Synthesis and Biological Evaluation of Novel Aryl-2H-pyrazole Derivatives as Potent Non-purine Xanthine Oxidase Inhibitors

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A series of aryl-2H-pyrazole derivatives were synthesized and evaluated for inhibitory activity against xanthine oxidase in vitro as potent xanthine oxidase inhibitors. Among them, 2 aryl-2H-pyrazole derivatives showed significant inhibitory activities against xanthine oxidase. Compound 19 emerged as the most potent xanthine oxidase inhibitor (IC_{50}=9.8µM) in comparison with allopurinol (IC_{50}=9.5µM). The docking study revealed that compound 19 might have strong interactions with the active site of xanthine oxidase. This compound is thus a new candidate for further development for the treatment of gout.

Key words  pyrazole; xanthine oxidase; inhibitory

As a key enzyme in the purine metabolic pathway, xanthine oxidase (XO) catalyzes the hydroxylation of hypoxanthine and xanthine in the last two steps in urate biosynthesis.\(^\text{1,2}\) There is an overwhelming acceptance that XO serum levels are increased in various pathological states like hepatitis and xanthine oxidase inhibitors have been reported.\(^\text{7}\) Therefore, there is a life-threatening side effects of these purine based xanthine oxidase inhibitors.\(^\text{14}\) Based on the importance of the two scaffolds that are found to be most active ones against XO with IC_{50} ranging from 9.8 to 12.4\(\mu\)M. For R2, the acetylaminophenol derivatives were developed as potential purine xanthine oxidase inhibitors. In some cases, however, severe life-threatening side effects of these purine based xanthine oxidase inhibitors have been reported.\(^\text{7}\) Therefore, there is a great need for developing novel non-purine xanthine oxidase inhibitors with smaller side effect. Y-700,\(^\text{8}\) a 1-phenylpyrazole derivative, flavonoids,\(^\text{9}\) FYX-051,\(^\text{10}\) a 3,5-diaryltriazole derivative and curcumin\(^\text{11}\) have been reported as non-purine XO inhibitors.

The reported papers showed that 2H-pyrazole derivatives have strong bioactivity, such as anticancer activity.\(^\text{12,13}\) 1-\{3-(Furan-2-yl)\}-4,5-dihydro-5-\{(pyridin-4-yl)pyrazol-1-yl\}-ethanone,\(^\text{14}\) a aryl-2H-pyrazole derivative was reported as non-purine xanthine oxidase inhibitor. Oxygen-bearing heterocycle such as the benzodioxole group is widely used in synthetic medicinal chemistry as a component of enzyme inhibitors.\(^\text{15}\) Based on the importance of the two scaffolds that is, the aryl-2H-pyrazole and benzodioxole, we hypothesized that both the two scaffolds in a single molecular would have promising XO inhibitory activity. Herein, we designed and synthesized a series of aryl-2H-pyrazole derivatives and tested their XO inhibitory activities.

Results and Discussion

Chemistry The synthetic route for the novel 3,5-diphenyl-4,5-dihydro-1H-pyrazole derivatives 13–26 is outlined in Chart 1. These compounds 14, 15–26 were synthesized from substituted 5\{benzo[d][1,3]dioxol-5-yl\}-3-phenyl-4,5-dihydro-1H-pyrazole 7b–13b with substituted nicotinic acid 3b–e and 2-phenylacetic acid 3d–i, respectively. Firstly, 3,4-dihydroxybenzaldehyde was dissolved in cool acetonitrile (CH_{3}CN), then K_{2}CO_{3} was added, and after that dibromomethane was added. The reaction mixture was then stirred at 90°C for 24 h, and benzo[d][1,3]dioxole-5-carbaldehyde 2a was obtained. Secondly, 2a and substituted acetophenone 1–6 were dissolved in alcohol, with that sodium hydroxide (NaOH) was added, the reaction mixture was then stirred at room temperature overnight to give compounds 7–13. Thirdly, to a solution of 7–13 in alcohol, with that NH_{2}–NH_{2}·H_{2}O was added, the reaction mixture was then stirred at 80°C for 4 h to gain compounds 7b–13b. At last, 3b–i were dissolved in CH_{3}Cl_{2}, and HOBt, EDC·HCl were then added, with that obtained 7b–13b was added immediately. The reaction mixture was then stirred at room temperature overnight to get the desired compounds 14–26 (Table 1). All of the synthetic compounds 14–26 gave satisfactory elemental analytical and spectroscopic data.

Biological Evaluation of Synthesized Compounds for Xanthine Oxidase Inhibitory Activity In vitro screening of the 3,5-diphenyl-4,5-dihydro-1H-pyrazole derivatives (14–26) using bovine milk xanthine oxidase enzymatic assay was tested in triplicate. Among the series of 13 compounds, 19 and 20 were found to be most active ones against XO with IC_{50} ranging from 9.8 to 12.4\(\mu\)M (Table 2). The inhibitory activity (IC_{50}=9.8\(\mu\)M) of the most potent compound was found to be comparable to that of allopurinol (IC_{50}=9.5\(\mu\)M).

Structure–Activity Relationship (SAR) The in vitro screening of the 3,5-diphenyl-4,5-dihydro-1H-pyrazole derivatives revealed some interesting aspects about the structure inhibitory relationship. The inhibitory potential of the compounds was sensitive according to the properties as well as positions of the substituent R1 and R2. For R1, in general activating group methoxy result in dilution of the activity (compare 20 with 26, 12.4<29.1; 17 with 15, 28.3<40.6). For R2, the activity decrease when the number of mehoxyl group increases (compare 19 with 20, 9.8<12.4).

Molecular Modeling In order to understand the binding conformation of the most potent XO inhibitor 19, its flexible molecular docking was carried out into the salicylic acid XO active site using the docking software Discovery Studio 3.5. The docking results showed that compound 19 could bind to...
the xanthine oxidase with the CDDOCKER interaction energy of $-58.7$ kcal/mol.

The binding modes of compound 19 and xanthine oxidase were depicted in Fig. 2. The amino acid residue which had interaction with xanthine oxidase were labeled in Fig. 2A. In the binding mode, compound 19 was nicely bound to the salicylic acid XO active site via two hydrogen bond and one cation–π interaction. The oxygen atom of ketonic and methoxy groups of 19 formed two hydrogen bond with the amino of Thr B: 354 and Lys B: 256, respectively. The benzene ring with methoxy group formed one cation–π interaction. This molecular docking results and the biological assay data suggested that compound 19 was a potential inhibitor of xanthine oxidase.

**Conclusion**

We have designed, synthesized and assessed *in vitro* XO inhibitory activity of 13, 3,5-diphenyl-4,5-dihydro-1H-pyrazole derivatives, all of them were novel. SAR study revealed that properties of substituent R1 and R2 greatly affected the XO inhibitory activity. Docking simulations were performed to position most active compound 19 into the XO active site to determine the probable binding conformation and the results confirmed that the compound was a potential inhibitor of XO and were in agreement with the previous reported studies.

**Experimental**

**Chemistry** Melting points (mp) were determined on a XT4MP apparatus (Taike Corp., Beijing, China). Electrospray
ionization (ESI) mass spectra were obtained on a Mariner System 5304 mass spectrometer, and 1H-NMR spectra were recorded on a DPX300 spectrometer at 25°C with tetramethylsilane (TMS) and solvent signals allotted as internal standards. Chemical shifts were reported in ppm (δ). Elemental analyses were performed on a CHN-O-Rapid instrument and were within 0.4% of the theoretical values.

General Procedure for Synthesis of Compounds (2a)
Firstly, a solution of 3,4-dihydroxybenzaldehyde (13.8 g, 100 mmol) in 250 mL cool acetonitrile (CH$_3$CN), was added with K$_2$CO$_3$ (40 g), dibromomethane (120 mmol, 10.4 mL). The reaction mixture was then stirred at 90°C for 24 h. After the reaction completed, reaction mixture was filtered and concentrated, then the concentration was extracted with ethyl acetate and water. The combined extracts were dried with anhydrous Na$_2$SO$_4$ and concentrated. The pure white solid benzo[d][1,3]-dioxole-5-carbaldehyde (2a) was gained with the yield of 85%.

General Procedure for Synthesis of Compounds (7–13)
Compounds 7–13 were synthesized by coupling of 2a (5 mmol) with substituted acetophenone 1–6 (5 mmol) using alcohol (20 mL) and NaOH (1 mmol). The reaction mixture was then stirred at room temperature overnight, then, filtered, the residue was purified by recrystallization with a yield of 90%.

General Procedure for Synthesis of Compounds (7b–13b)
A solution of 7–13 (3 mmol) in alcohol (15 mL), with that NH$_2$–NH$_2$·H$_2$O (2 × 3 mmol) was added, the reaction mixture was then stirred at 80°C for 4 h. Put the completed reaction mixture into ice-water and continued reacting for 3 h and filtered to gain compounds 7b–13b, yielding 80%.

General Procedure for Synthesis of Compounds (14–26)
The desired compounds 14–26 were synthesized, starting by dissolving 3b–i (1 mmol) in CH$_2$Cl$_2$ (10 mL), with that HOBt, and EDC·HCl were added, then theobtained 7b–13b (1 mmol) were added immediately. The reaction mixture was then stirred at room temperature overnight. Reaction mixture was concentrated under reduced pressure, then alcohol (5 mL) was added and move the new mixture to ice-water overnight, filtered to gain compounds 14–26 and purified by recrystallization with a yield of 30–45%.

1-(5-(Benzo[d][1,3]dioxol-5-yl)-3-(4-bromophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-2-(pyridin-3-yl)ethan-1-one (14)
Yellow powders, yield 40.6%. mp: 173–175°C. 1H-NMR (400 MHz, chloroform-d$_3$ (CDCl$_3$)-d$_6$) δ: 9.27 (s, 1H), 8.71 (dd, J = 4.8, 1.4 Hz, 1H), 8.27 (d, J = 7.9 Hz, 1H), 7.65–7.47 (m, 4H), 7.43–7.30 (m, 1H), 6.80 (dd, J = 20.3, 7.9 Hz, 3H), 5.93 (s, 2H), 5.73 (dd, J = 11.7, 4.9 Hz, 1H), 3.77 (dd, J = 17.8, 11.8 Hz, 1H), 3.19 (dd, J = 17.8, 5.0 Hz, 1H). MS (ESI): 451.29 (M$^+$H)+. Anal. Calcd for C$_{22}$H$_{16}$BrN$_3$O$_3$: C, 58.68; H, 3.58; N, 9.33. Found: C, 58.65; H, 3.57; N, 9.32.

1-(5-(Benzo[d][1,3]dioxol-5-yl)-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl)-2-(4-fluorophenyl)ethan-1-one (15)
Yellow powders, yield 42.5%. mp: 177–179°C. 1H-NMR (400 MHz, chloroform-d$_3$ (CDCl$_3$)-d$_6$) δ: 7.89–7.74 (m, 2H), 7.57–7.41 (m, 2H), 7.41–7.24 (m, 2H), 6.80 (dd, J = 7.9 Hz, 1H), 7.65–7.47 (m, 4H), 7.43–7.30 (m, 1H), 6.80 (dd, J = 20.3, 7.9 Hz, 3H), 5.93 (s, 2H), 5.73 (dd, J = 11.7, 4.9 Hz, 1H), 3.77 (dd, J = 17.8, 11.8 Hz, 1H), 3.19 (dd, J = 17.8, 5.0 Hz, 1H). MS (ESI): 451.29 (M$^+$H)+. Anal. Calcd for C$_{22}$H$_{16}$BrN$_3$O$_3$: C, 58.68; H, 3.58; N, 9.33. Found: C, 58.65; H, 3.57; N, 9.32.
1-(5-Benzoyl)[1,3]dioxol-5-yl)-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl)-2-(4-chlorophenyl)ethan-1-one (16) Yellow powders, yield 44.5%. mp: 182–184°C. 1H-NMR (400 MHz, DMSO-d$_6$) δ: 7.88–7.71 (m, 2H), 7.53–7.40 (m, 3H), 7.34 (q, J = 8.4 Hz, 4H), 6.88–6.74 (m, 1H), 6.68–6.53 (m, 2H), 5.96 (d, J = 4.3 Hz, 2H), 5.47 (dd, J = 11.7, 4.5 Hz, 1H), 4.07 (q, J = 14.6 Hz, 2H), 3.82 (dd, J = 18.1, 11.7 Hz, 1H), 3.11 (dd, J = 40.1, 20.1 Hz, 1H). MS(EI): 419.88 (M+H)$^+$.$^8$ Anal. Caled for C$_{24}$H$_{19}$BrN$_2$O$_3$: C, 62.20; H, 4.11; N, 6.03. Found: C, 62.80; H, 4.54; N, 6.67.

1-(5-Benzoyl)[1,3]dioxol-5-yl)-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl)-2-(4-bromophenyl)ethan-1-one (17) Yellow powders, yield 45.4%. mp: 185–187°C.$^7$ 1H-NMR (400 MHz, DMSO-d$_6$) δ: 7.81 (dd, J = 6.6, 3.0 Hz, 2H), 7.48 (dd, J = 5.7, 2.7 Hz, 5H), 7.26 (d, J = 8.4 Hz, 2H), 6.81 (d, J = 7.9 Hz, 1H), 6.68–6.57 (m, 2H), 5.96 (d, J = 3.4 Hz, 2H), 5.47 (dd, J = 11.7, 4.6 Hz, 1H), 4.05 (q, J = 14.6 Hz, 2H), 3.91–3.72 (m, 1H), 3.14 (dd, J = 18.2, 4.6 Hz, 1H). MS(EI): 464.33 (M+H)$^+$.$^8$ Anal. Caled for C$_{24}$H$_{19}$BrN$_2$O$_3$: C, 62.20; H, 4.13; N, 6.05. Found: C, 62.20; H, 4.11; N, 6.03.

1-(5-Benzoyl)[1,3]dioxol-5-yl)-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl)-2-(4-(trifluoromethyl)phenyl)ethan-1-one (18) Yellow powders, yield 41.6%. mp: 171–173°C.$^7$ 1H-NMR (400 MHz, DMSO-d$_6$) δ: 7.83 (dd, J = 6.7, 2.9 Hz, 2H), 7.73–7.61 (m, 2H), 7.52 (d, J = 8.1 Hz, 2H), 7.50–7.46 (m, 3H), 6.81 (d, J = 7.9 Hz, 1H), 6.63 (d, J = 10.2, 2.2 Hz, 2H), 5.96 (d, J = 2.1 Hz, 2H), 5.48 (dd, J = 11.7, 4.5 Hz, 1H), 4.19 (q, J = 14.8 Hz, 2H), 3.83 (dd, J = 18.2, 11.8 Hz, 1H), 3.15 (dd, J = 18.2, 4.6 Hz, 1H). MS(EI): 453.43 (M+H)$^+$.$^8$ Anal. Caled for C$_{24}$H$_{19}$F$_2$N$_2$O$_3$: C, 66.37; H, 4.23; N, 6.19. Found: C, 66.36; H, 4.21; N, 6.18.

1-(5-Benzoyl)[1,3]dioxol-5-yl)-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl)-2-(3-methoxyphenyl)ethan-1-one (19) Yellow powders, yield 42.4%. mp: 179–181°C.$^7$ 1H-NMR (400 MHz, DMSO-d$_6$) δ: 7.85–7.76 (m, 2H), 7.52–7.42 (m, 3H), 7.20 (dd, J = 9.8, 6.3 Hz, 1H), 6.87 (dd, J = 13.9, 7.4 Hz, 2H), 6.83–6.75 (m, 2H), 6.68–6.58 (m, 2H), 5.96 (d, J = 2.6 Hz, 2H), 5.48 (dd, J = 11.7, 4.5 Hz, 1H), 4.03 (q, J = 14.1 Hz, 2H), 3.81 (dd, J = 18.1, 11.8 Hz, 1H), 3.70 (s, 3H), 3.13 (dd, J = 18.1, 4.6 Hz, 1H). MS(EI): 415.46 (M+H)$^+$.$^8$ Anal. Caled for C$_{24}$H$_{19}$F$_2$N$_2$O$_3$: C, 72.45; H, 5.35; N, 6.76. Found: C, 72.43; H, 5.32; N, 6.73.
1H), 6.07 (d, J = 9.6 Hz, 2H), 3.89 (s, 1H), 3.81 (s, 2H), 3.76 (s, 2H), 3.71–3.57 (m, 9H). MS(ESI): 475.51 (M + H)+. Anal. Caled for C27H25ClN2O6: C, 67.17; H, 5.83; N, 5.40. Found: C, 67.16; H, 5.81; N, 5.38.

1-(5-(Benzof[d][1,3]dioxol-5-yl)-3-(4-chlorophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-2-(3,4,5-trimethoxyphenyl)ethan-1-one (25) White powders, yield 43.3%. mp: 191–193°C. 1H-NMR (400 MHz, DMSO-d6): δ: 7.76 (d, J = 8.7 Hz, 2H), 7.00 (d, J = 8.7 Hz, 2H), 4.63 (s, 1H), 4.61 (s, 1H), 4.51 (d, J = 8.9 Hz, 1H), 3.94 (d, J = 3.2, 13.8 Hz, 2H), 3.18 (s, 3H), 3.75 (s, 1H), 3.69 (d, J = 9.6 Hz, 6H), 3.09 (d, J = 17.5 Hz, 1H). MS(ESI): 474.51 (M + H)+. Anal. Caled for C27H25ClN2O6: C, 67.34; H, 5.52; N, 5.90. Found: C, 67.32; H, 5.51; N, 5.88.

**Biology: Xanthine Oxidase Assay** The assay method for compounds against XO activity was modified from a recent report. The experiment was performed by using 96 wells plate, and the allopurinol and dimethyl sulfoxide (DMSO) were used as the positive and negative control. The enzyme assay mixture conducted with 50 mM phosphate buffer (pH 7.6), 75 μM xanthine (Sigma) and 0.08 units of xanthine oxidase (Sigma). The enzyme was preincubated for 5 min, with test compound, dissolved in DMSO (1% v/v), and the reaction was started by the addition of xanthine. The reaction was monitored for 6 min at 293 nm on a micro-plate reader (Tecan Safire) at 25°C. The active compounds were tested at five concentrations diluted two times and duplicate assays were repeated three times. Compared to the absorbance value (A) of the blank (A_blank), the inhibitory percentage against XO (%) was calculated for the test sample (A_sample) by the following equation:

\[
\text{inhibition (\%)} = \left(\frac{1 - A_{\text{sample}}}{A_{\text{blank}}}\right) \times 100
\]

**Molecular Docking** Molecular docking of compound 19 into the three dimensional X-ray structure of bovine milk XO (PDB code: 1hlf) was carried out using the Discovery Studio (version 3.5) as implemented through the graphical user interface DS-CDOCKER protocol. The three-dimensional structure of the compound was constructed using Chem. 3D ultra 12.0 software [Chemical Structure Drawing Standard; Cambridge Soft Corporation, U.S.A. (2010)], then it was energetically minimized by using MMFF94 with 5000 iterations and minimum-RMS gradient of 0.10. The crystal structure of protein complex was retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). All bound waters and ligands were eliminated from the protein and the polar hydrogen was added to the proteins. Molecular docking of compound 19 was then carried out using the Discovery Studio (version 3.5) as implemented through the graphical user interface CDOCKER protocol. CDOCKER is an implementation of a CHARMM based molecular docking tool using a rigid receptor.

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**Conflict of Interest** The authors declare no conflict of interest.

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