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Xanthine oxidase inhibitory properties and anti-inflammatory activity of 2-amino-5-alkylidene-thiazol-4-ones

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ABSTRACT

Thirty 2-amino-5-alkylidene-thiazol-4-ones were assayed 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). 4-((2-Benzylamino-4-oxothiazol-5(4*H*)-ylidene)-methyl)benzonitrile showed the most potent inhibitory effect against commercial XO (IC_{50} = 17.16 μg/mL) as well as against rat liver XO (IC_{50} = 24.50 μg/mL). All compounds containing the 4-cyanobenzylidene group or (indol-3-yl)methylene group at the position 5 of thiazol-4-one moiety were moderately potent inhibitors of commercial XO. The assayed compounds were docked into the crystal structures of XO enzyme complexes with three diverse inhibitors (PDB codes: 1FIQ, 1VDV, and 1V97) using OEDocking software. Our results strongly point to a correlation between the data on inhibitory activity against commercial XO and data on antioxidant activity of studied compounds, screened using a lipid peroxidation (LP) method. 2-(Benzylamino)-5-((thiophen-2-yl)methylene)thiazol-4(5*H*)-one showed the highest anti-inflammatory response on PBMCs, exerted most probably through the NF-κB inhibition. Studied 2-amino-5-alkylidene-thiazol-4-ones obey the "Rule of five" and meet all criteria for good solubility and permeability.

1. Introduction

Xanthine oxidase (XO) is a highly versatile flavoprotein enzyme, ubiquitous among species (from bacteria to human) and within the various tissues of mammals [1]. It catalyzes the last two steps in the purine degradation pathway prior to formation of uric acid, that is, hydroxylation of hypoxanthine to xanthine, and then to uric acid [2]. There is an overwhelming acceptance that XO serum levels are significantly increased in various pathological states like hepatitis, inflammation, ischemia–reperfusion, carcinogenesis and aging, and that ROS generated in the enzymatic process are involved in oxidative damage. Thus, the inhibition of this enzymatic pathway could be beneficial in a number of mentioned conditions [1]. ROS generated by XO activity may activate redox-associated transcriptional factors, among them one of the most important is NF-κB. 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 [3].

2-Amino-5-alkylidene-thiazol-4-one is a privileged scaffold in drug discovery [4,5] as its derivatives show a variety of biological activities, such as antimicrobial [6,7], antioxidant [8], antiviral [9], anti-inflammatory [10], and cardiotonic [11]. We have synthesized a library of 30 diverse 2-amino-5-alkylidene-thiazol-4-ones (**1**–**30**) [12] and recently we have investigated their antimicrobial [7] and antioxidant activity [8], as well as cytotoxicity [7]. The antioxidant activity of some of studied compounds was comparable with activity of standard antioxidants (trolox, quercetin, caffeic acid and L-ascorbic acid) [8]. The important feature of these compounds is their low level of influence on cell viability, as

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0009-2797/

assayed by the HEK-293 metabolic activity assay [7]. As a part of a detailed biological screening of our 2-amino-5-alkylidene-thiazol-4-ones library, in the present study **1**–**30** 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). The molecular docking studies were performed in order to examine the binding mode of studied compounds in the XO active site. Additionally, the physico-chemical properties of studied compounds were calculated using Molinspiration tool [13].

2. Materials and methods

2.1. Synthesis

The synthesis of the studied compounds (**1**–**30**) were performed by an innovative one-pot tandem reaction using microwave-assisted synthesis, as described in our previous study [12].

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 spectrophotometric measurement uric acid formation at 293 nm (method slightly modified by [14]).

The inhibition was studied in a series of test-tubes with the reaction mixture (total volume 2150 μL), prepared in a following order: (i) *test samples* contained 0.01 units of XO, one of the studied compounds (**1**–**30**) diluted in DMSO (the final concentration of DMSO in the assay was 4.65% v/v), 232.5 μM of xanthine (Serva), and 46.5 mM TRIS–HCl buffer (pH 7.8); (ii) *solvent control samples* contained the same amount of XO, 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; (iv) *test substrate samples* were group of samples which contained only XO in the reaction mixture, one of the studied compounds (**1**–**30**) diluted in DMSO, and TRIS–HCl buffer, in order to test if the compounds **1**–**30** are XO substrates. The tubes were allowed to incubate at 37 °C for 15 min, together with the corresponding duplicate blank aliquots, where the enzyme was omitted. After incubation, the reaction was stopped by adding 100 μL of perchloric acid; afterwards the XO was added in corresponding blank samples in duplicate. The percentage of enzyme inhibition was determined by measuring the difference in absorbance that correlates with uric acid formation; it was calculated as a percentage of specimen absorbance vs. absorbance of the solvent control samples which involves only the absorbance of DMSO. All samples were assayed for XO inhibitory activity at concentrations of 50 μg/mL. Those showing inhibition greater than 50% at this concentration were tested in a broader concentration range to allow calculation of IC_{50} values. IC_{50} curves were generated using four concentrations of studied compounds (50, 40, 25 and 5 μg/mL). Allopurinol was used as positive control. All experiments were performed in triplicate and averaged.

2.2.2. Inhibition of rat liver xanthine oxidase

Inhibition of XO activity in rat liver homogenate was evaluated using a spectrophotometric method (slightly modified by [14]). 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**–**30**) 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.

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%, as determined using Trypan blue stain exclusion. After washing in PBS, obtained PBMC were resuspended in RPMI 1640 medium containing 10% of FCS.

2.4. Detection of NF-κB

All chemical were purchased from Sigma while the antibodies were purchased from Santa Cruz Biotechnology. Anti-inflammatory activity of compounds **1**–**30** dissolved in DMSO (the final concentration of studied compounds was 1 μg/mL, while the final concentration of DMSO in the assay was 0.1% v/v) was examined via quantification of NF-κB, as described in our previous papers [3,14]. 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 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 further 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 that were treated with primary and secondary antibodies only. The fluorescence intensity of cells indicated for both up or down regulated populations for each compound used, compared to control samples with the appropriate amounts of DMSO, and calculated as a percent change of the control PBMCs which involves the effect of appropriate amount of DMSO. All experiments were conducted in triplicate and averaged.

2.5. Molecular docking studies

2.5.1. Ligand preparation

The molecules were built with ChemBioDraw Ultra 13.0 (PerkinElmer, Inc.) and their geometries optimized with ChemBio 3D Ultra 13.0 (PerkinElmer, Inc.) using MM2 force field until a minimum 0.100 Root Mean Square (RMS) gradient was reached. The optimized structure was refined with GAMESS interface using the semi-empirical AM1 method, QA optimization algorithm and Gasteiger Hückel charges for all atoms for 100 steps. FRED requires

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a set of input conformers for each ligand, which were generated with OMEGA (OMEGA version 2.5.1.4. OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com), with maximum number of conformations set to 100 [15,16]. All the other options were left as default values.

2.5.2. Receptor preparation and docking protocol

The crystal structures of three XO enzyme complexes with known inhibitors (PDB codes: 1FIQ, 1VDV, and 1V97) were taken as starting points. The ligands were taken as reference structures and all cofactors included were left as "receptor" to avoid overlap with their binding sites. For each crystal structure, one grid box was created with volumes of 13,277, 12,212, and 12,779 $Å^3$, and outer contours of 1932, 2056, and 1966 $Å^2$ using Make Receptor 3.0.1.

The docking software FRED (OEDocking version 3.0.1. OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com) was used for docking studies with the default settings, and number of poses, which was set to 50 [17]. The proposed five binding poses with the highest rank of the docked inhibitors were evaluated using final score and relative position to the native ligand. The graphical representations of the calculated binding poses were obtained using VIDA (VIDA version 4.2.1. OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com).

2.5.3. Validation of the docking protocol

For all three diverse crystal structures, we have tried to reproduce the pose of the specific inhibitor (PDB codes: 1FIQ, 1VDV, and 1V97). In each case, one of the top five docking poses of the docked inhibitor were within 1.5 Å root mean square deviation (RMSD) of the ligand crystal structure. In case of the 1VDV crystal structure, the predicted top score pose was the one that best fitted the crystal structure of the ligand, overlapping it almost completely, which offers the proof of the protocol validation (Fig. 1).

3. Results and discussion

3.1. Xanthine oxidase inhibition

3.1.1. Assays on xanthine oxidase inhibition

19 of 30 studied 2-amino-5-alkylidene-thiazol-4-ones inhibit commercial bovine milk XO with an IC_{50} below 50 μg/mL (Table 1). The inhibitory activity of **1**–**30** on XO were further tested in rat liver homogenate and only 8 compounds inhibited XO with an IC_{50} lower than 50 μg/mL (Table 2). 4-((2-Benzylamino-4-oxothiazol-5(4*H*)-ylidene)-methyl)benzonitrile (compound **14**) showed the most potent inhibitory effect against commercial XO (IC_{50} = 17.16 μg/mL) as well as against rat liver XO (IC_{50} = 24.50 - μg/mL). All compounds containing the 4-cyanobenzylidene group or (indol-3-yl)methylene group at the position 5 of thiazol-4-one moiety inhibited commercial XO with IC_{50} values below 50 μg/mL. Compounds with 4-cyanobenzylidene group (with the exception of compound **11** containing piperidinyl group as the 2-amino substituent) inhibit rat liver XO with an IC_{50} lower than 50 μg/mL. On the other hand, compounds with (indol-3-yl)methylene substituent at position 5 exhibited lower than the threshold inhibitory activity (50%) against rat liver XO at concentrations of 50 μg/mL. Allopurinol, a widely used XO inhibitor and drug to treat gout, exhibited stronger inhibitory effect on commercial XO (IC_{50} = 0.26 μg/mL) as well as rat liver XO (IC_{50} = 0.79 μg/mL) than **1**–**30**.

3.1.2. Binding pose prediction

All assayed compounds were docked into the crystal structures of XO enzyme complexes with three diverse inhibitors (PDB codes: 1FIQ, 1VDV, and 1V97, [18–20]) using OEDocking software (Release 3.0.1, OpenEye Scientific Software, Inc.). The structures of known inhibitors vary significantly (Fig. 2), so we have used them as three distinct starting points to allow higher pose variability, as MakeReceptor (OEDocking version 3.0.1. OpenEye Scientific

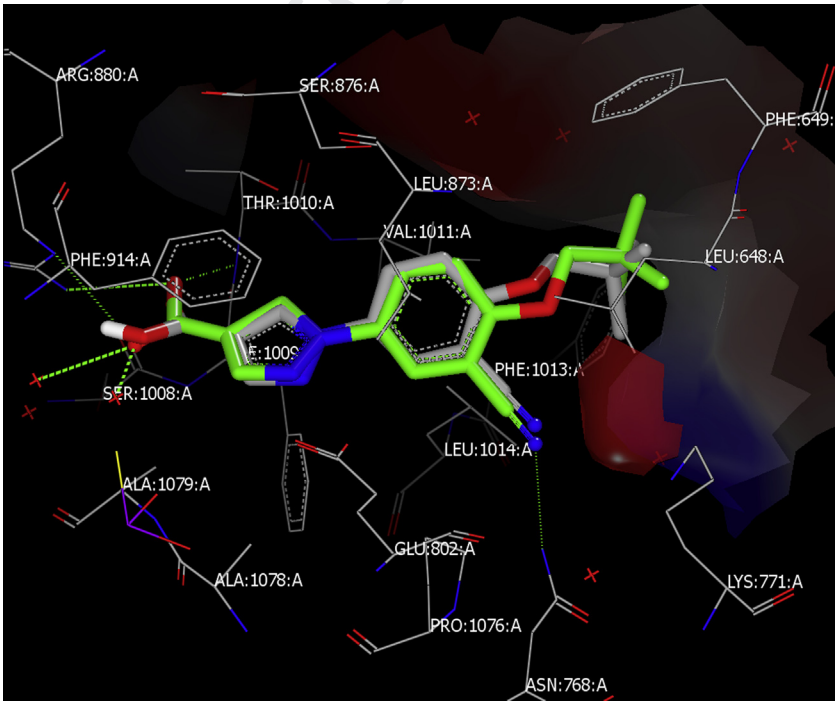

Fig. 1. Crystal structure of the XO inhibitor (niraxostat (**32**); 1-[3-cyano-4-(neopentyloxy)phenyl]-1*H*-pyrazole-4-carboxylic acid, carbons shown as green sticks) in complex with XO (PDB code: 1VDV), and top docked pose of the same inhibitor docked in the XO active site using FRED (shown as stick colored by atom type). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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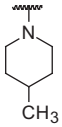
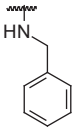
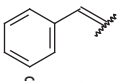
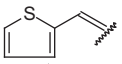
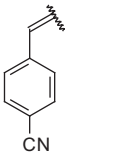
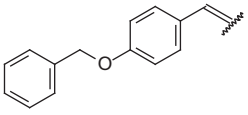
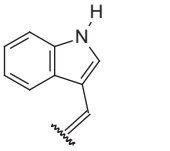
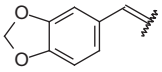
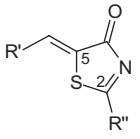
Table 1
In vitro screening of the synthesized compounds (**1**–**30**) for inhibitory activity against commercial XO.

Substituent at position 5	Substituent at position 2 Entry (Entry[a]) IC_{50} (μg/mL)					
		**1** (**8**[a]) 43.45	**2** (**9**[a]) 26.67	**3** (**10**[a]) >50	**4** (**11**[a]) >50	**5** (**12**[a]) >50
		**6** (**13**[a]) >50	**7** (**14**[a]) 25.82	**8** (**15**[a]) >50	**9** (**16**[a]) 37.57	**10** (**17**[a]) 39.41
		**11** (**18**[a]) 33.18	**12** (**19**[a]) 24.73	**13** (**20**[a]) 25.98	**14** (**21**[a]) 17.16	**15** (**22**[a]) 32.53
		**16** (**23**[a]) >50	**17** (**24**[a]) >50	**18** (**25**[a]) >50	**19** (**26**[a]) 31.77	**20** (**27**[a]) >50
		**21** (**28**[a]) 21.43	**22** (**29**[a]) 23.17	**23** (**30**[a]) 27.03	**24** (**31**[a]) 33.62	**25** (**32**[a]) 44.08
		**26** (**33**[a]) 27.24	**27** (**34**[a]) >50	**28** (**35**[a]) >50	**29** (**36**[a]) 39.77	**30** (**37**[a]) 33.85
Standard XO inhibitor Allopurinol	IC_{50} (μg/mL) 0.26					

[a] Entries as in the original article [12].

Software, Santa Fe, NM. http://www.eyesopen.com) builds the docking grid based on the native inhibitor in the crystal structure.

As expected, the "receptors" constructed from 1VDV and 1V97 reproduced more credible validation results as a consequence of larger inhibitor molecules that match the size of the assayed compounds. 1VDV-based receptor was used for further studies, and the results obtained indicate two possible binding modes for the assayed compounds that depend upon the nature of the "amine" part of the molecule:

- the "amine-out" mode; molecules with the tertiary (cyclic) amine are oriented with the arylidene part toward the Arg880 of the XO binding pocket, while the amine part protrudes toward solvent, and
- the "amine-in" mode; XO accommodates molecules with benzylamine moiety so that the benzylamine moiety points to Arg880.

The scores obtained (not shown) do not match exactly the potency observed on isolated XO enzyme, but might explain the general tendency that emerged out of the inhibition data:

- indole derivatives are generally potent inhibitors of the isolated XO enzyme, due to high steric complementarity with XO binding site, if the molecule follows the "amine-out" binding mode,
- "amine-in" binding mode is predicted for compounds with benzylamine fragment, where the arylidene moiety points toward surface (Fig. 3).

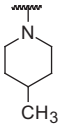
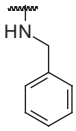
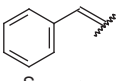
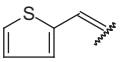
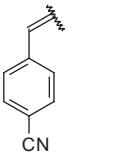
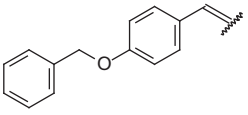
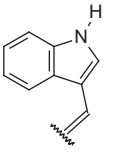
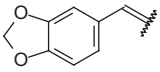
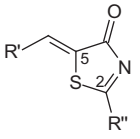
Compound **14** displayed the highest observed potency on isolated enzyme, which suits before mentioned binding hypothesis. Namely, compound **14** is predicted to follow the "amine-in" binding mode, with benzylamine moiety buried deeply into binding site, while the polar cyano group of the 4-cyanobenzilidene moiety makes contact with the solvent thus diminishing the entropic penalty during binding (Fig. 3).

3.2. Anti-inflammatory activity

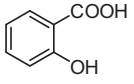
2-(Benzylamino)-5-((thiophen-2-yl)methylene)thiazol-4(5*H*)-one (**9**) showed the highest anti-inflammatory response on PBMCs, exerted through the NF-κB inhibition (Table 3).

Also, **3**, **5**, **16** and **21** at concentrations of 1 μg/mL caused a potent NF-κB downregulation. **14**, which was the most potent XO inhibitor in both used assays, showed a significant anti-inflammatory activity. Also, this compound showed very strong LP inhibitory effect [8], comparable to the effect of standard antioxidants. The compounds containing the 2-(benzylamino) group, such as compounds **9** and **14**, are secondary amines in contrast to the other studied compounds which are tertiary amines. Hydrogen atom of secondary amino group enables two tautomeric forms that could play an important role for NF-κB inhibition. However, it is difficult to get any clear SAR conclusions based on the results presented in the Table 3. Song et al. [10] reported that some 2-amino-5-alkylidene-thiazol-4-ones are potent cyclooxygenase-2 (COX-2) inhibitors. Within their thiazolone series they investigated the effect of

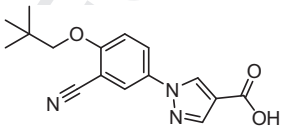
Table 2
In vitro screening of the synthesized compounds (**1**–**30**) for inhibitory activity against XO in rat liver homogenate.

Substituent at position 5	Substituent at position 2 Entry (Entry[a]) IC_{50} (μg/mL)					
		**1** (**8**[a]) 47.71	**2** (**9**[a]) 42.02	**3** (**10**[a]) >50	**4** (**11**[a]) >50	**5** (**12**[a]) >50
		**6** (**13**[a]) >50	**7** (**14**[a]) >50	**8** (**15**[a]) >50	**9** (**16**[a]) 40.87	**10** (**17**[a]) >50
		**11** (**18**[a]) >50	**12** (**19**[a]) 33.98	**13** (**20**[a]) 29.25	**14** (**21**[a]) 24.50	**15** (**22**[a]) 48.16
		**16** (**23**[a]) >50	**17** (**24**[a]) >50	**18** (**25**[a]) >50	**19** (**26**[a]) >50	**20** (**27**[a]) >50
		**21** (**28**[a]) >50	**22** (**29**[a]) >50	**23** (**30**[a]) >50	**24** (**31**[a]) >50	**25** (**32**[a]) >50
		**26** (**33**[a]) 48.56	**27** (**34**[a]) >50	**28** (**35**[a]) >50	**29** (**36**[a]) >50	**30** (**37**[a]) >50
Standard XO inhibitor Allopurinol	IC_{50} (μg/mL) 0.79					

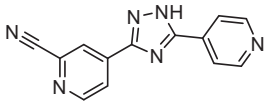
[a] Entries as in the original article [12].



31, inhibitor from 1FIQ



32, inhibitor from 1VDV



33, inhibitor from 1V97

Fig. 2. Structural formulae of the known XO inhibitors in complex with (PDB codes: 1FIQ, 1VDV, and 1V97).

the substituent at the position 2 of the thiazol-4-one moiety in detail and found that the nature of the chemical bond between the nitrogen atom and the substituents attached to it is critical for potency and selectivity of COX-2 inhibition. Namely, an N—O bond gives compounds which are potent and selective COX-2 inhibitors. N—H bond leads to weak COX-2 inhibition. An N—C and N—N bonds lead to loss of potency [10].

NF-κB is a redox-associated transcription factor that is required for maximal transcription of a wide array of pro-inflammatory mediators. It is well known that ROS stimulate the NF-κB pathway in the cytoplasm through IκB (inhibitor of kappa B) degradation. Overexpression of the antioxidant proteins was shown to inhibit NF-κB activation [21]. We have previously proposed the electron transfer (SET) mechanism as the most probable one to explain the observed antioxidant activity of 2-amino-5-alkylidenethiazol-4-ones [8]. Accordingly, this might be one of the mechanisms of NF-κB inhibition by studied compounds. Xu et al. [22] have found NF-κB binding site on human xanthine dehydrogenase (XDH) gene, and it is known that XDH conversion to XO may represent a feed-forward mechanism for stimulation of ROS production [23]. Accordingly, we speculate that NF-κB may directly affect the XO activity and ROS production.

3.3. Physico-chemical properties and potential reactivity of studied compounds

The above sections demonstrate the promising inhibitory activities of studied 2-amino-5-alkylidene-thiazol-4-ones toward commercial and rat liver XO. However, in view of future medical application, other important features should be also taken into account – favorable pharmacokinetic behavior in living organisms, providing the required bioavailability and transportation through different membranes to the site of action, optimal process of metabolism and elimination. For this reason preliminary screening of molecular physico-chemical properties such as lipophilicity, molecular size, flexibility and presence of hydrogen-donor and

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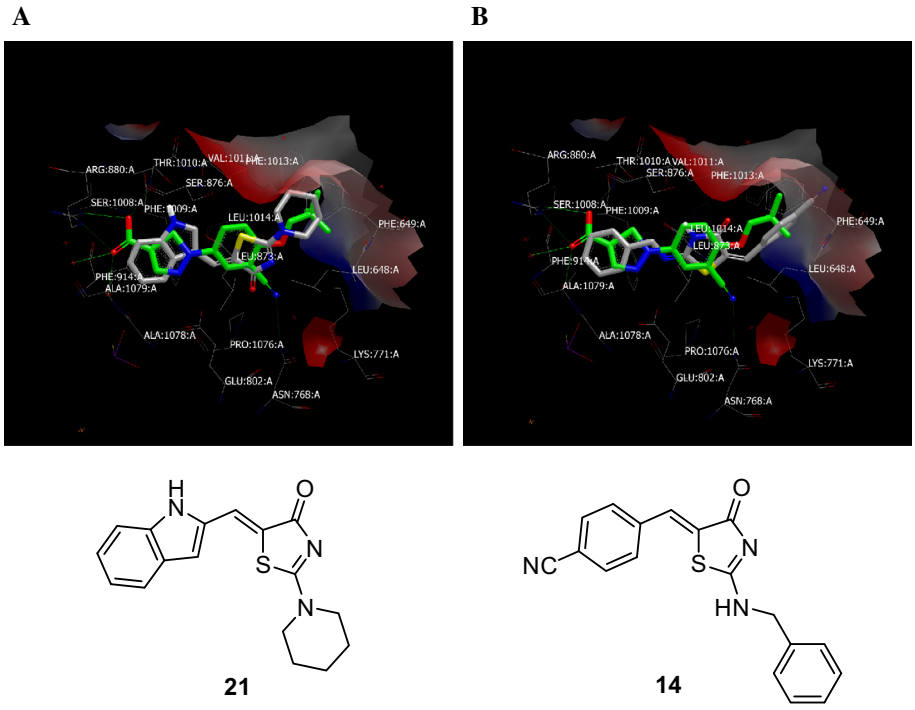
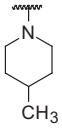
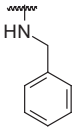
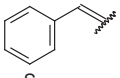
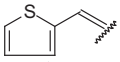
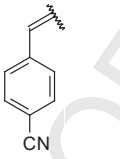
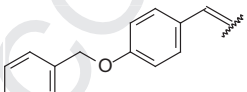
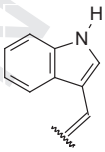
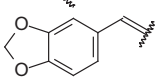


Fig. 3. Two proposed binding modes for assayed 2-amino-5-alkylidene-thiazol-4-ones: (A) "amine-out" mode of 21, and (B) "amine-in" mode of 14. The figures present docking results into 1VDV crystal structure with the known XO inhibitor rendered as green sticks and the docked molecule rendered as sticks colored by atom type. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Effect of studied 2-amino-5-alkylidene-thiazol-4-ones (sample concentration 1 μg/mL) on quantitative expression of NF-κB.

Substituent at position 5	Substituent at position 2 Entry (Entry[a]) % of control PBMCs with 0.1% DMSO					
		**1** (**8**[a]) 123.14 ± 15.28	**2** (**9**[a]) 95.56 ± 14.81	**3** (**10**[a]) 43.95 ± 6.10	**4** (**11**[a]) 72.21 ± 17.95	**5** (**12**[a]) 47.67 ± 20.37
		**6** (**13**[a]) 70.43 ± 17.32	**7** (**14**[a]) 72.00 ± 13.94	**8** (**15**[a]) 91.55 ± 24.16	**9** (**16**[a]) 26.18 ± 9.28	**10** (**17**[a]) 91.95 ± 23.77
		**11** (**18**[a]) 68.64 ± 23.14	**12** (**19**[a]) 81.22 ± 28.42	**13** (**20**[a]) 50.20 ± 14.39	**14** (**21**[a]) 54.41 ± 2.56	**15** (**22**[a]) 97.04 ± 7.63
		**16** (**23**[a]) 42.06 ± 32.70	**17** (**24**[a]) 70.46 ± 5.84	**18** (**25**[a]) 67.34 ± 27.32	**19** (**26**[a]) 66.26 ± 18.60	**20** (**27**[a]) 66.28 ± 5.19
		**21** (**28**[a]) 41.69 ± 10.80	**22** (**29**[a]) 54.05 ± 11.33	**23** (**30**[a]) 74.02 ± 12.43	**24** (**31**[a]) 92.34 ± 51.20	**25** (**32**[a]) 64.92 ± 27.93
		**26** (**33**[a]) 67.30 ± 1.87	**27** (**34**[a]) 51.60 ± 5.39	**28** (**35**[a]) 99.14 ± 32.89	**29** (**36**[a]) 66.75 ± 17.85	**30** (**37**[a]) 74.69 ± 30.96

[a] Entries as in the original article [12].

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Table 4
Calculated molecular properties of compounds **1**–**30** for assessment of the drug-likeness.

Compd. No. Rule	m_i log P[a] <5	TPSA[b]	N_{atoms}[c]	MW[d] <500	N_{ON}[e] <10	N_{OHNH}[f] <5	$N_{viol.}$[g]	$N_{rotb.}$[h] (<10)	Vol[i]
1	3.17	33	19	272	3	0	0	2	246
2	2.11	42	19	274	4	0	0	2	239
3	3.41	33	20	286	3	0	0	2	263
4	3.42	42	21	294	3	1	0	4	261
5	2.66	33	18	258	3	0	0	2	230
6	3.07	33	18	278	3	0	0	2	237
7	2.01	42	18	280	4	0	0	2	229
8	3.31	33	19	292	3	0	0	2	254
9	3.31	42	20	300	3	1	0	4	252
10	2.56	33	17	264	3	0	0	2	220
11	2.92	57	21	297	4	0	0	2	263
12	1.86	66	21	299	5	0	0	2	255
13	3.17	57	22	311	4	0	0	2	280
14	3.17	66	23	319	4	1	0	4	278
15	2.42	57	20	283	4	0	0	2	247
16	4.82	42	27	378	4	0	0	5	344
17	3.76	52	27	380	5	0	0	5	336
18	5.06	42	28	392	4	0	1	5	360
19	5.07	51	29	400	4	1	1	7	358
20	4.32	42	26	364	4	0	0	5	327
21	3.32	49	22	311	4	1	0	2	275
22	2.26	58	22	313	5	1	0	2	268
23	3.56	49	23	325	4	1	0	2	292
24	3.57	58	24	333	4	2	0	4	290
25	2.82	49	21	297	4	1	0	2	259
26	3.06	52	22	316	5	0	0	2	270
27	2.00	61	22	318	6	0	0	2	263
28	3.30	52	23	330	5	0	0	2	287
29	3.31	60	24	338	5	1	0	4	285
30	2.55	52	21	302	5	0	0	2	254

[a] Octanol–water partition coefficient, calculated by the methodology developed by [13].
[b] Polar surface area.
[c] Number of nonhydrogen atoms.
[d] Molecular weight.
[e] Number of hydrogen-bond acceptors (O and N atoms).
[f] Number of hydrogen-bond donors (OH and NH groups).
[g] Number of "Rule of five" violations.
[h] Number of rotatable bonds.
[i] Molecular volume.

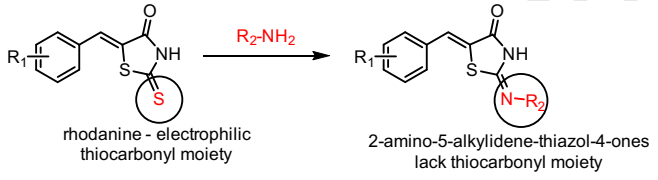

Fig. 4. The synthesis of 2-amino-5-alkylidene-thiazol-4-ones eliminates thiocarbonyl moiety as an electrophilic center of the rhodanine.

acceptors facilitate considerably the development of new pharmaceuticals and outline the usefulness of new drug candidates.

Physico-chemical properties of 2-amino-5-alkylidene-thiazol-4-ones **1**–**30**, calculated using Molinspiration tool [13], are shown in Table 4. Data indicate that none of the compounds is above the critical limits established by the Lipinski "Rule of five" [24]. Molinspiration methodology for calculation of m_i log P implements fragment-based contributions and correlation factors which makes it robust and applicable to virtually all organic and organometallic compounds. The m_i log P values of **1**–**30** show favorable physicochemical profiles for oral bioavailability [24]. Variation of the type of the ring (phenyl, thienyl, indolyl) attached to the alkylidene group does not lead to a dramatic change in the lipophilicity of the molecules studies, except when a polar (CN) or bulky (benzyloxy) group is present in the ring. Thus the compounds with CN group **11**–**15** show the lowest m_i log P values compared to the other corresponding compounds, while **16**–**20**, containing benzyloxy group, are the most lipophilic. The largest derivatives **18** and **19** with molecular weight of about 400 are the only compounds approaching the critical limit of 5.

The amine moiety show greater impact on the lipophilicity of **1**–**30**. Smaller rings such as pyrrolidine and more polar ones such as the morpholine significantly lower the lipophilicity of the molecules. The variation of the substitution pattern of the amine moiety (secondary acyclic benzylamine fragment vs. tertiary cyclic piperidinyl fragments) contributes to mild tuning of the lipophilicity. On the other hand, all compounds containing benzylamine moiety show higher conformational flexibility manifested through their greater number of rotatable bonds.

The number of rotatable bonds is an important factor for the efficient binding to receptors and channels as well as for the oral bioavailability [25]. Molecules with more than 10 rotatable bonds tend to show poor oral bioavailability. All studied 2-amino-5-alkylidene-thiazol-4-ones, including the benzylamine containing derivatives, satisfy this criterion and most of them have low conformational flexibility with only 2 rotatable bonds. Another descriptor for the oral bioavailability [25] and drug transport properties [26,27] is the polar surface area. It is expressed here as topological surface area (TPSA) which is a sum of the surface areas occupied by the oxygen and nitrogen atoms and the hydrogens attached to them. TPSA represents the hydrogen bonding capacity

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of the molecules. Molecules with TPSA less than 140 Å^2 are recognized to have good intestinal absorption, and those with TPSA less than 60 Å^2 show good blood–brain barrier penetration [26,27]. As could be seen in Table 4, all presented 2-amino-5-alkylidene-thiazol-4-ones are expected to have good intestinal absorption and most of them also good blood–brain barrier penetration. Hydrogen bonding capacity of the drug candidates is also described by the number of H-bond donors and acceptors. The compounds in the series show 3–6 H-bond acceptors. H-bond donors are present only in the 2-amino-5-alkylidene-thiazol-4-ones containing indolyl and benzylamine fragments. Molecular volumes of the compounds in the series are less than 300 Å^3 with a few exceptions due to the large benzyloxy group in **16**–**20**. Summarizing the physico-chemical properties of studied 2-amino-5-alkylidene-thiazol-4-ones, we could conclude that they obey the "Rule of five" and meet all criteria for good solubility and permeability.

Recent publication by Baell and Walters raised an interesting issue on pan-assay interference compounds, or PAINS, and we quote: "Repeated identification of the same types of molecule as promising hits against different proteins is polluting the chemical literature" [28]. We are aware that rhodanine derivatives with exocyclic double bond are frequent PAINS by 2 alternative mechanisms, i.e., they might act as covalent modifiers and metal complexers [28,29]. Our series of 2-amino-5-alkylidene-thiazol-4-ones indeed are rhodanine derivatives, and since we have already published their activity on other targets [7,8], we were particularly interested to determine whether our compounds might act as PAINS. Our data indicate that not all compounds act as XO inhibitors below 50 μg/mL threshold, and that the results for **11**–**15** correlate in two independent assays, namely in inhibition of commercial XO and in rat liver homogenate assays. The latter is performed in the tissue homogenate, where non-specific interactions (aggregation-based mechanism, covalent modifier mechanism, metal complexing mechanism) with numerous protein components and/or metal ions of the homogenate would undoubtedly reduce the potency/activity of our compounds. Since this was not the case, we believe that our study proves that the measured activity is the consequence of XO inhibition.

Carter et al. have reported similar compounds to covalently modify the TNFRc1 in a light-dependent fashion [30]. We envisaged 2 functional groups in these molecules that could lead to covalent modification, namely exocyclic double bond as a Michael-type acceptor, and a thiocarbonyl moiety. The mechanism of covalent binding via Michael addition for the present compounds would probably result in significant difference in reactivity depending on whether an electron donating and withdrawing group is attached to the benzylidene group adjacent to 2-amino-5-alkylidene-thiazol-4-one core. This was not found to be the case, as both 4-cyanobenzylidene (**11**–**15**, electron withdrawing) and indole-3-methylidene (**21**–**25**, electron donating) derivatives have displayed similar potency in inhibiting commercial XO. It is however true that compounds can have similar IC_{50} values and very different mechanisms/modes of action, so a definite conclusion cannot be drawn by comparing compounds electronic properties. Since all the compounds reported by Carter et al. possess thiocarbonyl moiety, we believe it is responsible for covalent bond formation as it is known to react with nucleophiles via addition–elimination mechanism [31]. Our compounds have rhodanine carbonyl sulfur substituted with an amine, as it was the synthetic route by which our compounds were synthesized [12]. Therefore, the absence of covalent modulatory activity of our compounds is probably due to the elimination of the reactive part of the rhodanine molecule, as depicted in the Fig. 4. Substitution of thiocarbonyl moiety with the primary or secondary amine could therefore be an attractive approach to circumvent the undesired or even toxic properties of the rhodanine-based compounds.

4. Conclusion

The present study identifies 2-amino-5-alkylidene-thiazol-4-ones as a new class of XO inhibitors. The binding mode of studied compounds with XO was studied by predicting binding pose with molecular docking. 4-((2-Benzylamino-4-oxothiazol-5(4*H*)-ylidene)-methyl)benzonitrile (**14**) showed the most potent inhibitory effect against commercial and rat liver XO, as well as a significant anti-inflammatory activity. It was interesting to note that the most potent compound has a different substitution pattern on thiazol-4-one core (secondary vs. tertiary amine, substituted with benzylamine moiety), which enables alternative tautomeric form of the compound **14**. Namely, all the compounds with benzylamine moiety (**4**, **9**, **14**, **24**, **29**) have shown quite potent inhibition of the isolated bovine XO. Furthermore, our data point out that 4-cyanobenzylidene moiety at position 5 is optimal for XO inhibition against both commercial and rat liver XO (**11**–**15**). We therefore conclude that these two moieties are optimal for XO inhibition, and that the compound **14**, as well as several other studied compounds, offer a good starting point toward drugs for the treatment of gout and other excessive uric acid production disorders.

2-Amino-5-alkylidene-thiazol-4-ones under study obey the "Rule of five" and meet all criteria for good solubility and permeability in such a way that they allow further structural modification for achieving desired pharmacological properties combined with appropriate pharmacokinetic behavior. Although we did not assay our compounds in vivo, the assay on PBMCs requires compounds to undergo passive diffusion prior action, so the observed compound activity is the direct proof that compounds do possess suitable physicochemical properties that enable passive diffusion. Furthermore, our study suggests that 2-amino-5-alkylidene-thiazol-4-ones do not undergo the same mechanism of target covalent modification as is the case for rhodanine-based compounds with thiocarbonyl moiety, and therefore do not fall in PAINS category. Additional research to obtain more potent XO inhibitors is in progress in our laboratories.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

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