

XANTHINE OXIDASE INHIBITORY PROPERTIES AND *IN SILICO* STUDY OF THREE N-(α -BROMOACYL)- α -AMINO ESTERS

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Three noncyclic N-(α -bromoacyl)- α -amino esters, methyl 2-(2-bromo-3-methylbutanamido)-pentanoate (**1**), methyl 2-(2-bromo-3-methylbutanamido)-2-phenylacetate (**2**) and methyl 2-(2-bromo-3-methylbutanamido)-3-phenylpropanoate (**3**), were assayed for inhibitory activity against commercial enzyme xanthine oxidase (XO) *in vitro* and XO in rat liver homogenate. The assayed compounds did not show any significant inhibitory effect against commercial XO, nor against rat liver XO, at the tested concentration (50 μ g/ml). The absence of significant XO inhibitory activity might be caused by basically noncyclic molecular structure of compounds **1-3**, what is in accordance with the presented proposal about depsipeptides that the cyclic structure is important and required for the biological activity. On the other hand, the favorable pharmacokinetic behavior and toxicological properties of the assayed esters, predicted by *in silico* study, may represent a beneficial prerequisite for their implementation in rational carrier-linked prodrug strategies and design. *Acta Medica Medianae* 2016;55(4):14-20.

Key words: N-(α -bromoacyl)- α -amino esters, xanthine oxidase inhibition, *in silico* study, prodrug

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Introduction

Xanthine oxidase (XO) (EC 1.1.3.22) is a versatile metalloflavoprotein enzyme, ubiquitous among species (from bacteria to humans) and in various mammalian tissues. In mammals, the liver and intestine have the highest XO activity. This terminal enzyme of purine catabolism in men catalyses the oxidative hydroxylation of hypoxanthine to xanthine and xanthine to uric acid (1). The over-production of uric acid leads to a gout-causing hyperuricemia, and XO is considered the most

promising target in the treatment of this condition. The inhibition of this enzyme would be beneficial, knowing that its serum levels are significantly increased in various pathological states, like inflammation, ischemia-reperfusion, vascular diseases and carcinogenesis (1, 2). Over the past years, the progress in the knowledge of the structure of this enzyme has been made as well as the efforts towards the development of new xanthine oxidase inhibitors (1). Allopurinol, the prototypical potent XO inhibitor with purine moiety, has been the cornerstone of the clinical management of gout and conditions associated with hyperuricemia for several decades, despite its problematic side effect profile that includes gastrointestinal distress, hypersensitivity reactions, renal toxicity (2). In 2008 and 2009, the European Medicines Agency (EMA) and Food and Drug Administration (FDA) approved febuxostat, a novel selective and non-purine xanthine oxidase inhibitor, more potent than allopurinol, for the treatment of hyperuricemia in gout patients (3). There has been a constant demand that novel non-purine alternatives to allopurinol, with potent XO inhibitory activity and fewer adverse and side effects, should be identified and developed. The identification, isolation and synthesis of some excellent non-purine XO inhibitors have been reported in the recent literature: Y-700 (4), curcumin (5), selenazoles (6), isoxazoles (7), 2-(indol-5-yl)-thiazoles (8), 2-amino-5-alkylidene-thiazol-4-ones (9), 3-phenyl-5-pyridyl-1,2,4-triazoles (10), isocytosines (11-14), N-(1,3-diaryl-3-oxo-

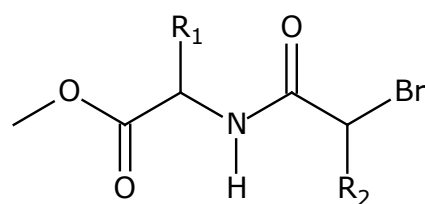
propyl)-amides (15), N-1-acetyl-3,5-diaryl-4,5-dihydro-(1H)-pyrazoles (16), thiazolo-pyrazolyl derivatives (17), 1-thiazolyl-2-pyrazoline derivatives (18), 7-methyl-2-(phenoxyethyl)-5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-5-ones (19), 4,6-diaryl/heteroaryl-pyrimidin-2(1H)-ones (20), naphthoflavones (21), azaflavones (22), naphthopyrans (23), xanthenes (24), 1-hydroxy/methoxy-4-methyl-2-phenyl-1H-imidazole-5-carboxylic acid derivatives (25).

Cyclodepsipeptides are known to exhibit a broad spectrum of biological activities (26). Recently, we assayed three cyclodidepsipeptides, 3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione, 3,6-di-(propan-2-yl)-4-methyl-morpholine-2,5-dione and 6-(propan-2-yl)-3-methyl-morpholine-2,5-dione, for inhibitory activity against commercial enzyme XO *in vitro* and XO in rat liver homogenate (27, 28). These three cyclodidepsipeptides were evaluated as excellent novel inhibitors of XO. Based on the molecular docking study, the binding modes of these compounds with the enzyme were clarified and recommendations for future structure-guided design of new morpholine-dione inhibitors of XO were made. Therefore, the results of our previous studies may suggest that 6-(propan-2-yl)-morpholine-2,5-diones are likely to be adopted as candidates for gout treatment and may be considered for further evaluation in *in vivo* studies (27, 28).

The part of our further investigation was also the synthesis of three novel N-(α -bromoacyl)- α -amino esters, methyl 2-(2-bromo-3-methylbutanamido)-pentanoate (**1**), methyl 2-(2-bromo-3-methylbutanamido)-2-phenylacetate (**2**) and methyl 2-(2-bromo-3-methylbutanamido)-3-phenylpropanoate (**3**), as well as the evaluation of their cytotoxicity on HeLa and RAW264.7 cells, antibacterial and anti-inflammatory activity (29). The potential of these esters for incorporation in acyclovir prodrugs was estimated and their physico-chemical properties were calculated. On the basis of calculated values of physico-chemical properties, all the assayed compounds were shown to meet all the criteria for good solubility and permeability. It was concluded that the low level of cytotoxicity, absence of anti-inflammatory and antibacterial activity, might be a beneficial prerequisite for their incorporation in prodrugs (of acyclovir, and also of other drugs containing hydroxy, thiol and amino groups) that would allow the modification of drug properties, such as a gradual adjustment of the lipophilicity, prodrug stability and pharmacological activity (29).

Aim

The aim of this study was to evaluate the inhibitory activity of three synthesized noncyclic esters, **1-3** (Figure 1), against commercial enzyme xanthine oxidase (XO) *in vitro* and XO in rat liver homogenate. The assay of XO inhibition was performed in order to assess whether the results



- (1) $R_1 = -CH_2CH_2CH_3$; $R_2 = -CH(CH_3)_2$
 (2) $R_1 = -C_6H_5$; $R_2 = -CH(CH_3)_2$
 (3) $R_1 = -CH_2C_6H_5$; $R_2 = -CH(CH_3)_2$

Figure 1. Chemical structures of the assayed compounds

will be in accordance with the suggestion that the ring form is required for biological activity of cyclodepsipeptides (30). In order to gain a better insight and more complete information about the assayed esters as a potentially promising scaffold for implementation in rational carrier-linked prodrug design, pharmacokinetic and toxicological properties of these compounds were predicted *in silico* using admetSAR tool (31).

Materials and Methods

Synthesis

The synthesis of compounds **1-3** was performed as described in our previous study (29).

Evaluation of xanthine oxidase inhibition

Inhibition of commercial xanthine oxidase

Commercial bovine milk XO, purchased from Sigma-Aldrich, was employed for *in vitro* evaluation of enzyme inhibition, by spectrophotometric measurement of uric acid formation at 293 nm, method by Smelcerovic et al. 2015 (9).

The inhibition was studied in a series of test-tubes with the reaction mixture (total volume 2150 μ l), prepared in the following order: (i) test samples contained 0.01 units of XO, one of the studied compounds (**1-3**) diluted in DMSO (the final concentration of DMSO in the assay was 4.65 % v/v), 232.5 μ M of xanthine (Serva), and 46.5 mM TRIS-HCl buffer (pH 7.8); (ii) solvent control samples contained the same amount of XO, an appropriate amount of DMSO, xanthine and TRIS-HCl buffer; (iii) control samples contained the same amount of XO, xanthine and TRIS-HCl buffer adjusted to the same volume. The corresponding blank samples were prepared for each group in the same way as the test solutions (i-iii). The tubes were allowed to incubate at 37°C for 15 min. After incubation, the reaction was stopped by adding 100 μ l of perchloric acid. The percentage of enzyme inhibition was determined by measuring

the difference in absorbance that correlates with uric acid formation. It was calculated as a percent change of the control which contained the appropriate amount of DMSO. All samples were assayed for XO inhibitory activity at concentration of 50 µg/ml. Allopurinol was used as a positive control. All experiments were performed in triplicate and averaged.

Inhibition of rat liver xanthine oxidase

Inhibition of XO activity in rat liver homogenate was evaluated using the spectrophotometric method by Smelcerovic *et al.* 2015 (9).

The reaction mixture (total 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. The 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. Allopurinol was used as a positive control. All experiments were performed in triplicate and averaged.

In silico pharmacokinetic and toxicological study

With physico-chemical characteristics taken into account, some other important features related to the pharmacokinetic behavior and toxicological properties of compounds **1-3** were predicted by admetSAR software (31).

Results and Discussion

The assayed compounds (**1-3**) did not inhibit commercial bovine milk XO with an IC₅₀ below 50 µg/ml. The inhibitory activity was further tested on XO in rat liver homogenate in comparison with allopurinol. The IC₅₀ values of the assayed compounds were higher than 50 µg/ml.

Cyclic depsipeptides and cyclodipeptides represent a rich variety of compounds, having unique structures and intriguing biological activities, of great interest in the field of medicinal chemistry (26). Structural determination revealed unique structural features of these compounds (30). One of the characteristic features of cyclic peptides would be their conformational rigidity and stability *in vivo*, in contrast to their linear counter-

parts (32). It has been suggested that cyclic depsipeptide structure is important and required for biological activity, because the linear homologues have been shown to be inactive (30). The suitable cyclization point has thus been of great importance for the progress in the field of synthetic strategies and methodologies (32). Synthesis of cyclodipeptides is achieved by terminal intermolecular cyclization. It is closely related to the ring-closure strategies applied in the synthesis of larger cyclodipeptide family members (30, 33). 6-(Propan-2-yl)-morpholine-2,5-diones are potent XO inhibitors (27, 28). It was therefore supposed that the reported absence of significant *in vitro* XO inhibitory activity might be caused by basically noncyclic molecular structure of the assayed compounds **1-3**.

In order to evaluate the possibility of future medical application, many important features of compounds such as physico-chemical properties, pharmacokinetic behavior in living organisms, bioavailability and transportation through different membranes, optimal process of biotransformation and elimination should be taken into account. Pharmacokinetic and toxicological properties of compounds **1-3** were predicted *in silico* using admetSAR tool (31) (Table 1).

Preliminary screening of molecular physico-chemical properties such as lipophilicity, molecular size, flexibility and presence of hydrogen-donors and acceptors facilitates considerably the development and outlines the usefulness of newly emerging scaffolds in medicinal chemistry. Based on the analysis of a large number of drugs, the established values of four physico-chemical properties were set into the Lipinski "Rule of five". More than one violation of the rule is the critical limit for acceptable drug-likeness (34). Summarizing the previously calculated physico-chemical properties of compounds **1-3**, it was concluded that the substances obey the Lipinski "Rule of five" and meet all criteria for good solubility and permeability (29). A conclusion may be drawn that the assayed compounds obey also the Veber rules (35) and are considered to possess the ability to penetrate biological membranes, which is a common requirement for bioavailability.

The data obtained by admetSAR software (31) indicated that the assayed compounds might show good intestinal absorption and blood-brain barrier penetration. Therefore, on the basis of previously calculated physico-chemical properties and predicted absorption, compounds **1-3** might have good oral bioavailability and membrane transport properties. Based on the calculations it is not likely that assayed compounds will act neither as substrates/inhibitors of P-glycoprotein, nor as inhibitors of renal organic cation transporter. Those estimations are of importance not only from the absorption point of view, but also from the viewpoints of elimination and potential interactions in biotransformation pathways. Low CYP inhibitory promiscuity goes in favor of a reduced probability

Table 1. Absorption, metabolic and toxicological properties of compounds **1-3** predicted *in silico* using admetSAR tool (31)

Predicted properties	Compound 1	Compound 2	Compound 3
Absorption			
Blood-brain barrier penetration	+	+	+
Human intestinal absorption	+	+	+
Caco2 permeability	+	+	+
P-glycoprotein substrate	-	-	-
P-glycoprotein inhibitor	-	-	-
Renal organic cation transporter inhibitor	-	-	-
Metabolism			
CYP450 2C9 substrate	-	-	-
CYP450 2D6 substrate	-	-	-
CYP450 3A4 substrate	+	-	+
CYP450 1A2 inhibitor	-	+	+
CYP450 2C9 inhibitor	-	-	-
CYP450 2D6 inhibitor	-	-	-
CYP450 2C19 inhibitor	-	-	-
CYP450 3A4 inhibitor	-	-	-
CYP inhibitory promiscuity	low	low	low
Toxicity			
AMES toxicity	-	-	-
Carcinogens	-	-	-
Fish toxicity	high	high	high
<i>Tetrahymena pyriformis</i> toxicity	high	high	high
Honey bee toxicity	low	low	low
Biodegradation	not ready	not ready	not ready
Acute oral toxicity	III	III	III
Carcinogenicity (Three-class)	non-required	non-required	non-required

of drug interactions. Additionally, the three assayed compounds were predicted as non-AMES toxic and non-carcinogens. From the ecotoxicological and environmental safety point of view, these compounds were evaluated as not readily biodegradable, with low honey bee toxicity, but high fish and *Tetrahymena pyriformis* toxicity. Compounds **1-3** were predicted as Category III for acute oral toxicity risk, including the compounds with LD₅₀ values greater than 500 mg/kg, but less than 5000 mg/kg. According to TD₅₀ values, the test compounds were assigned as a "non-required" rat carcinogenicity class, which meant these were non-carcinogenic chemicals.

In the previous study, the hypothetical products obtained by coupling of esters **1-3** to acyclovir were shown to be more lipophilic than acyclovir itself (29). Based on the site of conversion into a pharmacologically active agent, acyclovir belongs to the group of prodrugs metabolized intracellularly at the cellular targets of its therapeutic actions (36). Therefore, this gradual adjustment of lipophilicity, allowed by coupling of compounds **1-3** to acyclovir, is considered to be beneficial. Generally, the major groups of carrier-linked prodrugs are esters and amides. Ester prodrugs are most frequently used to enhance lipo-

philicity and passive membrane transport by masking polar groups (36).

Conclusion

The assayed compounds did not show any significant inhibitory effect against commercial XO and rat liver XO at the tested concentration (50 µg/ml). These results are in accordance with the suggestion about depsipeptides that the cyclic structure is important and required for biological activity (30). The absence of significant *in vitro* XO inhibitory activity reported here might be caused by noncyclic molecular structure of the assayed compounds **1-3**. On the other hand, favorable pharmacokinetic behavior and toxicological properties of these compounds, predicted by *in silico* study, may represent a beneficial prerequisite for their implementation in rational carrier-linked prodrug approaches, strategies and design, and can be used for the development of compounds with better drug-like properties, with improved stability and/or pharmacological activity. The observed absence of significant inhibitory activity against XO represents an important step forward that might be helpful for more thorough investigation of the

possibility of potential incorporation of the assayed esters in carrier-linked prodrugs. However, for such a claim to be made further research is required.

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doi:10.5633/amm.2016.0402**INHIBICIJA KSANTIN-OKSIDAZE I IN
SILICO STUDIJA TRI N-(α -BROMACIL)- α -AMINO
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Tri aciklična N-(α -bromacil)- α -amino estera, metil-2-(2-brom-3-metilbutanamido)-pentanoat (**1**), metil-2-(2-brom-3-metilbutanamido)-2-fenilacetat (**2**) i metil-2-(2-brom-3-metilbutanamido)-3-fenilpropanoat (**3**) bila su podvrgnuta testu inhibicije komercijalne ksantin-oksidge *in vitro* i ksantin-oksidge u homogenatu jetre pacova. Ispitivana jedinjenja nisu pokazala značajniji inhibitorni efekat niti na komercijalnom, niti na enzimu u homogenatu jetre pacova u ispitanoj koncentraciji (50 μ g/ml). Potencijalni uzrok zabeleženog odsustva značajnije inhibicije ksantin-oksidge može predstavljati aciklična struktura supstanci **1-3**, što je u saglasnosti sa datom pretpostavkom za depsipeptide da je ciklična struktura neophodna za ispoljavanje biološke aktivnosti. Sa druge strane, povoljni farmakokinetički i toksikološki profili, predviđeni *in silico* studijom, mogu predstavljati dobar preduslov za potencijalno implementiranje ispitivanih estara u racionalne strategije i dizajn nosača u sintezi prolekova. *Acta Medica Medianae* 2016;55(4):14-20.

Ključne reči: N-(α -bromacil)- α -amino estri, in silico studija, inhibicija ksantin-oksidge, prolek