Bioorganic & Medicinal Chemistry Letters 21 (2011) 3074–3077

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Synthesis and in vitro antitumor activity of new butenolide-containing dithiocarbamates

Xiao-Juan Wang [a,b], Hai-Wei Xu [a,c], Lin-Lin Guo [a,c], Jia-Xin Zheng [a,c], Bo Xu [a,c], Xiao Guo [a,c], Chen-Xin Zheng [a,c], Hong-Min Liu [a,b,c,*]

[a] New Drug Research & Development Center, Zhengzhou University, Zhengzhou 450001, PR China
[b] Department of Chemistry, Zhengzhou University, Zhengzhou 450052, PR China
[c] School of Pharmaceutical Sciences, Zhengzhou University, No. 100, Avenue Kexue, Zhengzhou 450001, PR China

ARTICLE INFO

Article history:
Received 23 December 2010
Revised 22 February 2011
Accepted 9 March 2011
Available online 17 March 2011

Keywords:
Butenolides
Dithiocarbamate
Anti-tumor activity

ABSTRACT

Three series of butenolide-containing dithiocarbamates were designed and synthesized. Their anti-tumor activity in vitro was evaluated. Among them compound **I-14** exhibited broad spectrum anti-cancer activity against five human cancer cell lines with IC_{50} <30 μM. Structure–activity relationship analysis showed that the introduction of dithiocarbamate side chains on the C-3 position of butenolide was crucial for anti-tumor activity.

One of the main objectives of organic and medicinal chemistry is the design, synthesis and production of molecules having value as human therapeutic agents. During the past decade, number of dithiocarbamates were synthesized and evaluated,[1] by which some privileged structures receiving special attention have been found to possess excellent anti-tumor activity.[2,3] For example, structure modification of Brassinin (**1**)[3] (Fig. 1), a dithiocarbamate isolated from cruciferous plants, led to the design and synthesis of a potential cancer chemopreventive agent Sulforamate (**2**)[4] (Fig. 1). Furthermore, the dithiocarbamate portion of the brassinin is a crucial moiety for the anti-tumor activity.[3]

Butenolides (**3**) (Fig. 1) are ubiquitous chemical moieties occurring in a large number of natural products and known to be associated with several biological activities.[5] Many of the butenolide-containing compounds can be considered as potential anti-cancer agent, bactericides, fungicides, etc.[6] There are also a wide variety of pharmacologically active non-natural products bearing the heterocycle as the active site.[7]

In an effort to look for the possible anti-tumor agents, we were interested in the incorporation of dithiocarbamate moiety with butenolide. In addition, it is suspected that there is a close relationship between the position of dithiocarbamates side chain on butenolide and their biological activities. So three series of butenolide containing dithiocarbamates were designed and synthesized in order to investigate the structure–activity relationship and obtain significant insight into the impact of activity on molecular.

The butenolide derivatives **I** with dithiocarbamates side chain at C-3 position were obtained by the reaction of 3-bromomethyl butenolide (**9**) with CS_2 and various amines in the presence of $Na_3PO_4{\cdot}11H_2O$ in acetone[8] as shown in Scheme 1. 3-bromomethyl butenolide (**9**) was derived from γ-butyrolactone (**4**) by five-step reaction according to the reported method.[9] The synthesized derivatives were summarized in Table 1.

The butenolide derivatives **II** with dithiocarbamate side chain at C-4 position were obtained by the reaction of 4-bromomethyl butenolide (**12**) with CS_2 and various amines. Compound **12** was prepared by an intramolecular substitution reaction of compound **11** which could be obtained from starting material **10** with 70% yield as shown in Scheme 2.[11]

Fortunately, a new scaffold of spirothiazolidine-2-thiones **III** was obtained during the reaction of compound **12** with primary amines. The compounds **II** and the novel spirothiazolidine-2-thiones **III** were shown in Table 2.

All of these compounds were tested for anti-tumor activity against five different human cancer cell lines in vitro by MTT [3-(4,5-dimethylthiao-2-yl)-2, 5-diphenyl-tetrazolium bromide] cell proliferation assay.[14] The anti-tumor drug 5-fluorouracil was used as positive control. The results are summarized in Tables 3 and 4

Analysis of the MTT assay results suggest that the position of dithiocarbamate side chain is crucial for the activity. Butenolides **I** with dithiocarbamate side chain on C-3 position were generally more potent than series **II** with dithiocarbamate side chain on C-4 position, compounds **II** had no or unconspicuous cytotoxic activity towards all these five cell lines. The bioassay results also suggest that except compound **III-3**, the spirothiazolidine-2-thiones

* Corresponding author. Tel./fax: +86 371 67781739.
E-mail address: liuhm@zzu.edu.cn (H.-M. Liu).

0960-894X/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2011.03.029

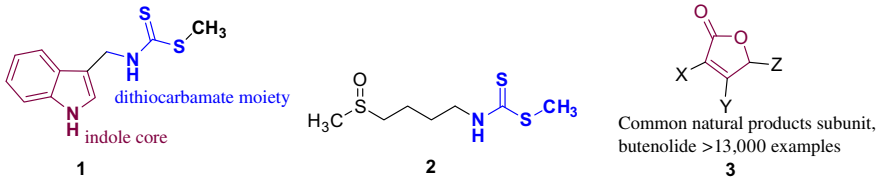
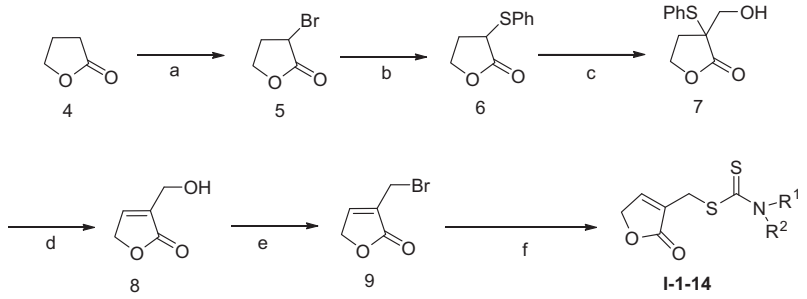
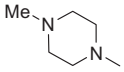
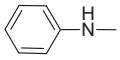
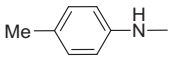
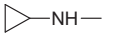
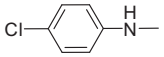
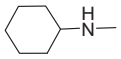
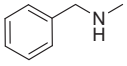


Figure 1. Structures of brassinin (**1**) sulforamate (**2**) and butenolides (**3**).

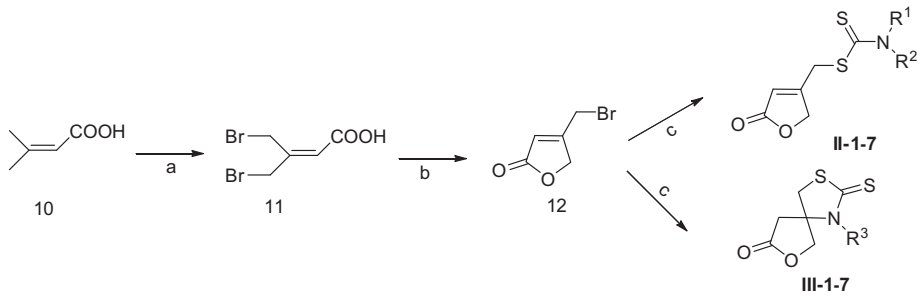


Scheme 1. The synthesis of butenolide derivatives **I**. Reagents and conditions: (a) Br_2, P, 69%; (b) PhSNa, CH_3OH, 88%; (c) CH_3ONa, CH_3OH, $(CH_2O)_n$, TMEDA, 95%; (d) (1) *m*CPBA, CH_2Cl_2, 0 °C; (2) $CaCO_3$, CCl_4, reflux, 82% two steps; (e) PBr_3, Et_2O, 0 °C, 83%; (f) CS_2, NHR^1R^2, $Na_3PO_4{\cdot}11H_2O$, acetone, 0.5–1 h.

Table 1
The butenolide derivatives **I** with dithiocarbamate side chain at C-3 position

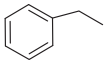
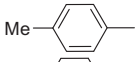
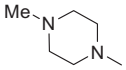
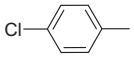
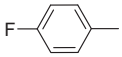
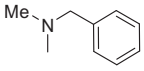
Products[a]	$-NR^1R^2$	Yield[b] (%)	Products[a]	$-NR^1R^2$	Yield[b] (%)
I-1	CH_3NH-	83	I-8		90
I-2	C_2H_5NH-	85	I-9		93
I-3	$(CH_3)_2N-$	81	I-10		86
I-4	$(C_2H_5)_2N-$	77	I-11		75
I-5	$(CH_3)_2CHNH-$	80	I-12		77
I-6		87	I-13		81
I-7		86	I-14[10]		77

[a] All the products were characterized by IR, NMR and mass spectral data.
[b] Refer to isolated pure products.



Scheme 2. Reagents and conditions: (a) 2.2 equiv NBS, $Ph(CO)_2O_2$, CCl_4, reflux, 95%; (b) 5% aq NaOH, rt 12 h, 73%; (c) CS_2, amines, $Na_3PO_4{\cdot}11H_2O$, acetone, 0.5–1 h.

Table 2
The compounds **II** and the novel spirothiazolidine-2-thiones **III**

Products[a]	$-NR^1R^2$	Yield[b] (%)	Products[a]	R^3	Yield[b] (%)
II-1[12]	$(CH_3)_2N-$	81	III-1[13]		87
II-2	$(C_2H_5)_2N-$	77	III-2		88
II-3	$((CH_3)_2CH)_2N-$	76	III-3		75
II-4		87	III-4		81
II-5		93	III-5		90
II-6		86	III-6		86
II-7		80	III-7		75

[a] All the products were characterized by IR, NMR and mass spectral data;
[b] Refer to isolated pure products.

Table 3
Inhibitory results of compounds **I** to 5 human cancer cell lines

No.	IC_{50} (μM)[a]				
	EC-9706[b]	HeLa[c]	PC-3[d]	SPCA1[e]	MCF-7[f]
I-1	85.41 ± 3.4	36.05 ± 2.4	76.50 ± 2.7	43.89 ± 3.2	83.1 ± 2.4
I-2	44.41 ± 0.8	nd[h]	30.62 ± 0.3	nd[h]	80.08 ± 1.3
I-3	181.50 ± 3.7	114.41 ± 2.2	146.57 ± 1.0	–[g]	–
I-4	135.89 ± 2.4	1.39 ± 0.8	57.05 ± 2.3	86.96 ± 1.5	121.32 ± 3.5
I-5	59.42 ± 1.6	136.71 ± 2.4	49.42 ± 1.2	107.64 ± 2.3	133.91 ± 2.2
I-6	53.57 ± 1.3	24.14 ± 1.9	116.86 ± 2.4	57.50 ± 1.3	94.92 ± 2.4
I-7	–[g]	150.03 ± 2.1	–	–	179.54 ± 2.3
I-8	14.36 ± 1.3	2.63 ± 0.2	113.68 ± 3.7	31.35 ± 1.5	56.41 ± 1.2
I-9	14.26 ± 1.5	0.77 ± 0.3	40.50 ± 2.6	53.73 ± 1.7	86.52 ± 2.6
I-10	26.02 ± 1.2	2.43 ± 0.2	48.83 ± 1.3	37.87 ± 1.7	60.30 ± 2.3
I-11	16.22 ± 2.3	111.23 ± 1.2	186.05 ± 2.9	95.31 ± 2.9	89.91 ± 1.7
I-12	90.99 ± 1.7	5.44 ± 0.3	93.49 ± 1.4	63.82 ± 1.4	97.37 ± 2.9
I-13	nd[h]	nd[h]	nd[h]	nd[h]	nd[h]
I-14	34.26 ± 1.4	15.08 ± 0.8	13.72 ± 0.2	20.14 ± 1.2	31.93 ± 1.3
5-Fu	20.30 ± 1.3	41.46 ± 2.5	29.30 ± 1.9	26.92 ± 1.4	7.54 ± 0.7

[a] Inhibitory activity was assayed by exposure for 72 h to substances and expressed as concentration required to inhibit tumor cell proliferation by 50% (IC_{50}). Data are expressed as mean ± SE from the dose–response curves of at least three independent experiments. Differences between groups were examined for statistical significance using one-way ANOVA analysis with SPSS 16.0 for WINDOWS. In all cases, P <0.05 was considered significant.
[b] Human esophageal cancer cell line.
[c] Human cervical carcinoma cell line.
[d] Human prostate cancer cell line.
[e] Human lung cancer cell line.
[f] Human breast cancer cell line.
[g] IC_{50} values greater than 50 μg/mL were considered as inactive and omitted here.
[h] No detection.

showed weaker or no inhibition activity compared with chain dithiocarbamates.

In series **I**, compound bearing benzylamino (**I-14**) showed broad spectrum anti-cancer activity to five different cell lines with IC_{50} <30 μM. While compounds **I-11**–**13** containing anilinos, exhibited weaker activity towards most cell lines tested, except compound **I-11** with IC_{50} = 16.22 μM to EC9706 cell line and compound **I-12** with IC_{50} = 5.44 μM to HeLa cell line. Compounds **I-8**–**10** which possess heterocyclic amino groups showed selective and significant cytotoxicity, particularly with respect to HeLa cell line, with IC_{50} values less than 3 μM, better than that of the control compound fluorouracil. Comparing compound **I-6** with **I-7**, both of them possess exocyclic amino groups, the result indicates that the ring size is very important for activating effect in vitro, compound **I-6** containing cyclopropylamino has stronger activities than compound **I-7** containing cyclohexylamino. Compounds **I-1** to **I-5** showed moderate cytotoxicity, and no matter secondary amines or tertiary amines showed negligible affect to their cytotoxicity.

In summary, a new family of butenolides-containing dithiocarbamates were synthesized and evaluated for their antitumor activity in vitro. Structure–activity relationship for the antitumor effect of them showed that the introduction of dithiocarbamate side chains on the C-3 position of butenolide was crucial for anti-tumor activity. Especially compound **I-14** exhibited broad spectrum anti-cancer activity in vitro. This result is valu-

Table 4
Inhibitory results of compounds **II** and **III** to **5** human cancer cell lines

No.	IC_{50} (μM)				
	EC-9706	HeLa	PC-3	SPCA1	MCF-7
II-1	7.55 ± 0.7	—	—	—	159.29 ± 3.5
II-2	—[a]	21.01 ± 1.7	—	57.05 ± 2.7	—
II-3	49.25 ± 0.3	28.51 ± 0.4	—	—	—
II-4	105.74 ± 1.4	—	—	60.03 ± 1.1	168.77 ± 0.5
II-5	35.83 ± 1.6	—	—	—	—
II-6	—	—	—		—
II-7	—	—	—	—	—
III-1	—	28.73 ± 0.9	—	—	—
III-2	—	—	—	—	—
III-3	128.33 ± 2.4	18.03 ± 0.5	—	100.70 ± 2.3	134.21 ± 2.4
III-4	—	—	98.08 ± 2.4	—	—
III-5	32.61 ± 1.2	—	—	—	—
III-6	—	—	—	—	—
III-7	—	—	—	—	—

[a] IC_{50} values greater than 50 μg/mL were considered as inactive and omitted here.

able for the construction of compound libraries and the screening of lead compound. Further investigation of the synthesis of compound **I-14** derivatives and in vivo anti-tumor activity are in progress.

Acknowledgments

We are grateful for the financial support from the National Natural Science Foundation of China (Project No. 20902086), as well as support from China Postdoctoral Science Foundation funded project (Project No. 20080430864).

References and notes

1. (a) Bowden, K.; Chana, R. S. *J. Chem. Soc., Perkin Trans. 2* **1990**, 2163; (b) Erian, A. W.; Sherif, S. M. *Tetrahedron* **1999**, *55*, 7957; (c) Caldas, E. D.; Hosana Conceição, M.; Miranda, M. C. C.; Souza, L. C. K. P.; Lima, J. F. *J. Agric. Food Chem.* **2001**, *49*, 4521; (d) Goel, A.; Mazur, S. J.; Fattah, R. J.; Hartman, T. L.; Turpin, J. A.; Huang, M.; Rice, W. G.; Appella, E.; Inman, J. K. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 767.
2. (a) Walter, W.; Bode, K. D. *Angew. Chem., Int. Ed. Engl.* **1967**, *6*, 281; (b) Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1887; (c) Elgemeie, G. H.; Sayed, S. H. *Synthesis* **2001**, 1747; (d) Ronconi, L.; Marzano, C.; Zanello, P.; Corsini, M.; Miolo, G.; Maccà, C.; Trevisan, A.; Fregona, D. *J. Med. Chem.* **2006**, *49*, 1648.
3. Gaspari, P.; Banerjee, T.; Malachowski, W. P.; Muller, A. J.; Prendergast, G. C.; Duhadaway, J.; Bennett, S.; Donovana, A. M. *J. Med. Chem.* **2006**, *49*, 684.
4. (a) Gerhäuser, C.; You, M.; Liu, J. F. *Cancer Res.* **1997**, *57*, 272; (b) Hou, X. L.; Ge, Z. M.; Wang, T. M.; Guo, W.; Cui, J. R.; Cheng, T. M.; Lai, C. S.; Li, R. T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4214; (c) Moriarty, R. M.; Naithani, R.; Kosmeder, J.; Prakash, O. *Eur. J. Med. Chem.* **2006**, *41*, 121; (d) Zahran, M. A. H.; Salem, T. A. R.; Samaka, R. M.; Agwa, H. S.; Awad, A. R. *Bioorg. Med. Chem.* **2008**, *16*, 9708; (e) Huang, W.; Ding, Y.; Miao, Y.; Liu, M. Z.; Li, Y.; Yang, G. F. *Eur. J. Med. Chem.* **2009**, *44*, 3687; (f) Qian, Y.; Ma, G. Y.; Yang, Y.; Cheng, K.; Zheng, Q. Z.; Mao, W. J.; Shi, L.; Zhao, J.; Zhu, H. L. *Bioorg. Med. Chem.* **2010**, *18*, 4310; (g) Cao, S. L.; Wang, Y.; Zhu, L.; Liao, J.; Guo, Y. W.; Chen, L. L.; Liu, H. Q.; Xu, X. Z. *Eur. J. Med. Chem.* **2010**, *45*, 3850.
5. For examples: (a) Ishikawa, T.; Nishigaya, K.; Uchikoshi, H.; Chen, I. S. *J. Nat. Prod.* **1998**, *61*, 534; (b) Guo, Y. W.; Gavagnin, M.; Mollo, E.; Trivellone, E.; Cimino, G. *J. Nat. Prod.* **1999**, *62*, 1194; (c) Brown, S. P.; Goodwin, N. C.; MacMillan, D. W. C. *J. Am. Chem. Soc.* **2003**, *125*, 1192; (d) Bagal, S. K.; Adlington, R. M.; Baldwin, J. E.; Marquez, R. *J. Org. Chem.* **2004**, *69*, 9100; (e) Lee, S. S.; Lin, Y. S.; Chen, C. K. *J. Nat. Prod.* **2009**, *72*, 1249; (f) Liu, X. Q.; Gao, W. Y.; Guo, Y. Q.; Zhang, T. J.; Yan, L. L. *Chin. Chem. Lett.* **2007**, *18*, 1075.
6. a WIPO Patent Application WO/ 1999 /052888; .; (b) Lattmann, E.; Ayuko, W. O.; Kinchinaton, D.; Langley, C. A.; Singh, H.; Karimi, L.; Tisdale, M. J. *J. Pharm. Pharmacol.* **2003**, *55*, 129; (c) Braun, M.; Hohmann, A.; Rahematpura, J.; Bühne, C.; Grimme, S. *Chem. Eur. J.* **2004**, *10*, 4584; (d) Xavier, N. M.; Rauter, A. P. *Carbohydr. Res.* **2008**, *343*, 1523; (e) Pimentel-Elardo, S. M.; Kozytska, S.; Bugni, T. S.; Ireland, C. M.; Moll, H.; Hentschel, U. *Mar. Drugs* **2010**, *8*, 373.
7. (a) Kupchan, S. M.; Fessler, D. C.; Eakin, M. A.; Giacobbe, T. J. *Science* **1970**, *168*, 37; (b) Kupchan, S. M.; Eakin, M. A.; Thomas, A. M. *J. Med. Chem.* **1971**, *14*, 1147; (c) Bourguignon, J. J.; Schoenfelder, A.; Schmitt, M.; Wermuth, C. G.; Hechler, V.; Charlier, B.; Maitre, M. *J. Med. Chem.* **1988**, *31*, 893.
8. Cao, S. L.; Feng, Y. P.; Jiang, Y. Y.; Liu, S. Y.; Ding, G. Y.; Li, R. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1915. General procedure for the synthesis of analogues **I**: CS_2 (5 equiv) was added dropwise to the solution of amine (1 equiv) and $Na_3PO_4{\cdot}11H_2O$ (0.6 equiv) in acetone, The reaction mixture was stirred at room temperature for 0.5 h. Then α,β-unsaturated ketones or esters (1 equiv) was added to the mixture, the reaction mixture was stirred at room temperature for 0.5 h (monitored by TLC). Upon completion, the reaction mixture was filtered and the filter liquor concentrated under reduced pressure, extracted with $CHCl_3$ (3 × 15 mL), dried over sodium sulfate, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel or recrystallized (in $CHCl_3/CH_3OH$ 1/1) to afford the product..
9. (a) Morgan, B.; Dolphin, D.; Jones, R. H.; Jones, T.; Einstein, F. W. B. *J. Org. Chem.* **1987**, *52*, 4631; (b) Yang, H. S.; Qiao, X. X.; Cui, Q.; Xu, X. H. *Chin. Chem. Lett.* **2009**, *20*, 1023. Compound **8**, red liquid, yield 83%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 4.11 (dd, J = 1.5 Hz, J = 3.0 Hz, 2H), 4.86 (dd, J = 1.6 Hz, J = 3.3 Hz, 2H), 7.52 (td, J = 1.3 Hz, J = 2.8 Hz, 1H)..
10. Compound **I-14**: yellow solid (77%), mp = 84.8–85.3 °C. IR (KBr, cm^{-1}) v: 3231, 2918, 1736, 1529, 1427, 1367, 1205, 1088, 1040, 929, 827, 739, 694; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65–7.31 (m, 6H), 4.92 (d, J = 5.1 Hz, 2H), 4.84 (d, J = 1.4 Hz, 2H), 4.18 (s, 2H), 1.62 (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 196.09, 173.33, 148.07, 135.71, 130.08, 128.95, 128.29, 128.02, 70.22, 51.26, 29.01. HRMS (ESI) Calcd for $C_{13}H_{13}NO_2S_2Na$ $[M+Na]^+$: 302.0388. Found: 302.0387.
11. (a) Robert, K. I.; Boeckman, J.; Soo, S. K. *J. Am. Chem. Soc.* **1982**, *104*, 1033; (b) Wang, E. S.; Choy, Y. M.; Henry, N. C. W. *Tetrahedron* **1996**, *52*, 12137.
12. Compound **II-1**: white solid (82%). Mp = 141.2–141.8 °C. IR (KBr, cm^{-1}) v: 3079, 2926, 1744, 1634, 1377, 1254, 1176, 1021, 986, 886, 718; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.04 (s, 1H), 4.87 (s, 2H), 4.43 (s, 2H), 3.57 (s, 2H), 3.41(s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 194.43, 173.22, 165.59, 117.86, 72.60, 46.08, 41.56, 30.94. HRMS (ESI) Calcd for $C_8H_{11}NO_2S_2Na$ $[M+ Na]^+$: 240.0129. Found: 240.0130.
13. Compound **III-1**: yellow solid (87%). Mp = 88.2–89.2 °C. IR (KBr, cm^{-1}) v: 2990, 2923, 1773, 1397, 1337, 1265, 1224, 986, 839, 697; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.52 (dd, J = 25.0, 10.1 Hz, 2H), 3.51–2.37 (m, 3H), 2.77 (d, J = 17.8 Hz, 1H), 2.56–2.37 (m, 1H), 1.21–1.00 (m, 3H), 0.97–0.81 (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 200.12, 173.13, 76.11, 72.53, 38.47, 37.76, 27.89, 9.01, 8.32. HRMS (ESI) Calcd for $C_{10}H_{15}NO_3S_2Na$ $[M+Na+CH_3OH]^+$: 284.0391. Found: 284.1132.
14. (a) Horowitz, D.; King, A. G. *J. Immunol. Methods* **2000**, *244*, 49; (b) Putnam, K. P.; Bombick, D. W.; Doolittle, D. *J. Toxicol. In vitro* **2002**, *16*, 599; (c) Russell, C. A.; Vindelov, L. L. *J. Immunol. Methods* **1998**, *217*, 165. *Materials:* All cancer cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mm glutamine, 50 units/mL penicillin, and 50 mg/mL streptomycin. Phosphate buffer saline (PBS) could be prepared by dissolving KH_2PO_4, $Na_2HPO_4{\cdot}12H_2O$, NaCl, KCl in distilled water. *Method*: Exponentially growing cells were seeded into 96-well flat-bottomed plates at a concentration of 5×10^3 cells per well. After 24 h incubation at 37 °C, removed the culture medium and replaced with fresh medium containing the studied compounds in different concentrations to the wells, and the cells were incubated for another 72 h. Afterwards, 10 μL of MTT solution (5 mg/mL in phosphate buffered solution) was added to all wells and incubated for 4 h at 37 °C. Pipetted out the spent media along with suspension of cultured cells and unconverted MTT, 150 μL of dimethyl sulfoxide (DMSO) was added to each well and shaked the plates to dissolve the dark blue crystals (formazan); their absorbance was measured using a spectrophotometric microplate reader at a wavelength of 490 nm. Each concentration was analyzed in triplicate and the experiment was repeated three times. The average 50% inhibitory concentration (IC_{50}) was determined from the dose-response curves according to the inhibition ratio for each concentration..