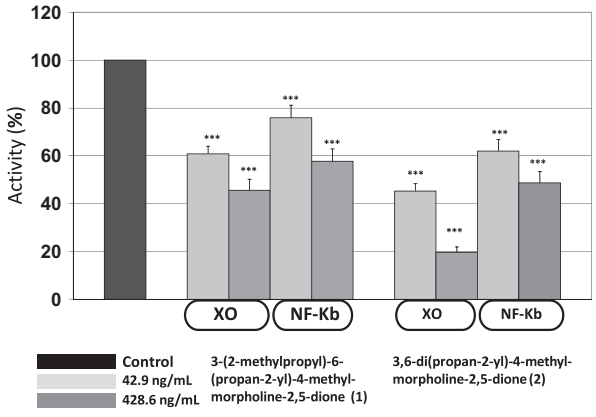

Fig. 2. Percentual dose-dependent effect of **1** and **2** on commercial bovine milk XO activity and PBMC NF-κB downregulation.

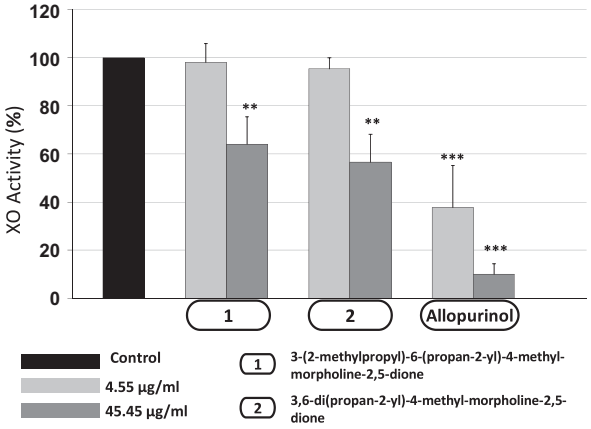

Fig. 3. Percentual dose-dependent effect of **1** and **2** on rat liver XO activity.

carbonyl groups that could provide favorable interactions with enzyme, and they could be potentially accommodated in the o/into the salicylic acid XO active site.

Considering the promising biological results, in order to examine the binding mode of **1** and **2** with XO, molecular docking was carried out into the salicylic acid active site of XO (PDB entry code 1FIQ) (Enroth et al., 2000). The docking conformations of **1** and **2** are shown in Figs. 4A and 5A.

Both ligands fit well in the active site pocket. Ligand **1** enters deeper in the cavity than **2** and has smaller exposure to the solvent. In case of **1** the ester carbonyl group has strong interaction with the dioxothiomolybdenum (MOS) moiety of molybdenum-pterin center (Fig. 4B). **2** is not directly interacting with MOS, but efficiently blocks the approach of potential substrates toward the metal. A number of lipophilic interactions with neighboring amino acid residues are stabilizing the ligand-pocket binding of both compounds (Figs. 4B and 5B).

The binding modes of **1** and **2** resemble those found by crystallographic studies on complexes of XO with inhibitors not forming a covalent bond with the molybdenum atom such as salicylic acid (Enroth et al., 2000) and febuxostat (Okamoto et al., 2003). It is known that the salicylic acid binds to the XO with the carboxylate atoms forming hydrogen bonds with Arg880 and Thr1010, the hydroxyl atom associated to Thr1010 also by hydrogen bonds, and the phenyl ring aligned parallel to the ring of Phe914 and perpendicular to the ring of Phe1009 (Fig. S1 in Supplementary materials). The binding of febuxostat in the enzyme involve interaction with similar amino acid residues.

As it could be seen from the 2D representations of the ligands in the pocket, introduction of protonodonor or acceptor groups in the side-chains of **1** and **2** could enhance further the interactions with the enzyme via hydrogen bonding and thus provide better inhibitor potency.

3.3. Anti-inflamatory activity

Compound **2** showed higher anti-inflammatory response on PBMCs, exerted through the NF-κB inhibition, than compound **1**. The activation of NF-κB is a double-edged sword. While needed for proper immune system function, inappropriate NF-κB activation can mediate inflammation and tumorigenesis. That duality is especially striking in relation to cancer, a proinflammatory disease (Balkwill and Mantovani, 2001). Extensive research over the last few years has shown that most inflammatory agents mediate their effects through the activation of NF-κB, with the expression of proinflammatory genes. Inhibition of NF-κB at the onset of inflammation leads to decreased inflammatory response (Lee et al., 2008).

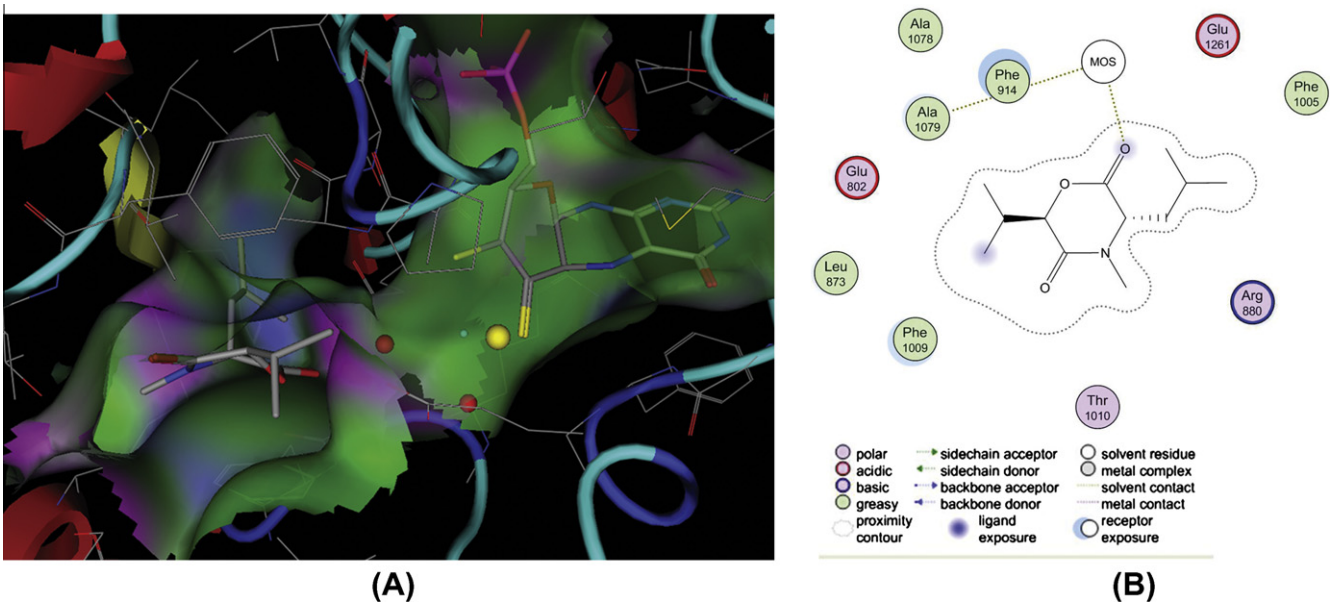

Fig. 4. Docking conformation of **1** (A) and 2D representation of the ligand interactions (B) in the pocket.

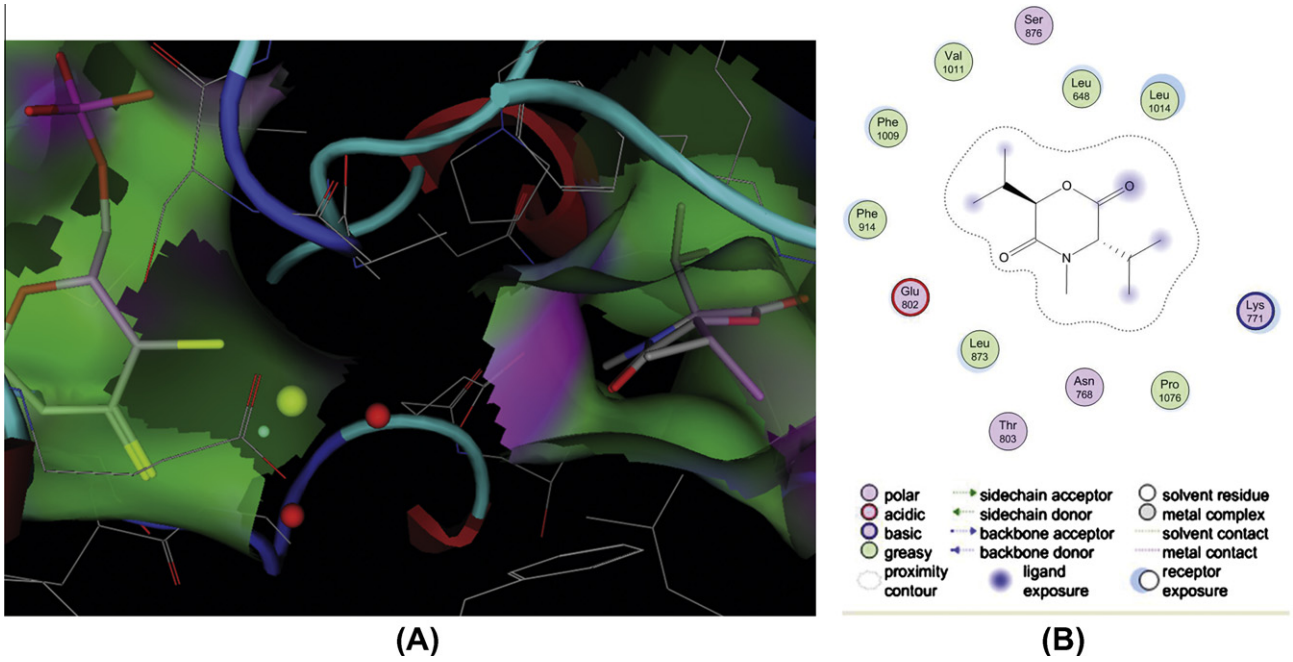

Fig. 5. Docking conformation of **2** (A) and 2D representation of the ligand interactions (B) in the pocket.

The urate crystals are capable of up-regulating effect and are capable of activating MyD88, the extracellular signal-regulated kinase 1 and 2 (ERK1/2), leading to NF-κB activation (Jaramillo et al., 2004a,b). These findings showed that two studied cyclodidepsipeptides suppress the NF-κB activation in a dose-dependent manner and suggest that **1** and **2** can show anti-inflammatory effects.

4. Conclusions

Two cyclodidepsipeptides, **1** and **2**, were documented to be the novel XO inhibitors. Based on molecular docking study, the binding modes of **1** and **2** with XO were clarified and recommendations for future structure-guided design of new morpholine-dione inhibitors of XO were drawn. Allopurinol were found to be more active against rat liver XO than **1** and **2**. The effect of **1** and **2** on antiinflammatory cascade was observed by testing the NF-κB response on PBMCs and a significant NF-κB downregulation was observed to occur in a dose-dependent manner. Both **1** and **2** were tested and confirmed as non-toxic in thymocytes (Pavlovic et al., 2012) so that studied morpholine-diones derivatives exerting XO inhibition and anti-inflammatory effect may give a promise to be used in the treatment of gout and other excessive uric acid production or inflammatory conditions. The *in vivo* study will be part of our further investigation, which will elucidate possibility of application of this novel class of non-purine XO inhibitors in therapy.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2013.01.052.

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