Bioorganic & Medicinal Chemistry Letters 20 (2010) 6416-6420

Contents lists available at ScienceDirect



Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl



Synthesis and biological evaluation of furoxan-based nitric oxide-releasing derivatives of glycyrrhetinic acid as anti-hepatocellular carcinoma agents

Yisheng Lai^{a,*}, Lihong Shen^{a,b}, Zhenzhen Zhang^c, Wenqing Liu^a, Yihua Zhang^a, Hui Ji^{c,*}, Jide Tian^d

^a Center of Drug Discovery, China Pharmaceutical University, Nanjing 210009, PR China

^b Chemistry Department, Handan College, Handan 056005, PR China

^c Department of Pharmacology, China Pharmaceutical University, Nanjing 210009, PR China

^d Department of Molecular and Medical Pharmacology, University of California Los Angeles, Los Angeles, CA 90095, USA

ARTICLE INFO

Article history: Received 18 August 2010 Revised 10 September 2010 Accepted 14 September 2010 Available online 17 September 2010

Keywords: NO donor Furoxan Glycyrrhetinic acid Hybrid compound Anti-hepatocellular carcinoma agent NO-releasing

ABSTRACT

A series of novel furoxan-based nitric oxide (NO)-releasing derivatives of glycyrrhetinic acid (GA) were designed, synthesized, and evaluated for their in vitro cytotoxicity against human hepatocellular carcinoma (HCC) and non-tumor liver cells. Five furoxan/GA hybrids, **7b–d**, **7f**, and **7g**, displayed potent cytotoxicity against HCC cells (IC₅₀: 0.25–1.10 μ M against BEL-7402 cells and 1.32–6.78 μ M against HepG2 cells), but had a little effect on the growth of LO2 cells, indicating that these compounds had selective cytotoxicity against HCC cells. Furthermore, these compounds produced high concentrations of NO in HCC cells, but low in LO2 cells and treatment with hemoglobin partially reduced the cytotoxicity of the hybrid in HCC cells. Apparently, the high concentrations of NO produced by NO donor moieties and the bioactivity of GA synergistically contribute to the cytotoxicity, but the NO is a major player against HCC cells in vitro. Potentially, our findings may aid in the design of new chemotherapeutic reagents for the intervention of human HCC at clinic.

© 2010 Elsevier Ltd. All rights reserved.

Nitric oxide (NO) is a critical regulator of the biological process involved in many physiological functions.¹ However, high concentrations of NO can induce cell cycle arrest and apoptosis, particularly for some tumor cells.^{2–4} Indeed, the NO-based anti-cancer reagents have been investigating for their potential application for cancer therapy at clinic.^{5–7} Furoxans, an important class of NO donors, can produce high concentrations of NO and exhibit strong anti-cancer activity.^{8–11} However, due to their broad biological effects, furoxan-based anti-cancer reagents may result in severe adverse effect. Therefore, development and discovery of new derivatives of furoxan that can selectively produce high concentrations of NO in tumor cells will be of great significance in the intervention of human tumors, such as hepatocellular carcinoma (HCC) with little adverse effect.

Glycyrrhetinic acid (GA) is the pentacyclic triterpene aglycone of glycyrrhizic acid. GA and glycyrrhizic acid have been shown to possess anti-inflammatory, anti-oxidative, anti-ulcer, anti-allergenic, anti-cancer, and immunomodulatory activities.¹² Furthermore, GA at a high concentration can induce apoptosis of HCC cells.¹³ More importantly, GA can bind to the epidermal growth factor receptor (EGFR)¹⁴ and steroid receptors¹⁵ with high affinity and protect hepatocytes from the carbon tetrachloride and other toxicant-induced hepatotoxicity in mice and rats.^{16–18} The high affinity of GA binding to hepatocytes and its protective activity make GA as an ideal leading compound for the design of novel furoxan/GA hybrids, which may selectively produce high concentrations of NO in HCC cells, leading to potent cytotoxicity against HCC cells, but little side effect on healthy hepatocytes.

To generate fruoxan/GA hybrids, the phenylsulfonylfuroxans **1a–h** were synthesized from benzenethiol in a four-step sequence, as previous reports.¹⁹ Simultaneously, GA solution of methanol was refluxed in the presence of *p*-toluenesulfonic acid (*p*-TSA) to yield glycyrrhetinic acid methyl ester **2**,²⁰ which was in turn esterified with succinic anhydride in the presence of DMAP to form 3-O-hemisuccinate GA methyl ester **3**.²¹ Subsequently, compound **3** was further reacted with **1a–h** in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and 4-dimethylaminopyridine (DMAP) to generate the target compounds **4a–h** in 65–70% yields (Scheme 1).²²

Given that coupling with an amino acid can help in delivering the compounds to tumor cells,^{23–25} other target compounds were designed using glycine as a linker and synthesized, as illustrated in Scheme 2 and 3. First, compounds **1a–g** were reacted with *N*-Boc-glycine in the presence of dicyclohexylcarbodiimide (DCC) and DMAP to form *N*-Boc-glycinates **5a–g** in 55–80% yields. Subsequently, the Boc group was removed with trifluoroacetic acid to generate the glycinates **6a–g**, which were coupled with GA in the presence of EDCI and DMAP to generate **7a–g** in 45–55% yields (Scheme 2).^{26,27}

^{*} Corresponding authors. Tel.: +86 25 83271285; fax: +86 25 86635503 (Y.L.); tel.: +86 25 86021369 (H.J.).

E-mail addresses: lcpu333@yahoo.com.cn (Y. Lai), huijicpu@163.com (H. Ji).



Scheme 1. The synthetic pathway of 4a-h. Reagents and conditions: (a) CH₃OH, *p*-TSA, reflux; (b) succinic anhydride, DMAP, dry CH₂Cl₂, reflux, 15 h; (c) 1a-h, DCC, DMAP, dry CH₂Cl₂, rt, 24 h.



Scheme 2. The synthetic pathway of 7a-g. Reagents and conditions: (a) Boc-NHCH₂CO₂H, DCC, DMAP, dry CH₂Cl₂, rt, 24 h; (b) CF₃CO₂H, dry CH₂Cl₂, rt, 2 h; (c) GA, EDCI, DMAP, DMF, rt, 24 h.

The condensation of GA with **1h** in the presence of EDCI and DMAP produced **8h** in 48% yield. 3-O-Acetylation of GA with acetic anhydride in the presence of DMAP furnished **9** in 98% yield,²⁸ which was coupled with **1h**, **6d**, or **6e** in the presence of EDCI and DMAP to produce compounds **10h**, **11d**, and **11e**, respectively. Alternatively, **9** was treated with oxalyl chloride to form acyl chloride derivative **12**, which was subsequently esterified with **1a**, **1b**, **1d**, **1e**, or **1g** in the presence of triethylamine (Et₃N) to form **13a**, **13b**, **13d**, **13e**, and **13g**, respectively (Scheme 3).²⁹

All of the final products were purified by column chromatography and their structures were characterized by infrared (IR), mass spectra (MS), ¹H NMR, and elemental analyses.

The cytotoxicity of the target compounds and GA against human HCC cells (HepG2, BEL-7402) was evaluated by MTT assay using adriamycin as a positive control (Table 1).³⁰ GA exhibited a weak cytotoxicity against HCC cells tested, consistent with previous reports.^{13,31} In contrast, most of the new furoxan/GA hybrids displayed potent anti-HCC activity, and some of them were stronger than that of adriamycin. For example, the IC₅₀ value of **7d** against HCC BEL-7402 cells was near fourfold less than that of adriamycin (0.25 vs 0.90 μ M). More importantly, the hybrids **7b–d**, **7f**, and **7g** had stronger anti-proliferatory activity against BEL-7402 cells than that of their corresponding NO donor moieties **1b–d**, **1f**, and **1g** (IC₅₀ = 6.52–13.26 μ M), suggesting a synergistic effect of furoxan and GA on inhibition of HCC cells proliferation. However, other hybrids displayed various cytotoxicities against HCC cells.

Next, we examined whether the new furoxan/GA hybrids could selectively inhibit the proliferation of HCC cells by MTT assays.³⁰ We found that the NO donor moieties **1b–d**, **1f** and **1g**, like adriamycin, inhibited the proliferation of both HCC BEL-7402 cells and non-tumor human liver LO2 cells with a similar level of inhibitory activity at the concentration of 10 μ M (Fig. 1). In contrast, the corresponding hybrids **7b–d**, **7f** and **7g** selectively inhibited the proliferation of BEL-7402 cells, but with a little effect on LO2 cells. Evidentially, treatment with **7b–d**, **7f** or **7g** inhibited the proliferation of BEL-7402 cells by 83.52–96.50%, respectively, and the inhibitory effects of these compounds on LO2 cells were less than 18.00%. These suggest that these new hybrids may have little adverse effect and be safe for the intervention of human HCC.

To understand their selectivity and diverse cytotoxicity against HCC and LO2 cells, we examined the production of NO by individual compounds in HCC and LO2 cells by the Griess assay (Fig. 2).³² The furoxan moieties produced similar concentrations of NO in HCC and LO2 cells, while treatment with hybrids **7b–d**, **7f** or **7g** generated high concentrations of NO in HCC cells, which were 3–5-fold higher than that in LO2 cells. The significantly higher concentrations of NO produced in HCC cells may contribute to the selective cytotoxicity of these new hybrids against HCC cells.



Scheme 3. The synthetic pathway of 8h, 10h, 11d, 11e, 13a, 13b, 13d, 13e, and 13g. Reagents and conditions: (a) 1h, EDCI, DMAP, DMF, rt, 24 h; (b) Ac₂O, DMAP, reflux, 15 h; (c) 6d or 6e, EDCI, DMAP, DMF, rt, 24 h; (d) oxalyl chloride, dry CH₂Cl₂, rt, 4 h; (e) 1a, 1b, 1d, 1e, or 1g, Et₃N, dry THF, reflux, 12 h.

Table 1									
The inhibitory	effects	of the	target	compounds	on	the	proliferation	of human	HCC
cells									

Compound	IC ₅₀	^a (μM)	Compound	$IC_{50}^{a}(\mu M)$		
	HepG2	BEL-7402		HepG2	BEL-7402	
ADM ^b	2.03	0.90	7d	6.78	0.25	
GA ^c	>50	>50	7e	5.18	3.78	
4a	18.18	7.85	7f	1.32	0.32	
4b	13.41	9.22	7g	3.39	0.84	
4c	26.03	6.03	8h	7.40	2.47	
4d	36.52	8.20	10h	23.72	14.16	
4e	15.67	19.92	11d	8.39	4.84	
4f	7.90	7.37	11e	6.40	9.47	
4g	11.55	13.41	13a	14.71	3.84	
4h	2.90	2.94	13b	11.52	15.35	
7a	9.06	1.38	13d	9.83	5.04	
7b	3.79	0.43	13e	31.45	21.38	
7c	3.02	1.10	13g	7.50	14.96	

^a IC_{50} = compound at a concentration required to inhibit tumor cell proliferation by 50%. Data are expressed as the mean IC_{50} from the dose–response curves of at least three independent experiments.

^b Adriamycin.

^c Glycyrrhetinic acid.

To further determine the role of NO in cytotoxicity of these hybrids against HCC cells, HCC BEL-7402 cells were pre-treated with varying concentrations of hemoglobin, a well-known NO scavenger, and then treated with different concentrations of hybrid compound **7d** for the detection of their cytotoxicity and NO production in Figure 3. As expected, **7d** produced high concentration of NO and displayed a strong cytotoxicity against the HCC cells without pre-treatment with hemoglobin. In contrast, pre-treat-



Figure 1. Cytotoxicity of the furoxan moieties and target compounds against HCC BEL-7402 cells and non-tumor liver LO2 cells. BEL-7402 and LO2 cells were treated with 10 μ M of the indicated compounds and their cytotoxicities were determined by MTT. Data are expressed as mean% ± SD of the cytotoxicity of individual compounds in both cells from three separate experiments. *p* <0.05, determined by X² tests.

ment with different concentrations of hemoglobin dramatically reduced the concentrations of NO and inhibited the cytotoxicity of **7d** against the BEL-7402 cells. Furthermore, the inhibitory effects of different concentrations of hemoglobin were dose-dependent and negatively correlated with the concentrations of NO ($R^2 = 0.87$, p < 0.01, determined by logistic regression analysis). More importantly, the cytotoxicities of **7d** were positively correlated with the concentrations of NO ($R^2 = 0.79$, p < 0.01). Notably, treatment with 20 µM of hemoglobin only reduced the cytotoxicity



Figure 2. Variable levels of NO produced by the compounds in human HCC cells and non-tumor liver cells. Cells were treated in triplicate with each compound at 100 μ M for 30–300 min. The cell lysates were prepared for determining the contents of NO by Griess assay. Data are expressed as mean of individual compounds in each type of cells after treatment with the compounds for 300 min. Similar patterns of NO production were detected at other time points (data not shown).



Figure 3. The concentrations of NO produced by **7d** were correlated with the cytotoxicities against HCC cells. BEL-7402 cells were cultured in 24-well plates and pre-treated in triplicate with the indicated concentrations (0, 1, 5, 10, or 20 μ M) of hemoglobin for 1 h. The cells were treated with 5 or 10 μ M of **7d** for 24 h, and the cytotoxicity and NO production of **7d** were determined by MTT and Griess assays, respectively. Data are expressed as mean% of the cytotoxicity and mean concentrations of NO in individual experimental conditions. The intra-group and inter-experimental variations were less than 10% and the indicated concentrations of hemoglobin used did not affect the cell growth (<5%).

of **7d** by near 66%. Apparently, the high concentrations of NO produced by NO donor moieties and the bioactivity of GA synergistically contribute to,³³ but the NO is a major player in the cytotoxicity of these new hybrids against HCC cells in vitro.

Analysis of structure and activity relationship (SAR) revealed that the hybrids with NO moieties coupling to 30-COOH of GA in general had stronger cytotoxicity than that of 3-OH hybrids (e.g., **7a** or **13a** vs **4a**, **7b** vs **4b**, **7c** vs **4c**, **7d**, **11d** or **13d** vs **4d**, **7e** or **11e** vs **4e**, **7f** vs **4f**, **7g** vs **4g**). Furthermore, the coupling model of the NO donor moiety with GA also affected the cytotoxic activity of these hybrids. For example, compound **4h** containing an amide bond was more active than its corresponding analog **4a** containing two ester bonds. Interestingly, the hybrids **7a**–**g** with a glycine as a linker were more cytotoxic to HCC cells than their corresponding analogs without glycine residue (e.g., **7a** vs **13a**, **7b** vs **13b**, **7d** vs **13d**, **7e** vs **13e**, **7g** vs **13g**), supporting the notion that introduction of a glycine residue into compounds can significantly enhance their cytotoxicity.^{23–25} Thus, new hybrid compounds generated by coupling NO donor moieties to 30-COOH of GA with a glycine as a linker should have potent cytotoxicity selectively against HCC cells.

In summary, a series of furoxan/GA hybrids were designed, synthesized and evaluated for their cytotoxicity against human HCC and non-tumor liver cells in vitro. Our data indicated that **7b–d**, **7f** and **7g** displayed selective cytotoxicity against human HCC cells, which was associated with higher concentrations of NO production in HCC cells and potentially synergistic effect of NO donor moieties and the bioactivity of GA. Potentially, our findings may aid in the design of new chemotherapeutic reagents for the intervention of human HCC at clinic.

Acknowledgments

The authors thank the Center of Analysis & Measurement of China Pharmaceutical University for the characterization of the compounds and Ms. Yao Liu for her technical support. The authors are also grateful to Drs. Xiaolei Ye, Zhangjian Huang and Lei Fang for their helpful discussions.

References and notes

- 1. Mocellin, S. Curr. Cancer Drug Targets 2009, 9, 214.
- Coulter, J. A.; McCarthy, H. O.; Xiang, J.; Roedl, W.; Wagner, E.; Robson, T.; Hirst, D. G. Nitric Oxide 2008, 19, 192.
- 3. Wink, D. A.; Ridnour, L. A.; Hussain, S. P.; Harris, C. C. Nitric Oxide 2008, 19, 65.
- 4. Hirst, D.; Robson, T. Curr. Pharm. Des. 2010, 16, 411.
- Yasuda, H.; Yamaya, M.; Nakayama, K.; Sasaki, T.; Ebihara, H.; Kanda, A.; Asada, M.; Inoue, D.; Suzuki, T.; Okazaki, T.; Takahashi, H.; Yoshida, M.; Kaneta, T.; Ishizama, K.; Yamanda, S.; Tomita, N.; Yamasaki, M.; Kikuchi, A.; Kubo, H.; Sasaki, H. J. Clin. Oncol. 2006, 24, 688.
- 6. Seimens, D. R.; Heaton, J.; Adams, M.; Graham, C. Nitric Oxide 2007, 17, S15.
- Siemens, D. R.; Heaton, J. P.; Adams, M. A.; Kawakami, J.; Graham, C. H. Urology 2009, 74, 878.
- 8. Cerecetto, H.; Porcal, W. Mini-Rev. Med. Chem. 2005, 5, 57.
- 9. Moharram, S.; Zhou, A.; Wiebe, L. I.; Knaus, E. E. J. Med. Chem. 2004, 47, 1840.
- Maksimovic-Ivanic, D.; Mijatovic, S.; Harhaji, L.; Miljkovic, D.; Dabideen, D.; Fan Cheng, K.; Mangano, K.; Malaponte, G.; Al-Abed, Y.; Libra, M.; Garotta, G.; Nicoletti, F.; Stosic-Grujicic, S. *Mol. Cancer Ther.* **2008**, *7*, 510.
- Boiani, M.; Cerecetto, H.; González, M.; Risso, M.; Olea-Azar, C.; Piro, O. E.; Castellano, E. E.; López de Ceráin, A.; Ezpeleta, O.; Monge-Vega, A. Eur. J. Med. Chem. 2001, 36, 771.
- 12. Asl, M. N.; Hosseinzadeh, H. Phytother. Res. 2008, 22, 709.
- 13. Hibasami, H.; Iwase, H.; Yoshioka, K.; Takahashi, H. Int. J. Mol. Med. 2006, 17, 215.
- 14. Kimura, M.; Inoue, H.; Hirabayashi, K.; Natsume, H.; Ogihara, M. Eur. J. Pharmacol. 2001, 431, 151.
- 15. Armanini, D.; Karbowiak, I.; Funder, J. W. Clin. Endocrinol. **1983**, 19, 609.
- Nose, M.; Ito, M.; Kamimura, K.; Shimizu, M.; Ogihara, Y. Planta Med. 1994, 60, 136.
- 17. Lin, G.; Nnane, I. P.; Cheng, T. V. Toxicon 1999, 37, 1259.
- Gumpricht, E.; Dahl, R.; Devereaux, M. W.; Sokol, R. J. J. Biol. Chem. 2005, 280, 10556.
- Li, R. W.; Zhang, Y. H.; Ji, H.; Yu, X. L.; Peng, S. X. Acta Pharm. Sin. 2001, 36, 821.
 Su, X.; Lawrence, H.; Ganeshapillai, D.; Cruttenden, A.; Purohit, A.; Reed, M. J.; Vicker, N.; Potter, B. V. Bioorg. Med. Chem. 2004, 12, 4439.
- Dean, P. D.; Halsall, T. G.; Whitehouse, M. W. J. Pharm. Pharmacol. 1967, 19, 682.
- 22. General procedure for the synthesis of the target compounds 4a-h: DMAP (0.1 mmol) and appropriate phenylsulfonylfuroxans 1a-h (0.31 mmol) were added to a mixture solution of 3-0-hemisuccinate GA methyl ester 3 (0.26 mmol) and DCC (0.33 mmol) in dry dichloromethane. The reaction mixture was stirred at room temperature for 24 h. Filtration and removal of the solvent in vacuo afforded the crude product, which was subsequently purified by column chromatography using (PE/EtOAc = 3:1) to give pure 4a-h in 65-70% yields.
- Song, X.; Lorenzi, P. L.; Landowski, C. P.; Vig, B. S.; Hilfinger, J. M.; Amidon, G. L. Mol. Pharm. 2005, 2, 157.
- 24. Deshmukh, M.; Chao, P.; Kutscher, H. L.; Gao, D.; Sinko, P. J. J. Med. Chem. 2010, 53, 1038.
- Ling, Y.; Ye, X.; Ji, H.; Zhang, Y.; Lai, Y.; Peng, S.; Tian, J. Bioorg. Med. Chem. 2010, 18, 3448.
- 26. General procedure for the synthesis of the target compounds 7a-g: A solution of N-Boc-glycine (1.0 mmol), DCC (1.25 mmol), DMAP (0.125 mmol) and appropriate phenylsulfonylfuroxans 1a-g (1.1 mmol) in dry CH₂Cl₂ was stirred at room temperature for 24 h. After filtration, the filtrate was evaporated in vacuo and the crude product was purified by column chromatography (PE/EtOAc = 3:1) to afford N-Boc-glycinates 5a-g. A solution of appropriate 5a-g (0.43 mmol) and trifluoroacetic acid (1 mL) in dry CH₂Cl₂

(4 mL) was stirred at room temperature for 2 h. The solvent was removed in vacuo. The crude residue was dissolved in the solution of DMF (15 mL) and DMAP (1.60 mmol) with stirring at room temperature for 0.5 h, followed by the addition of GA (0.43 mmol) and EDCI (1.30 mmol) to the reaction mixture for another 24 h with stirring at room temperature. The mixture was diluted with water (200 mL) and extracted with EtOAc (3×50 mL). The combined organic layer was washed with saturated NaCl solution, dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The crude product was purified by column chromatography (PE/EtOAc = 2.1) to yield **7a–g**.

27. Data for selected compounds: 7b: yield 50%, white solid, mp 87-89 °C; IR (KBr, cm⁻¹): 3442, 2958, 2867, 1751, 1652, 1616, 1550, 1525, 1452, 1384; ¹H NMR (300 MHz, CDCl₃): δ 0.80–1.43 (m, 21H, 7 × CH₃), 2.33 (s, 1H, CHC=O), 2.78 (br s, 1H, OH), 3.20-3.25 (m, 1H, CHOH), 3.74-3.76 (m, 2H, NHCH2), 4.38 (t, 2H, J = 6.0 Hz, OCH₂), 4.53 (t, 2H, J = 6.0 Hz, OCH₂), 5.72 (s, 1H, CH=), 6.17 (br s, 1H, NH), 7.61–7.66 (m, 2H, Ar-H), 7.74–7.79 (m, 1H, Ar-H), 8.05 (d, 2H, J = 7.2 Hz, Ar-H); MS (ESI) $m/z = 810 [M+1]^+$; Anal. Calcd for C₄₃H₅₉N₃O₁₀S: C, 63.76; H, 7.34; N, 5.19. Found: C, 63.66; H, 7.38; N, 5.25. Compound **7c**: yield 55%, white solid, mp 96–98 °C; IR (KBr, cm⁻¹): 3450, 2960, 2869, 1741, 1649, 1625, 1548, 1519, 1452, 1386; ¹H NMR (300 MHz, CDCl₃): δ 0.80–1.45 (m, 21H, 7 × CH₃), 2.33 (s, 1H, CHC=O), 2.78 (br s, 1H, OH), 3.20-3.25 (m, 1H, CHOH), 4.07 (m, 2H, NHCH₂), 4.11 (t, 2H, J = 6.0 Hz, OCH₂), 4.51 (t, 2H, J = 6.0 Hz, OCH₂), 5.71 (s, 1H, CH=), 6.17 (br s, 1H, NH), 7.62-7.65 (m, 2H, Ar-H), 7.72-7.77 (m, 1H, Ar-H), 8.05 (d, 2H, J = 7.4 Hz, Ar-H); MS (ESI) m/z = 824 [M+1]⁺; Anal. Calcd for C44H61N3O10S: C, 64.13; H, 7.46; N, 5.10. Found: C, 64.05; H, 7.66; N, 4.94. Compound 7d: yield 50%, white solid, mp 80-82 °C; IR (KBr, cm⁻¹): 3429, 2956, 2869, 1749, 1654, 1616, 1552, 1450, 1384; ¹H NMR (300 MHz, CDCl₃): δ 0.80-1.49 (m, 21H, 7 × CH₃), 2.33 (s, 1H, CHC = 0), 2.78 (br s, 1H, OH), 3.20-3.25 (m, 1H, CHOH), 4.04–4.10 (m, 2H, NHCH₂), 4.27 (t, 2H, J = 6.0 Hz, OCH₂), 4.46 (t, 2H, J = 6.0 Hz, OCH₂), 5.72 (s, 1H, CH=), 6.18 (br s, 1H, NH), 7.60–7.65 (m, 2H, Ar-H), 7.74–7.79 (m, 1H, Ar-H), 8.05 (d, 2H, J = 7.2 Hz, Ar-H); MS (ESI) m/z = 822[M-1]⁻; Anal. Calcd for C₄₄H₆₁N₃O₁₀S: C, 64.13; H, 7.46; N, 5.10. Found: C, 64.25; H, 7.55; N, 5.02. Compound 7f: yield 50%, white solid, mp 113-115 °C; IR (KBr, cm⁻¹): 3436, 2950, 2867, 1751, 1654, 1610, 1548, 1450, 1359; ¹H NMR (300 MHz, CDCl₃): δ 0.80-1.45 (m, 21H, 7 × CH₃), 2.35 (s, 1H, CHC=O), 2.78 (br s, 1H, OH), 3.20-3.25 (m, 1H, CHOH), 4.05-4.20 (m, 2H, NHCH2), 4.74 (m, 2H, OCH2), 5.05 (m, 2H, OCH2), 5.20-5.25 (m, 2H, 2 × CH=), 5.70 (s, 1H, CH=), 7.60-7.65 (m, 2H, Ar-H), 7.74–7.79 (m, 1H, Ar-H), 8.06 (d, 2H, J = 7.5 Hz, Ar-H); MS (ESI) $m/z = 822 [M+1]^+$; Anal. Calcd for $C_{44}H_{59}N_3O_{10}S$: C, 64.29; H, 7.23; N, 5.11. Found: C, 63.93; H, 7.34; N, 5.22. Compound 7g: yield 55%, white solid, mp 91-93 °C; IR (KBr, cm⁻¹): 3436, 2956, 2867, 1757, 1652, 1620, 1544, 1454, 1359;

¹H NMR (300 MHz, CDCl₃): δ 0.80–1.43 (m, 21H, 7 × *CH*₃), 2.33 (s, 1H, *CHC*=O), 2.78 (br s, 1H, OH), 3.20–3.25 (m, 1H, *CHO*H), 4.03–4.20 (m, 2H, NH*CH*₂), 4.82 (s, 2H, *CH*₂), 5.11 (s, 2H, *CH*₂), 5.71 (s, 1H, *CH*=), 6.13 (br s, 1H, *NH*), 7.61–7.66 (m, 2H, Ar-H), 7.75–7.80 (m, 1H, Ar-H), 8.07 (d, 2H, *J* = 7.5 Hz, Ar-H); MS (ESI) m/z = 820 [M+1]^{*}; Anal. Calcd for C₄₄H₅₇N₃O₁₀S: C, 64.45; H, 7.01; N, 5.12. Found: C, 64.25; H, 7.06; N, 5.03.

- 28. Sakano, K.; Ohshima, M. Agric. Biol. Chem. 1986, 50, 763.
- 29. General procedure for the synthesis of the target compounds 13a, 13b, 13d, 13e and 13g: 3-Acetoxy-glycyrrhetinic acid 9 (6.0 mmol) dissolved in dry dichloromethane (20 mL), was added oxalyl chloride (3.0 mL) by slow dropwise. The mixture was stirred at room temperature for 4 h, followed by concentration under reduced pressure to 3-acetoxy-11-oxo-olean-12-en-30-oyl chloride 12. A mixture of 12 (0.52 mmol) and appropriate phenylsulfonylfuroxans 1a-g (0.53 mmol) in dry THF (20 mL) was refluxed in presence of triethylamine (0.2 mL) for 12 h. The solution was cooled to room temperature. After filtration, the filtrate was evaporated in vacuo and the crude product was purified by column chromatography (PE/EtOAc = 5:1) to obtain pure 13a, 13b, 13d, 13e and 13g in 80-85% yield.
- 30. In vitro cell growth inhibition assay: The human hepatocellular carcinoma (HepG2, BEL-7402) and non-tumor liver (LO2) cells (all from Shanghai Institutes for Biological Science, Chinese Academy of Sciences) were cultured in 10% heat inactivated fetal calf serum DMEM medium (Gibco, USA) at 37 °C, 5% CO₂. The inhibitory effects of different compounds on the growth of these cells were tested by MTT assay. Briefly, cells at 3×10^5 /well were cultured in 96-well plates overnight and treated in triplicate with different concentrations of individual compounds for 24 h. Cells cultured in medium alone or treated with vehicle were used as negative controls. During the last 4 h incubation, the cells were dissolved in 200 μ M DMSO, followed by measuring at 570 nm on a microplate reader (Thermo, USA).
- Chintharlapalli, S.; Papineni, S.; Jutooru, I.; McAlees, A.; Safe, S. Mol. Cancer Ther. 2007, 6, 1588.
- 32. Nitrate/nitrite measurement in vitro: The human hepatocellular carcinoma (HepG2, BEL-7402) and non-tumor liver (LO2) cells were cultured in the 24-well plates for 24 h and treated in triplicate with 100 μ M of individual compounds for 30–300 min. The cells were harvested longitudinally and their lysates were prepared. The levels of nitrate/nitrite in the lysates were determined using the nitrate/nitrite colorimetric assay kit, according to manufacturers' instruction (Beyotime, China).
- 33. Lee, C. S.; Kim, Y. J.; Lee, M. S.; Han, E. S.; Lee, S. Life Sci. 2008, 83, 481.