Bioorganic & Medicinal Chemistry Letters 21 (2011) 6662–6666

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Synthesis and anti-tumor activities of *N*′-benzylidene-2-(4-oxothieno[2,3-*d*]pyrimidin-3(4*H*)-yl)acetohydrazone derivatives

Jiangping Lou [a,†], Zhen Liu [b,†], Yan Li [b], Mi Zhou [b], Zhengxi Zhang [b], Shu Zheng [a], Renxiao Wang [b,*], Jian Li [a,*]

[a] School of Pharmacy, East China University of Science and Technology, 130 Mei Long Road, Shanghai 200237, People's Republic of China
[b] State Key Laboratory of Bioorganic Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 345 Lingling Road, Shanghai 200032, People's Republic of China

ARTICLE INFO

Article history:
Received 26 July 2011
Revised 15 September 2011
Accepted 16 September 2011
Available online 21 September 2011

Keywords:
Thienopyrimidine
Acetohydrazone
Cytotoxicity
Apoptosis inducer
Anti-tumor compound

ABSTRACT

A compound with a cyclic thienopyrimidine moiety and an aceto-hydrazone moiety in its chemical structure was discovered in a cell-based screening to have noticeable cytotoxicity on several tumor cell lines. A total of 38 derivatives of this compound were synthesized at five steps with high yields. These compounds were tested in standard MTT assays, and several compounds exhibited improved cytotoxic activities. The most potent compounds have IC_{50} values of 10–20 μM on A549, HeLa, and MBA-MD-231 tumor cells. Flow cytometry analysis of several active compounds and subsequent examination of caspase activation indicate that they induce caspase-dependent apoptosis in tumor cells. In addition, these compounds do not have obvious effect on a normal cell line HEK-293T, demonstrating the desired selectivity against tumor cells. Results from a fluorescence polarization-based in vitro binding assay indicate that this class of compounds does not significantly interrupt the interactions between Mcl-1 and Bid. Their cytotoxicity is achieved presumably through other mechanisms.

The discovery and development of effective anti-cancer therapies has been accelerated in recent years by various molecular targeted techniques and strategies.[1] Nevertheless, in vitro screening with tumor cell lines is still an essential approach to the identification of compounds with desired potency.[2] One famous example is the NCI-60 platform, which is a panel of 60 human tumor cell lines made available by the US National Cancer Institute.[3] Millions of compounds have been screened on the NCI-60 platform so far, and such efforts have led to the discovery of many compounds with clear pharmaceutical implications.

As part of our efforts on discovering new potential anti-cancer compounds, we screen the compounds in our inventory, which are either purchased from a commercial source or synthesized by ourselves, whenever enough samples are available. In our cell-based screening, each compound is tested at a single concentration of 20 μM in a standard MTT assay on three selected tumor cell lines, including A549 (human alveolar epithelial cell), HeLa (human cervical tumor cell), and MBA-MD-231(human breast tumor cell). Recently, a compound, that is, **1** in Figure 1, was discovered in our screening, which exhibited notable cytotoxicity at the tested concentration. This compound was originally purchased from the Specs catalog (Specs ID = AF-399/40713992). The IC_{50} values of this compound in the MTT assay were 40 and 16 μM on HeLa and MDA-MB-231 cells, respectively, but its effect on A549 cells was rather low (Fig. 1). Considering the biological potency and synthetic accessibility of compound **1**, it may serve as a reasonable lead compound for further developments.

From the viewpoint of a medicinal chemist, two distinctive moieties are included in the chemical structure of this compound: a cyclic thienopyrimidine moiety and an acetohydrazone moiety (**2** in Fig. 2). Our literature survey indicates that some known compounds containing such moieties are indeed associated with anti-cancer activities. A number of tetrahydrobenzothieno-pyrimidine (**3** in Fig. 2) and arylthienopyrimidine (**4** in Fig. 2) derivatives were reported as anti-cancer, anti-bacterial and anti-microbial agents.[4–6] The molecular targets of these compounds are often unclear. At present, two possible molecular targets of these compounds have been reported, that is, 5-HT_{1A} receptor[7] and 17-β-hydroxysteroid dehydrogenase.[8,9] Both of them are related to cancers. The thienopyrimidine moiety is therefore a valuable scaffold for developing new anti-cancer compounds. Our literature survey also found several acetohydrazone derivatives that exhibit broad-spectrum anti-tumor and anti-microbial activities. The oxoquinoxaline derivatives (**5** in Fig. 2) are reported to show anti-tubercular, anti-bacterial and anti-fungal activities.[10] Such compounds with an aryl group instead of oxoquinoxaline will cause distinct cytotoxic activities.[11] In particular, an arylpiperazinyl aceto-hydrazone derivative (PAC-1, **6** in Fig. 2) was demonstrated to induce apoptosis in cancer cells by activating procaspase-3 to caspase-3.[12] Given these facts, it is reasonable to

* Corresponding authors.
E-mail addresses: wangrx@mail.sioc.ac.cn (R. Wang), jianli@ecust.edu.cn (J. Li).
† These authors made equal contributions to this work.

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doi:10.1016/j.bmcl.2011.09.061

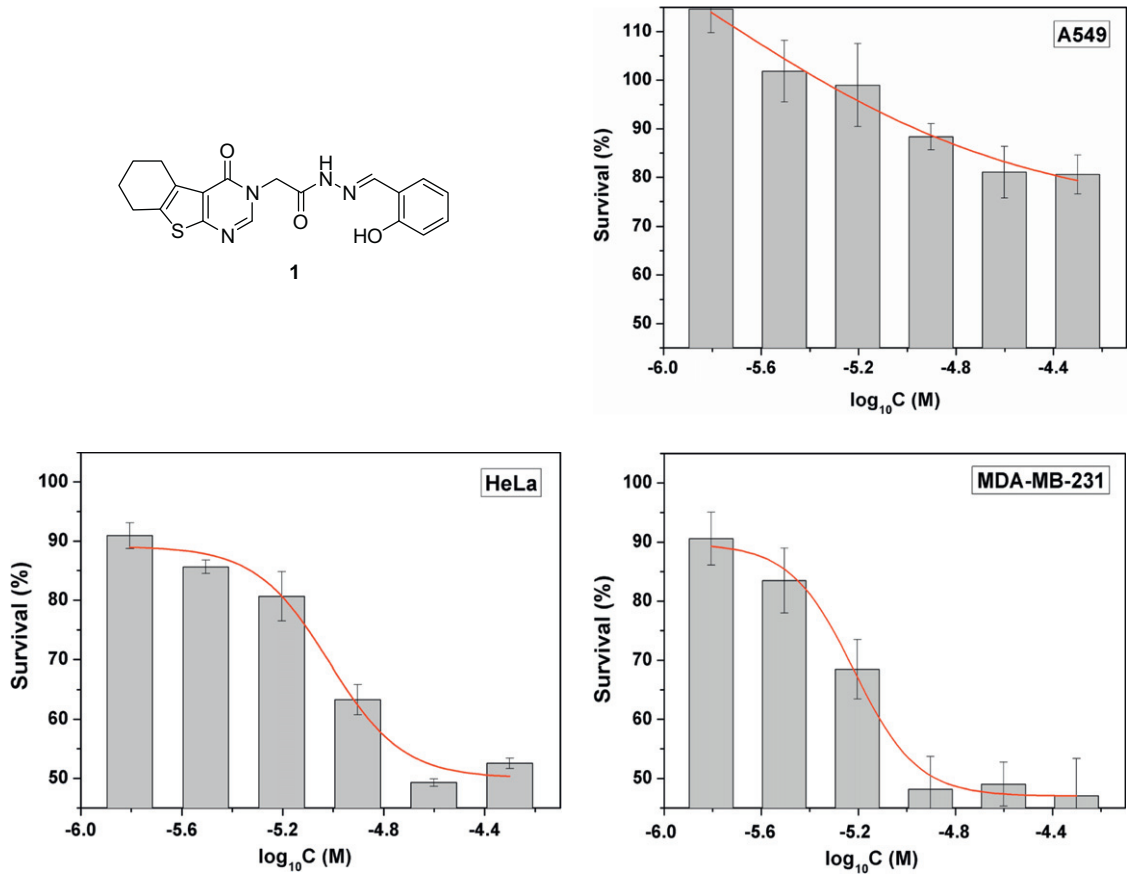


Figure 1. The lead compound (**1**) identified in our screening and its dose-dependent cytotoxicity on three selected tumor cell lines.

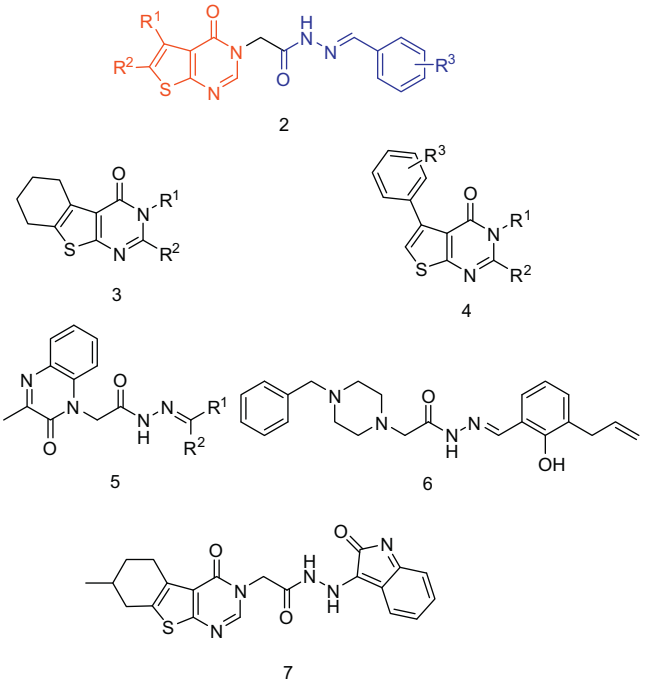
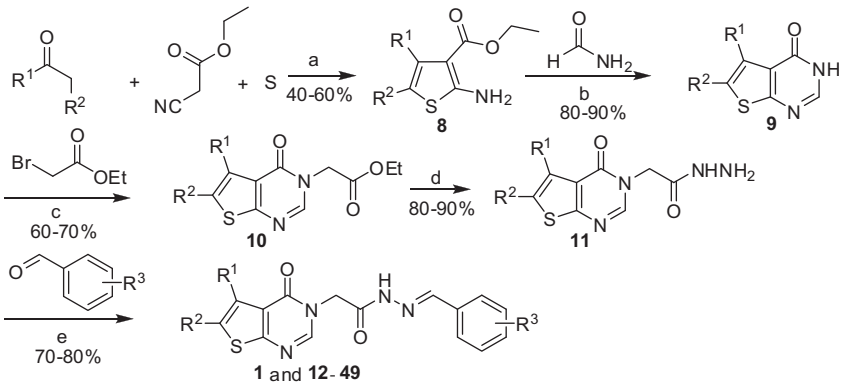


Figure 2. The structural scaffold of the compounds synthesized in this study (**2**) and some structurally similar compounds reported in literature (**3**–**7**).

expect that a compound combining these two moieties may have anti-tumor activities. In fact, one known compound (**7** in Fig. 2) is close to compound **1**. According to the record in the PubChem BioAssay database (CID: 5711212),[13] this compound inhibits the interactions between Mcl-1 and Bid proteins and thus may induce apoptosis in tumor cells as other effective Mcl-1 inhibitors.

The above analysis provides additional supports that the active compound identified in our screening, that is, compound **1**, is worth further developments. In this study, we have synthesized a total of 38 derivatives (**12–49**) of compound **1**. Chemical synthesis of this class of compounds can be completed relatively efficiently in high yields (10–30% total yield over five steps). The synthetic methods for the preparation of compounds **1** and **12–49** is illustrated in Scheme 1. Using our method described previously,[14] ketones, cyanoacetates, and sulfur were mixed and subjected to microwave irradiation for several minutes to produce 2-amino-thiophene (**8**). In this reaction, basic aluminum oxide was used as solid support and morpholine as base catalyst. Compound **8** was cyclized to thieno[2,3-*d*]pyrimidine (**9**) by condensation with formamide.[15] Compound **10** was the obtained by reacting ethyl bromoacetate and **9**, which then was converted to the hydrazide **11**, the key intermediate, by refluxing with 85% hydrazine monohydrate in ethanol. Various aromatic aldehydes were reacted with compound **11** to produce the target compounds **1** and **12–49**.

All of the synthesized compounds (Table 1) were then evaluated at a single concentration of 20 μM on A549, HeLa, and MDA-MB-231 cells. Inhibitions of cell growth were determined using a standard MTT assay after 48-hour treatment. Among them, compounds **24**, **33**, **35**, **37** and **45** exhibited inhibition ratio over 50% on at least one tumor cell line (Table 1). These compounds were then tested at multiple concentrations to determine their cytotoxicity quantitatively. In this experiment, cell line HEK-293T, that is, human embryonic kidney cells transformed with adenovirus 5 DNA and



Scheme 1. The synthetic route for obtaining the class of compounds reported in this study. Reagents and conditions: (a) MWI, morpholine, basic Al_2O_3, 140 W, 15 min; (b) 200 °C, 1.5 h; (c) K_2CO_3, acetone, 55 °C; (d) $N_2H_4.H_2O$ (85%), C_2H_5OH, reflux; (e) *n*-butanol, reflux.

Table 1
Chemical structures of compounds **1** and **12**–**49** and their cytotoxicity on three tumor cell lines

Compd	R^1	R^2	R^3	Inhibition ratio[a] (%) at 20 μM		
				A549	HeLa	MDA-MB-231
1	$-(CH_2)_4-$		2-OH	18	34	29
12	$-(CH_2)_4-$		3-F, 4-Cl	26	41	39
13	$-(CH_2)_4-$		4-CN	N.A[b]	N.A	N.A
14	$-(CH_2)_4-$		3-NO_2, 4-Cl	10	27	38
15	$-(CH_2)_4-$		4-CF_3	23	34	30
16	$-(CH_2)_3-$		2-OH	22	23	36
17	$-(CH_2)_3-$		4-CN	N.A	N.A	N.A
18	$-(CH_2)_3-$		3-F, 4-Cl	47	54	29
19	$-(CH_2)_3-$		3-NO_2, 4-Cl	N.A	34	41
20	$-(CH_2)_3-$		3-OH	N.A	N.A	N.A
21	$-(CH_2)_5-$		2-OH	29	36	40
22	$-(CH_2)_5-$		4-CN	10	11	14
23	$-(CH_2)_5-$		3-F, 4-Cl	43	50	28
24	$-(CH_2)_5-$		3-NO_2, 4-Cl	N.A	96	66
25	$-(CH_2)_5-$		3-OH	N.A	N.A	N.A
26	$-(CH_2)_3-$		4-OH	27	29	25
27	$-(CH_2)_5-$		4-OH	N.A	N.A	N.A
28	Phenyl	H	3-F, 4-Cl	N.A	N.A	N.A
29	4-Cl-phenyl	H	3-F, 4-Cl	N.A	N.A	N.A
30	4-Cl-phenyl	H	2-OH	48	43	39
31	Phenyl	H	2-OH	26	31	30
32	4-Cl-phenyl	H	4-CN	N.A	N.A	N.A
33	4-Cl-phenyl	H	3-NO_2, 4-Cl	58	74	78
34	Phenyl	H	4-CN	N.A	N.A	N.A
35	Phenyl	H	3-NO_2, 4-Cl	66	64	29
36	CH_3	H	3-F, 4-Cl	N.A	N.A	N.A
37	3-CH_3-4-Cl-phenyl	H	3-F, 4-Cl	51	52	74
38	CH_3	H	2-OH	N.A	N.A	30
39	CH_3	H	4-CN	N.A	N.A	N.A
40	CH_3	H	3-NO_2, 4-Cl	13	18	13
41	3-CH_3-4-Cl-phenyl	H	2-OH	35	33	37
42	Naphthalen-2-yl	H	3-F, 4-Cl	N.A	N.A	N.A
43	Naphthalen-2-yl	H	2-OH	44	22	36
44	Naphthalen-2-yl	H	4-CN	N.A	N.A	N.A
45	Naphthalen-2-yl	H	3-NO_2, 4-Cl	69	69	55
46	4-Cl-phenyl	H	3-NO_2	N.A	N.A	N.A
47	4-Cl-phenyl	H	3-OH, 4-OH	N.A	N.A	N.A
48	Naphthalen-2-yl	H	3-NO_2	N.A	N.A	N.A
49	Naphthalen-2-yl	H	3-OH, 4-OH	N.A	N.A	N.A

[a] Cell growth inhibition ratio were measured after 48-hour treatment.
[b] No significant activity observed at the tested concentration.

SV40 large T-antigen, was added as a model of normal cell. The concentrations at 50% inhibition (IC_{50}) of these compounds on all four cell lines are summarized in Table 2. As one can see here, compounds **33**, **35**, **37** and **45** exhibited dose-dependent cytotoxic profiles on all three tumor cell lines with IC_{50} values ranging from 11 to 32 μM. Compound **24** also exhibited μM-level potency on HeLa and MDA-MB-231 cells, but it was not effective on A549 cells (IC_{50} >50 μM). It is encouraging to observe that all five selected compounds did not exhibit obvious cytotoxicity on HEK-293T cells, demonstrating the desired selectivity between tumor cells and normal cells.

Some typical apoptotic phenotypes, such as blebbing and cell shrinkage, were observed on tumor cells after treated with the selected compounds. Thus, the pro-apoptotic activity of the most

Table 2
Cytotoxicity profiling of the most potent compounds

Compd	IC_{50}[a] (μM)			
	A549	HeLa	MDA-MB-231	HEK-293T
1	>50	40	16	>50
24	>50	6	23	>50
33	20	11	32	>50
35	16	11	20	>50
37	17	15	16	>50
45	13	24	20	>50

[a] Cytotoxicity was determined in MTT assay after 48-s treatment.

potent compounds identified in our MTT assays (Table 2) were further examined in a flow cytometry assay. After treated by the selected compounds at 20 μM for 24 h, HeLa cells were stained with propidium iodide, and then were analyzed by flow cytometry. The sub-diploid DNA contents of cells (apoptotic sub-G1 area) increased from 6% in the control cells to 22%, 27%, 20% upon the treatment of compounds **33**, **37**, and **45** (Fig. 3), indicating the obvious apoptotic activity of these compounds. In contrast, compounds **24** and **35** induced 6% and 8% cells to apoptosis, which was not significantly different from the negative control in this experiment.

Activation of caspases and the consequent cleavage of PARP is one of the indicators of cell apoptosis. The three compounds exhibiting apparent apoptotic signals in the flow cytometric analysis, that is, **33**, **37**, and **45**, were further examined in such an assay on HeLa cells. As shown by the immunoblotting results (Fig. 4), all three compounds clearly increased the level of cleaved caspase-3 and cleaved PARP as compared with a negative control. This observation confirms the results of flow cytometry analysis that compounds **33**, **37**, and **45** are able to induce caspase-dependent apoptosis.

As mentioned earlier, we noticed one compound in literature (**7** in Fig. 1) which is close to the compounds obtained and tested in our study. This compound is reported to interrupt the interactions

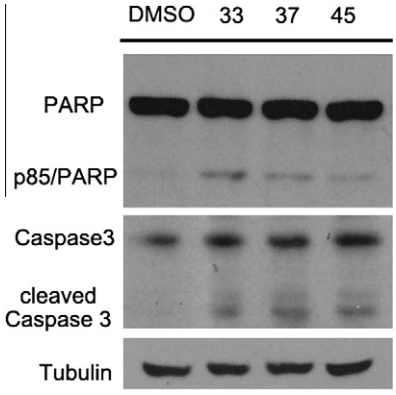


Figure 4. Activation of caspase-3 and PARP by three selected compounds. HeLa cells were treated by these compounds at 20 μM for 12 h, and then the expression levels of caspase-3 and PARP were examined by western blot.

between Mcl-1 and Bid proteins and thus has a potential to induce apoptosis in tumor cells with Mcl-1 over-expression. In order to verify if our compounds are also potential Mcl-1 inhibitors, compounds **1** and **12–49** were tested in a fluorescence polarization-based in vitro binding assay to measure their binding affinity to the Mcl-1 protein. Here, a FAM-labeled Bid-BH3 peptide was used as the fluorescence tracer. Competitive binding of a given compound was characterized quantitatively by monitoring the resulting changes in FP signals. This assay has been successfully applied in our previous study of small-molecule inhibitors of the Bcl-2 family proteins.[16]

As a preliminary screening, all compounds (**1** and **12–49**) were tested at three typical concentrations, that is, 1, 10, and 50 μM. None of these compounds was observed to achieve a significant binding to Mcl-1 even at 50 μM (data not shown here). Thus, these compounds were not examined at other concentrations. Only compounds **37**, **38**, and **49** exhibited inhibition ratio around 40%

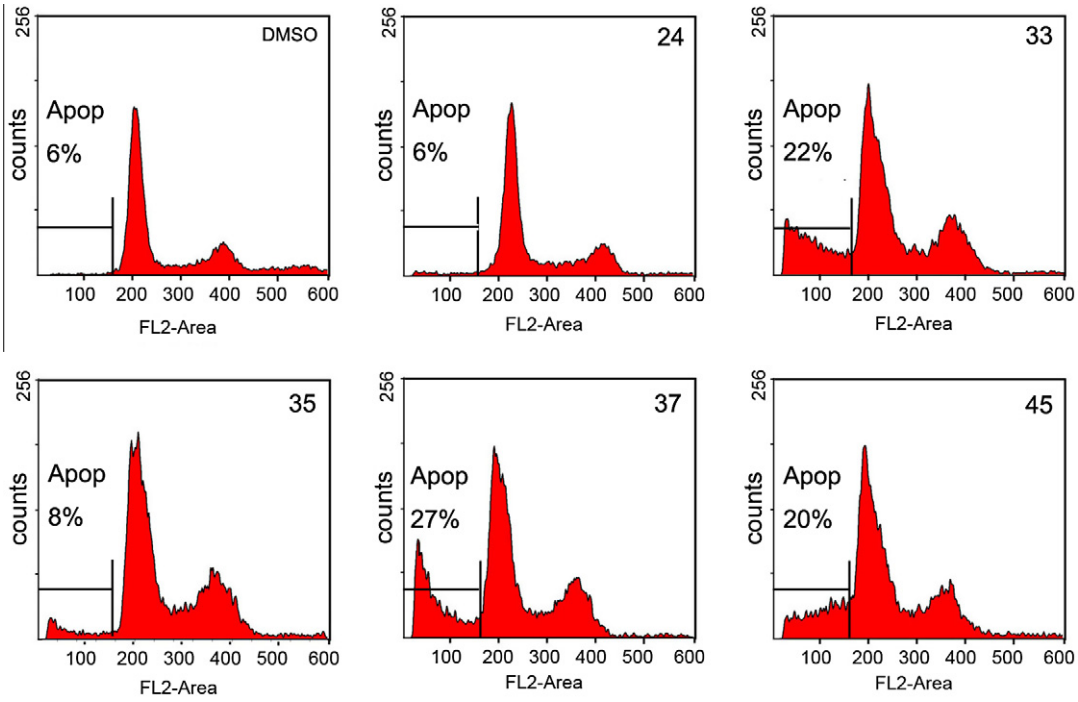


Figure 3. Results of flow cytometric analysis on HeLa cells for five selected compounds. Cells were treated by these compounds at 20 μM for 24 h. Apoptosis was analyzed by propidium iodide staining. DMSO was used as the negative control in this assay.

at 50 μM. Note that this level of binding affinity to Mcl-1 of **37** apparently cannot explain the potency of this compound observed in the MTT assay (IC_{50} = 10–20 μM). In addition, compounds **38** and **49** were basically inactive on the tumor cell lines used in our study (Table 1). Thus, we conclude that cytotoxicity of this class of compounds is not the consequence of Mcl-1 inhibition but rather some other unknown mechanisms.

It is difficult to give a clear structure–activity relationship for this class of compounds based on the results obtained so far. Nevertheless, some preliminary conclusions can be drawn here. First of all, cyclization of R^1 and R^2 (**2** in Fig. 2) into an aliphatic ring will almost abolish the biological potency of the resulting compounds (**1** and **12–27**). The lead compound pursued in our study, that is, compound **1**, has this cyclic moiety in its structure, and thus the first batch of compounds synthesized by us (**12–27**) followed this idea. Unfortunately, they were not very successful in terms of achieving the desired cytotoxicity on tumor cells. One such compound, that is, **24**, exhibited noticeable cytotoxicity on HeLa and MDA-MB-231 cells but not on A549 cells just as compound **1** (Table 2). On our second batch of compounds (**28–49**), R^1 is a separate branch group. Interestingly, this modification leads to the cytotoxicity on A549 cells at least on some of the corresponding compounds. Second, it seems that R^1 has to be a bulky aromatic group to maintain the biological potency. Several compounds with a methyl group as R^1, such as **36**, **38**, **39**, and **40**, are not active. Third, the best option for substituent group R^3 for maintaining the desired cytotoxicity is a combination of 3-NO_2 and 4-Cl, such as **33**, **35**, and **45**. It seems to be important that the *meta*- and the *para*-position on this phenyl ring should be occupied simultaneously. The role of R^3 needs to be explored more thoroughly in the future.

In conclusion, we noticed the cytotoxicity of a compound containing a thienopyrimidine and an aceto-hydrazone moiety (**1**) on several tumor cell lines. Based on this compound, a total of 38 derivatives of this compound were synthesized in five steps. All obtained compounds were tested in MTT assays, and several compounds exhibit improved cytotoxicity as compared to the lead compound. The most potent compounds have IC_{50} values ranging from 10–20 μM on A549, HeLa, and MBA-MD-231 cells. Flow cytometry analysis and detection of caspase activation of several active compounds indicate that they induce caspase-dependent apoptosis in tumor cells. In addition, these compounds do not have obvious effects on a normal cell line HEK-293T (IC_{50} >50 μM), demonstrating the desired selectivity against tumor cells. The results from a fluorescence polarization-based in vitro binding assay indicate that this class of compounds does not significantly interrupt the interactions between Mcl-1 and Bid. Their cytotoxicity is achieved presumably through other mechanisms.

Acknowledgments

The authors are grateful to the financial supports from the Chinese National Natural Science Foundation (Grants No. 20772149, No. 90813006, No. 21072213, No. 21002117 & No. 20921091), the Chinese Ministry of Science and Technology (Grants No. 2009ZX09501-002 and 2011ZX09102-005-02), and in particular, a supporting grant to Professor Jian Li by the State Key Laboratory of Bioorganic Chemistry at the Shanghai Institute of Organic Chemistry.

Supplementary data

Supplementary data (the synthetic methods and spectroscopic data of the compounds as well as the details of the MTT assay, flow cytometry analysis, western blot detection, and fluorescence polarization-based binding assay) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl. 2011.09.061.

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