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Syntheses of diacyltanshinol derivatives and their suppressive effects on macrophage foam cell formation by reducing oxidized LDL uptake

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ABSTRACT

A series of diacyltanshinol derivatives were synthesized by esterifying the corresponding *o*-hydroquinones of tanshinones. The suppressive effects of the synthesized compounds on oxidized low-density lipoprotein (oxLDL) uptake and oxLDL-induced macrophage-derived foam cell formation were evaluated. Our results indicated that the nicotinate derivatives **1a** and **2a**, modified from tanshinone IIA and cryptotanshinone, showed stronger suppressive activity on oxLDL uptake and the resultant foam cell formation relative to tanshinone IIA. Western Blot analysis indicated that derivatives **1a** and **2a** could dose-dependently inhibit the expression of oxLDL-induced LOX-1, implying that the suppressive effects of **1a** and **2a** on oxLDL uptake and foam cell formation could be at least partially attributed to the inhibition of LOX-1 expression in macrophages.

1. Introduction

Atherosclerosis, the leading cause of morbidity and mortality [1], is a systemic cardiovascular disease characterized by the accumulation of lipids, extracellular matrix, and various inflammatory cells in the subendothelium, resulting in the narrowing of the vessel lumen [2]. In the early stages of atherosclerosis, the uptake of modified low-density lipoprotein (LDL), such as oxidized LDL (oxLDL), by scavenger receptors (SRs) causes the lipoproteins accumulation in macrophages, resulting in the formation of foam cell [3–5]. It has been suggested that the down-regulation of SRs expression and activity may inhibit the macrophage-derived foam cell formation, and plays an important role in the prevention and treatment of atherogenesis [6,7].

Tanshinones are major lipophilic pharmacologically active compounds isolated from the *Salvia miltiorrhiza* Bunge, a well-known traditional Chinese medicinal herb that has been widely used in Asian countries for the prevention and management of cardiovascular diseases including atherosclerosis [8]. Tanshinone IIA is one of the major tanshinones that have been intensively investigated in recent years. It was recently reported that tanshinone IIA could down-regulate the expression of SR lectin-like oxLDL receptor-1 (LOX-1) at both the mRNA and protein level [9], and thereby suppress oxLDL uptake and prevent atherogenesis in several animal models [10–12]. However, the efficacy of tanshinone IIA is limited by its present poor oral bioavailability due to the relatively high lipophilic properties (the absolute oral bioavailability of tanshinone IIA is only about 3% in rats) [8]. To improve the bioavailability of tanshinones, the chemical modification of tanshinones has attracted much attention in the past decade. Tian et al. found that the acetylated product of the corresponding *o*-hydroquinone of tanshinone IIA showed improved aqueous solubility and activity [13]. However, in our screening study, the acetylated product showed lower suppressive activity against oxLDL uptake than the parent compound tanshinone IIA.

Nicotinic acid is clinically used to regulate lipid metabolism for more than 50 years [14]. Nicotinic acid can decrease LDL-cholesterol, total cholesterol and lipoprotein levels, and increase HDL-cholesterol in plasma [14–18]. We designed to acylate the *o*-hydroquinones of tanshinones with nicotinic anhydride derivatives to obtain the potential protective agents against atherosclerosis. Here, we report the syntheses of the acylated derivatives, named as diacyltanshinol derivatives, and their suppressive effects on oxLDL uptake in macrophages and formation of macrophage-derived foam cells.

Abbreviations: LDL, low-density lipoprotein; oxLDL, oxidized low-density lipoprotein; HDL, high-density lipoprotein; SRs, scavenger receptors; LOX-1, lectin-like oxidized low-density lipoprotein receptor 1; BSA, Bovine Serum Albumin; DiI, l,l′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; DAPI, 4′,6-diamidino-2-phenylindole; DMAP, 4-dimethylaminopyridine.

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2. Results and discussion

2.1. Chemistry

As shown in Scheme 1, Tanshinone IIA (**1**) was Pd/C catalytically hydrogenated into the corresponding *o*-hydroquinone intermediate, which was subsequently acylated with excess nicotinic anhydride to give the colorless solid **1a** in 70%. HRMS indicated that compound **1a** had a formula $C_{31}H_{26}N_2O_5$. In the ^{1}H NMR spectrum, the three signals at 8.17 (d) ppm, 7.63 (d) ppm and 7.52 (q) ppm and the six signals between 3.6 ppm and 1.36 ppm indicated the presence of tanshinone IIA skeleton. ^{13}C NMR spectrum and the colorless appearance implied the disappearance of *o*-quinone structure. ^{1}H NMR and ^{13}C NMR spectra indicated that compound **1a** contained two nicotinoyl groups. All spectra of compound **1a** proved its 1,6,6-trimethyl-6,7,8,9-tetrahydrophenanthro[1,2-*b*] furan-10,11-diyl dinicotinate structure.

To assess the effect of pyridinyl fragment and tanshinone scaffold on the activity, nicotinic anhydride analogues and tanshinone analogues, including tanshinone IIA, cryptotanshinone (**2**) and tanshinone I (**3**), were used as materials to give the target compounds **1a–d**, **2a–d** and **3a** (Scheme 1), respectively. Since compound **3** and **3a** showed high cytotoxicity against macrophage cells, only one compound, **3a**, derived from tanshinone I was synthesized. All diacyltanshinol derivatives were characterized by HRMS, ^{1}H NMR and ^{13}C NMR spectra.

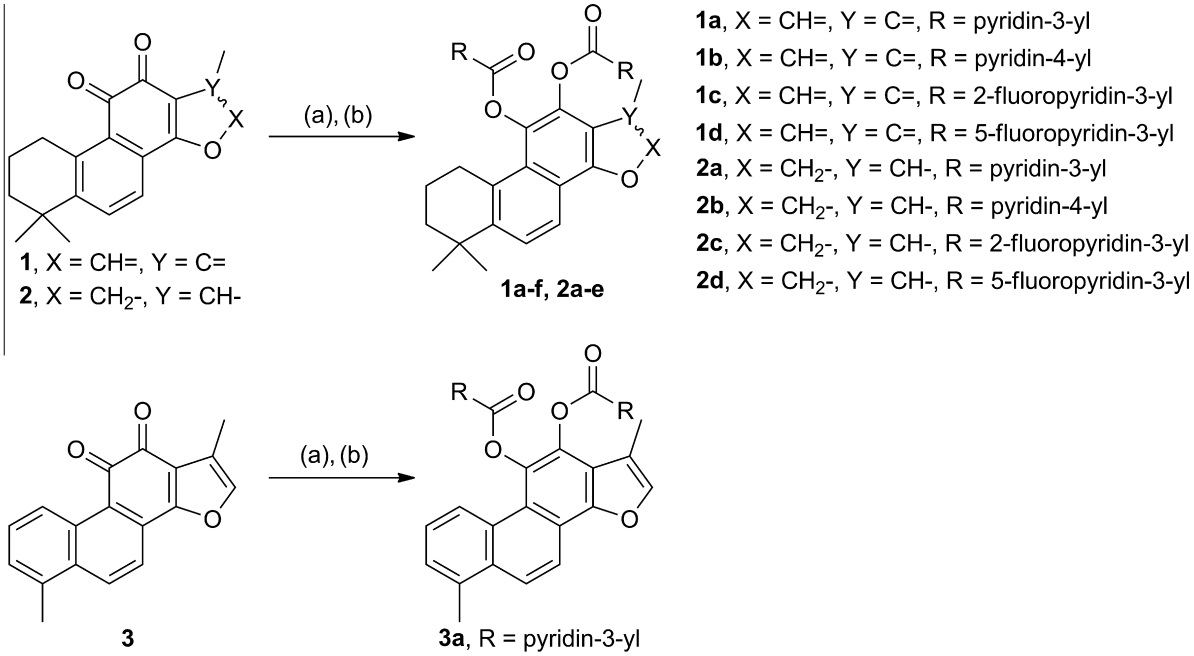
2.2. Biological activities

The diacyltanshinol derivatives at 5 μM concentration were pre-screened for their suppressive activity on oxLDL-induced macrophage-derived foam cell formation by the oil red O staining assay (Fig. S1, Supporting Information). The results indicated that the lipid accumulation was attenuated by pretreatment with compounds **1a–1e**. Although cryptotanshinone (**2**) showed no effects, its derivatives **2a–2d** could suppress lipid accumulation to some content, implying that it might be the diacyltanshinol pharmacophore to exert the suppressive effect. The derivatives from tanshinone IIA generally showed more potency than that from cryptotanshinone, as well as the substituent of fluorine atom on the pyridinyl ring did not improve their potency. Among them, compounds **1a** and **2a** showed stronger activity. To confirm their suppressive effects, the effects in various concentrations of compounds **1a** and **2a** were studied. As shown in Fig. 1, lipid accumulation in RAW264.7 macrophages was observed and the cells displayed typical foamy appearances after incubation with oxLDL for 24 h. After treatment with compounds **1**, **1a** and **2a** for 12 h, lipid accumulation and foam cell formation was attenuated dose-dependently.

To further confirm and quantify the effects of compounds on lipid accumulation and foam cell formation, DiI-oxLDL uptake assays of compounds **1a** and **2a** were conducted. The cells were pretreated with compounds followed by incubation with DiI-oxLDL (20 μg/ml) for 6 h. Cellular uptake of DiI-oxLDL was detected with confocal microscopy, and quantified by flow cytometry. As shown in Fig. 2, pretreatment with compounds dose-dependently inhibited the uptake of DiI-oxLDL in RAW264.7 macrophages, and their suppressive effects were significantly higher than that of compound **1** at 10 μM concentration. Compounds **1a** and **2a** (10 μM) suppressed DiI-oxLDL uptake by 91% and 87%, respectively.

LOX-1 is one of the crucial types of SRs involved in the uptake of modified LDL into macrophages [19]. Furthermore, the up regulation of LOX-1 has been demonstrated to be closely associated with the macrophage-derived foam cell formation and subsequent development of atherosclerosis. Fig. 3 showed that, similar to compound **1** [9], compounds **1a** and **2a** could markedly reduce oxLDL-induced LOX-1 protein expression dose-dependently. Although their suppressive effects on LOX-1 protein expression were similar to that of tanshinone IIA, compounds **1a** and **2a** showed significantly stronger suppressive activity than tanshinone IIA in oxLDL uptake, implying that their suppression effects on LOX-1 expression might partially contribute to the decrease in oxLDL uptake.

To evaluate the cytotoxicity of diacyltanshinol derivatives and their protection on the oxLDL-induced cytotoxicity in RAW264.7



Condition and reagents: (a) Pd/C, H_2, AcOEt, 10 min; (b) anhydrides, DMAP, DMF, refluxing, 4 h.

Scheme 1. The syntheses of diacyltanshinol derivatives.

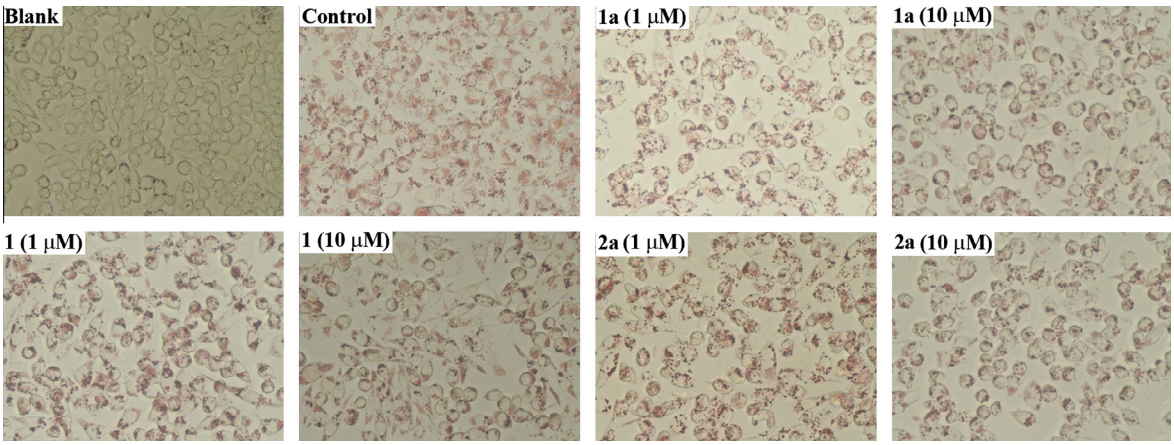


Fig. 1. Effects on oxLDL-induced lipid accumulation and foam cell formation in RAW264.7 macrophages. The macrophages were pre-incubated with indicated compounds for 12 h, followed with the treatment of oxLDL (50 μg/ml) for 24 h. The lipid laden macrophages (foam cells) were stained with oil red O. Blank: normal cells (without compound and oxLDL treatment). Control: foam cells (only with oxLDL treatment).

macrophages, MTT assay was performed. The results indicated that the IC_{50} values of the compounds **1a–d** and **2a–d** (6.25–50 μM) were more than 50 μM after incubation for 48 h. At the concentration of 10 μM, compounds **1**, **1a** and **2a** showed low cytotoxicity with the percentage of viable cells >84% by pretreatment for 12 h (Fig. 4). The oxLDL could significantly reduce the viability of RAW264.7 macrophages by 70%. However, compounds **1**, **1a** and **2a** could increase the macrophage cell viability up to 55%, 43% and 60%, respectively. This result indicated that compounds **1**, **1a** and **2a** had protective effect against oxLDL-induced cytotoxicity in RAW264.7 macrophages.

3. Conclusion

In summary, we synthesized a series of diacyltanshinol derivatives, which potently suppress oxLDL uptake and oxLDL-induced macrophage-derived foam cell formation to some content. Among these compounds, **1a** and **2a** showed higher efficacy than tanshinone IIA. Our experiments also revealed that their effects might be partially attributed to the inhibition of LOX-1 expression in macrophages, underscoring their potential protective effects against atherosclerosis. Pharmacological evaluation of the anti-atherosclerotic potential and underlying mechanisms of these compounds are underway in our laboratory.

4. Experimental section

4.1. Chemistry

All solvents were obtained from commercial suppliers and used without further purification unless mentioned. Niacin analogues were purchased from Aladdin Industrial Inc. Pd/C (10%) was purchased from Acros Organics. Tanshinone IIA (**1**), Cryptotanshinone (**2**) and Tanshinone I (**3**) were isolated from the Chinese medicinal herb, *S. miltiorrhiza* Bunge by our lab [20]. Their purities analyzed by HPLC are 95% (**1**), 96% (**2**) and 96% (**3**), respectively. Silica gel column chromatography was carried out with silica gel (200–300 mesh). Melting points were measured in open capillary tubes on a SRS-OptiMelt automated melting point instrument without correction. Nuclear magnetic resonance spectra were recorded on Bruker AVANCE III 400 MHz spectrometer using tetramethylsilane as the internal standard. Mass spectra were analyzed on an Agilent LC-MS 6120 (Quadrupole LC-MS) mass spectrometer. High-resolution mass spectra (HRMS) were analyzed on a SHIMADZU LCMS-IT-TOF mass spectrometer. The purities of the tested compounds were more than 95%, analyzed by High Performance Liquid Chromatography (HPLC) on SHIMADZU LC-20A equipped with a reverse-phase Waters Sunfire C_{18} column, 5 μm, 4.6 × 250 mm.

4.1.1. Synthesis of nicotinic anhydride derivatives

The anhydride derivatives were prepared according to the reported method with slight modification [21]. To a 500 ml three-necked round-bottomed flask, nicotinic acid derivative (0.081 mol) and new distilled toluene (275 ml) were added. To remove traces of moisture introduced with the nicotinic acid derivative, the mixture was heated until about 75 ml of toluene had distilled. The mixture was cooled to 0–5 °C with an ice-water bath, and added with new distilled triethylamine (8.65 g, 0.086 mol). To the resulting clear solution, a solution of thionyl chloride (0.043 mol) in toluene (200 ml) was added dropwise over a period to make sure the temperature of the reaction mixture was lower than 7 °C. White precipitate appeared along with the addition. After the addition of thionyl chloride solution, the resulting suspension was stirred at room temperature for 45 min, and then heated to about 60 °C. The precipitate was removed by filtration under slightly reduced pressure while hot. The precipitate was washed with warm toluene (60 °C, 25 ml × 3). The combined toluene solution was concentrated by reduced pressure. The resulting residue was simmered with new distilled warm toluene (75 ml). The precipitate was removed with filtration. The filtrate was allowed to stand for 3 h at room temperature. The white crystalline product was collected with filtration and washed with cold new distilled toluene (4 ml × 2). The dry anhydride product was used immediately.

4.1.2. General syntheses of diacyltanshinol derivatives

To a solution of tanshinone (0.60 g) in ethyl acetate (150 ml), palladium charcoal (10%, 0.12 g) was added. The mixture was stirred at room temperature for 10 min in a hydrogen atmosphere. And then, a solution of excess anhydride (5 equivalents) and DMAP (0.80 mg, 6.6 mmol) in DMF (20 ml) was dropwise added into the reaction solution. The mixture solution was stirred and refluxed for 4 h. The reaction solution was filtered. The filtrate was washed with water (50 ml × 4), saturated aqueous $NaHCO_3$ (50 ml) and water (50 ml), respectively, and dried over anhydride Na_2SO_4. Solvent was evaporated under vacuum. The residue was purified by silica gel column chromatography using ethyl acetate/petroleum ether (1:1–1:9) mixture as eluent.

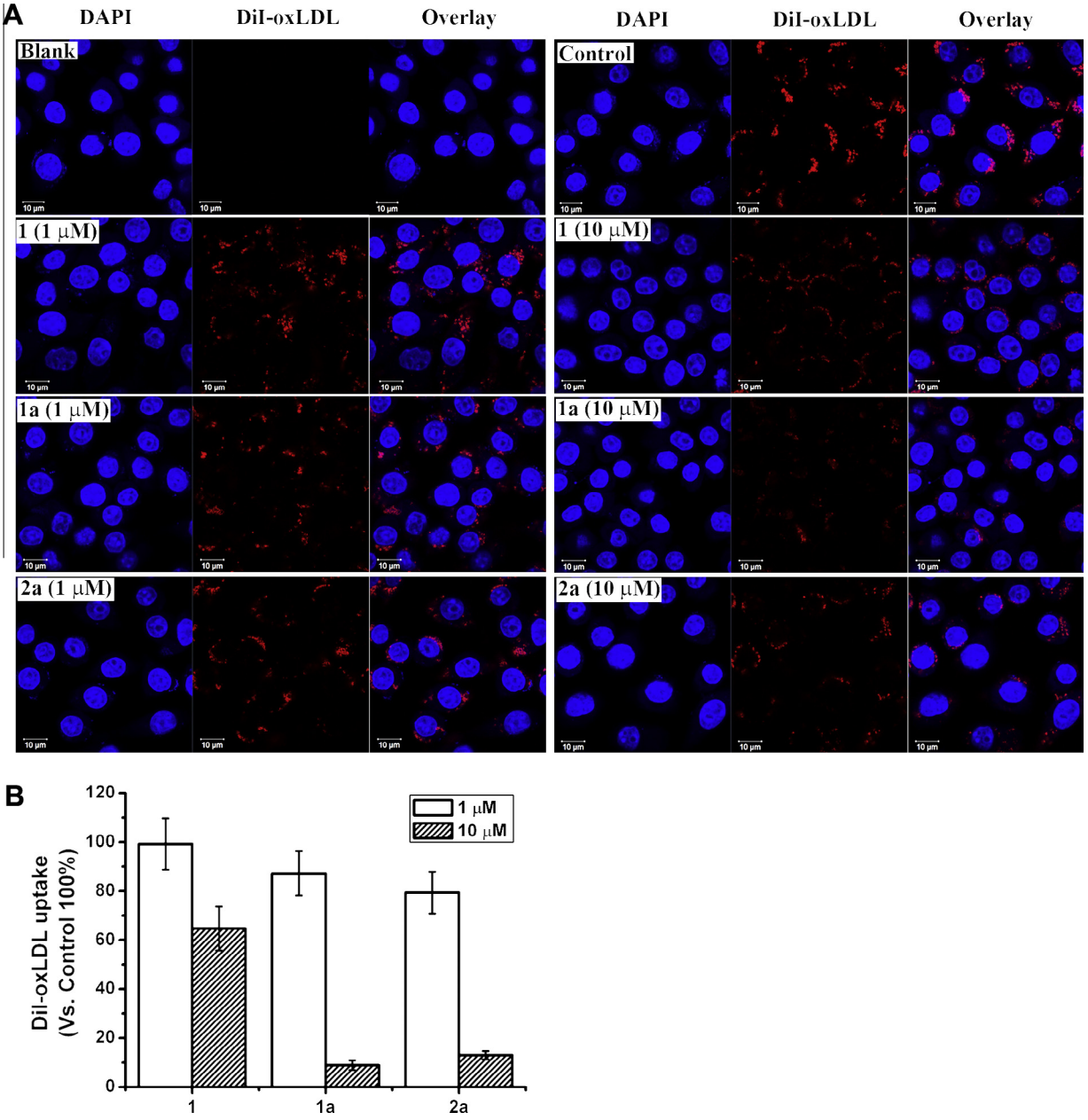


Fig. 2. (A) Effects of compounds **1**, **1a** and **2a** on DiI-oxLDL uptake by RAW264.7 macrophages. The cells were serum starved for 12 h and pre-incubated with indicated compounds for 12 h, and then treated with DiI-oxLDL for additional 6 h. Subsequently, cells were fixed and analyzed by confocal microscopy. Blank: cells incubated without DiI-oxLDL. Control: cells incubated with DiI-oxLDL and vehicle (0.1% DMSO). (B) Quantitation of DiI-oxLDL uptake by flow cytometry analysis from panel A.

*4.1.2.1. 1,6,6-trimethyl-6,7,8,9-tetrahydrophenanthro[1,2-b]furan-10, 11-diyl dinicotinate (**1a**).* Colorless solid, 0.72 g, 70% yield. Mp = 189.6–193.0 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.34 (s, 1H), 9.27 (s, 1H), 8.78–8.75 (m, 2H), 8.40 (d, J = 7.6 Hz, 1H), 8.33 (d, J = 8.0 Hz, 1H), 8.17 (d, J = 8.8 Hz, 1H), 7.63 (d, J = 8.8 Hz, 1H), 7.52 (q, J = 1.2 Hz, 1H), 7.39 (dd, J = 7.6, 4.8 Hz, 1H), 7.34 (dd, J = 8.0, 4.8 Hz, 1H), 3.59–3.50 (m, 1H), 2.95–2.80 (m, 1H), 2.18 (d, J = 1.2 Hz, 3H), 1.75–1.70 (m, 2H), 1.66–1.60 (m, 2H), 1.36 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.7, 163.3, 154.2, 154.1, 151.2, 151.1, 150.6, 144.6, 141.9, 137.7, 137.5, 134.8, 134.6, 131.5, 126.8, 125.0, 124.4, 124.3, 123.7, 123.6, 119.6, 118.5, 117.5, 115.5, 38.1, 34.9, 31.9, 30.1, 20.0, 9.0. ESI-MS m/z: 507.3 $[M+H]^+$. HRMS (ESI) m/z: calcd for $C_{31}H_{27}N_2O_5$ 507.1914 $[M+H]^+$; found: 507.1933.

*4.1.2.2. 1,6,6-trimethyl-6,7,8,9-tetrahydrophenanthro[1,2-b]furan-10, 11-diyl diisonicotinate (**1b**).* Colorless solid, 0.60 g, 58% yield. Mp = 184.5–187.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.79 (d, J = 5.6 Hz, 2H), 8.74 (d, J = 5.6 Hz, 2H), 8.18 (d, J = 8.8 Hz, 1H), 7.93 (dd, J = 4.4, 1.6 Hz, 2H), 7.86 (dd, J = 4.4, 1.6 Hz, 2H), 7.64 (d, J = 8.8 Hz, 1H), 7.52 (q, J = 1.2 Hz, 1H), 3.59–3.46 (m, 1H), 2.86–2.76 (m, 1H), 2.17 (d, J = 1.2 Hz, 3H), 1.74–1.68 (m, 2H), 1.65–1.60 (m, 2H), 1.36 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.7, 163.3, 150.99, 150.96, 150.7, 144.8, 142.1, 136.1, 135.5, 131.4, 127.0, 124.2, 123.0, 122.9, 119.7, 118.6, 117.4, 115.5, 38.1, 35.0, 31.9, 30.2, 20.0, 9.0. ESI-MS m/z: 507.2 $[M+H]^+$; HRMS (ESI) m/z: calcd for $C_{31}H_{27}N_2O_5$ 507.1914 $[M+H]^+$; found: 507.1924.

*4.1.2.3. 1,6,6-trimethyl-6,7,8,9-tetrahydrophenanthro[1,2-b]furan-10, 11-diyl bis(2-fluoronicotinate) (**1c**).* Colorless solid, 0.72 g, 65% yield. Mp = 130.2–132.4 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (ddd, J = 9.4, 7.6, 2.0 Hz, 1H), 8.48 (ddd, J = 9.4, 7.6, 2.0 Hz, 1H), 8.43–8.39 (m, 2H), 8.16 (d, J = 8.8 Hz, 1H), 7.62 (d, J = 8.8 Hz, 1H),

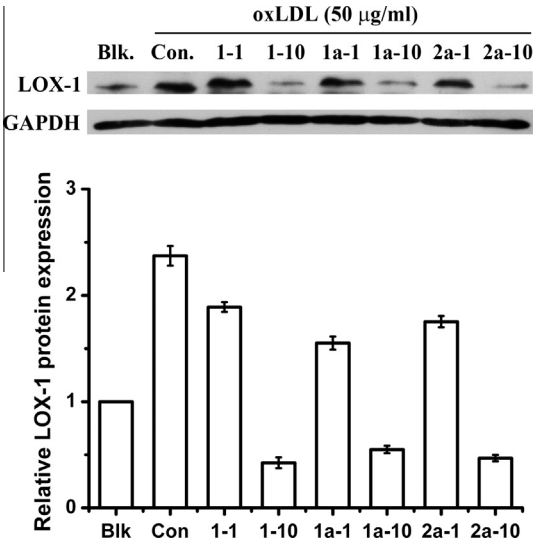


Fig. 3. Effects of compounds **1**, **1a** and **2a** on LOX-1 protein expression. Blk.: normal cells (without compound and oxLDL treatment). Con.: cells treated with oxLDL.

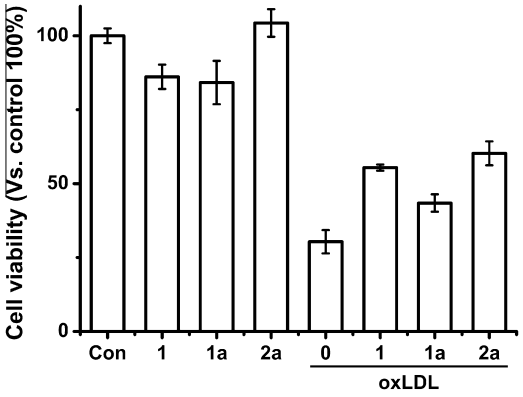


Fig. 4. Effects of compounds **1**, **1a** and **2a** on RAW264.7 macrophage cell viability in the absence or presence of oxLDL. In the absence of oxLDL, cells were serum starved for 12 h and pre-incubated with indicated compounds for 12 h. In the presence of oxLDL, cells were serum starved for 12 h and pre-incubated with compounds for 12 h, and then treated with oxLDL (50 μg/ml) for 24 h. The cell viability was measured by MTT assay.

7.51 (q, J = 1.2 Hz, 1H), 7.35–7.28 (m, 2H), 3.64–3.46 (m, 1H), 2.98–2.74 (m, 1H), 2.21 (d, J = 1.2 Hz, 1H), 1.76–1.70 (m, 2H), 1.67–1.62 (m, 2H), 1.36 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.7 (d, J^1_{C-F} = 249.4 Hz), 162.2 (d, J^1_{C-F} = 249.3 Hz), 161.6 (d, J^3_{C-F} = 8.5 Hz), 161.3 (d, J^3_{C-F} = 8.5 Hz), 152.7 (d, J^3_{C-F} = 15.3 Hz), 152.6 (d, J^3_{C-F} = 15.3 Hz), 150.6, 144.6, 143.7, 143.5, 142.0, 134.7, 134.4, 131.5, 126.9, 124.2, 121.82 (d, J^3_{C-F} = 4.3 Hz), 121.78 (d, J^3_{C-F} = 4.4 Hz), 119.6, 118.5, 117.4, 115.5, 112.8 (d, J^2_{C-F} = 24.3 Hz), 112.2 (d, J^2_{C-F} = 24.8 Hz), 38.1, 34.9, 31.9, 29.9, 20.0, 8.9. ESI-MS m/z: 543.2 $[M+H]^+$. HRMS (ESI) m/z: calcd for $C_{31}H_{25}F_2N_2O_5$ 543.1726 $[M+H]^+$; found: 543.1726.

*4.1.2.4. 1,6,6-trimethyl-6,7,8,9-tetrahydrophenanthro[1,2-b]furan-10,11-diyl bis(5-fluoronicotinate) (**1d**).* Colorless solid, 0.83 g, 75% yield. Mp = 171.4–172.7 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.10 (s, 1H), 9.03 (s, 1H), 8.60 (d, J = 2.8 Hz, 1H), 8.59 (d,J = 2.8 Hz, 1H), 8.10 (d, J = 8.8 Hz, 1H), 8.02 (ddd, J = 8.4, 2.8, 1.6 Hz, 1H), 7.97 (ddd, J = 8.4, 2.8, 1.6 Hz, 1H), 7.57 (d, J = 8.8 Hz, 1H), 7.45 (q, J = 1.2 Hz, 1H), 3.52–3.39 (m, 1H), 2.81–2.71 (m, 1H), 2.10 (d, J = 0.8 Hz, 3H), 1.68–1.61 (m, 2H), 1.58–1.55 (m, 2H), 1.29 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.6 (d, J^4_{C-F} = 2.1 Hz), 162.3 (d, J^4_{C-F} = 2.1 Hz), 159.1 (d, J^1_{C-F} = 258.5 Hz), 159.0 (d, J^1_{C-F} = 257.1 Hz), 150.7, 147.0 (d, J^4_{C-F} = 4.3 Hz), 146.8 (d, J^4_{C-F} = 4.2 Hz), 144.9, 143.4 (d, J^2_{C-F} = 22.9 Hz), 143.3 (d, J^2_{C-F} = 22.9 Hz), 142.1, 134.6, 134.4, 131.3, 127.0, 126.2 (d, J^3_{C-F} = 3.7 Hz), 125.6 (d, J^3_{C-F} = 3.7 Hz), 124.1 (d, J^2_{C-F} = 17.5 Hz), 123.9 (d, J^2_{C-F} = 19.6 Hz), 123.8, 119.6, 118.6, 117.3, 115.4, 38.1, 34.9, 31.9, 30.2, 20.0, 9.1.ESI-MS m/z: 543.2 $[M+H]^+$. HRMS (ESI) m/z: calcd for $C_{31}H_{25}F_2N_2O_5$ 543.1726 $[M+H]^+$; found: 543.1751.

*4.1.2.5. 1,6,6-trimethyl-1,2,6,7,8,9-hexahydrophenanthro[1,2-b]furan-10,11-diyl dinicotinate (**2a**).* Colorless solid, 0.79 g, 77% yield. Mp = 165.4–167.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.31 (s, br, 1H), 9.21 (d, J = 1.2 Hz, 1H), 8.76 (d, J = 3.6 Hz, 1H), 8.73 (d, J = 3.6 Hz, 1H), 8.37 (s, br, 1H), 8.28 (d, J = 7.6 Hz, 1H), 7.82 (d, J = 8.8 Hz, 1H), 7.48 (d, J = 8.8 Hz, 1H), 7.37 (dd, J = 7.4, 5.0 Hz, 1H), 7.31 (dd, J = 8.0, 4.8 Hz, 1H), 4.92 (t, J = 9.0 Hz, 1H), 4.36 (dd, J = 8.8, 6.4 Hz), 3.92–3.72 (m, 1H), 3.52–3.40 (m, 1H), 2.86–2.69 (m, 1H), 1.72–1.66 (m, 2H), 1.64–1.58 (m, 2H), 1.33–1.26 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.8, 162.5, 155.2, 154.1, 153.9, 151.1, 151.0, 145.3 137.7, 137.5, 132.0, 130.6, 127.3, 125.7, 125.1, 124.5, 123.6, 123.5, 119.9, 118.3, 118.2, 79.6, 38.1, 37.0, 34.9, 31.72, 31.68, 30.0, 19.9, 19.1. ESI-MS m/z: 509.2 $[M+H]^+$. HRMS (ESI) m/z: calcd for $C_{31}H_{29}N_2O_5$ 509.2071 $[M+H]^+$; found: 509.2094.

*4.1.2.6. 1,6,6-trimethyl-1,2,6,7,8,9-hexahydrophenanthro[1,2-b]furan-10,11-diyl diisonicotinate (**2b**).* Colorless solid, 0.72 g, 70% yield. Mp = 145.6–148.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.77 (d, J = 5.6 Hz, 2H), 8.71 (d, J = 6.0 Hz, 2H), 7.89 (s, br, 2H), 7.84–7.80 (m, 3H), 7.48 (d, J = 8.8 Hz, 1H), 4.93 (t, J = 8.8 Hz, 1H), 4.36 (dd, J = 8.8, 6.4 Hz, 1H), 3.90–3.72 (m, 1H), 3.52–3.38 (m, 1H), 2.79–2.69 (m, 1H), 1.72–1.59 (m, 7H), 1.33 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.8, 162.5, 155.3, 151.0, 150.9, 145.5, 137.5, 136.1, 135.5, 131.8, 130.9, 130.6, 128.9, 127.2, 125.9, 123.0, 122.8, 119.9, 118.4, 118.0, 79.6, 38.1, 37.0, 34.9, 31.72, 31.67, 30.00, 19.89, 19.03. ESI-MS m/z: 509.2 $[M+H]^+$; HRMS (ESI) m/z: calcd for $C_{31}H_{29}N_2O_5$ 509.2071 $[M+H]^+$; found: 509.2082.

*4.1.2.7. 1,6,6-trimethyl-1,2,6,7,8,9-hexahydrophenanthro[1,2-b]furan-10,11-diyl bis(2-fluoronicotinate) (**2c**).* Colorless solid, 0.78 g, 70% yield. Mp = 125.7–127.2 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.53–8.49 (m, 1H), 8.46–8.36 (m, 3H), 7.81 (d, J = 8.8 Hz, 1H), 7.47 (d, J = 9.2 Hz, 1H), 7.34–7.27 (m, 2H), 4.92 (t, J = 8.8 Hz, 1H), 4.36 (dd, J = 8.6, 6.2 Hz, 1H), 3.92–3.76 (m, 1H), 3.60–3.36 (m, 1H), 2.91–2.69 (m, 1H), 1.74–1.67 (m, 2H), 1.64–1.58 (m, 2H), 1.33 (s, br, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.7 (d, J^1_{C-F} = 249.6 Hz), 161.5 (d, J^1_{C-F} = 249.0 Hz), 161.7 (d, J^3_{C-F} = 8.7 Hz), 160.3 (d, J^3_{C-F} = 10.6 Hz), 155.3, 152.6 (d, J^3_{C-F} = 15.5 Hz), 152.5 (d, J^3_{C-F} = 15.4 Hz), 145.3, 143.6, 143.4, 137.4, 131.9, 130.6, 127.3, 125.8, 121.7 (d, J^3_{C-F} = 5.5 Hz), 121.6 (d, J^3_{C-F} = 5.6 Hz), 119.9, 118.4, 118.1, 112.9 (d, J^2_{C-F} = 24.2 Hz), 112.4 (d, J^2_{C-F} = 25.1 Hz), 79.6, 38.2, 37.1, 34.9, 31.8, 31.7, 29.7, 19.9, 19.0. ESI-MS m/z: 545.2 $[M+H]^+$. HRMS (ESI) m/z: calcd for $C_{31}H_{27}F_2N_2O_5$ 545.1883 $[M+H]^+$; found: 545.1897.

*4.1.2.8. 1,6,6-trimethyl-1,2,6,7,8,9-hexahydrophenanthro[1,2-b]furan-10,11-diyl bis(5-fluoronicotinate) (**2d**).* Colorless solid, 0.87 g, 78% yield. Mp = 148.6–150.8 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.14 (s, br, 1H), 9.04 (s, br, 1H), 8.67 (d, J = 2.8 Hz, 1H), 8.64 (d, J = 2.8 Hz, 1H), 8.11–8.03 (m, 1H), 7.99 (d, J = 7.6 Hz, 1H), 7.83 (d, J = 8.8 Hz, 1H), 7.49 (d, J = 8.8 Hz, 1H), 4.94 (t, J = 9.0 Hz, 1H), 4.37 (dd, J = 8.4, 6.4 Hz, 1H), 3.94–3.77 (m, 1H), 3.49–3.37 (m, 1H), 2.84–2.68 (m, 1H), 1.73–1.67 (m, 2H), 1.64–1.58 (m, 2H), 1.33 (s, br, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.8 (d, J^4_{C-F} = 4.8 Hz), 161.5 (d, J^4_{C-F} = 1.4 Hz), 159.0 (d, J^1_{C-F} = 258.8 Hz), 158.9 (d, J^1_{C-F} = 258.7 Hz), 155.4, 146.9 (d, J^4_{C-F} = 4.2 Hz), 146.8 (d,

J^4_{C-F} = 4.1 Hz), 145.6, 143.24 (d, J^2_{C-F} = 23.2 Hz), 143.15 (d, J^2_{C-F} = 23.1 Hz), 137.4, 131.7, 130.5, 127.2, 126.2 (d, J^3_{C-F} = 2.6 Hz), 125.9, 125.7 (d, J^3_{C-F} = 4.7 Hz), 124.0 (d, J^2_{C-F} = 19.3 Hz), 123.8 (d, J^2_{C-F} = 19.7 Hz), 119.9, 118.4, 118.0, 79.6, 38.1, 37.0, 34.9, 31.73, 31.68, 30.1, 19.9, 19.1. ESI-MS *m/z*: 545.2 $[M+H]^+$. HRMS (ESI) *m/z*: calcd for $C_{31}H_{27}F_2N_2O_5$ 545.1883 $[M+H]^+$; found: 545.1895.

*4.1.2.9. 1,6-dimethylphenanthro[1,2-b]furan-10,11-diyl dinicotinate (**3a**).* Colorless solid, 0.76 g, 72% yield. Mp = 179.3–180.2 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.45 (d, J = 1.6 Hz, 1H), 9.32(d, J = 1.6 Hz, 1H), 8.90 (d, J = 8.8 Hz, 1H), 8.83 (dd, J = 5.0, 1.4 Hz, 1H), 8.79 (dd, J = 4.8, 1.6 Hz, 1H), 8.48 (dt, J = 8.0, 2.0 Hz, 1H), 8.39 (dt, J = 8.0, 2.0 Hz, 1H), 8.37 (d, J = 9.2 Hz, 1H), 8.17 (d, J = 9.2 Hz, 1H), 7.63 (q, J = 1.2 Hz, 1H), 7.44–7.36 (m, 3H), 7.32 (dd, J = 8.4, 7.2 Hz, 1H), 2.79 (s, 3H), 2.26 (d, J = 1.2 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.5, 163.4, 154.4, 151.3, 151.2, 150.6, 142.8, 137.7, 137.6, 135.8, 135.3, 134.8, 131.6, 129.3, 128.0, 126.8, 124.9, 124.5, 124.4, 123.75, 123.69, 123.66, 121.1, 119.3, 118.8, 118.6, 115.6, 20.5, 9.1. ESI-MS *m/z*: 489.2 $[M+H]^+$. HRMS (ESI) *m/z*: calcd for $C_{30}H_{21}N_2O_5$ 489.1445 $[M+H]^+$; found: 489.1466.

4.2. Biological testing

4.2.1. Cell culture and MTT assay

RAW 264.7 cells were cultured in 96-well plates (Costar, Corning, NY) at a density of 5×10^4 cells/ml in RPMI 1640 medium (with 100 IU/ml of penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% (v/v) heat-inactivated fetal bovine serum), incubated in a humidified atmosphere of 5% CO_2 in incubator at 37 °C. To evaluate the cytotoxicity, cells were exposed to the synthesized compounds and measured using the MTT assay [22].

4.2.2. LDL isolation, modification, and labeling

The use of human plasma (from Guangzhou General Hospital of Guangzhou Military Command) conformed to the principle outlined in the Declaration of Helsinki. Human native-LDL (d = 1.019 to 1.063 g/ml) was prepared from the plasma of normolipidemic volunteers by sequential ultracentrifugation [23]. LDL was extensively dialyzed for 24 h at 4 °C in Tris buffer (20 mM Tris, 150 mM NaCl, 300 μM EDTA). LDL was oxidized with 10 μM $CuSO_4$ for 24 h to generate oxLDL followed by dialysis against PBS buffer (5 mM Tris, 50 nM NaCl) with 10 μM EDTA and then PBS buffer to remove EDTA. The quality of LDL and oxLDL were monitored by agarose gel electrophoresis. The relative electrophoretic mobility of oxLDL was 2.5–2.7 relative to native-LDL. The protein content of lipoprotein was determined by BCA Protein Assay Kit (Thermo) using BSA as a standard. All lipoproteins were filter sterilized, stored at 4 °C in dark and used within 3 weeks.

4.2.3. Treatment of compounds and oil red O staining

The RAW264.7 cells were seeded on 6-well tissue culture plates at a density of 5×10^5 cells/ml for 18 h, and then starved in RPMI 1640 medium without FBS for 12 h. The cells were pretreated with indicated compounds for 12 h in RPMI 1640 medium without FBS before treatment with oxLDL (50 μg/ml) for 24 h, while the cells in control group was pretreated with 0.1% DMSO for 12 h. Foam cell formation was revealed by oil red O staining according to the reported method [24]. Briefly, cells were fixed in phosphate buffered 10% formalin for 10 min and washed once with PBS buffer. After flushing with 60% isopropanol for 30 s at room temperature, the cells were stained with 0.5% oil red O (Sigma) in 60% isopropanol for 20 min (37 °C, in darkness) and washed once with 60% isopropanol for 5 s. Photomicrographs were taken by phase contrast microscopy with a X 40 objective (CKX41, Olympus, Japan).

4.2.4. Preparation and uptake of DiI-oxLDL

LDL was labeled with fluorescent probe, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI) according to the reported method with minor modification [25]. Briefly, LDL was incubated overnight at 37 °C under nitrogen and light protection with 50 μL of DiI (3 mg/ml in DMSO) for each milligram of LDL protein. Unbound dye was removed by ultracentrifugation at 46,000 rpm for 5.5 h at 4 °C. For the preparation of DiI-oxLDL, DiI-LDL (0.9 mg/ml) was incubated with 10 μM $CuSO_4$ (final concentration) at 37 °C in dark for 24 h. The DiI-oxLDL distributed in the middle layer was re-isolated and subjected to dialysis against PBS buffer containing 0.24 mM EDTA. The protein content of DiI-oxLDL was determined using BCA Protein Assay Kit (Thermo).

To assess the cellular DiI-oxLDL uptake, RAW264.7 macrophage cells were seeded overnight on confocal dishes (Corning, NY) at a density of 5×10^5 cells/ml, then were serum starved for 12 h and pre-incubated with the tested compounds or control (0.1% DMSO) for 12 h, and then loaded with DiI-oxLDL (20 μg/ml) for another 6 h at 37 °C. At the end of incubation period, cells were washed once with PBS buffer (with 2 mg/ml BSA) and twice with PBS buffer, then were fixed in phosphate buffered 10% formalin for 10 min. Thereafter, for nucleus staining, the cells were incubated for 20 min with 10 μg/ml DAPI (Sigma) and washed 3 times with PBS buffer. The cells were then placed for confocal microscopy with a X 100 oil immersion objective using a 546 nm filter set (LSM 710, ZEISS, Germany), and quantitatively analyzed by flow cytometry.

4.2.5. Western Blot analysis

RAW264.7 macrophage cells were pretreated with compounds for 6 h before stimulation with oxLDL (50 μg/ml) for 24 h. Cells were lysed in lysis buffer containing PMSF (Sigma), and 40 μg proteins were loaded and separated on a 10% SDS–PAGE, then transferred to PVDF membrane as described [23]. The membrane was probed with a rabbit anti-mouse LOX-1 antibody (1:1000, Biovision), followed by incubation with an HRP-conjugated anti-rabbit secondary antibody (1:3000, Cell Signaling Technology). Immunocomplexes were detected by ECL method (Pierce). GAPDH was used to normalize the protein loading.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2013.11.001.

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