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RESEARCH ARTICLE

5-Substituted *N*-(9*H*-purin-6-yl)-1,2-oxazole-3-carboxamides as xanthine oxidase inhibitors

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Abstract: Synthetic 6-substituted purine derivatives are known to exhibit diverse bioactivity. In this paper, a series of *N*-(9*H*-purin-6-yl)-1,2-oxazole-3-carboxamide derivatives were synthesized and evaluated *in vitro* against xanthine oxidase, an enzyme of purine catabolism. The introduction of aryl substituent at position 5 of the oxazole ring was found to increase the inhibition efficiency. Some of the inhibitors containing 5-substituted isoxazole and purine moieties were characterized by IC_{50} values in the nanomolar range. According to the kinetic data, the most active *N*-(9*H*-purin-6-yl)-5-(5,6,7,8-tetrahydronaphthalen-2-yl)-1,2-oxazole-3-carboxamide demonstrated a competitive type of inhibition with respect to the enzyme-substrate. Molecular docking was carried out to elucidate the mechanism of enzyme-inhibitor complex formation. The data obtained indicate that xanthine oxidase may be one of the possible targets for the bioactive purine carboxamides.

Keywords: *N*-(9*H*-purin-6-yl)-1,2-oxazole-3-carboxamides, synthesis, bioactivity, xanthine oxidase.

Introduction

Purine derivatives are known to possess a range of biological properties as inhibitors of kinases, sulfotransferases, phosphodiesterases, and other enzymes as well as ligands of some proteins [1]. It was reported that derivatives of 6-(*N*-benzoylamino)purine can be potent inhibitors of bromodomain-containing protein 4 (BRD4), which control the expression of genes related to inflammation, apoptosis, and cell proliferation [2-3]. Structural analogs of this compound with bulky biaryl substituent were found to be potential inhibitors of the cytosolic 5'-nucleotidase II, which regulates intracellular nucleotide pools and has been recognized as a therapeutic target for hematological cancers [4]. At the same time, 6-(*N*-benzoylamino)purine was described as an inhibitor of the purine catabolizing enzyme, xanthine oxidase [5]. This enzyme catalyzes the oxidation of hypoxanthine and xanthine to uric acid with the generation of superoxide radicals. The increased uric acid levels lead to hyperuricemia and gout, and overproduction of superoxide radicals and other reactive oxygen species can promote chronic inflammatory and cardiovascular diseases, cancer, and diabetes [6].

The inhibitors of xanthine oxidase can be represented by two groups, which include purine derivatives [7-8] and non-purine compounds. The purine analog allopurinol is widely used in clinical practice [9]. More effective non-purine inhibitors of xanthine oxidase have also been developed, such as derivatives of imidazole [10], pyrazole [11], isoxazole [12], selenazole [13], and thiazole [14]. Among them, febuxostat, with inhibition constants in the nanomolar range, was approved for the treatment of hyperuricemia and gout [15]. However, allopurinol and febuxostat are known to induce side effects [9, 16], and there is thus interest in new bioactive compounds targeting xanthine oxidase.

Introducing isoxazole fragment into organic molecules is considered as a strategy for designing bioactive compounds with anticancer, antimicrobial, anti-inflammatory, and other

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activities [17, 18]. Many of such compounds are represented by 3,5-substituted isoxazoles bearing other heterocyclic rings [19]. As an example, the hybrid molecules containing isoxazole, purine, and coumarin moieties were synthesized and tested *in vitro* as antioxidants and enzyme inhibitors [20]. In this paper, we synthesized substituted isoxazole-purine conjugates structurally similar to bioactive 6-(*N*-benzoylamino)purine. The 5-substituted derivatives of *N*-(9*H*-purin-6-yl)-1,2-oxazole-3-carboxamides were evaluated *in vitro* as inhibitors of xanthine oxidase.

Results and discussion

The synthesis of 5-substituted isoxazole acids was carried out using known synthetic methods [21] by the reaction of commercially available ketones with diethyl oxalate in the presence of sodium ethoxide [22]. The synthetic route included cyclization of ethyl 2,4-dioxobutanoates into ethyl isoxazole-3-carboxylates by the addition of hydroxylamine hydrochloride in ethanol at reflux [23] followed by saponification of the ester function with sodium hydroxide in ethanol. Corresponding acyl chlorides **3a-g** were synthesized in the reaction of the isoxazole acids and thionyl chloride in benzene and used without purification in acylation of adenine (**1**) or 8-aminoquinoline (**2**) (Scheme 1). All compounds were obtained in moderate to good yield. After crystallization of the crude products, the compounds **4a-g** and **5f** were characterized by ^{1}H NMR, IR spectra, and MS.

The inhibition activities of compounds **4a-g** against xanthine oxidase were assayed by monitoring the rate of enzymatic conversion of xanthine to uric acid. The IC_{50} values were defined as the concentration of the tested compound causing 50% inhibition of the enzyme with 50 μM xanthine as a substrate [24]. Allopurinol and 6-(*N*-benzoylamino)purine were used as reference inhibitors. Given the potential interest of the structures containing isoxazole and purine moieties, compound **4f** was also evaluated *in vitro* against purine nucleoside phosphorylase, however, no effect was observed on this enzyme.

Experimental data (Table 1) showed that compounds **4a** with 5-methyl-1,2-oxazole fragment displayed slightly decreased inhibitory activity as compared with 6-(*N*-benzoylamino)purine. The introduction of the aromatic group at 5-position of the isoxazole ring substantially increased the inhibitory potency of compounds **4b** and **4e**. Further increasing of xanthine oxidase inhibition was observed in the case of methyl or methoxy substituent at *para*-position of the phenyl ring of inhibitors **4c** and **4d**, respectively. Modification of the isoxazole ring by tetrahydronaphthalene fragment led to significant enzyme inhibition by compound **4f** with IC_{50} value of 14 nM which is approximately 280-fold more effective than that of allopurinol. Compound **4g** with more hydrophilic benzodioxinyl substituent demonstrated lower inhibitory potency. The importance of the purine part of the hybrid molecules in the inhibition mechanism was supported by compound **5f** which showed no activity.

Table 1. Xanthine oxidase inhibitory activity of *N*-(9*H*-purin-6-yl)-1,2-oxazole-3-carboxamides **4a-g**.

Compound	IC_{50}, μM[a]
6-(*N*-benzoylamino)purine	0.55±0.04
4a	0.75±0.18
4b	0.074±0.011
4c	0.048±0.013
4d	0.037±0.002
4e	0.078±0.011
4f	0.014±0.004
4g	0.044±0.003
Allopurinol	4.03±0.27

[a] IC_{50} values were calculated as the mean of 2-3 assays ± standard deviation.

Kinetic studies were performed for the most active compounds **4f** at different concentrations of substrate and the inhibitor to characterize the mechanism of inhibition. The double reciprocal Lineweaver-Burk plots indicated a competitive type of inhibition (Figure 1). This reveals that the inhibitor interacts with free enzyme competing with the substrate for the binding site. The calculated K_i value for compound **4f** was 7.46 ± 0.36 nM. It is known that allopurinol in complex with xanthine oxidase provides electron transfer to molecular oxygen with a generation of superoxide, but 6-(*N*-benzoylamino)purine does not exhibit such effect [5]. Compound **4f** was also not able to generate superoxide radical that was confirmed by a test with xanthine oxidase and reduced 2,6-dichlorophenolindophenol, controlled by absorbance at 605 nm. This result suggests that the purine part of the inhibitor with bulky 5-substituted isoxazole fragment is located near the molybdopterin center without electron transfer.

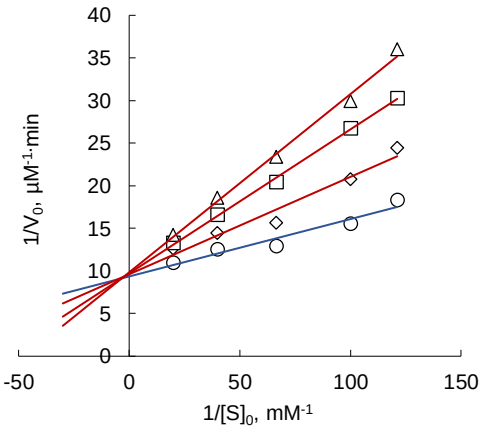
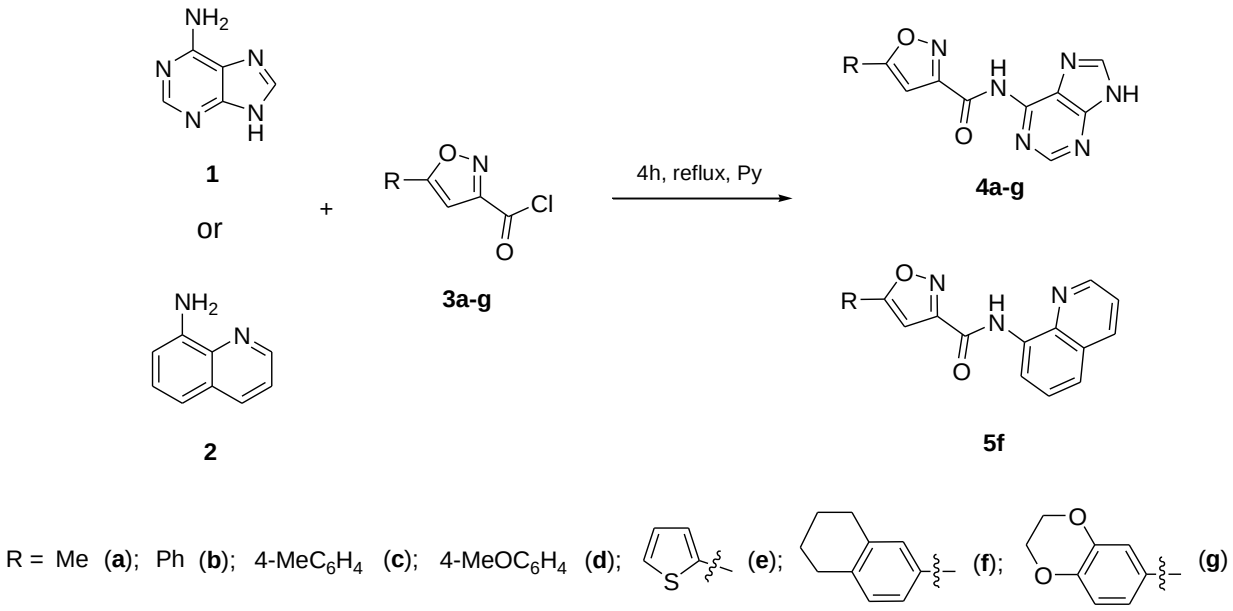


Figure 1. Lineweaver-Burk plots for inhibition of xanthine oxidase by compound **4f**. The inhibitor concentrations were 0 (○), 5 nM (●), 10 nM (□), and 15 nM (Δ).



Scheme 1. Synthesis of *N*-(9*H*-purin-6-yl)-1,2-oxazole-3-carboxamides **4a-g** and compound **5f**.

To elucidate the possible mechanism of the enzyme-inhibitor complex formation, computer modeling was performed. Molecular docking calculations using a modified version of Autodock 4.2 [25] showed that the *N*-7-protonated tautomer of purine inhibitor can be preferred for the formation of the enzyme-inhibitor complex. The calculated docking energy for *N*-7- and *N*-9-protonated tautomeric forms of compound **4f** were -9.09 kcal/mol and -8.02 kcal/mol, respectively. The docking results (Figure 2) showed that two NH groups of *N*-7-protonated tautomeric form participate in the formation of hydrogen bonds with carboxylate group of Glu802 which can be involved in protonation of the enzyme-substrate [26, 27]. At the same time, HOH1365 provides the interaction of *N*-9 atom of the purine ring of the inhibitor with Glu1261 which can act as a general base in the enzymatic reaction [28]. Purine fragment of the inhibitor is also stabilized by aromatic-aromatic interaction with Phe914 and Phe1009. The isoxazole ring can form hydrophobic and van der Waals contacts with Leu873 and Val1011. Tetrahydro-naphthalene substituent shows aromatic-sigma interaction with Val1011 as well as hydrophobic and van der Waals contacts with His875 and Phe649 at the site exit.

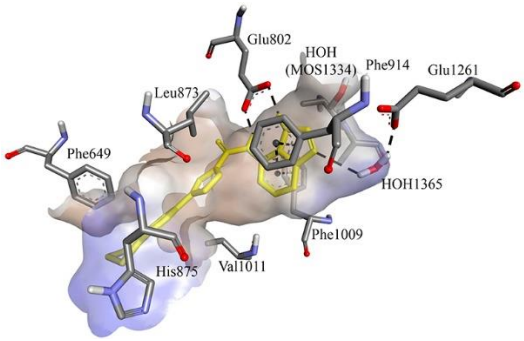


Figure 2. Possible binding mode of inhibitor **4f** in the active site of bovine milk xanthine oxidase.

Conclusions

In the present paper, 5-substituted *N*-(9*H*-purin-6-yl)-1,2-oxazoles were synthesized, and their inhibitory properties were evaluated *in vitro* against xanthine oxidase. The incorporation of a nonpolar bulky substituent at the isoxazole ring provided a better binding affinity to the enzyme active site. The most active compound, *N*-(9*H*-purin-6-yl)-5-(5,6,7,8-tetrahydronaphthalen-2-yl)-1,2-oxazole-3-carboxamide, was a competitive inhibitor of the enzyme with an inhibition constant in the nanomolar range. These data are helpful to consider xanthine oxidase as one of the possible targets for isoxazole-containing purine derivatives with diverse bioactivity.

Experimental section

The xanthine oxidase from bovine milk, bacterial purine nucleoside phosphorylase and xanthine were purchased from Sigma-Aldrich. Spectrophotometric measurements were performed on a Specord M-40. NMR spectra were obtained on a Bruker Avance DRX-500 instrument (^{1}H, 500 MHz) in a solution of DMSO-d_6 relative to internal TMS. The IR spectra were recorded on a Vertex 70 spectrometer from KBr pellets. The melting points were determined on a Fisher-Johns instrument. The LC/MS spectra were recorded on an Agilent 1100 series high-performance liquid chromatograph equipped by a diode matrix with an Agilent LC/MSD SL mass selective detector. The LC/MS parameters were set as follows: column, Zorbax SBC18 1.8 μm, 4.6x15 mm (PN 821975-932); solvents A, acetonitrile-water mixture (95:5), 0.1% trifluoroacetic acid and B, 0.1% aqueous trifluoroacetic acid; eluent flow rate, 3 ml/min; injection volume, 1 μl; UV detection, 215, 254, 265 nm; atmospheric-pressure chemical ionization (APCI) was used; scanning range, m/z 80-1000.

Synthesis

General procedure for the acylation of adenine

A suspension of adenine (**1**) or 8-aminoquinoline (**2**) (7.4 mmol) and corresponding acyl chloride (8 mmol) in pyridine (10 ml) was mixed at room temperature and then heated under reflux for 4 hours. The reaction mixture was cooled and water (20 ml) was added, the precipitate was filtered, washed with ethanol (5 ml). All the products **4a-g** and **5f** were purified by recrystallization from a mixture of DMF and water (1:1).

6-(N-Benzoylamino)purine was synthesized as described previously [29].

Yield 86%, mp 242-242.5 °C [29]. IR (KBr) *ν* 1521, 1552, 1581, 1599, 1621, 1686, 3256, 3369. ^{1}H NMR (500 MHz, DMSO-d$_6$) *δ* 7.56-7.65 (m, 3H), 8.03-8.15 (m, 2H), 8.51 (s, 1H), 8.75 (s, 1H), 11.09 (br s, 0.1H, NH), 11.52 (br s, 0.9H, NH), 12.37 (br s, 0.9H, NH), 13.47 (br s, 0.1H, NH). LC/MS (CI) *m/z* 240,2 (M+H)$^+$. Anal. Calcd. for $C_{12}H_9N_5O$: C, 60.25; H, 3.79; N, 29.27. Found: C, 60.13; H, 3.81; N, 29.19.

*5-Methyl-N-(9H-purin-6-yl)-1,2-oxazole-3-carboxamide (**4a**).*

Yield 60%, mp 270-271 °C. IR (KBr) *ν* 1556, 1597, 1632, 1704, 2900, 3111, 3215. ^{1}H NMR (500 MHz, DMSO-d$_6$) *δ* 2.35 (s, 3H), 7.40 (s, 1H), 8.52 (s, 1H), 8.74 (s, 1H), 11.00-12.25 (br s, 1H, NH), 12.25-14.00 (br s, 1H, NH). LC/MS (CI) *m/z* 245.2 (M+H)$^+$. Anal. Calcd. for $C_{10}H_8N_6O_2$: C, 49.18; H, 3.30; N, 34.41. Found: C, 49.02; H, 3.05; N, 34.23.

*3-Phenyl-N-(9H-purin-6-yl)-1,2-oxazole-5-carboxamide (**4b**).*

Yield 91%, mp 256-257 °C. IR (KBr) *ν* 1526, 1627, 1713, 3341. ^{1}H NMR (500 MHz, DMSO-d$_6$) *δ* 7.50-7.65 (s, 4H), 7.90-8.00 (m, 2H), 8.55 (s, 1H), 8.75 (s, 1H), 10.95-11.40 (br s, 0.2H, NH), 11.40-12.00 (br s, 0.8H, NH), 12.30-12.80 (br s, 0.8H, NH), 13.30-13.90 (br s, 0.2H, NH). LC/MS (CI) *m/z* 307.3 (M+H)$^+$. Anal. Calcd. for $C_{15}H_{10}N_6O_2$: C, 58.82; H, 3.29; N, 27.44. Found: C, 58.64; H, 3.18; N, 27.23.

*5-(4-Methylphenyl)-N-(9H-purin-6-yl)-1,2-oxazole-3-carboxamide (**4c**).*

Yield 86%, mp 255-256 °C. IR (KBr) *ν* 1523, 1559, 1610, 1718, 3354. ^{1}H NMR (500 MHz, DMSO-d$_6$) *δ* 2.37 (s, 3H), 7.37 (d, *J* 7.3, 2H), 7.51 (s, 1H), 7.84 (d, *J* 7.3, 2H), 8.54 (s, 1H), 8.75 (s, 1H), 10.80-12.10 (br s, 1H, NH), 12.10-13.00 (br s, 1H, NH). LC/MS (CI) *m/z* 321.3 (M+H)$^+$. Anal. Calcd. for $C_{16}H_{12}N_6O_2$: C, 60.00; H, 3.78; N, 26.24. Found: C, 60.01; H, 3.54; N, 26.21.

*5-(4-Methoxyphenyl)-N-(9H-purin-6-yl)-1,2-oxazole-3-carboxamide (**4d**).*

Yield 86%, mp 265-266 °C. IR (KBr) *ν* 1468, 1508, 1552, 1610, 1704, 3128, 3366. ^{1}H NMR (500 MHz, DMSO-d$_6$) *δ* 3.83 (s, 3H), 7.11 (d, *J* 7.3, 2H), 7.46 (s, 1H), 7.89 (d, *J* 7.3, 2H), 8.56 (s, 1H), 8.76 (s, 1H), 10.90-11.10 (br s, 0.2H, NH), 11.50-11.80 (br s, 0.8H, NH), 12.40-12.70 (br s, 0.8H, NH), 13.40-13.70 (br s, 0.2H, NH). LC/MS (CI) *m/z* 337.4 (M+H)$^+$. Anal. Calcd. for $C_{16}H_{12}N_6O_3$: C, 57.14; H, 3.60; N, 24.99. Found: C, 57.01; H, 3.34; N, 25.68.

*N-(9H-Purin-6-yl)-5-(thiophen-2-yl)-1,2-oxazole-3-carboxamide (**4e**).*

Yield 86%, mp 252-253 °C. IR (KBr) *ν* 1505, 1524, 1557, 1608, 1628, 1666, 1718, 3107, 3367. ^{1}H NMR (500 MHz, DMSO-d$_6$) *δ* 7.24-7.32 (m, 1H), 7.40 (s, 1H), 7.80-7.86 (m, 1H), 7.86-7.92 (m, 1H), 8.55 (s, 1H), 8.75 (s, 1H), 11.00-11.30 (br s, 0.2H, NH), 11.50-11.90 (br s, 0.8H, NH), 12.35-12.75 (br s, 0.8H, NH), 13.40-13.70 (br s, 1H, NH). LC/MS (CI) *m/z* 312.4 (M+H)$^+$. Anal. Calcd. for $C_{13}H_8N_6O_2S$: C, 50.00; H, 2.58; N, 26.91. Found: C, 49.88; H, 2.13; N, 26.67.

*N-(9H-Purin-6-yl)-5-(5,6,7,8-tetrahydronaphthalen-2-yl)-1,2-oxazole-3-carboxamide (**4f**).*

Yield 86%, mp 229-230 °C. IR (KBr) *ν* 1523, 1621, 1714, 2932, 3359. ^{1}H NMR (500 MHz, DMSO-d$_6$) *δ* 1.65-1.80 (m, 4H), 2.70-2.85 (m, 4H), 7.21 (d, *J* 8.4, 1H), 7.42 (s, 1H), 7.62 (br s, 2H), 8.47 (s, 1H), 8.69 (s, 1H), 10.50-13.50 (br s, 0.5H partially in exchange). LC/MS (CI) *m/z* 361.4 (M+H)$^+$. Anal. Calcd. for $C_{19}H_{16}N_6O_2$: C, 63.33; H, 4.48; N, 23.32. Found: C, 63.17; H, 4.21; N, 23.27.

*5-(2,3-Dihydro-1,4-benzodioxin-6-yl)-N-(9H-purin-6-yl)-1,2-oxazole-3-carboxamide (**4g**).*

Yield 86%, mp 269-270 °C. IR (KBr) *ν* 1506, 1556, 1576, 1624, 1716, 3127, 3356. ^{1}H NMR (500 MHz, DMSO-d$_6$) *δ* 4.27-4.35 (m, 4H), 7.02 (d, *J* 8.1, 1H), 7.40-7.50 (m, 3H), 8.55 (s, 1H), 8.75 (s, 1H), 10.95-11.15 (br s, 0.2H, NH), 11.50-11.75 (br s, 0.8H, NH), 12.40-12.65 (br s, 0.8H, NH), 13.45-13.65 (br s, 0.2H, NH). LC/MS (CI) *m/z* 365.4 (M+H)$^+$. Anal. Calcd. for $C_{17}H_{12}N_6O_4$: C, 56.05; H, 3.32; N, 23.07. Found: C, 56.10; H, 3.11; N, 23.14.

*N-(Quinolin-8-yl)-5-(5,6,7,8-tetrahydronaphthalen-2-yl)-1,2-oxazole-3-carboxamide (**5f**).*

Yield 78%, mp 166-167 °C. IR (KBr) *ν* 1498, 1577, 1615, 1729, 2937, 3129, 3411. ^{1}H NMR (500 MHz, DMSO-d$_6$) *δ* 1.65-1.75 (m, 4H), 2.7-2.85 (m, 4H), 7.18-7.77 (m, 7H), 8.44 (d, *J* 8.4, 1H), 8.71 (d, *J* 7.5, 1H), 8.98 (d, *J* 3.9, 1H), 10.95 (s, 1H, NH). LC/MS (CI) *m/z* 370.3 (M+H)$^+$. Anal. Calcd. for $C_{23}H_{19}N_3O_2$: C, 74.78; H, 5.18; N, 11.37. Found: C, 74.55; H, 5.05; N, 11.13.

Inhibition of xanthine oxidase

The enzymatic reaction was studied in sodium phosphate buffer (50 mM, pH 7.4) at 25 °C. The mixture contained xanthine (50 μM), inhibitor (from 2.5 nM to 50 μM), EDTA (0.1 mM), and 1% DMSO was incubated for 5 min and the reaction was initiated by addition of xanthine oxidase. The enzyme concentration was 0.008 units/mL. The reaction rate was monitored by the change in optical density at 293 nm. The IC$_{50}$ values were calculated from the plot of the inhibition percentage against inhibitor concentrations.

Purine nucleoside phosphorylase test

The reaction mixture contained sodium phosphate buffer (0.1 M, pH 7.4), guanosine (0.1 mM), bacterial purine nucleoside phosphorylase (0.071 units/mL), inhibitor (50 μM), EDTA (0.1 mM) and 0.5% DMSO. The enzymatic reaction was investigated at 25 °C. The reaction rate was monitored by the change in optical density at 258 nm.

Reduction of 2,6-dichlorophenolindophenol

The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.4), 15 μM 2,6-dichlorophenol-indophenol, and 10 μM xanthine or inhibitor. The reaction was initiated by the addition of xanthine oxidase (0.008 units/mL) to the reaction mixture and the absorbance change was monitored at 605 nm.

Molecular docking

The docking simulation was performed to analyze the probable binding mode of inhibitors at the active site of xanthine oxidase by using a modified version of AutoDock 4.2 [25]. The inhibitors were docked into the active site of xanthine oxidase using chain C (PDB code 3B9J) [30]. Before the docking calculations, other chains, ligand (2-hydroxy-6-methylpurine), and water molecules, with the exception of important for catalytic mechanism HOH1365 [31], were removed from the initial structure of the enzyme. The oxygen atom of the molybdopterin cofactor was replaced on a water molecule HOH1334. The structures of inhibitors were converted into three-dimensional ones and optimized in the MMFF94s force field by using program Avogadro [32]. AutoDock Tool (MGLTools 1.5.6) was used to prepare the docking files. The constraint position for the C_8 atom of the purine fragment was added to docking parameter files using the ATPOSCONSTR keyword [25]. A ligand's atom number from PDBQT file and its constrained coordinates (-57.055, -18.200 and 19.928 for x, y, and z, respectively) were included in this parameter with a maximal allowed distance of 3 Å. The Lamarckian genetic algorithm was applied to search for the optimum binding pose of the ligands [33]. The analysis of the binding mode of the inhibitors was performed using Discovery Studio 3.5 visualizer.

Notes

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The authors declare no conflict of interest.

Author contributions. **O. V. M**: the investigation of bioactivity, writing. **O. L. K**: molecular docking simulations, analysis of the experimental results, writing. **O. V. S**: synthesis of compounds, writing experimental section. **V. S. B**: synthesis of compounds, conceptualization. **A. I. V**: conceptualization, writing, and editing.

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5-Заміщені *N*-(9*H*-пурин-6-іл)-1,2-оксазол-3-карбоксаміди як інгібітори ксантиноксидази

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Резюме: У цій роботі нами синтезовано серію похідних *N*-(9*H*-пурин-6-іл)-1,2-оксазол-3-карбоксаміду та оцінено їх інгібувальну здатність щодо ксантиноксидази, ферменту пуринового катаболізму. Синтез 5-заміщених ізоксазолкарбонових кислот здійснено за допомогою відомих синтетичних методів. Для ацилювання аденіну використовували відповідні ацилхлориди, отримані реакцією ізоксазолкарбонових кислот з тіонілхлоридом. За результатами досліджень *in vitro* наявність фенільного замісника в положенні 5 оксазольного кільця підвищує ефективність інгібування ксантиноксидази. Подальше зростання інгібувального впливу спостерігалося при введенні метильної або метокси-групи в *пара*-положення фенільного кільця. Деякі з інгібіторів, що містять 5-заміщені ізоксазолові та пуринові фрагменти, характеризувались наномолярними значеннями IC_{50}. Згідно кінетичних даних, найбільш активний *N*-(9*H*-пурин-6-іл)-5-(5,6,7,8-тетрагідронафтален-2-іл)-1,2-оксазол-3-карбоксамід демонстрував конкурентний тип інгібування щодо субстрату з константою інгібування 7,46 ± 0,36 нМ. Для з'ясування механізму формування комплексу фермент-інгібітор було проведено молекулярний докінг. Результати моделювання показали, що *N*-7-таутомерна форма інгібітора може забезпечувати формування водневих зв'язків, гідрофобних і Ван-дер-Ваальсових контактів та донорно-акцепторних взаємодій. Отримані результати вказують на те, що ксантиноксидаза може бути однією з можливих мішеней для біоактивних карбоксамідних похідних пурину.

Ключові слова: *N*-(9H-пурин-6-іл)-1,2-оксазол-3-карбоксаміди, синтез, біоактивність, ксантиноксидаза.