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Development of 3,5-dinitrobenzoate-based 5-lipoxygenase inhibitors

Erchang Shang, Ying Liu *, Yiran Wu, Wei Zhu, Chong He, Luhua Lai

BNLMS, State Key Laboratory for Structural Chemistry of Unstable and Stable Species, College of Chemistry and Molecular Engineering, Center for Quantitative Biology, Peking University, Beijing 100871, China

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ABSTRACT

Human 5-lipoxygenase (5-LOX) is a well-validated target for anti-inflammatory therapy. Development of novel 5-LOX inhibitors with higher activities is highly demanded. In previous study, we have built a model for the active conformation of human 5-LOX, and identified naphthalen-1-yl 3,5-dinitrobenzoate (**JMC-4**) as a 5-LOX inhibitor by virtual screening. In the present work, 3,5-dinitrobenzoate-based 5-lipoxygenase inhibitors were developed. Twenty aryl 3,5-dinitrobenzoates, *N*-aryl 3,5-dinitrobenzamides and analogues were designed and synthesized. Several of them were found with significantly increased activities according to cell-free assay and human whole blood assay. The structure–activity relationship study may provide useful insights for designing effective 5-LOX inhibitors.

1. Introduction

5-Lipoxygenase (5-LOX) is a key enzyme in the metabolism of arachidonic acid (AA) to produce leukotrienes. It catalyzes the two-step conversion from AA to leukotriene A_4 (LTA_4).[1] LTA_4 is further metabolized by leukotriene A_4 hydrolase to leukotriene B_4, or by leukotriene C_4 synthase to leukotriene C_4 and then to leukotriene D_4 and E_4.[2] Leukotrienes are important mediators of inflammatory and allergic diseases, and also play roles in cardiovascular diseases and cancers.[3–6] The upstream enzyme of leukotrienes, 5-LOX, has been validated as a drug target for inflammation and related disorders therapy.[7] Several types of 5-LOX inhibitors have been reported, including redox, iron ligand, nonredox, and competitive inhibitors (for review see Ref. 8).[8] Up to now, only one 5-LOX inhibitor, zileuton, entered the market.[9] However, weak potency and short duration are the therapeutic problems of zileuton.[10] Development of safe and effective 5-LOX inhibitors is highly demanded.

We previously reported a model for the active conformation of human 5-LOX and new inhibitors identified by virtual screening using this model.[11] Among these inhibitors, naphthalen-1-yl 3,5-dinitrobenzoate (**JMC-4**), shows activity in the micromolar range and possesses novel structure compared to known 5-LOX inhibitors. Through analyzing of the docking conformation of **JMC-4** with 5-LOX, we recognized several key interactions between the compound and the enzyme. As shown in Figure 1, one nitro group makes a hydrogen bond with His600 of 5-LOX; the naphthyl group is crucial to form hydrophobic interaction with the pocket around Leu414. The ester linker of **JMC-4** is close to Tyr181 and Gln363, but no hydrogen bond is formed for the relative position. Guided by this interaction model, in the present work, we tried to improve the potency of **JMC-4**. Firstly, phenyls with different substituent

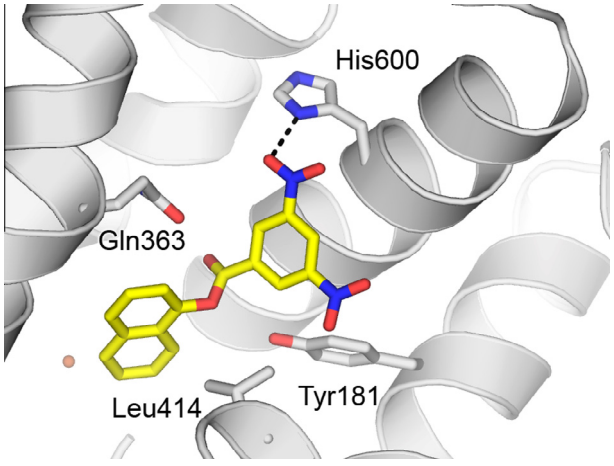

Figure 1. Binding mode of **JMC-4** with 5-LOX substrate binding site (predicted by molecular docking).

* Corresponding author. Tel.: +86 10 62751490; fax: +86 10 62751725.
E-mail address: liuying@pku.edu.cn (Y. Liu).

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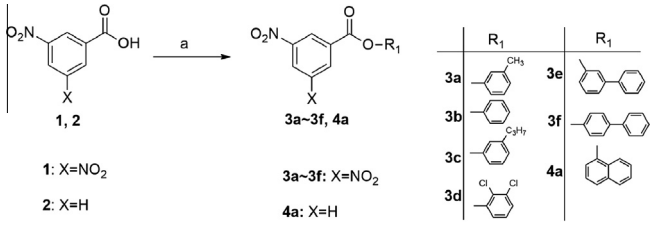
groups were introduced to search a more active hydrophobic moiety. Secondly, amide bond was used to replace the ester bond to test a linker with hydrogen bond donor. Finally, as the binding conformation shows only one nitro group makes a key interaction with the target, the mono-nitro group counterpart of **JMC-4** and the compounds with carboxyl group instead of nitro group were tested.

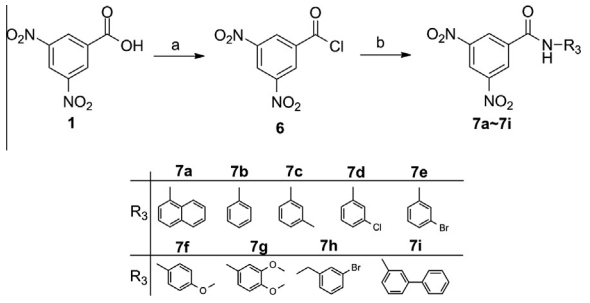
2. Results and discussion

2.1. Chemistry

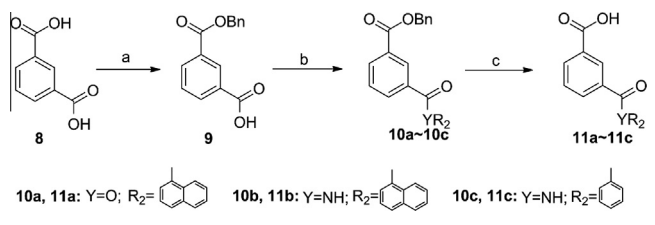
To prepare compounds **3a**–**3f** and **4a**, 3,5-dinitrobenzoic acid or 3-nitrobenzoic acid was reacted with different substituted phenols. Using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) as coupling agent and 4-dimethylaminopyridine (DMAP) as catalyst, the target compounds were obtained in moderate to high yields (Scheme 1). The amide analogs (**7a**–**7i**) were prepared using a two-step synthetic method, as shown in Scheme 2. The overall yields ranged from 60% to 80%. Preparation of the target compounds (**11a**–**11c**) were outlined in Scheme 3. The monobenzyl ester (**9**) was synthesized starting from isophthalic acid (**8**) according to the literature.[12] Acid chloride formation step followed by amidation or esterification gave the intermediates



Scheme 1. Synthesis of ester linker analogs of **JMC-4**. Reagents and conditions: (a) R_1OH, EDCI, DMAP, THF, rt.



Scheme 2. Synthesis of amide linker analogs of **JMC-4**. Reagents and conditions: (a) $SOCl_2$, reflux; (b) R_3NH_2, Et_3N, THF, rt.



Scheme 3. Synthesis of carboxyl derivatives of **JMC-4**. Reagents and conditions: (a) (i) Et_3N, MeOH, rt; (ii) BnBr, DMF, 100 °C; (b) (i) $SOCl_2$, reflux; (ii) R_2YH, THF, rt; (c) H_2, Pd/C, MeOH, rt.

(**10a**–**10c**). Then compounds (**11a**–**11c**) were obtained by cleavage of the benzyl ester of **10a**–**10c**.

2.2. Inhibition assay of 5-LOX in cell-free system

A fluorescence-based enzyme assay was used to test inhibition of the target compounds to human 5-LOX.[13] The fluorescent signal is measured during oxidation of 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) by human 5-LOX product. All compounds were dissolved in DMSO, and the inhibition values were tested at the concentration of 50 μM in preliminary test. Zileuton was used as the positive control and DMSO (4.5%, v/v) was used as vehicle control. The compounds of inhibition over 50% in the preliminary test were subjected to the determination of IC_{50} values (see Table 1).

2.3. Measuring LTB_4 generation in human whole blood

All the compounds were also evaluated in a human whole blood (HWB) assay, in which calcium ionophore A23187 was used to induce the 5-LOX pathway of AA metabolic network.[14] A LTB_4 ELISA kit was used to monitor the production of LTB_4 in HWB assay. As a preliminary test, all the compounds were tested at 50 μM concentration. Compounds with inhibition activity over 50% were then subjected to the determination of IC_{50} values. Zileuton was used as the positive control and DMSO (4.5%, v/v) was used as vehicle control.

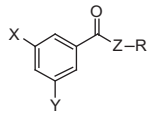
2.4. Structure–activity relationship studies

Among the newly prepared ester analogs with different hydrophobic groups, some compounds show improved activities. The introduction of 3-Me-phenyl as a hydrophobic moiety leads to a highly active compound 3-tolyl 3,5-dinitrobenzoate, **3a** (IC_{50} = 6 nM in cell-free assay and IC_{50} = 0.5 μM in HWB assay). Phenyl group at the same position leads to compound **3b** with activity comparable to the parent compound, but 3-propyl-phenyl (compound **3c**) results in a 5-fold loss of potency. Furthermore, replacement of phenyl with 2,3-dichlorophenyl (**3d**) loses the activity. Introduction of phenyl at the 3-position of phenyl (**3e**) results in about 9-fold and 4-fold loss of potency in cell-free and HWB assays, respectively. This tendency of potency reducing is enhanced while the phenyl group is substituted at the 4-position (**3f**). These results indicate that the shape of the hydrophobic moiety is crucial for inhibition activity: phenyl with small substituent group at 3-position performs best, while 2- or 4-substitution is less optimal.

Most amide analogs of the parent compound show enhanced inhibition activities to 5-LOX in cell-free assay. Interestingly, only one atom change from oxygen to nitrogen leads to the significant potency improvement of compound *N*-(naphthalene-1-yl)-3,5-dinitrobenzamide, **7a** (from 1.0 μM of **JMC-4** to 4 nM of **7a**). Besides, a series of phenyl analogues (**7c**, **7d**, **7f**, and **7i**) also show nanomolar inhibition. The significant potency increasing of amide analogs may due to the relatively rigid amide bond, which makes the two moieties maintain quasi-planar geometries. However, in HWB assay, only compounds *N*-phenyl 3,5-dinitrobenzamide (**7b**), *N*-(4-methoxyphenyl)-3,5-dinitrobenzamide (**7f**) and *N*-(3-bromobenzyl)-3,5-dinitrobenzamide (**7h**) can inhibit the production of LTB_4, while others cannot.

The mono-nitro derivative naphthalene-1-yl 3-nitrobenzoate (**4a**) has inhibition potency (IC_{50} = 7.1 μM) in the same magnitude with the parent compound in cell-free assay, and is also active in HWB (IC_{50} = 24.6 μM). This result is in good agreement with the prediction that only one nitro group is essential in the binding. A series of carboxyl analogs of the mono-nitro compound **4a** were

Table 1
Bio-evaluation of compounds



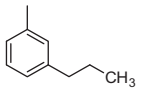
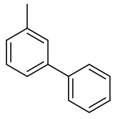
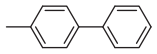
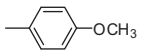
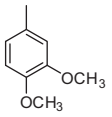
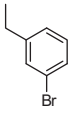
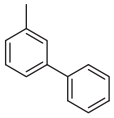
Compd	Structure				Cell-free[b] IC_{50} (μM)	HWB[b] IC_{50} (μM)
	X	Y	Z	R		
Zileuton[a,11]					1.1 ± 0.5	3.7 ± 0.4
JMC-4[11]	NO_2	NO_2	O		1.0 ± 0.2	8.6 ± 0.9
3a	NO_2	NO_2	O		0.006 ± 0.001	0.5 ± 0.2
3b	NO_2	NO_2	O		1.6 ± 0.1	15.8 ± 1.2
3c	NO_2	NO_2	O		7.4 ± 0.6	6.5 ± 1.4
3d	NO_2	NO_2	O		>50	>50
3e	NO_2	NO_2	O		8.7 ± 0.4	38.0 ± 4.5
3f	NO_2	NO_2	O		>50	>50
4a	NO_2	H	O		7.1 ± 1.6	24.6 ± 2.2
7a	NO_2	NO_2	NH		0.004 ± 0.002	>50
7b	NO_2	NO_2	NH		1.8 ± 0.7	15.2 ± 4.0
7c	NO_2	NO_2	NH		0.01 ± 0.01	>50
7d	NO_2	NO_2	NH		0.033 ± 0.005	>50

Table 1 (*continued*)

Compd	Structure				Cell-free[b] IC_{50} (μM)	HWB[b] IC_{50} (μM)
	X	Y	Z	R		
7e	NO_2	NO_2	NH		>50	>50
7f	NO_2	NO_2	NH		0.034 ± 0.004	2.7 ± 0.7
7g	NO_2	NO_2	NH		1.1 ± 0.1	>50
7h	NO_2	NO_2	NH		83.6 ± 6.4	14.4 ± 7.6
7i	NO_2	NO_2	NH		0.030 ± 0.003	>50
9	COOH	H	O		>50	>50
11a	COOH	H	O		>50	>50
11b	COOH	H	NH		>50	>50
11c	COOH	H	NH		>50	>50

[a] Positive control.
[b] Data are given as mean ± SE of $n \geqslant 3$ determinations.

also synthesized and evaluated for inhibition activity. However, all the carboxylic acid derivatives, **9** and **11a**–**11c**, have no inhibition activity to 5-LOX. The potency loss of these compounds may due to their undesirable pK_a profiles.

3,5-Dinitrobenzoate is always used as organic synthesis intermediate for its important scaffold. Some structures of our newly designed 5-LOX inhibitors were listed in Reaxys database.[15] Fortunately, we do not found any bio-activity report of the prepared compounds through an extensive literature search. Consequently, this is the first research about anti-inflammatory activities of these compounds.

To explain the increasing activities of the newly synthesized compounds, we docked ester analog **3a** and amide analog **7c** into the binding site of 5-LOX (Fig. 2). The two compounds share the same conformation of the linker, and both form an additional hydrogen bonds to Tyr181 of 5-LOX (**3a**, the ester oxygen atom; **7c**, the amide oxygen atom). Also for the hydrogen bond between nitro group and His600 of 5-LOX, which exists in **3a** (2.77 Å) and **7c** (2.59 Å) is stronger than that of **JMC-4** (3.11 Å). These interactions are likely to enhance the activities of the two compounds. Additionally, the relatively rigid amide bond may play key roles in the significant potency increasing of most amide analogs.

3. Conclusion

Twenty compounds with 3,5-dinitrobenzoate scaffold were synthesized and evaluated using cell-free and HWB assays for their 5-LOX inhibition activities. Compared to the parent compound, six compounds show significantly increased enzyme inhibition activities. The best compound, 3-tolyl 3,5-dinitrobenzoate (**3a**), showed high potency in cell free assay (IC_{50} = 6 nM) and in human whole blood assay (IC_{50} = 0.5 μM). SAR studies gave insights to the design of better 5-LOX inhibitors.

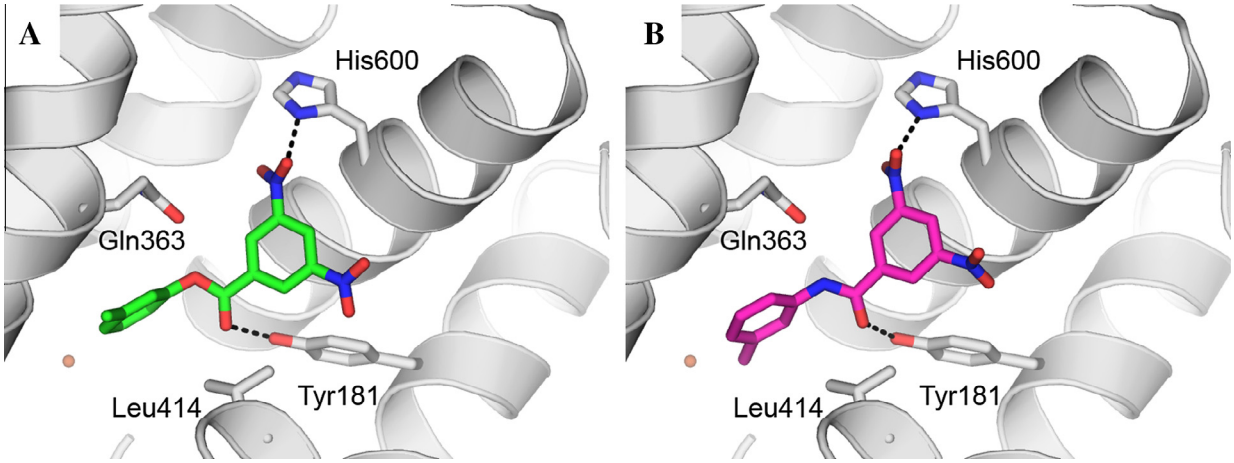


Figure 2. Inhibitors in 5-LOX substrate binding site (predicted by molecular docking). (A) Compound **3a** (green). (B) Compound **7c** (magenta).

4. Experiments

4.1. Chemistry

4.1.1. General conditions

The reagents and solvents were commercially available and purified according to conventional methods. Melting points were determined on an X6 microscopic melting point apparatus and were uncorrected. All new compounds gave satisfactory ^{1}H NMR, ^{1}H NMR spectra are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), integration. Representative compounds were measured their ^{13}C NMR spectra. ^{1}H (300 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Bruker Ascend 300/400 MHz spectrometer. Chemical shifts (δ) are relative to tetramethylsilane (0 ppm). HRMS were recorded on a Bruker Apex IV FTMS mass spectrometer using ESI (electrospray ionization). Compound **3d** and **7i** did not show the molecular ion peak in ESI test due to hardly ionization.

4.1.2. Synthesis of 3a–3g, and 4a

General method for the preparation of the dinitrobenzoates. To a stirred solution of 3,5-dinitrobenzoic acid (1.1 mmol) in THF (10 mL), phenol (1.0 mmol), EDCI (1.2 mmol) and DMAP (cat.) were added. The mixture was stirred at room temperature for 8 h. The solvent was eliminated in vacuo and the resulting residual was recrystallised from ethyl acetate/hexane to furnish the dinitrobenzoate.

3-Tolyl 3,5-dinitrobenzoate (3a). The compound **3a** was prepared according to the general procedure described above as a light yellow needle crystal in 40% yield. Mp/Lit.:[16] 169–171 °C (ethyl acetate/hexane)/165.4 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.32 (m, 3H), 7.37 (t, J = 6.0 Hz, 1H), 7.16 (d, J = 6.0 Hz, 1H), 7.07 (d, J = 6.0 Hz, 1H), 2.43 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 162.5, 151.1, 149.3, 140.4, 133.5, 130.3, 130.2 × 3, 128.1, 123.9, 122.9, 119.5, 21.7; HRMS (ESI): calcd for $C_{14}H_{10}N_2NaO_6$ [(M+Na)$^+$] 325.0431, found 325.0429.

Phenyl 3,5-dinitrobenzoate (3b). By use of the above-described procedure, the target compound was obtained as a gray needle crystal in 42% yield. Mp/Lit.:[16] 147–148 °C (ethyl acetate/hexane)/145.8 °C (enthanol). ^{1}H NMR (300 MHz, DMSO): δ 9.10 (m, 3H), 7.52 (t, J = 8.0 Hz, 2H), 7.39 (m, 3H); HRMS (ESI): calcd for $C_{13}H_8N_2NaO_6$ [(M+Na)$^+$], 311.0275, found 311.0280.

3-Propylphenyl 3,5-dinitrobenzoate (3c). The compound **3c** was prepared according to the general procedure described above as a gray lamellar crystal in 36% yield. Mp: 83–85 °C (ethyl acetate/hexane). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.30 (m, 3H), 7.39 (m, 1H), 7.17 (d, J = 9.0 Hz, 1H), 7.08 (d, J = 3.0 Hz, 2H), 2.65 (t, J = 6.0 Hz, 2H), 1.67 (m, J = 6.0 Hz, 2H), 0.97 (t, 3H); HRMS (ESI): calcd for $C_{16}H_{14}N_2NaO_6$ [(M+Na)$^+$] 353.0744, found 353.0744.

2,3-Dichlorophenyl 3,5-dinitrobenzoate (3d). The compound **3e** was prepared according to the general procedure described above as a light yellow needle crystal in 68% yield. Mp: 154–156 °C (ethyl acetate/hexane). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.13 (m, 3H), 7.72 (m, 1H), 7.64 (m, 1H), 7.56 (m, 1H).

Biphenyl-3-yl 3,5-dinitrobenzoate (3e). The compound **3f** was prepared according to the general procedure described above as a light yellow solid in 70% yield. Mp: 176–177 °C (ethyl acetate/hexane). ^{1}H NMR (300 MHz, DMSO): δ 9.12 (m, 3H), 7.72 (m, 4H), 7.61 (t, 1H), 7.50 (t, 2H), 7.41 (d, 2H); HRMS (ESI): calcd for $C_{19}H_{12}N_2NaO_6$ [(M+Na)$^+$] 387.0588, found 387.0596.

Biphenyl-4-yl 3,5-dinitrobenzoate (3f). The compound **3g** was prepared according to the general procedure described above as a light yellow solid in 78% yield. Mp: 229–230 °C (ethyl acetate/hexane). ^{1}H NMR (300 MHz, DMSO): δ 9.12 (m, 3H), 7.83 (d, J = 9.0 Hz, 2H), 7.80 (d, J = 9.0 Hz, 2H), 7.50 (m, 4H), 7.40 (t, J = 9.0 Hz, 1H); HRMS (ESI): calcd for $C_{19}H_{12}N_2NaO_6$ [(M+Na)$^+$] 387.0588, found 387.0579.

Naphthalene-1-yl 3-nitrobenzoate (4a). The compound **4a** was prepared according to the general procedure described above as a maple solid in 46% yield. Mp: 111–113 °C (ethyl acetate/hexane). ^{1}H NMR (300 MHz, DMSO): δ 8.90 (d, J = 3.0 Hz, 1H), 8.62 (m, 2H), 8.00 (d, J = 9.0 Hz, 1H), 7.97 (m, 3H), 7.60 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 164.0, 148.8, 147.0, 136.9, 135.1, 131.9, 131.2, 129.4, 129.0, 127.9, 126.6, 125.3, 121.8, 119.5; HRMS (ESI): calcd for $C_{17}H_{11}NNaO_4$ [(M+Na)$^+$] 316.0580, found 316.0583.

4.1.3. Synthesis of 7a–7i

General method for the preparation of the dinitrobenzamides. A solution of 3,5-dinitrobenzoic acid (1 mmol) in $SOCl_2$ (2 mL) was stirred and refluxed for 4 h. The solvent was evaporated under reduced pressure and added a solution of Et_3N (3 mmol) in THF (5 mL) to the resulting residual. After stirring for several minutes, a solution of aromatic amine (1 mmol) in THF (2 mL) was

added. The mixture was allowed to stir at room temperature for 8–12 h. The solvent was removed under reduced pressure and the resulting crude residual was recrystallised from ethyl acetate/hexane to furnish the dinitrobenzamide.

***N*-(Naphthalene-1-yl)-3,5-dinitrobenzamide (7a).** The compound **7a** was prepared according to the general procedure described above as a gray needle crystal in 68% yield. Mp: 265–267 °C (ethyl acetate/hexane). ^{1}H NMR (300 MHz, DMSO): δ 11.13 (s, 1H), 9.29 (d, J = 2.1 Hz, 2H), 9.06 (t, J = 2.1 Hz, 1H), 8.00 (m, 2H), 7.94 (d, J = 9.0 Hz, 1H), 7.60 (m, 4H); HRMS (ESI): calcd for $C_{17}H_{11}N_3NaO_5$ [(M+Na)$^+$] 360.0591, found 360.0598.

***N*-Phenyl 3,5-dinitrobenzamide (7b).** The compound **7b** was prepared according to the general procedure described above as a light yellow needle crystal in 60% yield. Mp/Lit.:[17] 241–242 °C (ethyl acetate/hexane)/235.8 °C. ^{1}H NMR (300 MHz, DMSO): δ 10.87 (s, 1H), 9.14 (d, J = 2.1 Hz, 2H), 9.01 (t, J = 2.1 Hz, 1H), 7.80 (d, J = 8.4 Hz, 2H), 7.42 (t, J = 8.4 Hz, 2H), 7.19 (t, J = 8.4 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 162.1, 149.0 × 2, 139.2, 138.3, 129.7 × 2, 128.9 × 2, 125.4, 122.0, 121.6 × 2; HRMS (ESI): calcd for $C_{13}H_9N_3NaO_5$ [(M+Na)$^+$] 310.0434, found 310.0431.

***N*-3-Tolyl 3,5-dinitrobenzamide (7c).** The compound **7c** was prepared according to the general procedure described above as a yellow solid in 55% yield. Mp/Lit.:[18] 281–283 °C (ethyl acetate/hexane)/263 °C. ^{1}H NMR (300 MHz, DMSO): δ 10.80 (s, 1H), 9.17 (d, J = 2.1 Hz, 2H), 9.01 (t, J = 2.1 Hz, 1H), 7.61 (d, 2 J = 7.8 Hz, H), 7.30 (t, J = 7.8 Hz, 1H), 7.01 (d, J = 7.8 Hz, 1H), 2.27 (s, 3H); HRMS (ESI): calcd for $C_{14}H_{11}N_3NaO_5$ [(M+Na)$^+$] 324.0591, found 324.0591.

***N*-(3-Chlorophenyl)-3,5-dinitrobenzamide (7d).** The compound **7d** was prepared according to the general procedure described above as a white needle crystal in 80% yield. Mp/Lit.:[18] 233–234 °C (ethyl acetate/hexane)/229 °C. ^{1}H NMR (300 MHz, DMSO): δ 10.99 (s, 1H), 9.15 (d, J = 2.1 Hz, 2H), 9.00 (t, J = 2.1 Hz, 1H), 7.95 (t, J = 2.1 Hz, 1H), 7.72 (m, 1H), 7.44 (t, J = 8.4 Hz, 1H), 7.24 (m, 1H); HRMS (ESI): calcd for $C_{13}H_8ClN_3NaO_5$ [(M+Na)$^+$] 344.0045, found 344.0044.

***N*-(3-Bromophenyl)-3,5-dinitrobenzamide (7e).** The compound **7e** was prepared according to the general procedure described above as a white needle crystal in 76% yield. Mp/Lit.:[18] 220–221 °C (ethyl acetate/hexane)/220 °C. ^{1}H NMR (300 MHz, DMSO): δ 10.97 (s, 1H), 9.17 (d, J = 2.1 Hz, 2H), 9.02 (t, J = 2.1 Hz, 1H), 8.10 (s, 1H), 7.79 (m, 1H), 7.39 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 162.4, 149.1 × 2, 140.8, 138.0, 131.7, 129.0 × 2, 128.1, 123.9, 122.4, 122.2, 120.3; HRMS (ESI): calcd for $C_{13}H_8BrN_3NaO_5$ [(M+Na)$^+$] 387.9540, found 387.9548.

***N*-(4-Methoxyphenyl)-3,5-dinitrobenzamide (7f).** The compound **7f** was prepared according to the general procedure described above as a yellow solid in 60% yield. Mp/Lit.:[18] 244–246 °C (ethyl acetate/hexane)/244 °C. ^{1}H NMR (300 MHz, DMSO): δ 10.76 (s, 1H), 9.17 (d, J = 2.1 Hz, 2H), 9.00 (t, J = 2.1 Hz, 1H), 7.70 (d, J = 9.0 Hz, 2H), 6.98 (d, J = 0.0 Hz, 2H), 3.77 (s, 3H); HRMS (ESI): calcd for $C_{14}H_{11}N_3NaO_6$ [(M+Na)$^+$] 340.0540, found 340.0539.

***N*-(3,4-Dimethoxyphenyl)-3,5-dinitrobenzamide (7g).** The compound **7g** was prepared according to the general procedure described above as a yellow solid in 75% yield. Mp: 255–257 °C (ethyl acetate/hexane). ^{1}H NMR (300 MHz, DMSO): δ 10.74 (s, 1H), 9.17 (d, J = 2.1 Hz, 2H), 9.00 (t, J = 2.1 Hz, 1H), 7.44 (d, J = 2.4 Hz, 1H), 7.38 (dd, J = 8.7 Hz, 2.4 Hz, 1H), 7.00 (d, J = 8.7 Hz, 1H), 3.78 (s, 3H), 3.76 (s, 3H); HRMS (ESI): calcd for $C_{15}H_{13}N_3O_7$ [M$^+$] 347.0748, found 347.0740.

***N*-(3-Bromobenzyl)-3,5-dinitrobenzamide (7h).** The compound **7h** was prepared according to the general procedure described above as a light yellow needle crystal in 72% yield. Mp: 181–182 °C (ethyl acetate/hexane). ^{1}H NMR (300 MHz, DMSO): δ 9.78 (t, J = 5.7 Hz, 1H), 9.10 (d, J = 2.1 Hz, 2H), 8.97 (t, J = 2.1 Hz, 1H), 7.57 (s, 1H), 7.49 (m, 1H), 7.37 (m, 2H), 4.55 (d, J = 5.7 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 163.1, 149.1 × 2, 142.5, 137.5, 131.5, 131.2, 130.8, 128.5 × 2, 127.6, 122.6, 121.8, 43.5; HRMS (ESI): calcd for $C_{14}H_{11}BrN_3O_5$ [(M+H)$^+$] 379.9877 found 379.9869.

***N*-(Biphenyl-3-yl)-3,5-dinitrobenzamide (7i).** The compound **7i** was prepared according to the general procedure described above as a gray solid in 67% yield. Mp: 228–229 °C (ethyl acetate/hexane). ^{1}H NMR (300 MHz, DMSO): δ 10.96 (s, 1H), 9.21 (d, J = 2.1 Hz, 2H), 9.02 (t, J = 2.1 Hz, 1H), 8.10 (s, 1H), 7.84 (m, 1H), 7.68 (m, 2H), 7.51 (m, 4H), 7.41 (m, 1H).

4.1.4. Synthesis of 9 and 11a–11c

3-(Benzyloxycarbonyl)benzoic acid (9). To a suspension of isophthalic acid (4.0 g, 24 mmol) in methanol (50 mL) and water (5 mL), the solution of triethylamine (2.5 g, 24 mmol) in methanol (25 mL) was added. The mixture was stirred at rt overnight. The solvent was evaporated under reduced pressure and the residual was dissolved in DMF (60 mL). Benzyl bromide (4.5 g, 27 mmol) was added to the DMF solution and the resulting mixture was heated to 100 °C for 2 h. After cooling to room temperature, the mixture was poured into $NaHCO_3$(aq) (5%, 120 mL). The pH was adjusted to around 3 by progressively dropping 1 M HCl(aq) and the resulting mixture was extracted with EtOAc (100 mL × 3). The combined organic phases were washed with saturated NaCl(aq) and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure and the residual was purified by chromatography on silica gel (MeOH/DCM) to provide a white solid **9** (2.1 g, 34%). ^{1}H NMR (300 MHz, DMSO): δ 13.37 (s, 1H), 8.50 (s, 1H), 8.22 (m, 2H), 7.69 (t, 1H), 7.45 (m, 5H), 5.39 (s, 2H); HRMS (ESI): calcd for $C_{15}H_{12}NaO_4$ [(M+Na)$^+$] 279.0628, found 279.0630.

3-((Naphthalene-1-yloxy)carbonyl)benzoic acid (11a). A solution of EDCI (0.38 g, 2.0 mmol) and 3-(benzyloxycarbonyl)benzoic acid (**9**) (0.38 g, 1.5 mmol) in THF (20 mL) was stirred at rt. After 0.5 h, DMAP (0.0018 g, 0.15 mmol) and 1-naphthol (0.22 g, 1.5 mmol) were added and the resulting mixture was stirred for 12 h at same temperature. The solvent was evaporated under reduced pressure and the residual was dissolved in water. The mixture was brought to pH = 2 with 2 M HCl, filtered and washed with ice ethyl acetate, then dried in a high vacuum oven to give a yellow solid benzyl naphthalene-1-yl isophthalate (0.40 g, 70% yield).

The solution of benzyl naphthalene-1-yl isophthalate (0.19 g, 0.50 mmol) and Pd/C (0.04 g) in 10 mL of methanol was put into a hydrogenation device. Setting the hydrogen pressure at 0.4 MPa, the solution was stirred at room temperature for 5 h. The mixture was then filter through Celite and the solvent was removed under reduced pressure. The resulting crude residual was recrystallised from ethyl acetate/hexane to give a gray solid 3-((naphthalene-1-yloxy)carbonyl)benzoic acid (**11a**) (0.11 g, 75% yield). Mp: 220–222 °C (ethyl acetate/hexane). ^{1}H NMR (300 MHz, DMSO): δ 13.51 (s, 1H), 8.77 (s, 1H), 8.51 (d, J = 8.1 Hz, 1H), 8.49 (d, J = 8.1 Hz, 1H), 8.07 (d, J = 7.2 Hz, 1H), 7.87 (m, 3H), 7.60 (m, 4H); ^{13}C NMR (100 MHz, DMSO) δ_C: 167.3, 165.1, 147.2, 135.6, 135.1, 135.0, 132.6, 131.4, 130.7, 130.1, 129.0, 127.9, 127.7, 127.2, 127.2, 126.7, 121.8, 119.6; HRMS (ESI): calcd for $C_{18}H_{12}NaO_4$ [(M+Na)$^+$] 315.0628, found 315.0629.

3-(Naphhthalen-1-ylcarbmoyl)benzoic acid (11b). The compound **11b** was prepared according to the procedure described above as a gray solid in 80% yield. Mp: 251–254 °C (ethyl acetate/hexane). ^{1}H NMR (300 MHz, DMSO): δ 13.29 (s, 1H), 10.67 (s, 1H), 8.67 (s, 1H), 8.33 (d, J = 7.8 Hz, 1H), 8.18 (d, J = 7.8 Hz, 1H), 7.99 (m, 2H), 7.87 (d, J = 6.3 Hz, 1H), 7.71 (t, J = 7.8 Hz, 1H), 7.59 (m, 4H); HRMS (ESI): calcd for $C_{18}H_{13}NO_3$ [(M+H)$^+$] 292.0968, found 292.0967.

3-(Phenylcarbamoyl)benzoic acid (11c). The compound **11c** was prepared according to the procedure described above as a gray solid in 70% yield. Mp: 258–260 °C (ethyl acetate/hexane). ^{1}H NMR (300 MHz, DMSO): δ 13.31 (s, 1H), 10.47 (s, 1H), 8.53 (s, 1H), 8.18 (m, 2H), 7.80 (d, J = 7.5 Hz, 2H), 7.70 (t, J = 7.8 Hz, 1H), 7.37 (t, J = 7.8 Hz, 2H), 7.12 (t, J = 7.5 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ_C: 167.8, 165.7, 139.9, 136.3, 133.1, 132.9, 132.0, 129.8, 129.6 × 2, 129.4, 124.8, 121.4 × 2; HRMS (ESI): calcd for $C_{14}H_{12}NO_3$ [(M+H)$^+$] 242.0812, found 242.0811.

4.2. Inhibition assay of 5-LOX in cell-free system

According to our reported protocol, a fluorescence-based enzyme assay was used to test the target compounds' inhibition to human 5-LOX. In 96-well microtiter plates (Costar, Corning Inc.), the compounds were incubated with enzyme and H2DCFDA (10 μM) in assay buffer (50 mM Tris–HCl, 0.2 mM ATP, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM $CaCl_2$) for 10 min. After preincubation, the reaction was initiated by the addition of AA (25 μM) and quickly monitored by excitation at 500 nm and emission at 520 nm using a multiwall fluorometer (Synergy4, BIOTEK). A kinetics mode program was used to record the fluorescence signals and the initial reaction rate was used to calculate the inhibition value. IC_{50} was calculated with a four parameter logistical model of the GraphPad using inhibition value at different inhibitor concentrations.

4.3. Measuring LTB_4 generation in human whole blood

Fresh blood from healthy volunteer was collected into Vacuette tubes (Greiner, containing lithium heparin) and was used within 1 h after collection. Fresh blood were aliquot into 200 μL in wells of 96-well plate preloaded with either 1 μL of DMSO (total activity) or 1 μL of test compounds DMSO solutions at different final concentrations. The plate was vortexed gently and incubated for 15 min at 37 °C. 1 μL calcium ionophore A23187 (12.5 mM in DMSO) was added and the incubation was continued under the same conditions for an additional 30 min. The plate was buried in ice-bath for 5 min to terminate the incubation. The samples were transferred to 200 μL EP tubes and plasma was collected after centrifugation (3000 rpm, 5 min, 4 °C). Plasma was kept at −80 °C until it was measured LTB_4 production using an LTB_4 ELISA kit (Cayman Chemical) according to the manufacturer's instructions.

4.4. Molecular docking

Molecular docking with flexible ligands and rigid receptor was performed with the program AutoDock 4.00.[19] Lamarckian genetic algorithm was used with following parameters: number of individuals in population, 300; maximum number of energy evaluations, 25,000,000; maximum of generations, 27,000; number of runs, 100. Docked conformations of the 100 runs were clustered with a rms tolerance of 2.0 Å, and the lowest energy conformation from the largest cluster was taken as docking result. The crystal structure of human 5-LOX is used in molecular docking (PDB ID: 3O8Y).[20]

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.03.008. These data include MOL files and InChiKeys of the most important compounds described in this article.

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