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Synthesis and anti-hepatocellular carcinoma activity of novel O^2-vinyl diazeniumdiolate-based nitric oxide-releasing derivatives of oleanolic acid

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[ABSTRACT] Considering that high levels of nitric oxide (NO) exert anti-cancer effect and the derivatives of oleanolic acid (OA) have shown potent anti-cancer activity, new O^2-vinyl diazeniumdiolate-based NO releasing derivatives (5a–l, 11a–l) of OA were designed, synthesized, and biologically evaluated in the present study. These derivatives could release different amounts of NO in liver cells. Among them, 5d, 5i, 5j, 11g, 11h, and 11j released more NO in SMMC-7721 cells and displayed stronger proliferative inhibition against SMMC-7721 and HepG2 cells than OA and other tested compounds. The most active compound **5j** showed almost 20-fold better solubility than OA in aqueous solution, released larger amounts of NO in liver cancer cells than that in normal ones, and exhibited potent anti-hepatocellular carcinoma activity but little effect on the normal liver cells. The inhibitory activity against the cancer cells was significantly diminished upon addition of an NO scavenger, suggesting that NO may contribute, at least in part, to the activity of **5j**.

[KEY WORDS] Oleanolic acid; O^2-Vinyl diazeniumdiolate; Cytochrome P450; NO release; Anti-hepatocellular carcinoma activity

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Introduction

Oleanolic acid (OA) and its derivatives are an important class of pentacyclic triterpenoids widely distributed in the plant kingdom. These compounds exhibit a wide variety of biological activities, including liver protection, anti-cancer activity, and immune regulation [1-5]. Some effects displayed by OA may be related to the cytochrome P450 (CYP) enzyme system in the human liver [6], which is an important metabolic organ responsible for the activation and deactivation of a large number of xenobiotics. It has been documented that the expression of CYP varies in normal and tumorous liver cells [7], and that the nitric oxide (NO) donor, O^2-vinyl diazeniumdiolate can be activated by CYP to release NO in liver [8-9]. Accordingly, it is likely that O^2-vinyl diazeniumdiolate-based derivatives of OA may release different amounts of NO in these cells.

Given that high levels of NO exert anti-cancer effect [10] and a number of OA derivatives synthesized by our group have shown potent anti-cancer activity[11-14], we introduced various O^2-vinyl diazeniumdiolates to the 3-position of OA to search for new NO-releasing OA derivatives with stronger anti-tumor activity than OA. Meanwhile, several water-soluble groups such as galactosyl, glucosyl and piperazine ring were respectively connected to the 28-position of OA to improve the solubility of these molecules. Herein, we report the synthesis and biological evaluation of the NO releasing derivatives of OA.

Materials and Methods

Chemical synthesis

General procedures

Melting points were determined on a melting point apparatus (1101D Mel-TEMP II, UK) which was uncorrected. ^{1}H NMR spectra were recorded with a 300 MHz spectrometer

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(Bruker Avance III, Switzerland) at 300 K, using TMS as an internal standard. MS spectra were recorded on a GC-MS 2010 (Shimadzu EI, Japen). Analytical and preparative TLC was performed on silica gel (200−300 mesh) GF/UV 254 plates and the chromatograms were visualized under UV light at 254 and 365 nm. All solvents used in the present study were reagent grade and, when necessary, were purified and dried by standard method. The purity of all compounds tested was characterized by the HPLC (LC-10A HPLC system consisting of LC-10ATvp pumps and SPD-10Avp UV detector) and high resolution mass spectrometry (Agilent technologies LC/MSD TOF, USA). Individual compounds with a purity of > 95% were used for subsequent experiments. Oleanolic acid (OA) was commercially available.

*Synthesis of compounds **5a–f***

DCC (70 mg, 0.34 mmol) and DMAP (41 mg, 0.34 mmol) were added to a solution of Compound **3a** (42.2 mg, 0.226 mmol) and **4a** (200 mg, 0.226 mmol) in dry CH_2Cl_2 (5 mL). The resulting mixture was stirred at room temperature for 12 h. The organic extracts were washed with saturated NaCl solution (10 mL × 3), and the organic fraction was dried over sodium sulfate. After removal of the solvent, the crude product was purified by column chromatography [1 : 3 (*V/V*) acetone−cyclohexane] to give **5a**–**f**.

Compound **5a**: Following the general procedure, the title compound was obtained in 81% yield as a white solid: m.p. 80-82℃.IR (KBr): 3434, 2923, 2851, 1733, 1641, 1163, 1073 cm^{-1}.ESI-MS: 1073 $[M + NH_4]^+$. ^1H NMR (300 MHz, $CDCl_3$), δ(ppm): 0.75 (3H, s, CH_3), 0.87 (3H, s, CH_3), 0.92 (3H, s, CH_3), 0.95 (3H, s, CH_3), 1.14 (3H, s, CH_3), 1.27 (3H, s, CH_3), 2.04 (4H, s, 2 × $COCH_2$), 2.03 (3H, s, $COCH_3$), 2.06 (6H, s, 2 × $COCH_3$), 2.19 (3H, s, $COCH_3$), 2.60−2.71 (2H, m, CH_2), 3.53−3.64 (4H, m, 2 × NCH_2), 3.80−3.83 (1H, m, CH_3), 4.04−4.09 (1H, m, H_5), 4.27−4.32 (1H, m, H_4), 4.44 (dd, *J* = 2.4, 6.6 Hz, 1H, CH=C<u>H</u>$_2$), 4.50−4.56 (1H, m, 3α-H), 4.88 (1H, *dd*, *J* = 2.4, 14.1 Hz, CH=C<u>H</u>$_2$), 4.97−4.97 (1H, ms, OCH), 5.24 (1H, d, *J* = 2.1 Hz, H_6), 5.34 (1H, brs, C_{12}-H), 5.60 (d, *J* = 8.1 Hz, H_1), 6.84 (1H, *dd*, *J* = 6.6, 14.1 Hz, C<u>H</u>=CH_2).

Compound **5b**: Following the general procedure, the title compound was obtained in 73% yield as a white solid: mp: 105−107 ºC.IR (KBr): 3 433, 2 921, 1 734, 1 640, 1 161, 1 069 cm^{-1}. ESI-MS: 1 073 $[M + NH_4]^+$.^1H NMR (300 MHz, $CDCl_3$), δ: 0.76 (3H, s, CH_3), 0.87 (6H, s, 2 × CH_3), 1.14 (3H, s, CH_3), 1.27 (6H, s, 2 × CH_3), 2.02 (12H, s, 4 × $COCH_3$), 4.00−4.05 (1H, m, H_3), 4.12−4.13 (1H, m, H_5), 4.44 (1H, dd, *J* = 2.4, 6.9 Hz, CH=C<u>H</u>$_2$), 4.53 (1H, t, *J* = 7.5 Hz, 3α-H), 4.90 (1H, dd, *J* = 2.4, 14.1 Hz, CH=C<u>H</u>$_2$), 4.96−4.98 (1H, m, OCH), 5.34 (1H, brs, C_{12}-H), 5.41−5.44 (1H, m, H_4), 5.57 (1H, d, *J* = 8.4 Hz, H_1), 6.84 (1H, dd, *J* = 6.6, 14.1 Hz, C<u>H</u>=CH_2). (Found: C, 62.80; H, 7.95; N, 4.08. $C_{55}H_{81}N_3O_{17}$ requires C, 62.54; H, 7.73; N, 3.98).

Compound **5c**: Following the general procedure, the title compound was obtained in 64% yield as a white solid: m.p. 89−91 ºC. IR (KBr): 3 431, 2 921, 1 735, 1 641, 1 162, 1 071cm^{-1}. ESI-MS: 1085 $[M + H]^+$. ^1H NMR (300 MHz, $CDCl_3$), δ: 0.50 (3H, s, CH_3), 0.62 (6H, s, 2 × CH_3), 0.69 (6H, s, 2 × CH_3), 0.89 (3H, s, CH_3), 1.19 (6H, s, 2 × CH_3), 1.80 (3H, s, $COCH_3$), 1.84 (9H, s, 3 × $COCH_3$), 2.41 (4H, s, 2 × $COCH_2$), 3.56 (d1H, d, *J* = 1.2, 6.3 Hz, H_3), 3.80−3.84 (1H, m, H_5), 4.04 (1H, dd, *J* = 4.5, 12.6 Hz, CH=C<u>H</u>$_2$), 4.18 (1H, d, *J* = 6 Hz, H_4), 4.27 (1H, t, *J* = 7.1 Hz, 3α-H), 4.63 (1H, dd, *J* = 2.4, 14.1 Hz, CH=C<u>H</u>$_2$), 4.81−5.05 (3H, m, 3H, H_2, H_6 and $H_{6'}$), 5.09 (1H, brs, C_{12}-H), 5.34 (1H, d, *J* = 7.8 Hz, H_1), 6.58 (1H, dd, *J* = 6.6, 14.1 Hz, C<u>H</u>=CH_2).

Compound **5d**: Following the general procedure, the title compound was obtained in 71% yield as a white solid: mp: 120−122 ºC. IR (KBr): 3 440, 2 927, 1 736, 1 641, 1 163, 1 072, 1 027 cm^{-1}. ESI-MS: 1 085 $[M + H]^+$. ^1H NMR (300 MHz, $CDCl_3$), δ: 0.75 (3H, s, CH_3), 0.83 (6H, s, 2 × CH_3), 0.90−0.94 (9H, m, 3 × CH_3), 1.13 (3H, s, CH_3), 1.25 (9H, s, 3 × CH_3), 1.90 (6H, s, 2 × $COCH_3$), 1.99 (3H, s, $COCH_3$), 2.17 (3H, s, $COCH_3$), 2.64 (4H, s, 2 × $COCH_2$), 3.55 (4H, brs, 2 × NCH_2), 4.01− 4.03 (1H, m, H_3), 4.09 (1H, s, H_5), 4.25 (2H, brs, OCH_2), 4.41 (1H, dd, *J* = 2.7, 6.9 Hz, CH=C<u>H</u>$_2$), 4.51 (1H, m, 3α-H), 4.86 (1H, dd, *J* = 2.7, 14.1 Hz, CH=C<u>H</u>$_2$), 5.08 (1H, dd, *J* = 3.3, 10.2 Hz, H_4), 5.32 (1H, brs, C_{12}-H), 5.38−5.39 (1H, m, $H_{6'}$), 5.41 (1H, d, *J* = 3.0 Hz, H_6), 5.54 (1H, d, *J* = 8.1 Hz, H_1), 6.80 (1H, dd, *J* = 6.9, 14.1 Hz, C<u>H</u>=CH_2).

Compound **5e**: Following the general procedure, the title compound was obtained in 81% yield as a white solid: m.p. 58−63ºC. IR (KBr): 3 441, 1 757, 1 640, 1 226, 1 080 cm^{-1}. ESI-MS: 1047 $[M + NH_4]^+$. ^1H NMR (300 MHz, $CDCl_3$), δ: 0.73 (3H, s, CH_3), 0.85 (6H, s, 2 × CH_3), 0.90−0.93 (9H, s, 3 × CH_3), 1.12 (3H, s, CH_3), 1.26 (3H, s, CH_3), 2.02 (9H, s, 3 × $COCH_3$), 2.07 (3H, s, $COCH_3$), 2.63 (4H, s, 2 × $COCH_2$), 2.802−.83 (1H, m, C_{18}-H), 3.14 (3H, s, NCH_3), 3.68 (2H, t, *J* = 5.4 Hz, $OCH_2C\underline{H}_2N$), 3.78−3.81 (1H, m, H_3), 4.03−4.07 (1H, m, H_4), 4.30 (2H, t, *J* = 5.4 Hz, $OC\underline{H}_2CH_2N$), 4.41 (1H, dd, *J* = 2.4, 6.9 Hz, CH=C<u>H</u>$_2$), 4.48 (1H, t, *J* = 8.4 Hz, 3α-H), 4.82−4.88 (1H, dd, *J* = 2.4, 14.1 Hz, CH=C<u>H</u>$_2$), 5.11−5.25 (3H, m, H_6, $H_{6'}$ and H_2), 5.32 (1H, brs, C_{12}-H), 5.57 (1H, d, *J* = 7.8 Hz, H_1), 6.81 (1H, dd, *J* = 6.6, 13.8 Hz, C<u>H</u>=CH_2).

Compound **5f**: Following the general procedure, the title compound was obtained in 67% yield as a white solid: mp: 102−104 ºC. IR (KBr): 3 442, 2 954, 1 755, 1 640, 1 224, 1 161, 1 074 cm^{-1}. ESI-MS: 1 047 $[M + NH_4]^+$. ^1H NMR (300 MHz, $CDCl_3$), δ: 0.70 (3H, s, CH_3), 0.84 (6H, s, 2 × CH_3), 0.86−0.87 (6H, m, 2 × CH_3), 0.89 (3H, s, CH_3), 1.80 (3H, s, CH_3), 1.95 (3H, s, $COCH_3$), 1.98 (6H, s, 2 × $COCH_3$), 2.59 (4H, s, 2 × $COCH_2$), 3.10 (3H, s, NCH_3), 3.64 (2H, t, *J* = 5.4 Hz, $HOCH_2C\underline{H}_2N$), 4.26 (2H, t, *J* = 5.4 Hz, $HOC\underline{H}_2CH_2N$), 4.36 (1H, dd, *J* = 2.7, 6.9 Hz, CH=C<u>H</u>$_2$), 4.47 (1H, t, *J* = 7.8 Hz, 3α-H), 4.81 (dd, *J* = 2.4, 14.1 Hz, CH=C<u>H</u>$_2$), 5.04 (1H, dd, *J* = 3.3, 10.4 Hz, H_4), 5.28 (1H, brs, C_{12}-H), 5.31−5.34 (1H, m, $H_{6'}$), 5.37 (1H, d, *J* = 8.1 Hz, H_6), 5.51 (1H, d, *J* = 8.1 Hz, H_1), 6.77 (1H, dd, *J* = 6.6, 14.1 Hz, C<u>H</u>=CH_2).

*Synthesis of compounds **5g–l***

Compounds **5a**–**f** were dissolved in a 1 : 1 mixture of dry