Bioorganic & Medicinal Chemistry Letters 21 (2011) 1097–1101

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Synthesis, structure–activity relationships and preliminary antitumor evaluation of benzothiazole-2-thiol derivatives as novel apoptosis inducers

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ARTICLE INFO

Article history:
Received 1 October 2010
Revised 16 December 2010
Accepted 28 December 2010
Available online 31 December 2010

Keywords:
Tumor
Benzothiazole-2-thiol
Structure–activity relationships (SAR)
Apoptosis

ABSTRACT

A series of novel benzothiazole-2-thiol derivatives were synthesized, and their anti-proliferative activities on HepG2 and MCF-7 cells were investigated. Most compounds had inhibitory effects on cell growth, and some of them were more effective than cisplatin. Compounds **6m** and **6t** displayed good inhibitory activities against a panel of different types of human cancer cell lines, with IC_{50} values in the low micromolar range. Further biological evaluation indicated that **6m** induced apoptosis in HepG2 cancer cells. Structure–activity relationships were also proposed.

Cancer is a notably complex, widespread and lethal disease that can affect almost every tissue lineage in the human body,[1] and poses great challenges to medical science. Although tumor cells are diverse and heterogeneous, they all share the property that they proliferate rapidly.[2] The discovery and development of novel antitumor drugs that could effectively inhibit proliferative pathways is needed urgently. Apoptosis, a physiological mode of cell death in higher eukaryotes, plays a crucial role in the prevention of tumor cell proliferation.[3] Apoptosis serves as a defense mechanism to remove unwanted and potentially dangerous cells, such as tumor cells. Its deregulation is widely believed to be involved in the pathogenesis of cancer, and there are many antitumor compounds that induce the apoptotic process in tumor cells.[4] Thus, development of drugs that can effectively trigger apoptosis in tumor cells has been receiving considerable attention. Whether newly developed antitumor agents can induce apoptosis is usually studied first during their preliminary biological evaluation.

Benzothiazole derivatives are known for different biological properties, including antitubercular,[5] antimalarial,[6] anticonvulsant,[7] antihelmintic,[8] analgesic,[9] anti-inflammtory,[10] antidiabetic[11] and antitumor[12] activities. In recent years, extensive research has focused on developing novel benzothiazole derivatives to improve antitumor activities. Among these attempts, modifying the benzothiazole moiety with some functional groups, such as benzenesulfonamide,[13] imidazole[14] and aryl,[15] were shown to be important, and the resulting compounds had an inhibitory effect against certain cancer cell lines. However, most investigators focused on designing new benzothiazole compounds by substituting 2-arylbenzothiazoles or 2-aminobenzothiazoles; only a few employed the benzothiazole-2-thiol as a functional group. Recent findings have indicated that compounds derived from benzothiazole-2-thiol could achieve good antitumor potency; but these compounds were not explored further.[16] Furthermore, as far as we are aware, none of the reported benzothiazole derivatives were reported to induce apoptosis in cancer cells.

In a previous cell-based screening study of anticancer activity of benzothiazole compounds, we found a hit compound **1** (Fig. 1), a benzothiazole-2-thiol derivative bearing amide linkages and phenyl rings, could inhibit HepG2 cell growth and induce its apoptosis in vitro. Compound **1** exhibited moderate inhibition against the

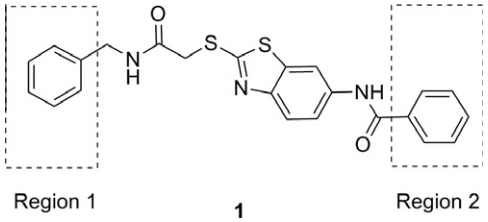

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Figure 1. The structure of compound **1**.

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doi:10.1016/j.bmcl.2010.12.124

human hepatocellular carcinoma cell line, HepG2 (IC_{50} = 34.2 μM), and the human mammary carcinoma cell line, MCF-7 (IC_{50} = 35.6 μM), in the MTT assay. To develop novel tumor growth inhibitors and apoptosis inducers as potential anticancer agents, we designed and synthesized a series of benzothiazole-2-thiol derivatives, based on compound **1**, employing a structure–activity relationship (SAR) study.

The two regions of compound **1** used for SAR evaluation are shown in Figure 1. The route adapted for the synthesis of compound **1** and other benzothiazole derivatives **6a–w** is outlined in Scheme 1, using a modified literature method.[17,18] The starting compounds **3a–c** were prepared from commercially available benzylamine derivatives **2a–c**, and 2-chloroacetyl chloride. Compound **3d** could be obtained through aqueous methylamine and 2-chloroacetyl chloride at room temperature. In the next step, **3a–d** were condensed with 6-aminobenzothiazole-2-thiol in acetone with refluxing, affording the amines, **5a–d**. Then, target compounds, **1** and **6a–w**, were obtained, respectively, by the reaction between **5a–d** and the corresponding acyl chloride in the presence of sodium bicarbonate, at room temperature, in dichloromethane. The yield of all reactions was satisfactory. All the products were characterized by ^{1}H NMR, ^{13}C NMR and MS data. The structures of two representative compounds, **6m** and **6t**, were further confirmed by IR data.[19]

Twenty-four benzothiazole-2-thiol derivatives were synthesized to survey the SAR by evaluating the cell growth inhibitory activities (IC_{50}) in cultures of HepG2 and MCF-7 cells, using the MTT assay.[20] As shown in Table 1, most compounds were able to increase the inhibitory activities against the two cancer cell lines, and some were even better than cisplatin, while five compounds had no activity.

For Region 1, the structure–activity relationships were not very clear among analogs that had the same substituent in Region 2. The activities of **6b**, **6f**, and **6q** were higher than their parent compounds, but the activities of **6a**, **6u** and **6v** were closed to their parent compounds, and the potency of **6r** even decreased. Replacement of 4-position on the left phenyl ring with chlorine or methoxy groups showed no clear SAR trends.

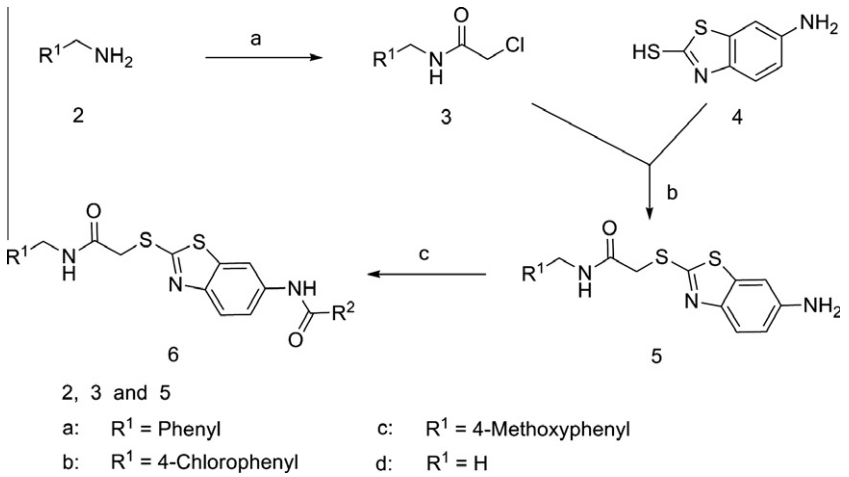
For Region 2, replacement of the hydrogen at the 4-position of the phenyl ring with chlorine altered slightly the activity against the tumor cells tested (compound **6d** vs **1**, **6e** vs **6a**, **6f** vs **6b**, **6g** vs **6c**). The increased potency could be attributed most probably to a $+\pi$ effect, a $+\sigma$ effect, or to a combination of $+\pi$ and $+\sigma$[21] Replacement of the hydrogen at the 4-position of the phenyl ring with an electron-attracting nitro group resulted in complete loss of activity (compounds **6i–l**). However, replacing the hydrogen at the 2-position of the phenyl ring with an electron-donating methoxy group resulted in the activities increasing significantly; for example, compounds **6m** and **6n**. Compound **6m** in particular was the most active analog, and, compared with compound **1**, enhanced the potency by approximately 57-fold against HepG2 cells, and 32-fold against MCF-7 cells.

In another variation, we replaced the substituted phenyl ring with methyl and chloromethyl groups at R^2 for Region 2, affording 6p–**s** and **6t–w**. As shown in Table 1, **6p–r** had considerably better activity than compound **1**, indicating methyl might be a good substitution for modification. However, the lost potency of compound **6s** suggested that the phenyl rings on both sides of the compound should not be removed simultaneously. In contrast, the potent activities of **6t–w**, especially **6t** (IC_{50} = 1.1 μM), suggested that chloromethyl could improve greatly the potency of the parent compound. Interestingly, when the phenyl ring in Region 2 was replaced by chloromethyl, the absence of another phenyl ring in Region 1 did not result in the loss of activity (**6w**). This observation confirmed further that chloromethyl could play an important role in the biological activities of benzothiazole derivatives.

Through the structure–activity relationship study, we found that chloromethyl could improve significantly the antitumor activity of this series of benzothiazole derivatives in vitro. Region 2 seemed to play a more important role in the activities of these compounds than Region 1; this observation provided us with important information for further molecular structure modification.

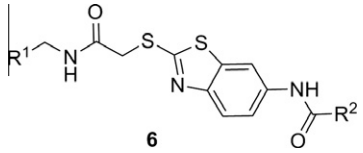
To further study the cytotoxic profile, the most potent analogs **6m** and **6t** were selected for evaluation of their inhibitory activities against a panel of different types of human cancer cell lines: colon cancer cell lines HCT-116, SW480 and SW620, lung cancer cell line A549, prostate cancer cell line PC-3, pancreatic cancer cell line BxPC-3, epidermoid cancer cell line A431, cervical cancer cell line HeLa, melanoma cancer cell line A375, ovarian cancer cell line SKOV-3, and breast cancer cell lines MDA-MB-468 and SKRB-3. As shown in Table 2, compounds **6m** and **6t** exhibited good antitumor activities against the different types of human cancer cell lines in vitro, with IC_{50} values ranging from 1.1 μM to 8.8 μM. This broad-spectrum anti-proliferative activity is encouraging for the development of new anticancer drugs, and adds to the current armamentarium that includes cisplatin, doxorubicin and paclitaxel.

As mentioned above, the antitumor efficacy of chemotherapeutic agents is correlated with their apoptosis inducing ability.



Scheme 1. Reagents and conditions: (a) **3a–c**: 2-chloroacetyl chloride, K_2CO_3, CH_2Cl_2, reflux, 4 h, 65–76%; **3d**: 2-chloroacetyl chloride, H_2O, 0–10 °C, 36%; (b) K_2CO_3, acetone, reflux, 4 h, 92–98%; (c) benzoyl chloride/4-chlorobenzoyl chloride/4-nitrobenzoyl chloride/2-methoxybenzoyl chloride/acetyl chloride/2-chloroacetyl chloride, $NaHCO_3$, CH_2Cl_2, rt, 4–26 h, 80–96%.

Table 1
SAR evaluation of benzothiazole derivatives, **6a–w**



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Compounds	R^1	R^2	Yield (%)	IC_{50}[a] (μM)	
				HepG2	MCF-7
1	Phenyl	Phenyl	96	34.2	35.6
6a	4-Chlorophenyl	Phenyl	90	30.1	33.7
6b	4-Methoxyphenyl	Phenyl	88	12.7	21.2
6c	H	Phenyl	85	26.5	34.4
6d	Phenyl	4-Chlorophenyl	94	25.4	25.9
6e	4-Chlorophenyl	4-Chlorophenyl	80	17.8	29.3
6f	4-Methoxyphenyl	4-Chlorophenyl	81	7.4	19.2
6g	H	4-Chlorophenyl	86	15.3	29.4
6h	Phenyl	4-Nitrophenyl	85	>100	>100
6i	4-Chlorophenyl	4-Nitrophenyl	82	>100	>100
6j	4-Methoxyphenyl	4-Nitrophenyl	83	>100	>100
6k	H	4-Nitrophenyl	86	>100	>100
6l	Phenyl	2-Methoxyphenyl	91	8.0	17.2
6m	4-Chlorophenyl	2-Methoxyphenyl	85	0.6	1.1
6n	4-Methoxyphenyl	2-Methoxyphenyl	92	2.1	9.3
6o	H	2-Methoxyphenyl	89	18.5	23.4
6p	Phenyl	Methyl	91	9.2	17.6
6q	4-Chlorophenyl	Methyl	92	6.7	10.2
6r	4-Methoxyphenyl	Methyl	91	11.2	20.5
6s	H	Methyl	87	>100	>100
6t	Phenyl	Chloromethyl	90	1.1	4.2
6u	4-Chlorophenyl	Chloromethyl	91	1.8	4.9
6v	4-Methoxyphenyl	Chloromethyl	92	2.0	5.1
6w	H	Chloromethyl	88	1.9	5.0
Cisplatin	—	—	—	16.3	8.4

[a] Values are means of three experiments. See Ref. 20.

Table 2
The anti-proliferative activities for compounds **6m** and **6t** against various cancer cell lines

Compounds	IC_{50}[a] (μM)											
	HCT116	SW480	SW620	A549	PC-3	BxPC-3	A431	HeLa	A375	SKOV-3	MDA-MB-468	SKRB-3
6m	1.5	5.4	4.2	2.0	4.6	4.4	1.1	4.2	2.2	6.0	2.3	1.2
6t	1.3	5.2	1.2	4.0	6.1	6.6	4.4	4.9	1.8	4.8	4.1	8.8
Cisplatin	17.9	20.2	17.0	27.2	23.1	17.6	15.0	25.9	9.7	12.6	14.4	18.1

[a] Values are means of three independent experiments. See Ref. 20.

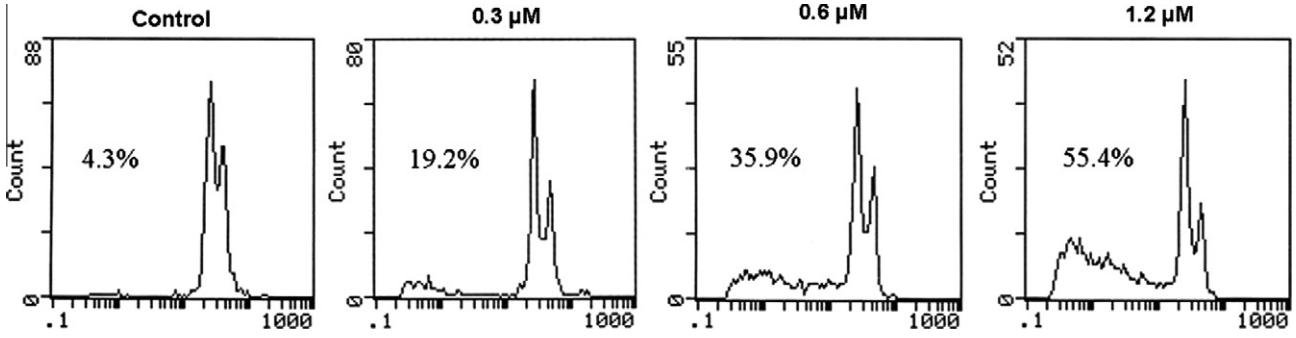

Figure 2. Induction of apoptosis in HepG2 cancer cells following treatment with **6m**. Cells were treated with various concentrations of **6m** for 24 h: 0 μM (control); 0.3 μM; 0.6 μM; 1.2 μM. The cells in the sub-G1 phase were considered as apoptotic cells ($n = 5$).

Therefore, we examined whether our compounds could induce the apoptosis. As compound **6m** exhibited the best potency against HepG2 cancer cells in vitro, we took it as a lead for further study.

To quantitatively assess apoptosis, we used flow cytometry analysis to identify sub-G1 cells/apoptotic cells, and to measure the percentage of sub-G1 cells after PI-staining.[22] The percentage

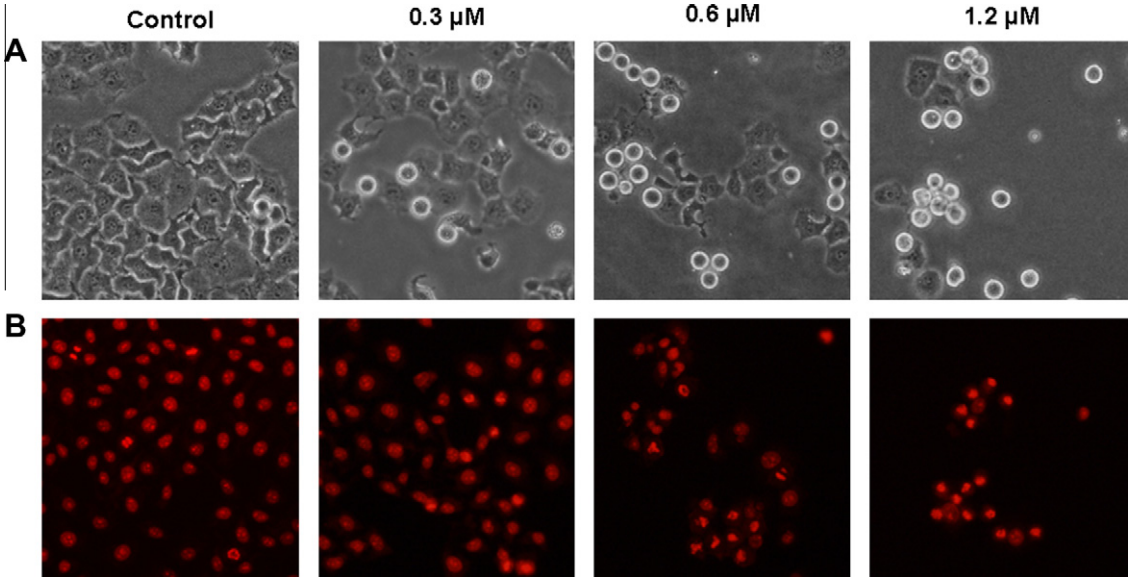

Figure 3. Effect of **6m** on cell morphology. Bright-field microscopy images (A), and fluorescence microscopic appearance of PI-stained nuclei (B) of HepG2 cancer cells, after incubation with **6m** for 24 h at varying concentrations: 0 μM (control); 0.3 μM; 0.6 μM; 1.2 μM (original magnification 400×). Apoptotic cells containing condensed and fragmented fluorescent nuclei are visible in **6m**-treated cells, but not in control cells.

of apoptotic cells were 19.2%, 35.9% and 55.4%, respectively, after treatment with 0.3 μM, 0.6 μM and 1.2 μM **6m** for 24 h (Fig. 2). The results suggested that **6m** inhibited the proliferation of HepG2 cells by inducing apoptosis in a concentration-dependent manner.

Furthermore, fluorescence microscopic examination of PI-stained cell confirmed the apoptosis inducing effect of **6m**.[23] The apoptotic cells are often characterized by DNA fragmentation, chromatin condensation, cell shrinkage, and membrane blebbing.[24] When treated with **6m** for 24 h, HepG2 cancer cells shrank and rounded up. Moreover, these phenomena became more significant as the **6m** concentration was increased. In contrast, the untreated HepG2 cells retained their normal size and shape, kept proliferating with time and became over-crowded by 24 h (Fig. 3A). The morphological changes were also characteristic of apoptosis after PI-staining: bright red fluorescent, condensed nuclei (intact or fragmented) were observed, and the change was concentration-dependent. In contrast, the untreated cells showed red, diffusely stained intact nuclei (Fig. 3B). These findings also indicated that **6m** induced apoptosis in HepG2 cancer cells.

In conclusion, a novel series of benzothiazole-2-thiol derivatives were synthesized and their anti-proliferative activities were evaluated in vitro. The data acquired by the MTT assay showed that most of the compounds exhibited good inhibitory activities, and some were even better than cisplatin. Through the SAR study, the inhibition was found to depend on substitution pattern of the side chains, especially in Region 2, and chloromethyl played a crucial role in this series of benzothiazole-2-thiol derivatives. Compounds **6m** and **6t** displayed good inhibitory activities against a panel of different types of human cancer cell lines with IC_{50} values in the low micromolar range. The results of flow cytometry analysis and morphological analysis indicated that compound **6m** induced apoptosis in HepG2 cancer cells in a concentration-dependent manner, suggesting a new method for the evaluation of the antitumor evaluation of benzothiazole derivatives. Our results will provide useful information for the design of novel benzothiazole derivatives with better potency as antitumor agents. The exact biological mechanism of these compounds is currently under investigation in our laboratory.

Acknowledgment

This work was supported by National Science and Technology Major Project of China (2009ZX09103-132). The authors gratefully thank Yong-Qiu Mao (State Key Laboratory of Biotherapy, Huaxi hospital, Sichuan University) for helpful technical assistance and discussion in the flow cytometry analysis.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.12.124.

References and notes

1. Varmus, H. *Science* **2006**, *312*, 1162.
2. Evan, G. I.; Vousden, K. H. *Nature* **1992**, *411*, 342.
3. Thompson, C. B. *Science* **1995**, *267*, 1456.
4. Cummings, J.; Ward, T. H.; Ranson, M.; Dive, C. *Biochim. Biophys. Acta* **2004**, *1705*, 53.
5. Palmer, P. J.; Trigg, R. B.; Warrington, J. V. *J. Med. Chem.* **1971**, *14*, 248.
6. Burger, A.; Sawhney, S. N. *J. Med. Chem.* **1968**, *11*, 270.
7. Chakole, R. D.; Amnerkar, N. D.; Khedekar, P. B.; Bhusari, K. P. *Indian J. Heterocycl. Chem.* **2005**, *15*, 27.
8. Jayachandran, E.; Bhatia, K.; Naragud, L. V. G.; Roy, A. *Indian Drugs* **2003**, *40*, 408.
9. Siddiqui, N.; Alam, M.; Siddiqui, A. A. *Asian J. Chem.* **2004**, *16*, 1005.
10. Gurupadayya, B. M.; Gopal, M.; Padmashali, B.; Vaidya, V. P. *Indian J. Heterocycl. Chem.* **2005**, *15*, 169.
11. Diaz, H. M.; Molina, R. V.; Andrade, R. O.; Coutino, D. D.; Franco, L. M.; Webster, S. P.; Binnie, M.; Soto, S. E.; Barajas, M. I.; Rivera, I. L.; Vazquez, G. N. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2871.
12. (a) Rana, A.; Siddiqui, N.; Khan, S. A. *Indian J. Pharm. Sci.* **2007**, *69*, 10; (b) Shi, D.-F.; Bradshaw, T. D.; Wrigley, S.; McCall, C. J.; Lelieveld, P.; Fichtner, I.; Stevens, M. F. G. *J. Med. Chem.* **1996**, *39*, 3375; (c) Hutchinson, I.; Chua, M. S.; Browne, H. L.; Trapani, V.; Bradshaw, T. D.; Westwell, A. D.; Stevens, M. F. G. *J. Med. Chem.* **2001**, *44*, 1446; (d) Kok, S. H. L.; Gambari, R.; Chui, C. H.; Yuen, M. C. W.; Lin, E.; Wong, R. S. M.; Lau, F. Y.; Cheng, G. Y. M.; Lam, W. S.; Chan, S. H.; Lam, K. H.; Cheng, C. H.; Lai, P. B. S.; Yu, M. W. Y.; Cheung, F.; Tanga, J. C. O.; Chan, A. S. C. *Bioorg. Med. Chem.* **2008**, *16*, 3626.
13. Kamal, A.; Khanl, M. N. A.; Reddyl, K. S. R.; Srikanthl, Y. V. V.; Sridhar, B. *Chem. Biol. Drug Des.* **2008**, *71*, 78.
14. (a) Trapani, G.; Franco, M.; Latrofa, A.; Reho, A.; Liso, G. *Eur. J. Pharm. Sci.* **2001**, *14*, 209; (b) Srimanth, K.; Rao, V. R.; Krishna, D. R. *Arzneimittelforschung* **2002**, *52*, 388.
15. Song, E. Y.; Kaur, N.; Park, M.-Y.; Jin, Y.; Lee, K.; Kim, G.; Lee, K.-Y.; Yang, J. S.; Shin, J. H.; Nam, K. Y.; No, K. T.; Han, G. *Eur. J. Med. Chem.* **2008**, *43*, 1519.

16. (a) Zhang, L.; Fan, J.; Vu, K.; Hong, K.; Le Brazidec, J.-Y.; Shi, J.; Biamonte, M.; Busch, D. J.; Lough, R. E.; Grecko, R.; Ran, Y.; Sensintaffar, J. L.; Kamal, A.; Lundgren, K.; Burrows, F. J.; Mansfield, R.; Timony, G. A.; Ulm, E. H.; Kasibhatla, S. R.; Boehm, M. F. *J. Med. Chem.* **2006**, *49*, 5352; (b) Fréchette, S.; Leit, S.; Woo, S. H.; Lapointe, G.; Jeannotte, G.; Moradei, O.; Paquin, I.; Bouchain, G.; Raeppel, S.; Gaudette, F.; Zhou, N.; Vaisburg, A.; Fournel, M.; Yan, P. T.; Trachy-Bourget, M.-C.; Kalita, A.; Robert, M.-F.; Lu, A.; Rahil, J.; MacLeod, A. R.; Besterman, J. M.; Li, Z.; Delorme, D. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1502; (c) Zhang, J.-T.; Qi, J.; Peng, H.; Dong, Z. WO 2010/048603 A1, 2010.
17. Baraldi, P. G.; Preti, D.; Tabrizi, M. A.; Fruttarolo, F.; Saponaro, G.; Baraldi, S.; Romagnoli, R.; Moorman, A. R.; Gessi, S.; Varani, K.; Borea, P. A. *Bioorg. Med. Chem.* **2007**, *15*, 2514.
18. Musso, D. L.; Cochran, F. R.; Kelley, J. L.; McLean, E. W.; Selph, J. L.; Rigdon, G. C.; Orr, G. F.; Davis, R. G.; Cooper, B. R.; Styles, V. L.; Thompson, J. B.; Hall, W. R. *J. Med. Chem.* **2003**, *46*, 399.
19. *Compound* **6m**: White solid, yield 85%, mp 163 °C; ^{1}H NMR (DMSO-d_6) δ: 3.91 (s, 3H), 4.19 (s, 2H), 4.31 (d, J = 6.0 Hz, 2H), 7.08 (t, J = 7.6 Hz, 1H), 7.20 (d, J = 8.4 Hz, 1H), 7.27–7.34 (m, 4H), 7.50–7.54 (m, 1H), 7.65 (dd, J = 7.6, 1.6 Hz, 1H), 7.70 (dd, J = 8.8, 2.0 Hz, 1H), 7.78 (d, J = 8.8 Hz, 1H), 8.53 (d, J = 2.0 Hz, 1H), 8.85 (t, J = 6.0 Hz, 1H), 10.35 (s, 1H); ^{13}C NMR (DMSO-d_6) δ: 36.49, 41.89, 55.87, 111.84, 111.97, 119.16, 120.48, 120.95, 124.76, 128.12, 128.95 (2C), 129.66 (2C), 131.33, 132.11, 135.40, 135.94, 138.09, 148.79, 156.48, 164.34, 164.58, 166.55; IR (KBr, cm^{-1}): 3413, 3318, 3066, 1678, 1653, 1577, 1534, 1482, 1445, 1401, 1294, 1235, 1009, 800, 750; ESI-MS m/z: 496.1 ($M-H^-$). *Compound* **6t**: White solid, yield 90%, mp 168 °C; ^{1}H NMR (DMSO-d_6) δ: 4.19 (s, 2H), 4.30 (s, 2H), 4.33 (d, J = 5.6 Hz, 2H), 7.21–7.29 (m, 5H), 7.56 (dd, J = 8.8, 2.0 Hz, 1H), 7.79 (d, J = 8.8 Hz, 1H), 8.37 (d, J = 2.0 Hz, 1H), 8.82 (t, J = 5.6 Hz, 1H), 10.55 (s, 1H); ^{13}C NMR (DMSO-d_6) δ: 36.52, 42.52, 43.55, 111.76, 118.73, 121.10, 126.76, 127.09 (2C), 128.19 (2C), 135.26, 135.46, 138.96, 149.01, 164.76, 164.84, 166.38; IR (KBr, cm^{-1}): 3268, 3043, 1635, 1574, 1537, 1443, 1403, 1226, 1012, 750, 697; ESI-MS m/z: 404.0 ($M-H^-$).
20. Briefly, cells (3×10^3/well) were seeded in 96-well plates and cultured for 24 h, followed by compounds treatment for 48 h. A volume of 10 μl of 10 mg/ml MTT was added per well and incubated for another 2 h at 37 °C, then the supernatant fluid was removed and DMSO was added 150 μl/well for 15–20 min. The light absorptions (OD) were measured at 570 nm with SpectraMAX M5 microplate spectrophotometer (Molecular Devices). The effect of compounds on tumor cells viability was expressed by IC_{50} of each cell line.
21. Topliss, J. G. *J. Med. Chem.* **1972**, *15*, 1006.
22. Briefly, cells were seeded in 6-well plates at the density of 1×10^5/well and cultured for 24 h, followed by **6m** treatment for another 24 h. At the indicated intervals, both attached cells and floating cells were harvested, sediments were resuspended in 1 ml hypotonic fluorochrome solution containing 50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100, and then analyzed by flow cytometer (ESP Elite, Beckman-Coulter, Miami, FL). Apoptotic cells appeared in the cell cycle distribution as cells with a DNA content of less than that of G1 cells and were estimated with Listmode software.
23. Briefly, cells (1×10^5/well) were seeded in 6-well plates and cultured for 24 h, followed by **6m** treatment for another 24 h. After rinsing with PBS, the cells were fixed using 75% of ethanol. The morphological change of cell was examined by inverted microscope, then the cells were stained with PI (1 μg/ml, in PBS) and analyzed under fluorescence microscope (Zeiss, Axiovert 200, Germany) to identify the apoptotic cells.
24. Fan, C. D.; Zhao, B. X.; Wei, F.; Zhang, G. H.; Dong, W. L.; Miao, J. L. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3860.