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Original Article

Synthesis, Cytotoxicity, Biological Assessment and Molecular Docking of a few Dihydropyridines as Xanthine Oxidase Inhibitors

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Abstract

Xanthine Oxidase (XO) inhibitors may contribute to the increased incidence of several inflammatory diseases such as gout. Inhibition of XO enzyme activity plays a significant role in the prevention and treatment of these types of diseases. The aim of present study were to synthesize, determine the cytotoxicity and biological activity, and molecular docking of a few Dihydropyridines as XO Inhibitors. The synthesis of a few 1, 4-dihydropyridine (DHP) derivatives (A-C) were evaluated for their cytotoxicity effects against MCF-7 and L929 cells. All the selected compounds / All the potential candidate compounds were also evaluated for their XO inhibitory activity. Molecular docking simulation was used to investigate the potential binding modes of DHP derivatives within XO binding site. The results of this study showed the superior cytotoxic activities of compound B against both tested cell lines, while no significant XO inhibitory activity was recorded for it. On the basis of molecular docking studies, different enzyme blocking activities might be attributed to the various binding sites with the enzyme active sites.

Keywords: Dihydropyridines; Cytotoxic; Xanthine Oxidase; Molecular Docking

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1. Introduction

Gout and other inflammatory diseases such as rheumatoid arthritis (RA), hypertension and diabetes mellitus are characterized by high uric acid level in blood (1, 2). These diseases are described by frequent occurrences of acute inflammatory arthritis caused by the precipitation of uric acid in the joints and neighboring soft tissues. The blood uric acid levels (as a final metabolite of purines) depend on the function of the kidneys especially in a purine-rich diet (2).

Xanthine oxidase (XO, EC 1.17.3.2) is the enzyme (MW 285 KD) which belongs to the family of xanthine oxidoreductase. This enzyme can catalyze hypoxanthine oxidation to xanthine, and further to uric acid and generates reactive oxygen species (ROS) such as free superoxide radicals and hydrogen peroxide (3, 4). Frequent and high intake of pruine-rich foods seem to be associated with activation and liberation of XO. Therefore, the treatment of gout and other related diseases can be achieved by inhibiting high activity of this enzyme (5). Allopurinol is the only available drug as a xanthine oxidase inhibitor that decreases the production of uric acid and has side effects such as Steven-Johnson syndrome (SJS), allopurinol hypersensitivity syndrome (AHS), and nephrotoxicity (3, 6). Therefore, the discovery and development of new XO inhibitors with fewer adverse and side effects is considered an important area of research within the contemporary drug discovery scenario.

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Some 1,4-dihydropyridine (DHP) derivatives have been shown to reduce the intracellular production of reactive oxygen species (ROS) (7). The aim of this study was to evaluate a few derivatives of DHP in terms of cytotoxicity and XO inhibitory potential (8-11). Previous investigations demonstrated several biological activities of DHP derivatives such as calcium channel blocker (CCBs) (12), antioxidant (13), anticonvulsant (14), antitumor (15) and anti-inflammatory (16) whereas some of them including amlodipine, nifedipine, lecarnidipine and cilinidipine have been widely applied as therapeutic agents (17). Despite the beneficial biological activities of DHPs, some reports indicated that these compounds possessed relatively intrinsic cytotoxicity. The results of some studies showed that the intrinsic cytotoxicity of DHPs could favorably be used against cancer cells (17, 18). By modifying the structure of some relevant derivatives, it might be possible to reduce the property of blocking of calcium channels effect and hence increase their level of selective toxicity against cancer cells (8, 17, 19, 20). Another application of DHPs and their derivatives is their usage as anti-multidrug resistance (MDR) agents. MDR is a serious health problem in simultaneous administration of multiple drugs for cancer treatment. The reason of MDR is the over-expression of P-glycoprotein in cancer cells, which leads to its more activity and then reduces the accumulation of drugs in cancer cells and finally increases the drug resistance cancer cells. Recently, it has been discovered that the new derivatives of DHPs have a significant role in inhibition of p-glycoproteins and hence can act as potential tumor chemo-sensitizers (20, 21). The use of computational methods to estimate and model the activity of different molecules increases the pace of drug discovery and development.

Today, molecular docking tools are considered essential and has been used widely for the rational design of drugs and this technique is based on molecular structure and interactions between two molecules (protein-protein or protein-ligand). In this technique, structure of a small molecule or drug is randomly changed in terms of translation, torsion and conformation search algorithms at the macromolecule level with the aim of finalizing the ideal location for connecting to macromolecule (22).

The interactions of the new derivatives of DHP as small molecule with XO as a macromolecule target have been explored through molecular docking (5, 6, 23, 24). In this study, the inhibitory activity and cytotoxicity of three 1,4-DHP derivatives (A-C) were assessed by spectrophotometric analysis and MTT assay on MCF-7 and L929 cells, respectively. Moreover, molecular docking studies were conducted on candidate structures to explore the binding features within XO binding sites.

2. Material and Methods

Two previously identified compounds (A and B) consist of 2,6-dimethyl-3,5-bis-N-(2-benzothiazolyl) carbamoyl-4-(4-methoxyphenyl)-1,4-dihydropyridine and 2,6-dimethyl-3,5-bis-N-(2-benzothiazolyl) carbamoyl-4-(3-nitrophenyl)-1,4-dihydropyridine were synthesized according to the Hantzsch reaction (11, 25), while 2,6-dimethyl-3,5-bis-N-(2-benzothiazolyl) carbamoyl-4-(4-chloro-3-nitrophenyl)-1,4-dihydropyridine (C; Fig 1) was synthesized at this study. For this purpose, in a 100 ml round-bottom flask 1 mmol 4-chloro-3-nitrophenyl was added to 2 mmol of N-aryl acetamide and 2 mmol AcONH4. Absolute ethanol (20 ml) was used as a solvent and the mixture was refluxed with stirring for 48 h at 80 °C. The progress of the reaction and purity of the products were monitored by thin-layer chromatography (TLC) which supported by Merck silica gel 60 F254 aluminum plates. After the reaction is completed, the mixture was condensed under reduced pressure and washed tree times with cooled ethanol. The final product was separated and re-crystallized three times from ethanol. The structure of the synthesized compound was confirmed by 1HNMR (A-380, Bruker, USA) and Fourier-transform infrared spectroscopy (FTIR) (8400S, Shimadzu, Japan) while for two other derivatives, spectroscopic properties were compared and confirmed with previous reports.

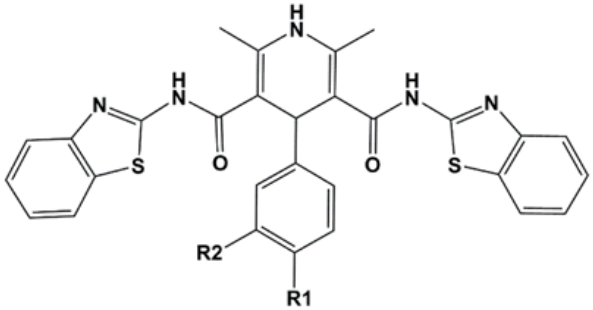

Figure 1. Chemical structure of assessed dihydropyridine derivatives (A: R_1=OCH_3, R_2=H, B: R_1=H, R_2=NO_2, C: R_1=Cl, R_2=NO_2)

2.1. Synthesis of 2,6-dimethyl-3,5-bis-N-(2-benzothiazolyl) carbamoyl-4-(4-chloro-3-nitrophenyl)-1,4 dihydropyridine (C)

Pale yellow powder, Yield 63%, FTIR (KBr, cm^{-1}) 3412 (N–H, DHP), 3066 (C–H, aliphatic), 1708 (C=O, amide), 1599 (C=C, alkene), ^{1}HNMR (DMSO-d6) δ (ppm) 12.10 (2H, brs, NH-amide), 9.19 (1H, brs, NH-DHP), 8.03 (1H, d, *J* = 7.6 Hz, CH-phenyl), 7.89 (IH, s, CH-phenyl), 7.82 (1H, d, *J* = 8 Hz, CH-phenyl), 7.76 (2H, d, *J* = 8.4 Hz, C4′H-benzothiazole), 7.56 (2H, d, *J* = 8.4 Hz, C7′H-benzothiazole), 7.52 (2H, t, *J* = 8.4 Hz, C6′H-benzothiazole), 7.38 (2H, t, *J* = 8 Hz, C5′H-benzothiazole), 5.53 (1H, s, C4H-DHP), 2.36 (6H, s, CH_3-DHP).

2.2. Biological Assessment

2.2.1. Methyl thiazolyl tetrazolium (MTT) Assay

Cell toxic activity of the synthesized compounds were assessed by their cytotoxic effects using MTT assay

method against two cell lines L929 (the parent L strain was derived from normal subcutaneous areolar and adipose tissue of a 100-day-old male C3H/An mouse, this cell line can be widely used for toxicity testing) and MCF-7 (as human breast cancer cell line) (8, 19, 26) which were purchased from Pasteur institute, Iran. MCF-7 and L929 cells were cultured in RPMI-1640 medium containing 10% FBS in a wet atmosphere of CO_2 (5%) at 37 °C, 100 U/ml penicillin, and 100 µg/mL streptomycin, then cells were trypsinized in and then seeded into plastic 96-well plates at a density of 5000-10000 cells per well and incubated for 24 and 48 hours. Serial concentrations of compounds (5, 10, 20, 40, 60, 80, and 100 µg/ml) were prepared in RPMI media using dimethyl sulfoxide (DMSO) as co-solvent. Samples were further incubated for 48 hours and the assay was started with 20 µl of MTT reagent (Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml) and incubated for 4 hours at 37 °C. After removing the reagent and cell culture medium, 200 µl of DMSO was added to dissolve the forming formazan crystals. The spectrophotometric absorbance of the samples was recorded using an ELISA reader at 570 nm (Thermo, USA).

2.2.2. Preparation of Xanthine Oxidase Enzyme

Enzyme preparation was conducted in accordance to the method of Johnson et al. (27). Briefly, the rat was killed by cervical dislocation, and then its liver was immediately separated and located in a mixture of water and ice containing isotonic potassium chloride solution (1.15% KCl w/v) and 0.1 mM EDTA. Then, the sample was homogenized at 4000-5000 rpm for 4 min at 4 °C using Potters homogenizer. The homogenate was heated in a steam bath at 55-57 °C for 15 min, cooled down to 4 °C and centrifuged at 15000 g for 45 min at 4 °C, and then the saturated solution of ammonium sulphate (50%, 35.3 g/100 ml) was added to supernatant fluid at 4 °C. The resulting suspension was centrifuged at 6000 g for 20 min at 4 °C. The precipitate was then dissolved in 500 ml of 0.1 mM EDTA solution and then was frozen until use. The Ethics Committee of Tabriz University of Medical Sciences approved the study (Code of ethics committee: IR.TBZMED.REC.1395.146).

2.2.3. Enzyme Assays

The XO enzyme activity and the inhibition activity of it by DHP derivatives were carried out at 37 °C using a Shimadzu 2550 UV/VIS spectrophotometer which was connected to cell temperature control unit (Shimadzu UV Probe personal software). XO activity was determined by using xanthine (50 mM) as a specific substrate, allopurinol (50 mM) as a xanthine oxidase inhibitor (control test) by monitoring absorbance changes at 296 nm. The substrate was dissolved in Sorenson's phosphate buffer (pH 7.0) containing 0.1 mM EDTA and the initial oxidation was carried out up to 2 min. Then, the inhibition activity of DHP derivatives (A, B, and C at 500 µl & 50 µM) were measured. The inhibition activity results were also compared with the inhibitory effect of allopurinol (500 µl and 50 µM) as a control.

2.3. Molecular Docking

The development in any scientific field, particularly biology, cheminformatics, and computers as well as the availability of the crystallographic structures of targets such as proteins for three-dimensional structure of bioactive agents like drugs, facilitated the control of the diseases by scientists. Molecular docking approach is a new computer-aided drug design method for predicting the preferred interactions and binding orientations of proteins with drugs that is usually compared with the experimental biological tests. It were considered into account to study the interactions between the active site of XO, ligands AutoDock (version 4.2) (28) and Gold (version 5.0) (29). Also the X-ray crystallographic structure of bovine XO was downloaded from Protein Data Bank (PDB) with the code 3NVW. The B, C, J, K, L chains, and all small molecules were removed from the protein structure, and finally the C-chain was selected for molecular docking procedure. Mode of molecular docking during this process was rigid receptor. The proteins and the ligands are considered respectively as rigid and flexible receptors (30). To perform docking process, several steps were done such as preparation of target and ligand files prior the analysis, grid box definition, ligands docking, and analyzing the results of docking experiments. Auto Grid analysis was used as an efficient computational method for calculating protein-ligand for atomic affinity. Docking was carried out using Lamarckian genetic algorithm (LGA) which is an adaptive local search method (31). To perform docking simulations, a grid box was defined to enclose the binding site of XO with dimensions of 60Å×60Å×60Å and the grid space of 0.375 Å. The other parameters were set as default. According to docking results, a cluster analysis for the obtained 100 structures based on root mean square with tolerance of 0.2 nm was subsequently performed and the lowest energy conformation of the more populated cluster was selected and taken into account. To generate the schematic 2D representations of the ligand-receptor hydrogen and hydrophobic interactions LIGPLOT software program was used (32).

3. Results and Discussion

A few DHP compounds were obtained by condensation of various aromatic aldehydes, 2-aminobenzothiazole and ammonium acetate under reflux conditions in ethanolic solution. The structure of the synthesized compound was confirmed by 1HNMR in DMSO solution. In vitro cytotoxic activities of the synthesized compounds (A, B, and C) were measured by MTT assay method on the MCF-7 and L929 cell lines at concentrations 5, 10, 20, 40, 60, 80, and 100 µg/ml. The percentage of cell viability of MCF-7 and L929 after 24, 48 hours incubation against concentrations of A, B, and

C derivatives for concentrations > 20 µM is shown in Table 1. It should be notified that no significant cell cytotoxicity could be observed at concentrations of less than 20 µM for DHP derivatives.

Table 1. Percentage of cell viability for MCF-7 and L929 cells in the presence of A, B, and C. The cells were seeded in 96 well plate and incubated for 24 & 48 hours. Cell viability was assessed using MTT assay

Compound and Incubation time (h)	A (24)	A (48)	B (24)	B (48)	C (24)	C (48)
MCF-7, %	57	64	33	5	60	55
L929, %	82	64	47	5	80	73

Table 2. Inhibition of Liver xanthine oxidase by compounds A and C and Allopurinol as a positive control

Compound	Inhibitor conc. (µM)	Substrate	IC50 (µM)	Inhibition, %
A	20	Xanthine	11.07 ± 1.46	88
C	20	Xanthine	8.96 ± 1.40	85
Allopurinol	100	Xanthine	2.10 ± 0.80	97.1

The results are expressed as percentage inhibition (mean ± SD and n = 3).

Table 3. Free binding energy, Ki, and Gold fitness score of 1,4-DHP derivatives (A-C and allopurinol)

Compound	Free binding energy (kcal/mol)	Ki	Gold fitness score
A	-8.05	1.26 µM	60.3251
B	-9.61	90.85 nM	65.2671
C	-7.93	1.55 µM	57.8462
Allopurinol	-4.47	545.56 µM	-

The percentages of cell viability in the presence of compound A were estimated as 57% and 82% at concentrations above 40 µg/ml for MCF-7 and L929 cells, respectively, after 24 hours incubation, whereas these amounts were found to be 64% for both types of cell lines after 48 hours incubation. Reduced toxic effect of the compound A on both cell lines after 48 hours incubation compared to 24-hours incubation may be due to drugs' low half-life and instability after 24 hours. Also the MTT assay results of compound B showed the viability percentages for both types of cell lines in the range of 30-35 percent at concentration above 10 µg/ml after 24 hours incubation. These amounts were 5 percent for both types of cell lines after 48 hours incubation. The evaluation of MTT assay results of compound C showed that the percentages of cell viability for both types of cell lines were at the range of 30-35 percent at concentration above 10 µg/ml after 24 hours incubation, while these amounts were 5 percent for both types of cell lines after 48 hours incubation. The MTT assay results for compound B within both cell lines showed that the toxicity of compound B was more than A and C compounds. Therefore, it might be possible to extend the scope of compound B and its derivatives as potential pharmaceutical agents.

3.1. Xanthine Oxidase Inhibition Assay

XO inhibitory activity of compounds A and C in rat liver homogenate was assessed using spectroscopic method (compound B did not show any inhibition of XO). The results of inhibitory activity assay have been tabulated in Table 2.

3.2. Molecular Docking

The best docking poses were ranked according to the scoring function and clustering analysis with regard to their relevant binding energy. In Table 3, binding free energy, Ki and Gold fitness score of XO inhibitors (A-C) were summarized. Binding energy of the compounds A-C were calculated by consideration of intermolecular energy, van der Waals' energy, hydrogen bonds, desolvation free energy, electrostatic hydrogen energy (kcal/mol), torsional free energy (kcal/mol) and unbound systems energy (kcal/mol). The energetic results of docking were shown in Table 3 and the ligand binding site of the enzyme was shown by the LIGPLOT software as a 2D visualization program (Fig 3).

The results of the docking process and 2D images of LIGPLOT showed that the binding site and the number of interactions of compound B was different from other two derivatives (Figs 2, 3 and 4 and Table 4). The observational evidence might explain to some extent the different behaviors of compound B vs XO in in vitro assay.

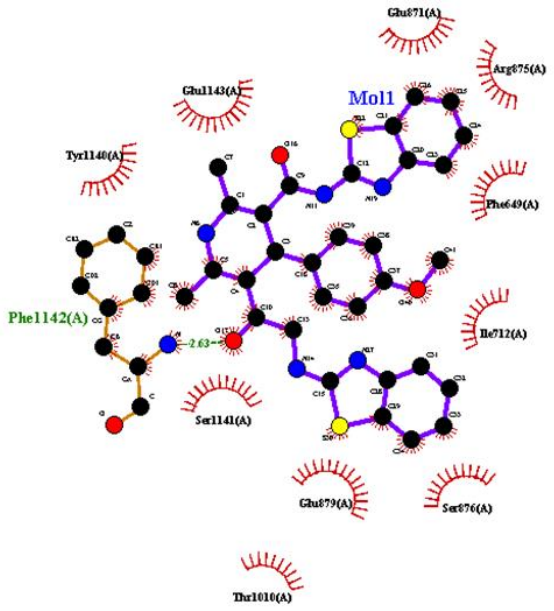

Figure 2. 2D scheme of binding interactions between A and XO generated by LIGPLOT, PDB deposition code: 3NVY

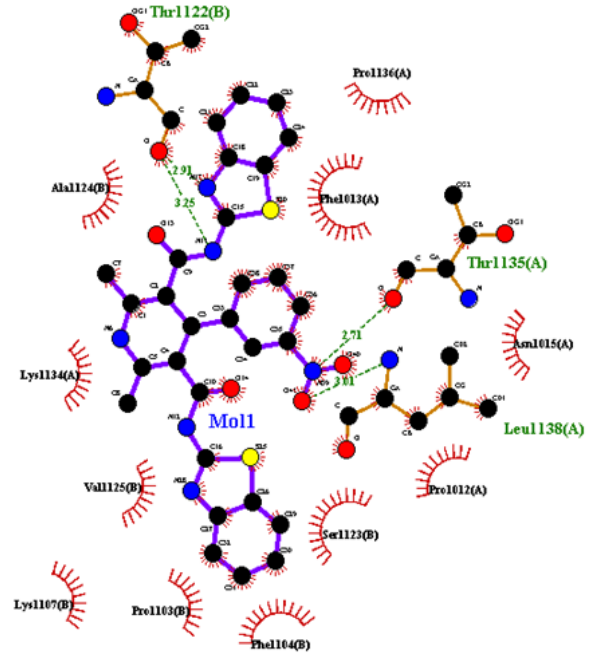

Figure 3. 2D scheme of binding interactions between B and XO generated by LIGPLOT, PDB deposition code: 3NVY

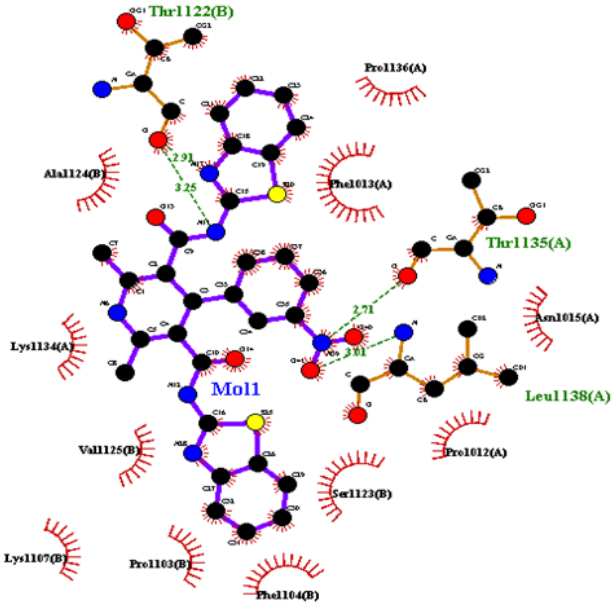

Figure 4. 2D scheme of binding interactions between C and XO generated by LIGPLOT, PDB deposition code: 3NVY

Table 4. Hydrogen bond distances (Å) and location for 1,4-DHP XO inhibitors

Involved amino acids	H bond distance (Å)	atom of the ligand
A		
Phe1142	2.63	(carbonyl) O
B		
Thr1122	3.25	(methylene) C
Thr1135	2.71	(NO_2) N
C		
Leu1138	3.01	(NO_2) O
Lys771	2.64	(NO_2) O
Leu648	3.27	(NO_2) N

4. Conclusions

In the present study, three derivatives of 1,4-DHPs were assessed for their cytotoxicity on MCF-7 and L929 cell lines from which compound B was found to be toxic on both cell lines within 24 and 48 incubation periods. Thus, compound B was identified as a candidate for further cancer care delivery studies. Moreover, the results of XO inhibition assay revealed that unlike the cytotoxicity assessment, compound B did not exhibit any inhibitory effect on XO enzyme which might be attributed to the different binding sites or/and number of interactions resulted from molecular docking simulation. Among the three derivatives, compound C was the superior XO-binding molecule which was also confirmed by the fitness score of GOLD software. Comparison of the binding free energies and XO inhibition constant values with that of allopurinol indicated that ΔGb of the three compounds were higher than allopurinol whereas the inhibition constant value of allopurinol was superior.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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