Brief Articles

Design, Synthesis, and Antihepatocellular Carcinoma Activity of Nitric Oxide Releasing Derivatives of Oleanolic Acid

Li Chen,[†,‡] Yihua Zhang,*[,†] Xiangwen Kong,[†] Edward Lan,[§] Zhangjian Huang,[†] Sixun Peng,[†] Daniel L. Kaufman,[§] and Jide Tian*[,§]

Center of Drug Discovery and Department of Phytochemistry, China Pharmaceutical University, Nanjing 210009, P. R. China, and Department of Molecular and Medical Pharmacology, University of California—Los Angeles, Los Angeles, California 90095

Received February 18, 2008

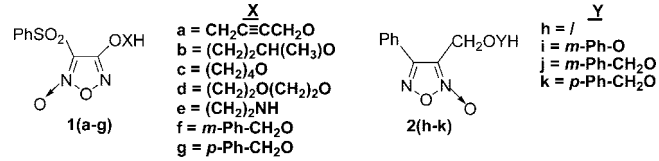
Novel furoxan-based nitric oxide (NO) releasing derivatives of oleanolic acid (OA) were synthesized for potential therapy of liver cancers. Six compounds produced high levels of NO in human hepatocellular carcinoma (HCC) cells and exhibited strong cytotoxicity selectively against HCC in vitro. Treatment with **8b** or **16b** significantly inhibited the growth of HCC tumors in vivo. These data provide a proof-in-principle that furoxan/OA hybrids may be used for therapeutic intervention of human liver cancers.

Introduction

Nitric oxide (NO[a]) is a key mediator involved in many physiological and pathological processes.[1] High levels of NO and its metabolic derivatives, the reactive nitrogen species (RNS) and reactive oxygen species (ROS), can modify functional proteins by S-nitrosylation, nitration, and disulfide formation, leading to bioregulation, inactivation, and cytotoxicity, particularly in tumor cells.[2] Indeed, some synthesized NO-releasing compounds have shown cytotoxic activity against human colon carcinoma cells in vitro, inhibiting the growth and metastasis of cancers in vivo.[3–6] However, the therapeutic effect of those compounds is limited, possibly because of low levels of NO release. Furoxans are thermally stable compounds and represent one class of NO donors that can produce high levels of NO; however, they have a variety of NO-related bioactivities in vivo.[7–9] To obtain tissue-specific NO-related function, new types of furoxan/drug hybrids need to be developed.[10]

Oleanolic acid (OA) is widely found in plants and has been demonstrated to inhibit hepatitis in humans without apparent side effects.[11,12] Furthermore, OA has been shown to protect rodent liver from CCl_4 and other toxicant-induced hepatotoxicity and chronic cirrhosis, which is associated with the distribution and metabolism of OA in the liver and modulation of OA on cytochrome p450 (CYP) activity.[12–15] On the basis of its therapeutic effects, liver-specific metabolism, and availability, OA may be an ideal lead compound for the design of novel furoxan-based NO-releasing compounds that produce high levels of NO specifically in the liver. We hypothesized that such furoxan/OA hybrids may have strong cytotoxicity selectively against HCC, one of the most frequent cancers in the world,[16] because of the efficient entry and metabolism of those compounds to produce high levels of NO in HCC cells.

Chart 1



To test this hypothesis, 32 novel furoxan-based NO-releasing derivatives of OA were designed and synthesized by coupling phenylsulfonyl- or phenyl-substituted furoxans to the 3- or 28-position of OA through various chemical linkers. Subsequently, their anti-HCC activities were evaluated in vitro and in vivo. Interestingly, some of the derivatives (**8a**−**e**, **16b**) showed strong cytotoxicity against HCC cells in vitro, which were associated with high levels of NO production selectively in HCC cells. Importantly, treatment with one of the active hybrids **8b** or **16b** inhibited the growth of HCC tumors in vivo. We discuss potential structure−activity relationships among the compounds and the implication of these findings.

Results and Discussion

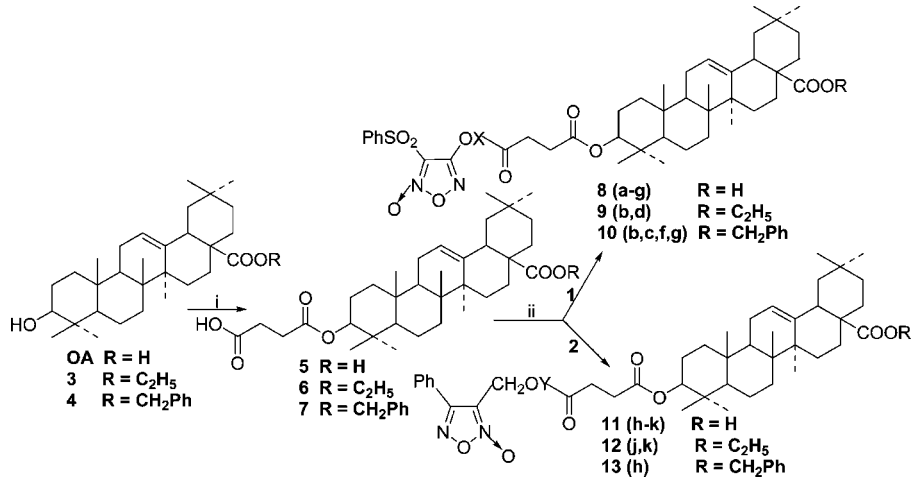
Chemistry. A total of seven phenylsulfonyl-substituted furoxans (**1**) and four phenyl-substituted furoxans (**2**) (Chart 1) were synthesized as described previously.[17,18] The preparation of 3-position derivatives of OA is outlined in Scheme 1. OA was first modified with ethyl bromide or benzyl bromide in the presence of Et_3N to give the corresponding C-28 esters (**3** or **4**) in 65−73% yields. Subsequently, OA, **3**, or **4** was reacted with succinic anhydride in the presence of 4-*N*,*N*-dimethylaminopyridine (DMAP) to form 3-*O*-acyl derivatives (**5**−**7**) in 52−60% yields. Furthermore, **5**−**7** were esterified with **1** or **2** in the presence of dicyclohexylcarbodiimide (DCC)/DMAP to generate target compounds (**8a**−**g**, **9b**,**d**, and **10b**,**c**,**f**,**g** or **11h**−**k**, **12j**,**k**, and **13h**). Thus, 20 target compounds were generated by coupling phenylsulfonyl- or phenyl-substituted furoxans to the 3-position of OA.

* To whom correspondence should be addressed. For Y.Z.: phone, +86-25-83271186; fax, +86-25-86635503; e-mail, zyhtgd@hotmail.com. For J.T.: phone, 310-206-3350; fax, 310-825-6267; e-mail, jtian@mednet.ucla.edu.
† Center of Drug Discovery, China Pharmaceutical University.
‡ Department of Phytochemistry, China Pharmaceutical University.
§ University of California—Los Angeles.

[a] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HCC, human hepatocellular carcinoma; LDH, lactate dehydrogenase; NO, nitric oxide; OA, oleanolic acid; PE, petroleum ether; OD, optical density.

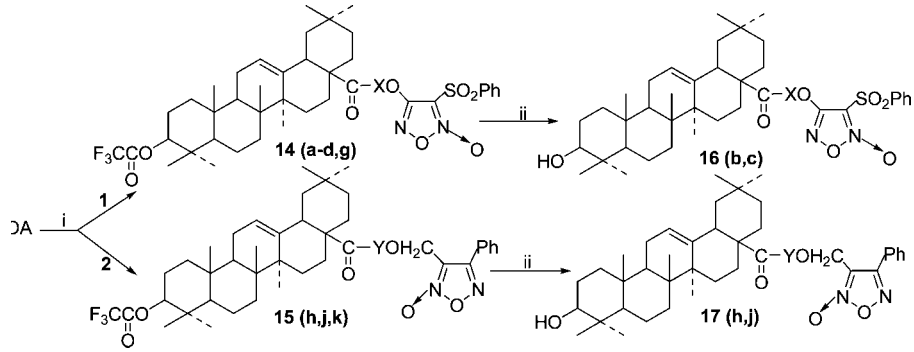
10.1021/jm800167u CCC: $40.75
Published on Web 07/04/2008

Scheme 1. Synthesis of Compounds **8**−**13**[a]



[a] Reagents and conditions: (i) succinic anhydride, DMAP, dry CH_2Cl_2, reflux; (ii) DCC/DMAP, dry CH_2Cl_2, **1** for **8**−**10**, **2** for **11**−**13**. The definitions of X and Y are indicated in Chart 1.

Scheme 2. Synthesis of Compounds **14**−**17**[a]



[a] Reagents and conditions: (i) $(CF_3CO)_2O$, toluene, reflux, 4 h, **1** for **14**, **2** for **15**; (ii) $KHCO_3$, MeOH, room temp. The definitions of X and Y are indicated in Chart 1.

The other 12 target compounds (**14a**−**d**,**g**, **15h**,**j**,**k**, **16b**,**c**, and **17h**,**j**) were synthesized by modifying the 28-COOH of OA with phenylsulfonylfuroxan or phenylfuroxan (Scheme 2). Direct esterification of OA with hydroxyl compound **1** or **2** failed perhaps because of the large steric hindrance and weak acidity of the 28-COOH. Alternatively, the carboxyl group of OA was activated by the mixed anhydride. OA was initially treated with trifluoroacetic anhydride (TFAA) to form a mixed anhydride in a quantitative yield, which was subsequently reacted with **1** or **2** to afford 3-*O*-trifluoroacetyl OA ester **14** or **15** in 63−75% yields. The trifluoroacetyl groups in **14** and **15** were removed in diluted $KHCO_3$ solution, without breakdown of other ester bonds, to produce **16** and **17**.

All of the compounds generated were further purified and characterized (see Supporting Information). The success in the synthesis of these compounds provided a base for the characterization of their anti-HCC activity in vitro and in vivo.

Biological Results. The bioactivities of these compounds were determined by the lactate dehydrogenase (LDH) assays (Table 1). While there was no obvious cytotoxicity against HCC cells for the parent OA at any of the tested concentrations, the anti-HCC activities of **8a**−**e** and **16b** against HepG2 appeared to be dose-dependent. For example, **8b** at a dose of 1 μM promoted 100% cell death and 0.01 μM of it induced 13.34% cell death. Similar patterns of cell death induced by those active compounds were achieved in Hep3b cells (Table 2S, Supporting Information), and **8b** displayed the most potent cytotoxicity against HepG2 and Hep3b cells. However, **8f**, **11h**, **14b**, and the other 23 synthesized compounds showed no cytotoxicity against HepG2 and Hep3b cells in our experimental system (data not shown). Interestingly, none of the tested compounds under the same experimental conditions showed any obvious cytotoxicity against HeLa, PC-3, MCF-7, and HEK 293 (data not shown). The strong cytotoxicity against HCC cells, but not other noncancer and nonliver cancer cell lines tested, suggests that those compounds may be selectively cytotoxic to HCC cells.

Table 1. Percentage of HepG2 Cell Death[a]

compd	1 μM	0.1 μM	0.01 μM	0.001 μM
OA	0	0	0	0
18	0	0	0	0
8a	67.41	13.68	10.07	0
8b	100.00	93.50	13.34	1.70
8c	98.16	56.35	10.96	2.60
8d	95.63	67.96	17.32	2.70
8e	74.53	13.01	8.51	0
8f	0	0	0	0
11h	0	0	0	0
14b	0	0	0	0
16b	89.21	55.11	6.67	0

[a] Data shown are the average percentages of cell death induced by the compound at 24 h post-treatment from three independent experiments. Intragroup variations were less than 10% of the values presented. Treatment of the cells with the compound for a longer period did not increase its cytotoxicity. The other 23 synthesized compounds tested showed no cytotoxicity against HepG2 in our experimental system (data not shown).

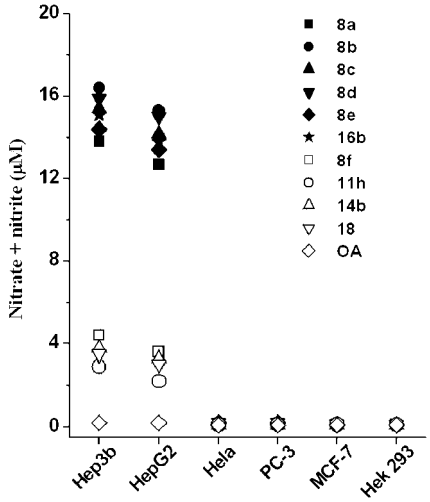


Figure 1. Variable levels of NO produced by furoxan/OA hybrids selectively in HCC cells. Individual values were obtained by subtracting the background (diluent treated wells) and calculated according to the standard curve. Data shown are the mean value of two experiments for each compound at 240 min post-treatment, and similar patterns of nitrate/nitrite levels in HCC cells were observed at other experimental time points (data not shown).

The furoxans can be metabolized predominately by CYP in liver cells to produce high levels of NO and RNS/ROS.[8,9] OA is mainly metabolized in the liver.[11] It is possible that the selective cytotoxicity of these compounds may stem from high levels of NO production selectively in the HCC cells. To test this possibility, HepG2, Hep3b, Hela, PC-3, MCF-7, and Hek 293 cells were exposed to each compound (100 μM) for varying durations (30−300 min). The levels of nitrite/nitrate in the lysates of different cell lines were characterized using the nitrite/nitrate colorimetric assay kit (Figure 1). First, treatment with OA did not induce a detectable level of nitrite/nitrate in any of the tested cells. Second, the kinetics of NO release by **8a**−**e** and **16b** were similar to that of the control, a nitrate-based derivative of ursodeoxycholic acid[19] (**18**, data not shown), suggesting that NO was slowly released. Furthermore, treatment with any of the furoxan/OA hybrids tested failed to promote significantly higher levels of nitrate/nitrite in nonhepatic cells. Importantly, treatment with **8a**−**e** and **16b**, but not **8f**, **11h**, or **14b**, induced high levels of nitrate/nitrite production in HepG2 and Hep3b cells and the levels of nitrate/nitrite were severalfold higher than that of **18** in HCC cells. Finally, although the levels of nitrate/nitrite induced by **8a**−**e** and **16b** were not significantly different, they appeared to be positively correlated to anti-HCC activities of those compounds. Therefore, treatment with those compounds promoted high levels of NO selectively in HCC cells in vitro, which may contribute to strong cytotoxicities of those compounds selectively against HCC in vitro.

Next, we examined the impact of treatment with **8b** or **16b** on animal survival and behaviors by acute toxicity assay, and the growth of HCC tumor in vivo in a mouse model of HCC. Groups of healthy male Balb/c mice that were treated intraperitoneally (ip) with **8b**, **16b**, or OA (15, 50, or 100 mg/kg) appeared healthy throughout a 15-day observation period with no significant differences in eating, drinking, body weight, and activity among those groups of mice (data not shown). These data suggest that administration of these compounds at these dosages tested did not have adverse effects. Furthermore, the average size and weight of HCC tumors in the mice that were inoculated with Hep3b cells and treated with OA were similar to that in mice treated with control diluent (Table 2), suggesting

Table 2. Treatment with **8b** or **16b** Inhibits the Growth of HCC Tumors in Vivo[a]

treatment	tumor size (mm^3)	tumor weight (g)
diluent	701 ± 157	1.031 ± 0.171
OA	668 ± 208	0.961 ± 0.168
8b	232 ± 77[b]	0.262 ± 0.082[b]
16b	289 ± 41[c]	0.332 ± 0.074[b]

[a] Data shown are the mean ± SD of group mice ($n = 8$). [b] $p < 0.01$ vs OA or diluent group, determined by Student t analysis. Experimental and control groups of mice were tested simultaneously. [c] $p < 0.02$ vs OA or diluent group, determined by Student t analysis. Experimental and control groups of mice were tested simultaneously.

that the growth of HCC tumors was not significantly affected by OA treatment. Importantly, the mean size and weight of the tumors from the mice treated with **8b** or **16b** were reduced dramatically. Interestingly, histological analysis of the liver tissues from those groups of mice showed no morphological differences (data not shown). Apparently, treatment with **8b** or **16b** significantly inhibited the growth of HCC tumors but did not result in an obvious change in the liver structures in mice.

Analysis of structure−activity relationships (SAR) among these compounds revealed that the 4-phenylsulfonylfuroxan was crucial for the anti-HCC cytotoxicity of furoxan/OA hybrids because the phenylsulfonylfuroxan-substituted OA (**8a**−**e** and **16b**), but not single phenylfuroxan-substituted OA, showed strong cytotoxicity selectively against HCC cells in vitro. Furthermore, the coupling site of OA with furoxans had a great impact on the cytotoxic potency of the derivatives. The compounds obtained by coupling 4-phenylsulfonylfuroxan to the 3-OH of OA were more active than those obtained by coupling the same furoxan to the 28-COOH (**8a** vs **14a**, **8b** vs **14b** or **16b**, **8c** vs **14c** or **16c**, and **8d** vs **14d**). Indeed, 5 out of 13 compounds with phenylsulfonylfuroxan to the 3-OH were bioactive, while only 1 out of 7 derivatives with phenylsulfonylfuroxan to the 28-COOH showed anti-HCC activity. Moreover, the type and length of the linkers, which connected NO donor moiety to the 3- or 28-position of OA, were important for compounds' activities. Target compounds that had aliphatic linkers showed strong cytotoxicity. For example, **8b** and **16b** with a linker bearing a four-carbon aliphatic chain, $(CH_2)_2$-$CH(CH_3)$, were highly active. However, those with aromatic linkers (**8f**,**g**) did not show any cytotoxicity. Finally, a free 28-COOH for compounds with furoxan coupling to the 3-position of OA appeared to be necessary, evidenced by the fact that all active compounds (**8a**−**e**) in this series had free 28-COOH while the compounds with corresponding ethyl or benzyl esters (**9b**,**d** and **10b**,**c**) had no cytotoxicity against HCC cells. Similarly, a free 3-OH was essential for the anti-HCC activity of 28-position derivatives (**16b** vs **14b**). The striking characteristics may be required for those active compounds to enter and metabolize effectively in HCC cells and produce toxic levels of NO and its derived RNS/ROS, leading to cytotoxicity. However, the precise SAR of these derivatives and the optimal compounds with potent anti-HCC activity remain to be further investigated.

Previous study has shown that **18** inhibits liver inflammation.[19] Our previous data demonstrated that nitrate-based derivatives of OA that produced lower levels of NO protected HCC cells from anti-Fas mediated apoptosis.[20] In this study, we showed that some furoxan-based NO-releasing derivatives of OA had strong cytotoxicity selectively against HCC cells in vitro, which was associated with higher levels of NO production in HCC cells. Treatment with a representative compound **8b** or **16b** significantly inhibited the growth of HCC tumors inoculated in vivo. Our data provide a proof-in-principle that NO is a dual functional regulator for the HCC cells and higher levels of NO

produced in HCC cells are cytotoxic while lower levels of it are protective.[21,22] Importantly, treatment with a furoxan/OA hybrid up to 100 mg/kg did not cause any apparent adverse effects and continual administration of the compound for 35 consecutive days did not result in morphological abnormalities in mouse liver. Together, our findings suggest that these active compounds may be promising drug candidates with potent cytotoxicity selectively against HCC cells and may potentially be used for therapeutic intervention of liver cancer in the clinic. The selective cytotoxicity of these compounds against HCC cells may be associated with the efficient entry and metabolism of these compounds in HCC cells, but not in other cells tested, to produce high levels of NO. However, the precise mechanism(s) underlying the selective effect of these compounds remains to be further determined.

In summary, a group of novel furoxan-based NO-releasing derivatives of OA were designed and synthesized by coupling phenylsulfonyl- or phenyl-substituted furoxans to the 3- or 28-position of OA through various chemical linkers, respectively. Compounds **8a**−**e** and **16b** exhibited strong cytotoxicity selectively against HCC cells, which appeared to be associated with high levels of NO production in HCC cells in vitro. Treatment with **8b** or **16b** greatly inhibited the growth of HCC tumors inoculated but did not cause obvious morphological changes in mouse liver. Our data demonstrate that high levels of NO are toxic to HCC cells. Our findings provide a base for the design of novel furoxan/OA hybrids for therapeutic intervention of human liver cancers.

Experimental Section

General. Melting points were determined using a capillary apparatus (RDCSY-I) and are reported directly. All of the compounds synthesized were purified by column chromatography (CC) on silica gel 60 (200−300 mesh) and thin-layer chromatography (TLC) on silica gel 60 F254 plates (250 μm; Qingdao Ocean Chemical Company, China). Subsequently, they were routinely analyzed by IR (Shimadzu FTIR-8400S), ^{1}H NMR (Bruker ACF-300Q, 300 MHz), MS (Hewlett-Packard 1100 LC/MSD spectrometer), and elemental analysis (Elementar Vario EL III instrument).

General Procedure for the Preparation of 8a−g, 9b,d, 10b,c,f,g, 11h−k, 12j,k, and 13h. A solution of **5**, **6**, or **7** (0.42mmol), **1** or **2** (0.42mmol), DCC (86.5 mg, 0.42mmol), and DMAP (5.1 mg, 0.042mmol) in dry CH_2Cl_2 (20 mL) was stirred at room temperature for 4−10 h. After filtration, the filtrate was evaporated to dryness in vacuo, and the crude product was purified by column chromatography (petroleum ether (PE)/EtOAc = 4:1 to 3:1) to yield the compounds (39−72%).

3-{[4-(1-Methyl-3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}propoxy)-4-oxobutanoyl]oxy}olean-12-en-28-oic Acid (8b). The title compound was obtained starting from **5** and **1b**. White solid, 48.8% yield. Mp 140−142 °C. ESI-MS: 875 $[M + Na]^+$. IR (KBr): 3398, 2943, 2879, 1731, 1694, 1621, 1556, 1370, 1155 cm^{-1}. ^{1}H NMR ($CDCl_3$), δ 0.73 (s, 3H, CH_3), 0.77 (s, 3H, CH_3), 0.81 (s, 6H, 2 × CH_3), 0.89 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 2.59 (s, 4H, 2 × $COCH_2$), 2.77−2.83 (m, 1H, C_{18}−H), 4.39 (brs, 1H, 3α-H), 4.43 (t, 2H, OCH_2, J = 6 Hz), 5.12−5.19 (m, 1H, OCH), 5.26 (brs, 1H, C_{12}−H), 7.58−7.63 (m, 2H, ArH), 7.70−7.75 (m, 1H, ArH), 8.05 (d, 2H, ArH, J = 8 Hz). Anal. ($C_{46}H_{64}N_2O_{11}S$) C, H, N.

General Procedure for the Preparation of 14a−d,g and 15h,j,k. OA (456 mg, 1mmol) dissolved in toluene was mixed with TFAA (0.82 mL, 5.87mmol) by stirring at room temperature for 10 min, followed by the addition of **1** or **2** (1mmol) to the reaction mixture and allowing the mixture to reflux for an additional 4 h with stirring. After cooling to room temperature, the mixture was gradually neutralized with 10% NaOH, and the organic layer was washed with water and saturated NaCl solution sequentially, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude product was purified by column chromatography (PE/EtOAc = 4:1 to 3:1) to give the title compounds (62−70%).

1-Methyl 3-{[5-Oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}-propyl-3-[(trifluoroacetyl)oxy]olean-12-en-28-oate (14b). The title compound was obtained starting from OA, TFAA, and **1b**. White solid, 70% yield. Mp 74−76 °C. ESI-MS: 866 $[M + NH_4]^+$. IR (KBr): 2935, 2870, 1779, 1720, 1614, 1550, 1450, 1373, 1163 cm^{-1}. ^{1}H NMR ($CDCl_3$), δ 0.71 (s, 3H, CH_3), 0.75 (s, 3H, CH_3), 0.87 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 1.04 (s, 3H, CH_3), 1.12 (s, 3H, CH_3), 2.84−2.86 (m, 1H, C_{18}−H), 4.45−4.47 (m, 2H, OCH_2), 4.65−4.69 (m, 1H, 3α-H), 5.26−5.27 (m, 1H, OCH), 5.28 (brs, 1H, C_{12}−H), 7.63−7.66 (m, 2H, ArH), 7.74−7.75 (m, 1H, ArH), 8.06−8.09 (m, 2H, ArH). Anal. ($C_{44}H_{59}F_3N_2O_9S$) C, H, N.

General Procedure for the Preparation 16b,c and 17h,j. Compound **14** or **15** was dissolved in MeOH, and their pH was adjusted slowly with diluted $KHCO_3$ solution to pH 8−9. The solution was kept stirring at room temperature for 1 week and concentrated under reduced pressure. The residue was diluted with water and extracted with EtOAc. The obtained organic layer was washed with water and saturated NaCl solution, dried, and evaporated. The crude product was purified by column chromatography (PE/EtOAc = 4:1 to 3:1) to give the title compounds (92−98%).

1-Methyl 3-{[5-Oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}-propyl-3-hydroxyolean-12-en-28-oate (16b). The title compound was obtained starting from **14b**. White solid, 92% yield. Mp 86−88 °C. ESI-MS: 775 $[M + Na]^+$. IR (KBr): 3438, 2947, 2869, 1720, 1616, 1551, 1451, 1367, 1170 cm^{-1}. ^{1}H NMR ($CDCl_3$), δ 0.66 (s, 3H, CH_3), 0.73 (s, 6H, 2 × CH_3), 0.87 (s, 6H, 2 × CH_3), 0.93 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 2.79−2.81 (m, 1H, C_{18}−H), 3.14−3.19 (m, 1H, 3α-H), 4.14−4.46 (m, 2H, OCH_2), 5.07−5.08 (m, 1H, OCH), 5.24−5.25 (brs, 1H, C_{12}−H), 7.59−7.64 (m, 2H, ArH), 7.71−7.73 (m, 1H, ArH), 8.03−8.06 (m, 2H, ArH). Anal. ($C_{42}H_{60}N_2O_8S$) C, H, N.

Biological Experiments. Cytotoxicity Assay in Vitro. The cytotoxicity of individual compounds screened was determined by the LDH assay using the cytotoxicity detection kit (Roche) according to the manufacturer's instructions. Briefly, human liver cancer cell lines (HepG2 and Hep3b), human cervical cancer cell line (HeLa), human prostate cancer cell line (PC-3), human breast cancer cell line (MCF-7), and human kidney epithelial cell line (HEK 293) were obtained from ATCC and were cultured in 10% fetal calf serum (FCS) Dulbecco's modified Eagle's medium (DMEM). The cells (10^4/well) were in triplicate exposed to varying concentrations of individual compound (diluted from the stock in DMSO) in 2% FCS phenol red free DMEM for 24−72 h. The cells exposed to the same concentration of DMSO in DMEM were used as negative controls (spontaneous releasing of LDH), and the cells exposed to 1% Triton X-100 in DMEM were used as positive controls (maximum releasing of LDH). A portion of the cell supernatant (50 μL) was harvested from each well, and LDH levels were determined by LDH assays at optical density (OD, 490/650). The percent specific cytotoxicity of each compound was determined based on [(OD experiments) − (OD negative controls)]/[(OD positive controls) − (OD negative controls)] × 100.

Nitrate/Nitrite Measurement in Vitro. The levels of nitrate and nitrite produced by the compounds were determined by the colorimetric assay using the nitrate/nitrite colorimetric assay kit (Cayman Chemical) according to the manufacturer's instructions. Briefly, HepG2, Hep3b, Hela, PC-3, MCF-7, and Hek 293 cells (5 × 10^6/well) were treated with 100 μM of one of the compounds (**8a**−**f**, **11h**, **14b**, **16b**, **18**, or OA) for varying periods (30−300 min). Subsequently, the cells were harvested and their cell lysates were prepared. Following microfuge ultrafiltration, a 40 μL sample was used for analysis at OD 540. The cells treated with diluent were used as negative controls for the background levels of nitrate/nitrite production, and nitrate at different concentrations was used as positive controls for the standard curve.

Acute Toxic Assay in Vivo. Groups of male Balb/c mice (n = 8 per group, Jackson Laboratory) were treated ip with **8b**, **16b**, or

control OA at 15, 50, or 100 mg/kg (100−250 μL, diluted from different concentrations of compound stock) daily for 3 consecutive days. A group of Balb/c mice received similar volumes of 10% DMSO. PBS was used as a sham control. Following injections, their body weights, feeding behavior (the amount of food and water consumed), and their activities were monitored daily. Fifteen days after injection, the mice were sacrificed and their livers were histologically examined.

HCC Model and Treatment in Vivo. Groups of male NOD/scid mice at 6−8 weeks of age (Taconic) were inoculated subcutaneously (sc) with 10^6 Hep3b cells and then injected ip with about 100 μL of **8b**, **16b**, OA (15 mg/kg) or the diluent daily for 35 consecutive days. The mice were sacrificed, and their tumor size and weight were measured by an external caliper or a balance in a blinded fashion.

Acknowledgment. The authors thank Drs. Louis Ignarro and Jon M. Fukuto for their advice. This work was partially supported by a grant from the National Institutes of Health (NIH) (Grant CA116846 to J.T.).

Supporting Information Available: General synthetic procedures; spectroscopic data of target compounds except for **8b**, **14b**, and **16b**; elemental analysis results of all target compounds; and data of cytotoxicity against Hep3b cells. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Ignarro, L. J. Signal transduction mechanisms involving nitric oxide. *Biochem. Pharmacol.* **1991**, *41*, 485–490.
(2) Fukuto, J. M.; Wink, D. A. Nitric oxide (NO): formation and biological roles in mammalian systems. *Met. Ions Biol. Syst.* **1999**, *36*, 547–595.
(3) Millet, A.; Bettaieb, A.; Renaud, F.; Prevotat, L.; Hammann, A.; Solary, E.; Mignotte, B.; Jeannin, J. F. Influence of the nitric oxide donor glyceryl trinitrate on apoptotic pathways in human colon cancer cells. *Gastroenterology* **2002**, *123*, 235–246.
(4) Dhar, A.; Brindley, J. M.; Stark, C.; Citro, M. L.; Keefer, L. K.; Colburn, N. H. Nitric oxide does not mediate but inhibits transformation and tumor phenotype. *Mol. Cancer Ther.* **2003**, *2*, 1285–1293.
(5) Postovit, L. M.; Adams, M. A.; Lash, G. E.; Heaton, J. P.; Graham, C. H. Nitric oxide-mediated regulation of hypoxia-induced B16F10 melanoma metastasis. *Int. J. Cancer* **2004**, *108*, 47–53.
(6) Findlay, V. J.; Townsend, D. M.; Saavedra, J. E.; Buzard, G. S.; Citro, M. L.; Keefer, L. K.; Ji, X.; Tew, K. D. Tumor cell responses to a novel glutathione *S*-transferase-activated nitric oxide-releasing prodrug. *Mol. Pharmacol.* **2004**, *65*, 1070–1079.
(7) Medana, C.; Ermondi, G.; Fruttero, R.; Di Stilo, A.; Ferretti, C.; Gasco, A. Furoxans as nitric oxide donors. 4-Phenyl-3-furoxancarbonitrile: thiol-mediated nitric oxide release and biological evaluation. *J. Med. Chem.* **1994**, *37*, 4412–4416.
(8) Moharram, S.; Zhou, A.; Wiebe, L. I.; Knaus, E. E. Design and synthesis of 3′- and 5′-*O*-(3-benzenesulfonylfuroxan-4-yl)-2′-deoxyuridines: biological evaluation as hybrid nitric oxide donor-nucleoside anticancer agents. *J. Med. Chem.* **2004**, *47*, 1840–1846.
(9) Medana, C.; Di Stilo, A.; Visentin, S.; Fruttero, R.; Gasco, A.; Ghigo, D.; Bosia, A. NO donor and biological properties of different benzofuroxans. *Pharm. Res.* **1999**, *16*, 956–960.
(10) Ekmekcioglu, S.; Tang, C. H.; Grimm, E. A. NO news is not necessarily good news in cancer. *Curr. Cancer Drug Targets* **2005**, *5*, 103–115.
(11) Liu, J. Pharmacology of oleanolic acid and ursolic acid. *J. Ethnopharmacol* **1995**, *49*, 57–68.
(12) Chen, Y. J.; Liu, J.; Yang, X. L.; Zhao, X. L.; Xu, H. B. Oleanolic acid nanosuspensions: preparation, in-vitro characterization and enhanced hepatoprotective effect. *J. Pharm. Pharmacol.* **2005**, *57*, 59–64.
(13) Liu, Y.; Hartley, D. P.; Liu, J. Protection against carbon tetrachloride hepatotoxicity by oleanolic acid is not mediated through metallothionein. *Toxicol. Lett.* **1998**, *95*, 77–85.
(14) Liu, J.; Liu, Y.; Madhu, C.; Klaassen, C. D. Protective effects of oleanolic acid on acetaminophen-induced hepatotoxicity in mice. *J. Pharmacol. Exp. Ther.* **1993**, *266*, 1607–1613.
(15) Kim, K. A.; Lee, J. S.; Park, H. J.; Kim, J. W.; Kim, C. J.; Shim, I. S.; Kim, N. J.; Han, S. M.; Lim, S. Inhibition of cytochrome P450 activities by oleanolic acid and ursolic acid in human liver microsomes. *Life Sci.* **2004**, *74*, 2769–2779.
(16) Parkin, D. M.; Bray, F.; Ferlay, J.; Pisani, P. Estimating the world cancer burden: Globocan 2000. *Int. J. Cancer* **2001**, *94*, 153–156.
(17) (a) Kenney, W. J.; Walsh, J. A.; Davenport, D. A. An acid-catalyzed cleavage of sulfoxides. *J. Am. Chem. Soc.* **1961**, *83*, 4019–4022. (b) Farrar, W. V. The 3, 4-bisarenesulphonylfuroxans. *J. Chem. Soc.* **1964**, (part I), 904–906. (c) Kelley, J. L.; Mclean, E. W.; Williard, K. F. Synthesis of bis(arylsulfonyl) furoxans from aryl nitromethyl sulfones. *J. Heterocycl. Chem.* **1977**, *14*, 1415–1416. (d) Li, R. W.; Zhang, Y. H.; Ji, H.; Yu, X. L.; Peng, S. X. Synthesis and anti-inflammatory activity of benzenesulfonylfuroxan-coupled diclofenac. *Acta Pharm. Sin.* **2001**, *36*, 821–826.
(18) (a) Gasco, A. M.; Fruttero, R.; Sorba, G.; Gasco, A. Phenylfuroxancarbaldehydes and related compounds. *Liebigs. Ann. Chem.* **1991**, *11*, 1211–1213. (b) Li, R. W.; Zhang, Y. H.; Ji, H.; Yu, X. L.; Peng, S. X. Synthesis and anti-inflammatory analgestic activities of phenylfuroxan-coupled diclofenac. *Acta Pharm. Sin.* **2002**, *37*, 27–32.
(19) (a) Fiorucci, S.; Mencarelli, A.; Palazzetti, B.; Del Soldato, P.; Morelli, A.; Ignarro, L. J. An NO derivative of ursodeoxycholic acid protects against Fas-mediated liver injury by inhibiting caspase activity. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 2652–2657. (b) Fiorucci, S.; Antonelli, E.; Morelli, O.; Mencarelli, A.; Casini, A.; Mello, T.; Palazzetti, B.; Tallet, D.; Del Soldato, P.; Morelli, A. NCX-1000, a NO-releasing derivative of ursodeoxycholic acid, selectively delivers NO to the liver and protects against development of portal hypertension. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 8897–8902.
(20) Chen, L.; Zhang, Y. H.; Kong, X. W.; Peng, S. X.; Tian, J. Synthesis and biological evaluation of nitric oxide-releasing derivatives of oleanolic acid as inhibitors of HepG2 cell apoptosis. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2979–2982.
(21) Xu, W. M.; Liu, L. Z.; Loizidou, M.; Ahmed, M.; Charles, I. G. The role of nitric oxide in cancer. *Cell Res.* **2002**, *12*, 311–320.
(22) Mocellin, S.; Bronte, V.; Nitti, D. Nitric oxide, a double edged sword in cancer biology: searching for therapeutic opportunities. *Med. Res. Rev.* **2007**, *27*, 317–352.

JM800167U