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Research Article

Identification of 1, 4-Dihydrothieno[3′, 2′:5, 6]thiopyrano [4, 3-c]pyrazole Derivatives as Human 5-Lipo-oxygenase Inhibitors

Jianshu Hu[1,†], Wei Zhu[1,†], Hu Meng[1], Ying Liu[1,*], Xin Wang[2,3] and Chun Hu[2,3,*]

[1]Beijing National Laboratory for Molecular Sciences, State Key Laboratory for Structural Chemistry of Unstable and Stable Species, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China
[2]Key Laboratory of Structure-Based Drug Design & Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang 110016, China
[3]School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Shenyang 110016, China
**Corresponding authors: Ying Liu, liuying@pku.edu.cn; Chun Hu, chunhu1999@163.com*
[†]Both authors contributed equally to this work.

A series of novel 1,4-dihydrothieno[3′,2′:5,6]thiopyrano [4,3-c]-pyrazole-3-carboxamide derivatives were synthesized and evaluated for their inhibitory activity to human 5-lipo-oxygenase (5-LOX). Compound 7c was found to exhibit significant inhibition to human 5-LOX with IC_{50} value of 5.7 ± 0.9 μM. Compound 7c was further studied using molecular docking in order to delineate its structure–activity relationship and to gain insight into the design of effective 5-LOX inhibitors.

Key words: bioassay, cross-reactivity, human 5-lipo-oxygenase inhibitors, molecular docking, synthesis

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Human lipo-oxygenases (LOXs) are a family of structurally related enzymes that catalyze the hydroperoxidation of polyunsaturated fatty acids with molecular oxygen. The three main isozymes of pharmacological interest are 5-lipo-oxygenase (5-LOX), 12-LOX, and 15-LOX, which are named according to their positional specificity on arachidonic acid (AA) (1).

5-LOX is a key enzyme in the biosynthesis of leukotriene (2). Its inhibitors have been shown to have potential therapeutic values for the treatment of asthma, allergic rhinitis, atherosclerosis, and cancer (3). Thus far, zileuton is the only 5-LOX inhibitor that has been approved by the FDA in USA for the treatment of asthma. Needless to say, the development of additional therapeutics with novel structures and effective activity against 5-LOX is still a much sought after endeavor.

In the AA metabolic network, 12-LOX and 15-LOX act upon AA and produce endogenous anti-inflammatory mediators (4–8). Therefore, to treat inflammation effectively, a 5-LOX inhibitor should have minimal affect against these two LOXs (9,10). We report herein a series of 1, 4-dihydrothieno [3′, 2′:5, 6]thiopyrano-[4, 3-c]pyrazole-3-carboxamide derivatives as potential inhibitors to 5-LOX. Previously, derivatives of the basic scaffold of these carboxamide derivatives have been reported as estrogen receptor antagonists (11).

Methods and Materials

Chemistry

All melting points (MPs) were measured in open capillary tubes and are uncorrected. Nuclear magnetic resonance spectra (NMR) were recorded in $CDCl_3$ or DMSO solutions, using Bruker 300 MHz spectrometers or 600 MHz spectrometers. The mass spectra (MS) were obtained by electronic impact (EI) at 70 eV in an Agilent spectrometer (with direct insertion probe) or by electrospray (ESI) in a Waters spectrometer. Infrared spectra (IR) were obtained using a Bruker IFS55 spectrometer. Chromatographic separations were performed on silica gel, using flash column chromatography, and compounds were detected using UV light (254 nm).

General synthetic procedure for 3-(thiophen-2-ylthio) propanoic acid (1)

A mixture of 2-mercapto thiophene (2.32 g, 0.02 mol), THF (40 mL), Et_3N (5.5 mL, 0.04 mol) and acrylic acid (1.65 mL, 0.024 mol) was heated at reflux under N_2. After 12 h, the solution was poured into dilute aqueous hydrochloride solutions and extracted with 20 mL EtOAc for three times. The organic extracts were dried, filtered, and concentrated to dryness. The residue was recrystallized from petroleum ether, and the solid was filtered to yield 3.01 g (80.0%) of **1**, which is a white solid. MP: 43–45 °C (reported 43–44 °C in 12).

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General synthetic procedure for 5,6-dihydro-4H-thieno[2,3-b]thiopyran-4-one (2)

Under N_2 in a three-neck was placed **1** (3.76 g, 0.02 mol), CH_2Cl_2 (20 mL) and two drops of DMF. To the solution was added dropwise oxalyl chloride (2.81 g, 0.022 mol) at ambient temperature. After 1 h, the solution was cooled to −10 °C, and a solution of $SnCl_4$ (2.57 g, 0.01 mol) in CH_2Cl_2 (10 mL) was added dropwise. The mixture was then stirred at 0 °C, and after 0.5 h, H_2O (20 mL) was added. The mixture was separated, and the organic extracts were washed with saturated Na_2CO_3-H_2O and brine, dried, filtered, and concentrated to dryness (13). The residue was recrystallized from petroleum ether, and the solid was filtered to yield 2.59 g (76.2%) of **2**, which is a white solid. MP: 59–60 °C (reported 65 °C in 14); ^{1}H-NMR (300 MHz, DMSO-d_6): δ2.77 (t, 2H), 3.47 (t, 2H), 7.35 (d, 1H, J = 5.4 Hz), 7.38 (d, 1H, J = 5.4 Hz).

General synthetic procedure for methyl oxo(4-oxo-5,6-dihydro-4H-thieno[2,3-b]thiopyran-5-yl)acetate (3)

Sodium (0.46 g) was dissolved in 20 mL of absolutely dry methanol which was removed by distillation. In the meantime, compound **2** (1.70 g, 0.01 mol) and dimethyl oxalate (2.36 g, 0.02 mol) were dissolved in 20 mL toluene and this solution was added to the sodium methoxide. The mixture was stirred for 24 h at room temperature and then poured into 100 mL water. The mixture was separated, and the organic phase was extracted with 50 mL of 10% NaOH. Then, the water phase was washed with 50 mL of diethyl ether and acidified with 1 N HCl until no precipitation appeared. The mixture was filtered and dried to give 1.5 g (59.0%) of **3**, which is a yellow solid. MP: 93–95 °C (11,15); ^{1}H-NMR (600 MHz, DMSO-d_6): δ 3.76 (s, 3H), 4.15 (s, 2H), 7.35 (d, 1H, J = 4.8 Hz), 7.41 (d, 1H, J = 4.8 Hz).

General synthetic procedure for methyl 1,4-dihydrothieno[3′,2′:5,6]thiopyrano[4,3-c]pyrazole-3-carboxylate (4)

A mixture of **3** (0.51 g, 2.0 mmol), 80% hydrazine hydrate (2 mL, 0.032 mol) and 10 mL of HOAc was heated at reflux for 12 h. Then, the solution was poured into 50 mL water and filtered to give 0.32 g (63.2%) of **4**, which is a white solid. MP: 94–96 °C (11,15); ^{1}H-NMR (300 MHz, $CDCl_3$): δ3.97 (s, 3H), 4.33 (s, 2H), 7.18 (d, 1H, J = 5.3 Hz), 7.40 (d, 1H, J = 5.3 Hz).

General synthetic procedure for methyl 7-bromo-1,4-dihydrothieno[3′,2′:5,6]thiopyrano[4,3-c] pyrazole-3-carboxylate (5)

To a solution of **4** (0.24 g, 1 mmol) in 10 mL glacial acetic acid, Br_2 (0.32 g, 2 mmol) was added at ice bath within 30 min. The mixture was stirred at room temperature for 12 h. Subsequently, the solution was poured into 50 mL water, and the solid was filtered to yield 26.4 mg (80.0%) of **5**, which is a white solid. MP: 182–184 °C; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 3.86 (s, 3H), 4.38 (s, 2H), 7.47 (s, 1H).

General synthetic procedure for 7-bromo-1,4-dihydrothieno[3′,2′:5,6]thiopyrano[4,3-c]pyrazole-3-carboxylic acid (6)

To a solution of **5** (0.24 g, 1 mmol) in 30 mL water, NaOH (0.1 g, 2.5 mmol) was added. The mixture was heated at reflux for 2 h and then cooled to room temperature. Subsequently, the solution was acidified with 1 N HCl until no precipitation appeared. The mixture was filtered and dried to give 0.30 g (95%) of **6**, which is a white solid. MP: 255–257 °C. ^{1}H-NMR (600 MHz, DMSO-d_6): δ 4.35 (s, 2H), 7.46 (s, 1H), 13.84 (s, 1H).

General synthetic procedure for 7-bromo-1,4-dihydrothieno[3′,2′:5,6]thiopyrano[4,3-c]pyrazole-3-carboxamides (7)

A mixture of **6** (0.32 g, 1 mmol), amine (1.1 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.24 g, 1.25 mmol), 1-hydroxybenzotriazole (0.05 g, 0.37 mmol), 1 mL of Et_3N and absolutely dry CH_2Cl_2 (20 mL) was stirred for 24 h at room temperature. The mixture was filtered, and the filtrate was washed with 20 mL of 1 N HCl for twice, saturated Na_2CO_3, H_2O, and brine; then it was dried, filtered, and concentrated to dryness. The residue was purified by column chromatography on silica with chloroform and methanol [V (chloroform): V (methanol) = 15:1] as the eluent to give **7** as a white solid.

Evaluating the enzyme activity of 5-LOX

The enzyme activity of 5-LOX was determined fluorescence spectrophotometrically by oxidation of the substrate H_2DCFDA to the highly fluorescent 2′, 7′-dichlorofluorescein product during 5-LOX's catalytic reaction (16). The reactions were initiated by the addition of AA as substrate and then monitored by excitation at 500 nm and emission at 520 nm utilizing a multiwall fluorometer (Synergy4; Biotek, Winooski, VT, USA). Fluorescence signals were recorded for 5 min with a kinetics mode program. Inhibition activities were measured as described previously (17). All inhibition values were tested at the concentration of 91 μM. Zileuton (reported IC_{50} = 0.9 μM in 18) was used as positive control.

Inspecting the selectivity of compound 7c

The rates of 5-LOX, 12-LOX, and 15-LOX were determined (19) by following the formation of product at 235 nm (ε = 25 000 M/cm). The enzyme was added into quartz 96-well microtiter plates in sodium phosphate buffer (100 mM, pH 7.4) and incubated with a compound (dissolved in DMSO) at room temperature for 1 min. The reac-

tions were initiated by adding the substrate AA. Then, absorbance of product (12-HpETE or 15-HpETE) was monitored via a plate reader (Synergy; Biotek).

Result and Discussion

Chemistry

The synthesis of 1,4-dihydrothieno[3′,2′:5,6]thiopyrano[4,3-c]pyrazole-3-carboxamide derivatives is illustrated in Scheme 1. 3-(Thiophen-2-ylthio)propanoic acid **(1)** was synthesized from 2-mercapto thiophene and acrylic acid (12). Treatment of **1** with oxalyl chloride and tin chloride under a nitrogen atmosphere resulted in ketone intermediate **2** (13,14), which was treated with dimethyl oxalate at room temperature in the presence of sodium hydride for 24 h to provide **3** (11,15). A mixture of **3** and hydrazine hydrate in acetic acid was then refluxed for 12 h to afford **4**. The direct bromination of **4** with bromine in glacial acetic acid resulted in intermediate **5**. Subsequent treatment of the intermediate **5** with sodium hydroxide and then hydrogen chloride gave **6** in nearly quantitative yield. Target compounds **7** were then obtained by the reaction of **6** with the respective amines, including diethylamine, t-butylamine, 1-arylpiperazine, and 1-benzylpiperazine, under general condensation conditions in the presence of EDC·HCl and HOBt.

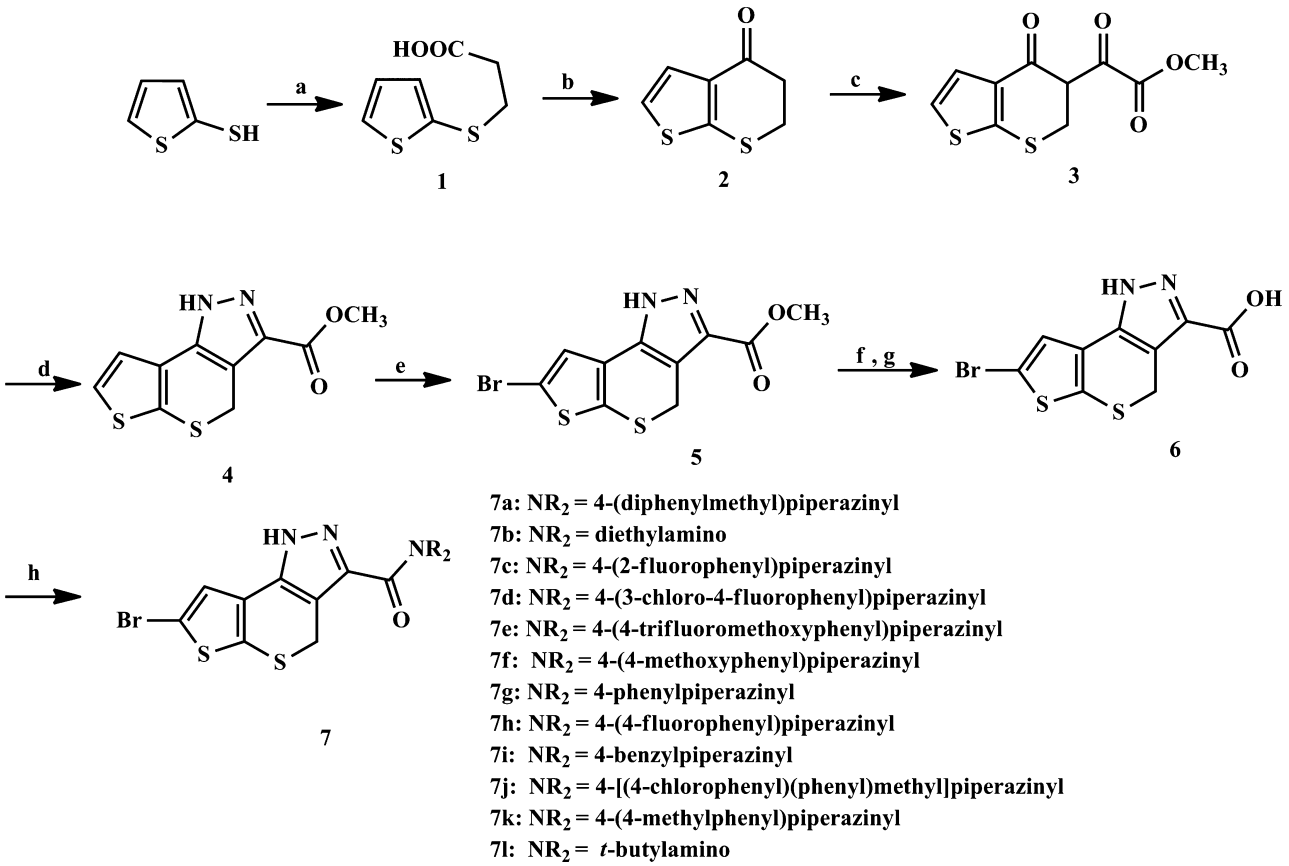
Inhibition activity against 5-LOX

The activities of 12 target compounds were evaluated using human 5-LOX in a cell-free assay (16). Three of the target compounds showed over 50% inhibition, and they were further tested for their dose–response behavior (17). The results were shown in Table 1.

Compound **7c** showed a significant activity against 5-LOX with an IC_{50} value of 5.7 ± 0.9 μM (Figure 1), which was comparable to zileuton (18) (IC_{50} 5.3 ± 1.2 μM in our assay).

The selectivity against 12-LOX and 15-LOX of compound 7c

The cross-reactivity of compound **7c** against 12-LOX (pellet type) and 15-LOX (leukocyte type) was evaluated to ascertain its selectivity (19). The results showed that compound **7c** only weakly inhibited 12-LOX (76% inhibition at



Scheme 1: Reagents and conditions: (a) acrylic acid, THF, Et_3N, reflux, 12 h; (b) oxalyl chloride, $SnCl_4$, CH_2Cl_2, r.t., 0.5 h; (c) oxalic acid dimethyl ester, sodium methoxide, toluene r.t., 24 h; (d) 80% hydrazine hydrate, HOAc, reflux, 12 h; (e) Br_2, AcOH, r.t., 12 h; (f) NaOH, H_2O, reflux, 2 h; (g) HCl; (h) amine, EDC, HOBt, CH_2Cl_2, r.t., 24 h.

Table 1: Inhibition of target compounds to 5-lipo-oxygenase[a]

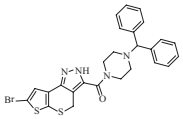
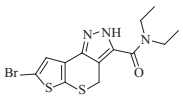
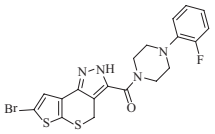
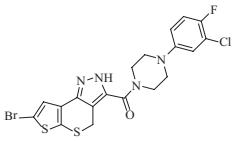
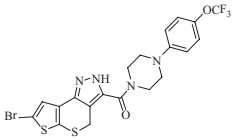
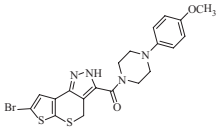
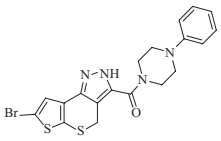
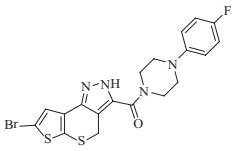
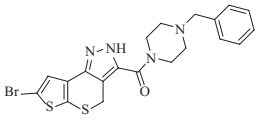
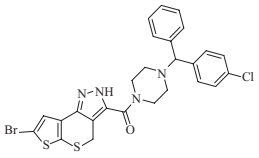
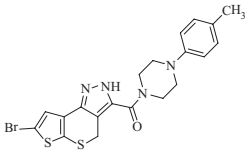
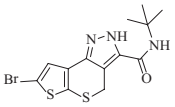
Compounds	Chemical structure	Inhibition at 91 μM (%)	IC_{50} (μM)
Zileuton			5.3 ± 1.2
7a		2.3 ± 0.3	n.m.[b]
7b		44 ± 5	n.m.
7c			5.7 ± 0.9
7d		28 ± 3	n.m.
7e			86 ± 5
7f		20 ± 3	n.m.
7g		7.4 ± 1.1	n.m.
7h		3.7 ± 0.4	n.m.
7i			21 ± 5
7j		26 ± 2	n.m.
7k		−20 ± 3	n.m.

Table 1: continued

Compounds	Chemical structure	Inhibition at 91 μM (%)	IC_{50} (μM)
7l		41 ± 6	n.m.

[a]Data shown represent the mean ± SEM (n = 3).
[b]n.m. means not measured.

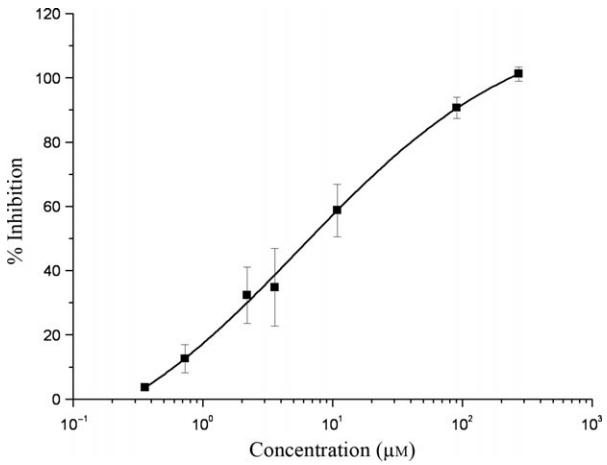


Figure 1: Dose–response curves of compound **7c.**

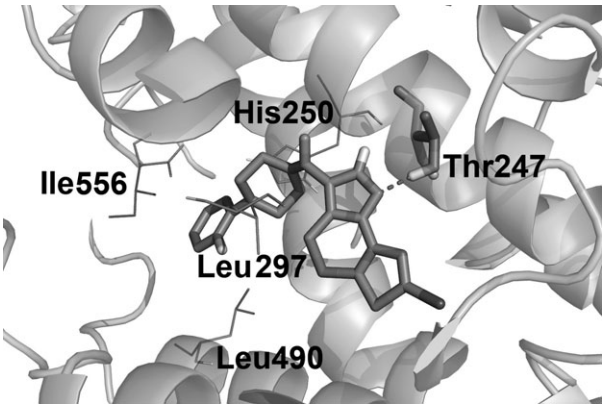


Figure 2: Binding mode of **7c** with 5-lipo-oxygenase substrate binding site (predicted by molecular docking).

100 μM, IC_{50} = 46 ± 7 μM) and had negligible activity against 15-LOX (<5% inhibition at 100 μM) in the cell-free assay.

Binding modes of compound 7c

Molecular modeling was used to illustrate the possible binding mode of the compounds that we have synthesized. Molecular docking studies was conducted by using AUTODOCK 4.0 (Autodock, version 4; The Scripps Research Institute, La Jolla, CA, USA, 2007). All LOXs are homologous in sequence and have the same two-domain structure: an N-terminal β-barrel domain (C2-like or PLAT domain) and a C-terminal catalytic domain (LOX domain). Because there is no crystal structure of human 5-LOX fitting drug-design studies, we use 5-LOX comparative model to study, which was built based on the closed conformation of 15-LOX by our group (17). Similar method is used to 15-LOX. For 12-LOX, the crystal structure (PDB ID: 3D3L) is used.

In the 5-LOX substrate-binding site, tricyclic fused-ring structure of compound **7c** was in the center of the pocket and positioned non-flat. A nitrogen atom of the pyrazole ring formed a hydrogen bond of 2.2 Å with Thr247, and the 4-(2-fluorophenyl) piperazinyl group located in the site comprising Leu297, Ile556 and Leu490 corresponding to the interaction result of the AUTODOCK TOOL (ADT) (Figure 2).

The molecular docking results provided insight for the further optimization of the 1,4-dihydrothieno[3′,2′:5,6]thiopyrano[4,3-c]pyrazole derivatives. It could be inferred from the modeling studies that the arylpiperazine moiety was too tightly bound in 5-LOX to allow for further substitution. On the other hand, further substitution in the piperazine moiety, being in the proximity of a larger pocket, was possible and might likely garner additional binding affinity. Considering that the arylpiperazine structure was around the hydrophobic area, replacing the piperazine by a triazine or hexahydrotriazine may form new hydrogen bonds to improve the interaction between the inhibitor and 5-LOX.

When compound **7c** was docked in the substrate-binding site of 12-LOX or 15-LOX, the 4-(2-fluorophenyl) piperazinyl moiety was also located in a hydrophobic site. However, **7c** did not show a tendency to form a hydrogen bond with either 12-LOX or 15-LOX (refer to supplementary data). It could account for the selectivity of **7c** to these three kinds of LOXs.

Conclusion

In this study, a series of 1,4-dihydrothieno[3′,2′:5,6]thiopyrano[4,3-c]pyrazole-3-carboxamide derivatives were synthesized and evaluated for their inhibitory activity against human 5-lipo-oxygenase *in vitro*. The interaction between

the inhibitor and 5-LOX was studied using molecular modeling, which provided insight into the further modification of the 1, 4-dihydrothieno[3′, 2′:5, 6]thiopyrano[4, 3-c]pyrazole derivatives and the design of effective human 5-LOX inhibitors.

Acknowledgments

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Conflict of interest

The authors have declared no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Physical properties and spectrum data of target compounds, binding mode of compound **7c** with 12-LOX, 15-LOX are provided.