Bulgarian Chemical Communications, Volume 47, Number 3 (pp. 783 – 787) 2015

Inhibition mechanism and molecular modeling studies of the interactions of 6-(propan-2-yl)-3-methyl-morpholine-2,5-dione with xanthine oxidase

Ž. Šmelcerović[1]*, M. Rangelov[2], E. Cherneva[3], G. Kocić[4], S. Stojanović[4], T. Jevtović-Stoimenov[1,4], Ž. Petronijević[5], D. Yancheva[2]

[1]*Center for Biomedicinal Science, Faculty of Medicine, University of Niš, Bulevar Dr Zorana Djindjica 81, 18000 Niš, Serbia*

[2]*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Build. 9, 1113 Sofia, Bulgaria*

[3]*Faculty of Pharmacy, Medical University of Sofia, 2 Dunav Str., 1000 Sofia, Bulgaria*

[4]*Institute of Biochemistry, Faculty of Medicine, University of Niš, Bulevar Dr Zorana Djindjica 81, 18000 Niš, Serbia*

[5]*Faculty of Technology, University of Niš, Bulevar Oslobodjenja 124, 16000 Leskovac, Serbia*

Received April 11, 2014, Revised April 27, 2015

Xanthine oxidase (XO) is an enzyme which catalyzes oxidation of hypoxanthine to xanthine and then to uric acid, and plays a key role in hyperuricemia. The inhibition of XO activity in rat liver homogenate by 6-(propan-2-yl)-3-methyl-morpholine-2,5-dione (**1**) was evaluated and compared with that of two previously studied cyclodidepsipeptides, 3,6-di(propan-2-yl)-4-methyl-morpholine-2,5-dione (**2**) and 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione (**3**), and allopurinol. Compound **1** showed significant inhibitory activity against rat liver XO (IC_{50} = 49.82 μg/mL), comparable with the activity of **2** and **3**. Allopurinol, a widely used XO inhibitor and drug to treat gout, exhibited a stronger inhibitory effect on rat liver XO than **1**-**3**. Compound **1** was synthesized as a mixture of two diastereoisomers, (*3S,6R*) and (*3S,6S*), and molecular docking studies were performed to gain an insight into their binding modes with XO. Lineweaver–Burk plot analysis of the inhibition kinetics data demonstrated that the studied compound (**1**) was a competitive inhibitor of XO. Both forms of **1** bind in the entrance of the narrow tunnel towards the dioxothiomolybdenum moiety of the active center of XO, blocking in this way the approach of the substrates toward the metal atom.

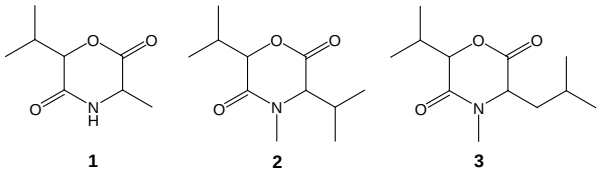
Keywords: inhibition kinetics; molecular modeling; 6-(propan-2-yl)-3-methyl-morpholine-2,5-dione; xanthine oxidase inhibition.

INTRODUCTION

Cyclodidepsipeptides are the simplest ones in the cyclodepsipeptide family containing one residue of amino acid and one residue of lactic, α-hydroxyisovaleric or other α-hydroxy acid. There are reports on their antioxidant [1], antimicrobial [2,3] and immunomodulatory [2,4,5] activities, as well as inhibitory activities towards acyl-CoA:cholesterol acyltransferase [6], α-glucosidase [7-9] and platelet aggregation [10]. Recently, we synthesized a novel cyclodidepsipeptide, 6-(propan-2-yl)-3-methyl-morpholine-2,5-dione (**1**; $C_8H_{13}NO_3$, M = 171.19) (Scheme 1), containing an alanine moiety [3]. Xanthine oxidase (XO) is a key enzyme which can catalyze oxidation of xanthine to uric acid causing hyperuricemia in humans [11] and recently, we evaluated two synthetic cyclodidepsipeptides 3,6-di-(propan-2-yl)-4-methyl-morpholine-2,5-dione (**2**; $C_{11}H_{19}NO_3$, M = 213.27) and 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione (**3**; $C_{12}H_{21}NO_3$, M = 227.30) [12] for inhibitory activity against commercial enzyme XO *in vitro* and XO in rat liver homogenate as well as anti-inflammatory response on human peripheral blood mononuclear cells (PBMCs) [13]. The two cyclodidepsipeptides were excellent inhibitors of XO and significantly suppressed the nuclear factor of κB (NF-κB) activation. Based on molecular docking study, the binding modes of **2** and **3** with XO were clarified and recommendations for future structure-guided design of new morpholine-dione inhibitors of XO were drawn [13].

In this paper, we investigated the inhibition of xanthine oxidase (XO) activity in rat liver homogenate by compound **1** and compared with that by cyclodidepsipeptides **2** and **3**, as well as allopurinol, a widely used XO inhibitor and drug to treat gout. Inhibition kinetics of compound **1** with XO was studied in order to determine the type of enzyme inhibition, while the molecular docking studies were performed in order to examine the binding mode of **1** with XO.

* To whom all correspondence should be sent:
E-mail: zsmelcerovic@yahoo.com



Scheme 1. Chemical structure of cyclodidepsipeptides **1**-**3**

EXPERIMENTAL

Evaluation of xanthine oxidase inhibition

Inhibition of XO activity in rat liver homogenate was evaluated using a spectrophotometric method [14], by measuring uric acid formation at 293 nm. The reaction mixture (volume 2200 μL) was prepared by allocating the following test sample groups: i) Test sample group contained 100 μL of 10 % rat liver homogenate, one of the studied compounds (**1**-**3**) diluted in DMSO (the final concentration of DMSO in the assay was 4.55 % v/v), 454.5 μM of xanthine (Serva), and 45.5 mM TRIS-HCl buffer (pH 7.8); ii) Solvent control group contained the same amount of rat liver homogenate, appropriate amount of DMSO, xanthine and TRIS-HCl buffer; iii) Control group contained the same amount of rat liver homogenate, xanthine and TRIS-HCl buffer adjusted to the same volume. Corresponding blank samples were prepared for each group 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 DMSO. All samples were assayed for XO inhibitory activity at concentration of 50 μg/mL. Those showing greater than 50 % inhibition at this concentration were tested further to ascertain the corresponding IC_{50} values. IC_{50} curves were generated using three concentrations of studied compounds (50, 40 and 25 μg/mL). Allopurinol was used as positive control. All experiments were performed in triplicate and averaged.

Lineweaver–Burk plots

To determine the mode of inhibition by compound **1**, Lineweaver–Burk plot analysis was performed. This kinetics study was carried out in the absence and presence of the inhibitor with varying concentrations of xanthine as the substrate. Commercial bovine milk XO, purchased from Sigma-Aldrich, was employed for *in vitro* evaluation of enzyme inhibition. The inhibition was studied in a series of test-tubes with the reaction mixture (total volumen 2150 μL) containing 0.01 units of XO and 46.5 mM TRIS-HCl buffer (pH 7.8). Xanthine concentration was varied (0, 18.6, 46.5, 93, 232.5 or 930 μM) at three series of fixed concentrations of compound **1** (0, 13.6 and 54.4 μM). Amount of generated uric acid formation was measured at 293 nm.

Molecular docking

Molecular docking was carried out into the salicylic acid active site of XO (PDB entry code 1FIQ) using MOE software [15]. 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. Docking was done using the induced-fit docking protocol allowing the side-chain and backbone movement in the receptor to accommodate the ligand. Scoring of docking poses was performed by affinity dG, calculated with MMFF94x force field.

RESULTS AND DISCUSSION

Compound **1** showed a significant inhibitory activity against rat liver XO (IC_{50} = 49.82 μg/mL (291 μM)), comparable with activity of **2** (IC_{50} = 41.88 μg/mL (196 μM)) and **3** (IC_{50} = 46.66 μg/mL (205 μM)). As cyclodidepsipeptides **1**-**3** have a common part of the structure (6-(propan-2-yl)-morpholine-2,5-dione core), it can be concluded that the presence of isopropyl groups at position 3 and a methyl group at position 4 of morpholine ring (structural characteristics of compound **2**) are more favorable for XO inhibition then the presence of isobutyl group at position 3 and a methyl group at position 4 of morpholine ring (structural characteristics of compound **3**) or the presence of a methyl group at position 3 and the absence of substituents at position 4 of morpholine ring (structural characteristics of compound **1**).

Allopurinol (IC_{50} = 0.79 μg/mL (5.8 μM)), a widely used XO inhibitor and drug to treat gout, exhibited stronger inhibitory effect on rat liver XO than **1**-**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 [16]. 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 [17]. 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) [18]. In the recent literature there are data on non-purine XO inhibitors [19-22].

We also determined the type of enzyme inhibition. The related inhibition type of the title compound on XO was identified from Lineweaver–Burk plots (Fig. 1). The compound **1** was a competitive inhibitor of XO.

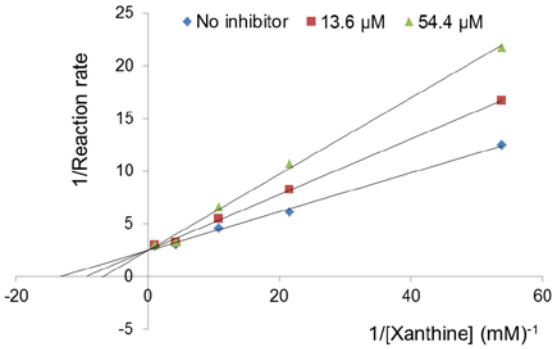


Fig. 1. Lineweaver–Burk plots for the inhibition of xanthine oxidase by compound **1** with xanthine as substrate (see the Methods and Materials section for details)

The interaction of the inhibitor with the XO was studied also by molecular docking into the salicylic acid active site of the bovine milk enzyme (PDB entry code 1FIQ) [23]. Based on recent spectral and computational studies, it is known that the keto form of the title compound is much more energetically favored than the corresponding enol form. The reported NMR and IR data show no evidences for enol formation neither in polar nor in nonpolar medium (3). For this reason only the keto forms of the two diastereoisomers of **1**, (*3S,6R*) and (*3S,6S*), were taken into consideration in the molecular docking studies. The docking poses of the ligands **1**(*3S,6R*) and **1**(*3S,6S*), are presented in Fig. 2.

Both forms of **1** bind in the entrance of the narrow tunnel towards the dioxothiomolybdenum moiety of the active center of XO, blocking in this way the approach of the substrates toward the metal atom (Fig. 2). As can be seen from the 2D 2D representation of the ligand interactions in the pocket (Fig. 3), the ligand-pocket binding of both diastereoisomers is stabilized via hydrogen bonds with Thr1010 and Arg880 and a number of lipophilic interactions with neighboring amino acid residues. In the case of **1**(*3S,6R*) the hydrogen bond is formed between the amide O-atom and Thr1010, while **1**(*3S,6S*) interacts with Thr1010 and Arg880 through its ester carbonyl group (Fig. 3). **1**(*3S,6S*) enters deeper in the cavity. The molecular interactions of title compound to XO resemble those found by crystallographic studies on complexes of XO with other inhibitors not forming a covalent bond with the molybdenum atom such as salicylic acid [23] and febuxostat [24]. These results demonstrate that both diastereoisomers have good binding capacity toward the XO.

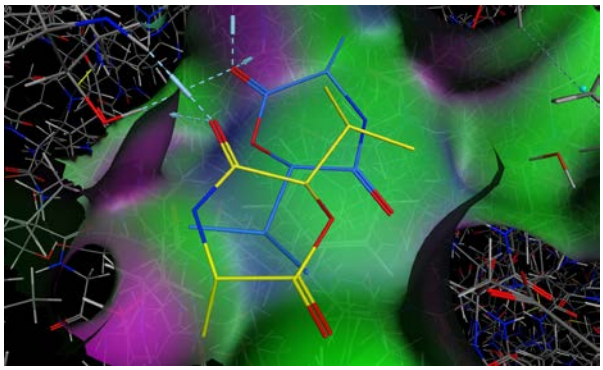


Fig. 2. Docking poses of: **1**(*3S,6R*) - yellow carbons; **1**(*3S,6S*) - blue carbons

1(*3S,6R*) **1**(*3S,6S*)

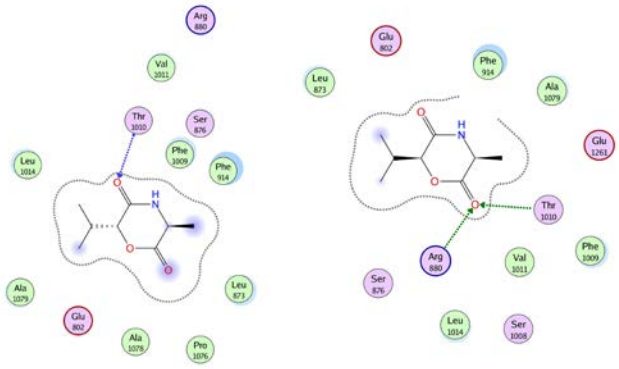


Fig. 3. 2D representation of the ligand interactions in the pocket.

CONCLUSIONS

Cyclodidepsipeptide **1** showed a potent inhibitory effect on XO in a competitive mode. Both diastereoisomers, **1**(*3S,6R*) and **1**(*3S,6S*), bind in the entrance of the narrow tunnel towards the dioxothiomolybdenum moiety of the active center of XO, blocking in this way the approach of the substrates toward the metal atom. Allopurinol was found to be more active against rat liver XO than **1-3.** Chronic allopurinol administration for the inhibition of XO is clinically effective against the hyperuricemia associated with gout, but undesirable side effects have prompted efforts to isolate or synthesise other types of XO inhibitors [25]. Therefore, the results of this study as well as results of our previous study [13] may suggest that 6-(propan-2-yl)-morpholine-2,5-diones are likely to be adopted as candidates to treat gout and may be

taken for further evaluation by using *in vivo* studies. The synthesis of new morpholine-diones derivatives as well as evaluation of their potential for inhibition of XO activity will be part of our further investigation.

Acknowledgements: *The financial support of this work by National Science Fund of Bulgaria (Contract DMU-03/66) and Ministry of Science and Technological Development of Serbia (Projects OI 172044 and TR 31060) is gratefully acknowledged.*

REFERENCES

1. V. Stankov-Jovanovic, J.C. Tabet, P. Dzodic, L. Daskalova, E. Cherneva, D. Yancheva, A. Smelcerovic, *Acta Chim. Slov.*, **59**, 939 (2012).
2. V. Pavlovic, A. Djordjevic, E. Cherneva, D. Yancheva, A. Smelcerovic, *Food Chem. Toxicol.*, **50**, 761 (2012).
3. D. Yancheva, L. Daskalova, E. Cherneva, B. Mikhova, A. Djordjevic, Z. Smelcerovic, A. Smelcerovic, *J. Mol. Struct.*, **1016**, 147 (2012).
4. M. Iijima, T. Masuda, H. Nakamura, H. Naganawa, S. Kurasawa, Y. Okami, M. Ishizuka, T. Takeuchi, Y. Iitaka, *J. Antibiot.*, **45**, 1553 (1992).
5. V. Pavlovic, E. Cherneva, D. Yancheva, A. Smelcerovic, *Food Chem. Toxicol.*, **50**, 3014 (2012).
6. K. Hasumi, C. Shinohara, T. Iwanaga, A. Endo, *J. Antibiot.*, **46**, 1782 (1993).
7. A. Arcelli, D. Balducci, A. Grandi, G. Porzi, M. Sandri, S. Sandri, *Monatsh. Chem.*, **135**, 951 (2004).
8. A. Arcelli, D. Balducci, A. Grandi, G. Porzi, M. Sandri, S. Sandri, *Tetrahedron: Asymmetry*, **16**, 1495 (2005).
9. A. Arcelli, D. Balducci, S.F.E. Neto, G. Porzi, M. Sandri, *Tetrahedron: Asymmetry*, **18**, 562 (2007).
10. T. Kagamizono, E. Nishino, K. Matsumoto, A. Kawashima, M. Kishimoto, N. Sakai, B.-M. He, Z.-X. Chen, T. Adachi, S. Morimoto, K. Hanada, *J. Antibiot.*, **48**, 1407 (1995).
11. J.-F. Hsieh, S.-H. Wu, Y.-L. Yang, K.-F. Choong, S.-T. Chen, *Bioorg. Med. Chem.*, **15**, 3450 (2007).
12. A. Smelcerovic, D. Yancheva, E. Cherneva, Z. Petronijevic, M. Lamshoeft, D. Herebian, *J. Mol. Struct.*, **985**, 397 (2011).
13. A. Smelcerovic, M. Rangelov, Z. Smelcerovic, A. Veljkovic, E. Cherneva, D. Yancheva, G.M. Nikolic, Z. Petronijevic, G. Kocic, *Food Chem. Toxicol.*, **55**, 493 (2013).
14. Z. Smelcerovic, A. Veljkovic, G. Kocic, D. Yancheva, Z. Petronijevic, M. Anderluh, A. Smelcerovic, *Chem.-Biol. Interact.*, **229**, 73 (2015).
15. Molecular Operating Environment (MOE), 2011.10; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2011.
16. H. Horiuchi, M. Ota, S. Nishimura, H. Kaneko, Y. Kasahara, T. Ohta, K. Komoriya *Life Sci.*, **66**, 2051 (2000).
17. H. Horiuchi, M. Ota, S. Kitahara, T. Ohta, M. Kiyoki, K. Komoriya, *Biol. Pharm. Bull.*, **22**, 810 (1999).
18. P. Pacher, A. Nivorozhkin, C. Szabo, *Pharmacol. Rev.*, **58**, 87 (2006).
19. K. Nepali, G. Singh, A. Turan, A. Agarwal, S. Sapra, R. Kumar, U.C. Banerjee, P.K. Verma, N.K. Satti, M.K. Gupta, O.P. Suri, K.L. Dhar, *Bioorg. Med. Chem.*, **19**, 1950 (2011).
20. K. Nepali, A. Agarwal, S. Sapra, V. Mittal, R. Kumar, U.C. Banerjee, M.K. Gupta, N.K. Satti, O.P. Suri, K.L. Dhar, *Bioorg. Med. Chem.*, **19**, 5569 (2011).
21. S. Sharma, K. Sharma, R. Ojha, D. Kumar, G. Singh, K. Nepali, P.M.S. Bedi, *Bioorg. Med. Chem. Lett.*, **24**, 495 (2014).
22. H. Singh, S. Sharma, R. Ojha, M.K. Gupta, K. Nepali, P.M.S. Bedi, *Bioorg. Med. Chem. Lett.*, **24**, 4192 (2014).
23. C. Enroth, B.T. Eger, K. Okamoto, T. Nishino, T. Nishino, E.F. Pai, *Proc. Natl. Acad. Sci. USA*, **97**, 10723 (2000).
24. K. Okamoto, B.T. Eger, T. Nishino, S. Kondo, E.F. Pai, T. Nishino, *J. Biol. Chem.*, **278**, 1848 (2003).
25. F. Borges, E. Fernandes, F. Roleira, *Curr. Med. Chem.*, **9**, 195 (2002).

МЕХАНИЗМИ НА ИНХИБИРАНЕ И МОЛЕКУЛНО МОДЕЛИРАНЕ НА НА ВЗАИМОДЕЙСТВИЯТА НА 6-(ПРОПАН-2-ИЛ)-3-МЕТИЛ-МОРФОЛИН-2,5-ДИОН С КСАНТИН ОКСИДАЗА

Ж. Шмелцерович[1]*, М. Рангелов[2], Е. Чернева[3], Г. Коцич[4], С. Стоянович[4], Т. Йевтович-Стоименов[1,4], Ж. Петрониевич[5], Д. Янчева[2]

[1]*Център за биомедицински науки, Медицински факултет, Университет в Ниш, Сърбия*
[2]*Институт по органична химия с Център по фитохимия, Българска академия на науките, 1113 София, България*
[3]*Факултет по фармация, Медицински университет в София, 1000 София, България*
[4]*Институт по биохимия, Медицински факултет, Университет в Ниш, Сърбия*
[5]*Технологичен факултет, Университет в Ниш, Сърбия*

Постъпила на 11 април2014 г.; коригирана на 27 април, 2015 г.

(Резюме)

Ксантин оксидазата (XO) е ензим, катализиращ окислението на хипоксантина до ксантин, а след това до пикочна киселина. С това той има ключова роля при хиперурицемията. Оценено е инхибирането на активността на XO в препарат от черен дроб на плъхове чрез 6-(пропан-2-ил)-3-метил-морфолин-2,5-дион (**1**) и е сравнено с това на две по-рано изследвани цикло-дипепсипептиди: 3,6-ди(пропан-2-ил)-4-метил-морфолин-2,5-дион (**2**) и 3-(2-метилпропил)-6-(пропан-2-ил)-4-метил-морфолин-2,5-дион (**3**) и алопуринол. Съединението **1** показва значителна инхибираща активност спрямо XO от плъхове (IC_{50} = 49.82 μg/mL), сравнима с активността на **2** и **3**. Алопуринолът, като широко използван инхибитор на XO и лекарство за лечение на подагра проявява по-силен инхибиращ ефект върху XO от съединенията **1**-**3**. Съединенията **1** са синтезирани като смес от два диастереоизомера (*3S,6R*) и (*3S,6S*). Молекулно моделиране е приложено за да даде поглед върху връзките им с ензимните активни центрове. Обработката на данните с помощта на координатите на Lineweaver–Burk показват, че съединение (**1**) е конкурентен инхибитор на XO. Двете форми на **1** се свързват с входа на тесен „тунел“ към диоксо-тиомолибденовата половина на активния център на XO, блокирайки по този начин достъпа на субстратите до металния атом.