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Synthesis and biological evaluation of nitric oxide-releasing derivatives of oleanolic acid as inhibitors of HepG2 cell apoptosis

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Abstract—A total of 106 nitric oxide-releasing derivatives of oleanolic acid were synthesized and their effects on the inhibition of anti-Fas-mediated HepG2 cell apoptosis were evaluated in vitro. Several compounds inhibited anti-Fas-mediated HepG2 cell apoptosis in a dose-dependent manner. Within this series of compounds, **8b** is the most potent inhibitor. The development of new NO-releasing derivatives of oleanolic acid may aid in the design of NO-based medicines for the intervention of human liver inflammatory diseases.

Nitric oxide (NO), synthesized from L-arginine by a family of constitutive and inducible NO synthases (cNOS and iNOS), is a small, diffusible, highly reactive molecule with dichotomous biological functions, such as blood vessel dilatation, neurotransmission, modulation of the hair cycle, and penile erections.[1] NO can also be generated from synthetic NO-releasing compounds, such as nitrate, furoxan, hydroxyguanidine, *S*-nitrosothiol, diazeniumdiolate, and others.[2] Higher concentrations of NO can promote the apoptosis of many types of cells, while lower concentrations of NO usually protect cells, such as hepatocytes, from inflammation-mediated apoptosis.[3] Several mechanisms have been proposed to explain the anti-apoptosis effects of NO, including increased cGMP production, inhibition of caspase activation as well as prevention of mitochondrial permeability transition that leads to the release of cytochrome *C* into the cytosol.[4]

Increased scientific evidence indicates that NO deficiency is implicated in many physiological and pathological processes within the mammalian body. This fact provides a plausible biologic basis for the application of NO replacement therapy in clinic. However, the diverse bioactivities of NO limit its clinical application. New types of NO-releasing compounds/drug hybrids need to be developed for obtaining tissue-specific NO-related function.

Oleanolic acid (OA) is a triterpenoid compound, widely found in natural plants.[5] Systemic treatment with the purified OA leads predominately to the distribution and metabolism of OA in the liver. OA has been shown to protect rodent liver from CCl_4 and many other toxicant-induced hepatotoxicity and chronic cirrhosis.[6] Importantly, OA has been clinically used as a safe non-prescription drug for treatment of hepatitis in China for more than 20 years,[7] although its therapeutic efficacy is limited. The therapeutic effect, the property of liver-specific metabolism, and wide availability make it to be an ideal base for the design of new NO-releasing compounds for the production of NO specifically in the liver. The combination of NO and OA probably provides synergic protection of hepatocytes from inflammation- and toxicant-mediated liver damage.

In the present studies, 106 novel NO-releasing derivatives of OA were synthesized by connecting NO-donating moiety to the OA-28-COOH/OA-3-OH through varying lengths of linkers. The various linkers containing anti-oxygen functionalities, such as ferulic acid, *p*-hydroxyl cinnamic acid, and vanillic acid,[8] were

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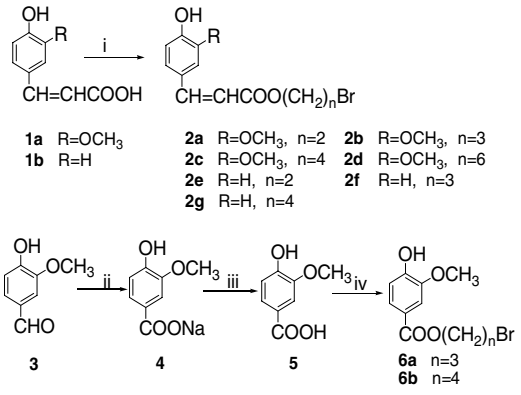
designed to increase the ability of these objective compounds to scavenge free radicals. The bioactivities of all derivatives of OA were evaluated. Several compounds were found to inhibit anti-Fas-mediated hepatocyte apoptosis and their anti-apoptotic effects were dose-dependent. One of the compounds, **8b**, inhibited hepatocyte apoptosis at a lower nanomolar level. The development of new NO-releasing derivatives of OA may aid in the design of NO-based new medicine for the intervention of human inflammatory liver diseases.

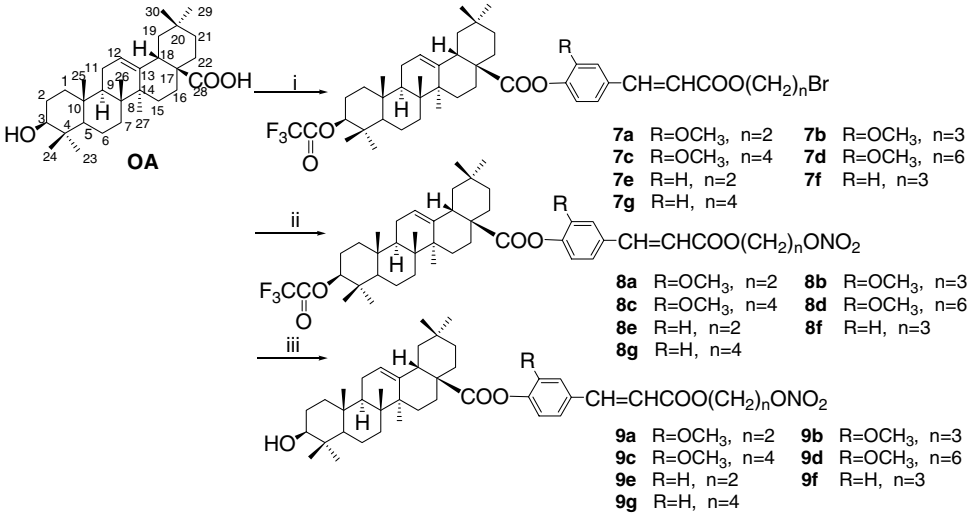
The synthetic routes of very important intermediates (**2a**–**2g**, **6a**, and **6b**) are outlined in Scheme 1. Ferulic acid (**1a**) or *p*-hydroxyl cinnamic acid (**1b**) was first treated with dibromoalkanes bearing two to six carbons in the presence of Et_3N and acetone at 50 °C to generate compounds **2a**–**2g** in 60–73% yields. In a similar way, compounds **6a** and **6b** were obtained in 61–65% yields by treatment of vanillic acid (**5**) with 1, 3-dibromopropane and 1, 4-dibromobutane, respectively.



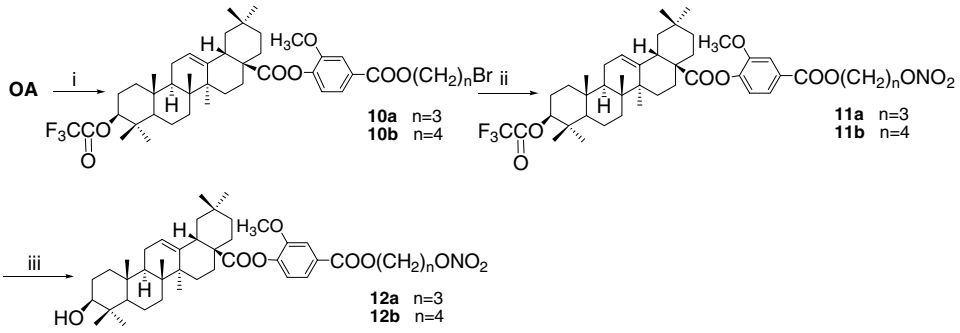
Scheme 1. Reagents and conditions: (i) $Br(CH_2)_nBr$, Et_3N, 50 °C, 4 h (60–73%); (ii) AgOH, NaOH (65%); (iii) HCl; (iv) $Br(CH_2)_nBr$ ($n = 3$ or 4), Et_3N, 50 °C, 4 h (61–65%).

The synthetic routes of OA-nitrate conjugates (**8a**–**8g**, **9a**–**9g**, **11a**, **11b**, **12a**, and **12b**) are summarized in Schemes 2 and 3. Several strategies were attempted to esterify OA-28-COOH directly with hydroxyl compounds, but failed, perhaps because of the large steric hindrance and weak acidity of OA-28-COOH. Alternatively, OA was first treated with trifluoroacetic anhydride to form a mixed anhydride in a quantitative yield after stirring at room temperature for 10 min. The resulting mixed anhydride was then reacted with hydroxyl compounds (**2a**–**2g** or **6a** and **6b**) in toluene to afford 3-*O*-trifluoroacetyl OA esters (**7a**–**7g** or **10a** and **10b**) in good yields (70–78%), respectively.[9,10] Compounds **7a**–**7g** or **10a** and **10b** were further converted to the corresponding nitrates **8a**–**8g** or **11a** and **11b**, respectively, with $AgNO_3$ in THF/CH_3CN. Compounds **9a**–**9g** or **12a** and **12b** were prepared by the hydrolysis of **8a**–**8g** or **11a** and **11b**, respectively, with diluted $KHCO_3$ to remove trifluoroacetyl group at C-3–OH without affecting other ester bonds. The resulting products were purified by column chromatography and their structures were characterized by IR, ^1H NMR, MS, and elemental analysis.[11]

Compounds **8a**–**8g**, **9a**–**9g**, **11a**, **11b**, **12a**, **12b**, and controls, OA and NCX-1000, were in vitro evaluated for their protective effects on anti-Fas-mediated HepG2 cell apoptosis determined by LDH assay.[12] As shown in Figure 1, treatment with different concentrations of OA failed to protect the HepG2 cells from anti-Fas-induced apoptosis as there was no significant difference in the percentage of survived HepG2 cells between the presence and absence of different concentrations of OA. Treatment with NCX-1000, a NO-releasing derivative of urodeoxycholic acid (UDCA) generated by adding an ONO_2 moiety through an antioxidant spacer, showed moderate protective effects and NCX-1000 at 10^{-6} M protected 33.4% of HepG2 cells from anti-Fas-induced apoptosis, consistent with previous report.[3] Furthermore, five of the tested compounds displayed great protective effects, which were 10- to 1000-fold



Scheme 2. Reagents and condition: (i) $(CF_3CO)_2O$, **2a**–**2g**, <90 °C, 6 h (70–78%); (ii) THF/CH_3CN, $AgNO_3$, reflux (67–75%); (iii) $KHCO_3$, rt (90–95%).



Scheme 3. Reagents and conditions: (i) $(CF_3CO)_2O$, **6a** and **6b**, <90 °C, 6 h (71–73%); (ii) THF/CH_3CN, $AgNO_3$, reflux (67–73%); (iii) $KHCO_3$, rt (90–95%).

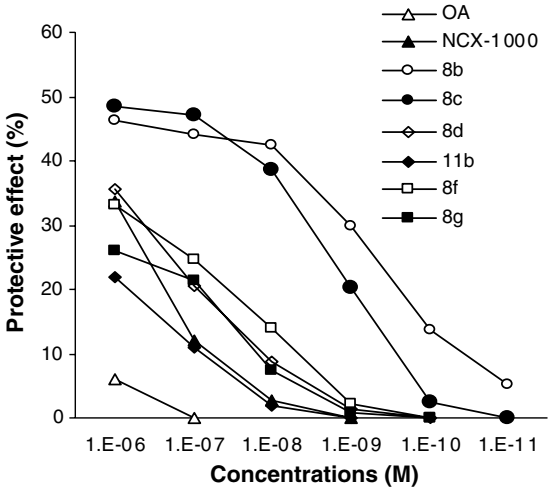


Figure 1. NO-releasing derivatives of OA-protected hepatocytes from anti-Fas-mediated apoptosis. The anti-apoptotic effects of NO-releasing derivatives of OA were evaluated by LDH assays. Briefly, HepG2 cells at 10^4/well were plated in triplicate in 96-well plates in 4% FCS and 2 μg/ml cycloheximide in phenol-red free DMEM. The cells were treated with anti-Fas (50 ng/ml for CH-11 or 1.5 μg/ml for DX2) in the presence or absence of indicated concentrations of OA, NCX-1000 or NO-releasing derivatives of OA at 37 °C 5% CO_2 for 18 h. Unmanipulated cells or the cells treated with 1% Triton X-100 were used as negative or positive controls for cytotoxicity. Their supernatants were harvested and their contained LDH activities were measured by LDH assays using the LDH cytotoxicity detection kit (Roche) and following the manufacturer's instructions at O.D. (490/650). The protective effect (%) was determined by the formula: the protective effect (%) = (1 − (O.D. experiments − O.D. negative controls)/(O.D. positive controls − O.D. negative controls)) × 100. Data presented as means of three independent experiments and the O.D. values of intra-group variations were less than 10%.

stronger than that of NCX-1000. The lowest concentration that significantly inhibited anti-Fas-induced HepG2 cell apoptosis was 10^{-10} M for **8b**, 10^{-9} M for **8c**, 10^{-8} M for **8f** or 10^{-7} M for **8d** and **8g**, respectively. A similar pattern of protective effects was observed when using the $p53^{-/-}$ Hep3b cells, indicating that the protective effects of these NO-releasing derivatives of OA were independent of p53. Given that Fas/FasL-mediated hepatocyte apoptosis was involved in many inflammatory- and toxicant-mediated liver diseases the potent anti-apoptotic effect of NO-releasing derivatives of OA suggests that these compounds may be potential for the treatment of inflammatory diseases in clinic.

As shown in Figure 2, these compounds produced low levels of NO (about 12 ng/ml for **8b**, 12 ng/ml for **8c**, 21 ng/ml for **8d**, 18 ng/ml for **8f**, 20 ng/ml for **8g**, and 28 ng/ml for **11b**, respectively) determined by in vitro Griess reaction. This is consistent with the notion that low dose of NO protects cells from inflammation-mediated apoptosis. Notably, some compounds that produced higher levels of NO in vitro showed potent cytotoxicity on human liver cancer cells (unpublished observations). In addition, our preliminary studies showed that oral treatment with **8b** at 128 mg kg^{-1} significantly inhibited CCl_4-induced hepatic fibrosis and liver damage in rats (data not shown). Collectively, NO-releasing derivatives of OA with low level of NO productivity protected human hepatocytes from anti-Fas-mediated apoptosis.

Analysis of structure–activity relationships suggests that the A-ring of OA may be a good target for further modification as the compounds **8b**–**8d**, **8f**, and **8g**, whose C-3-OH of A-ring was connected with trifluoroacetyl, showed potent anti-apoptotic activities, while the compounds **9b**–**9d**, **9f**, and **9g** with corresponding free

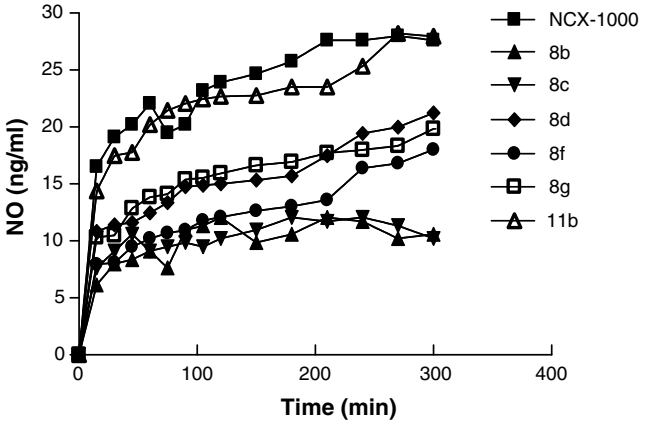


Figure 2. NO-releasing derivatives of OA produced low levels of NO in vitro. NO_2^- concentrations which represent the quantity of NO were determined by Griess assay. Griess reagent could combine with NO_2^- and form the chromophore after 10 min at 30 °C, the absorbance then was measured at 540 nm.

C-3-OH were inactive. Second, the length of the linkers connecting to ferulic acid or *p*-hydroxyl cinnamic acid appears crucial for their bioactivities. The **8b** with a 3-carbon long linker had 10- to 100-fold more potent anti-apoptotic activity than **8c** or **8d**, respectively, while **8a** was inactive. Similarly, the compounds **8f** and **8g**, but not **8e**, had anti-apoptotic activity. Apparently, a 3-carbon long linker was optimal, while compounds with longer linkers were accompanied by decreased bioactivities. Furthermore, the potent anti-apoptotic activities were governed by appropriate anti-oxygen reagent, like ferulic acid, at the C-28 of OA esters as compounds **8b** and **8c** showed higher anti-apoptotic activities than the corresponding compounds **8f**, **8g** and **11a**, **11b**, even if they had the same length of carbon chain. Finally, these OA-nitrate conjugates at less than 10^{-6} M, but not OA, inhibited anti-Fas-mediated HepG2 cell apoptosis in our experimental system, suggesting that the NO-releasing moieties may be responsible for the inhibitory activities of these derivatives of OA.

A series of OA-nitrate conjugates were synthesized and their biological functions were evaluated. Within the series of compounds, five of them showed to inhibit anti-Fas-mediated hepatocyte apoptosis in vitro and their anti-apoptotic effects were dose-dependent. Notably, the compound **8b** was the most potent inhibitor and protected hepatocytes from anti-Fas-mediated apoptosis at a lower nanomolar level. The preliminary SAR analysis of these compounds revealed that connection of the A-ring of OA with an optimal length of linker and the C-28 of OA esters with an anti-oxygen reagent was crucial for their anti-apoptotic activities. Together, these findings provide a new framework for the rational design of NO-based medicine for the intervention of human inflammatory liver diseases in clinic.

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References and notes

1. Grisham, M. B.; Jourdheuil, D.; Wink, D. A. *Am. J. Physiol.* **1999**, *276*, G315.
2. (a) Hou, Y. C.; Janczuk, A.; Wang, P. G. *Curr. Pharm. Des.* **1999**, *5*, 417; (b) Wang, P. G.; Xian, M.; Tang, X.; Wu, X.; Wen, Z.; Cai, T.; Janczuk, A. J. *Chem. Rev.* **2002**, *102*, 1091.
3. (a) Fiorucci, S.; Mencarelli, A.; Palazzetti, B.; Del Soldato, P.; Morelli, A.; Ignaro, L. J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 2652; (b) Ebisawa, Y.; Kono, T.; Yoneda, M.; Asama, T.; Chisato, N.; Sugawara, M.; Ishikawa, K.; Iwamoto, J.; Ayabe, T.; Kasai, S. *J. Surg. Res.* **2004**, *118*, 183; (c) Jenkins, D. C.; Charles, L. L.; Thomsen, D. W.; Moss, D. W.; Holmes, L. S.; Baylis, S. A.; Rhode, P.; Westmore, K.; Emson, P. C.; Moncada, S. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 4392.
4. Moncada, S.; Erusalimsky, J. D. *Nat. Rev. Mol. Cell. Biol.* **2002**, *3*, 214.
5. Liu, J. *J. Ethnopharmacol.* **1995**, *49*, 57.
6. (a) Liu, Y.; Hartley, D. P.; Liu, J. *Toxicol. Lett.* **1998**, *95*, 77; (b) Han, D. W.; Ma, X. H.; Zhao, Y. C.; Yin, L.; Ji, C. X. *J. Tradit. Chin. Med.* **1982**, *2*, 83; (c) Liu, J.; Liu, Y.; Madhu, C.; Klaassen, C. D. *J. Pharmacol. Exp. Ther.* **1993**, *266*, 1607; (d) Liu, Y.; Kreppel, H.; Liu, J.; Choudhuri, S.; Klaassen, C. D. *J. Pharmacol. Exp. Ther.* **1993**, *266*, 400.
7. (a) Liu, J.; Liu, Y.; Parkinson, A. *J. Pharmacol. Exp. Ther.* **1995**, *275*, 768; (b) Wang, L. X.; Han, G. X.; Liu, W. Y. *J. Pharm. Practice* **2001**, *19*, 104.
8. (a) Ozaki, Y. *Chem. Pharm. Bull.* **1992**, *40*, 954; (b) Hu, H. J.; Hang, B. Q. *J. China Pharm. Univ.* **1990**, *21*, 279; (c) Lin, Y. H.; Chen, W. W. *Acta Pharm. Sin.* **1994**, *28*, 267.
9. (a) Parish, R. C.; Stock, L. M. *Tetrahedron Lett.* **1964**, *20*, 1285; (b) Bourne, J. E.; Stacey, M.; Tatlow, J. C.; Tedder, J. M. *J. Chem. Soc.* **1949**, 2976.
10. Chen L. *Ph.D. dissertation*. China Pharmaceutical University at Nanjing in China, **2004**.
11. Analytical data for **8b**: mp 112–114 °C; IR (KBr, cm^{-1}) : 1779, 1750, 1715, 1635; ^1H NMR ($CDCl_3$, 300 MHz): δ 0.86 (s, 3H, CH_3), 0.92 (s, 6H, 2× CH_3), 0.94 (s, 3H, CH_3), 0.97 (s, 6H, 2× CH_3), 1.19 (s, 3H, CH_3), 2.10–2.18 (m, 2H, CH_2), 2.94–3.00 (m, 1H, C_{18}–H), 3.83 (s, 3H, OCH_3), 4.32 (t, 2H, OCH_2, J = 6.0 Hz), 4.61 (t, 2H, OCH_2, J = 6.0 Hz), 4.68–4.73 (m, 1H, H-3α), 5.33 (br s, 1H, H-12), 6.36 (d, 1H, =CH, J = 16.0 Hz), 6.95–6.97 (m, 1H, ArH), 7.07–7.12 (m, 2H, ArH), 7.65 (d, 1H, =CH, J = 16.0 Hz); ESI-MS: 854 $[M+Na]^+$; Anal. $C_{45}H_{60}F_3NO_{10}$. Found (%): N 1.56, C 65.34, H 7.31; Calcd (%): N 1.68, C 64.79, H 7.27.Analytical data for **9b**: mp 98–100 °C; IR (KBr, cm^{-1}) : 3523, 1748, 1715, 1634; ^1H NMR ($CDCl_3$, 300 MHz): δ (ppm): 0.82 (s, 3H, CH_3), 0.88 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.23 (s, 3H, CH_3), 2.96–3.02 (m, 1H, H-18), 3.25 (br s, 1H, H-3α), 3.85 (s, 3H, OCH_3), 4.35 (t, 2H, OCH_2, J = 6 Hz), 4.64 (t, 2H, OCH_2, J = 6.0 Hz), 5.36 (br s, 1H, C_{12}–H), 6.38 (d, 1H, =CH, J = 16.0 Hz), 6.99 (d, 1H, ArH, J = 7.8Hz), 7.11–7.16 (m, 2H, ArH), 7.68 (d, 1H, =CH, J = 16.0Hz); ESI-MS: 758 $[M+Na]^+$; Anal. $C_{43}H_{61}NO_9{\cdot}0.5H_2O$. Found (%): N 1.64, C 69.34, H 8.52; Calcd (%): N 1.88, C 69.31, H 8.32.
Analytical data for **11b**: mp 64–66 °C; IR (KBr, cm^{-1}) : 1779, 1756, 1720, 1632; ^1H NMR ($CDCl_3$, 300 MHz): δ 0.85 (s, 3H, CH_3), 0.92 (s, 6H, 2× CH_3), 0.94 (s, 3H, CH_3), 0.97 (s, 6H, 2× CH_3), 1.19 (s, 3H, CH_3), 2.94–3.00 (m, 1H, H-18), 3.85 (s, 3H, OCH_3), 4.36 (br s, 2H, OCH_2), 4.53 (br s, 2H, OCH_2), 4.68–4.73 (m, 1H, 3α-H), 5.33 (br s, 1H, C_{12}–H), 7.00 (d, 1H, ArH, J = 7.9 Hz), 7.61–7.64 (m, 2H, ArH); ESI-MS : 823 $[M+NH_4]^+$, 828 $[M+Na]^+$; Anal. $C_{44}H_{60}F_3NO_{10}$. Found (%): N 1.62, C 64.16, H 7.45; Calcd (%): N 1.71, C64.45, H 7.38.
12. Fernandez-Cruz, E.; Escartin, P.; Bootello, A.; Kreisler, M.; Segovia de Arana, J. M. *Clin. Exp. Immunol.* **1978**, *31*, 436.