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Research Article

Synthesis and Bioevaluation of Thieno[2,3-*d*]pyrimidinone Derivatives as Potential Tumor Cell Growth Inhibitors

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A series of novel cycloalkylthieno[2,3-*d*]pyrimidinone derivatives have been conveniently synthesized *via* three steps including G-3CR, heterocyclization, and alkylation reactions as a part of our ongoing search for new bioactive molecules. The newly synthesized compounds were evaluated for their potential tumor cell growth inhibitory activity by standard MTT assay. The preliminary results show that compound **6b** exhibited better inhibitory activities against HepG2, MCF-7, and BCG-823 cell lines compared to the control.

1. Introduction

In recent years, nitrogen-containing compounds are of considerable pharmacological and synthetic interest due to their extensive biological activities [1–4], in particular, which also are extremely versatile building blocks for the manufacture of bioactive compounds in pharmaceutical drug design and agrochemical industry [5–7]. Notably, among all the heterocyclic derivatives, the class of multicycle compounds bearing thienopyrimidines scaffold (Figure 1) exhibits a diversity of pharmacological effects such as kinase inhibition, antibacterial, antifungal, and immunosuppressive activity, and antidiabetic and anticancer activity [8–17]. Up to now, there are many different structures containing thienopyrimidines cores which have been synthesized, and Figure 1 describes the chemical structures of some representative thienopyrimidine derivatives used in research or clinical practice.

Despite the breadth of biological activities displayed by these agents, developing new nitrogen-containing heterocyclic derivatives as pharmaceuticals is still an important area of interest in the life sciences. The promising bioactive diversity of this class of heterocyclic compounds urges us to synthesize and biologically evaluate a series of novel structural variants of cycloalkylthieno[2,3-*d*]pyrimidinone derivatives.

In continuation of our research program which aimed at the search for novel bioactive molecules as potential anticancer agents, we wish to describe herein the convenient synthesis and pharmacological evaluation of novel series of cycloalkylthieno[2,3-*d*]pyrimidinone derivatives. We utilized thieno[2,3-*d*]pyrimidinone scaffold as key prototype structural unit, planed for the attachment of the hydrophobic cycloalkyl group to the core structure as shown in Figure 2, and explored the structure-activity relationship.

2. Materials and Methods

2.1. Instrumentation and Chemicals. All melting points (m.p.) were obtained using a digital model X-5 apparatus and were uncorrected. ^{1}H NMR spectra were recorded on a Brucker spectrometer at 400 MHz with $CDCl_3$ as the solvent and TMS as the internal standard. Chemical shifts are reported in δ (parts per million) values. Coupling constants ^nJ are reported in Hz. Standard abbreviations indicating multiplicity are used as follows: s is singlet, d is doublet, dd is doublet of doublets, t is triplet, q is quadruplet, m is multiplet and br is broad. Mass spectra were performed on a MicroMass Quattro *micro*-API instrument. Analytical thin-layer chromatography (TLC) was carried out on precoated plates, and spots were visualized with ultraviolet light. All chemicals or reagents used for syntheses were commercially available, were of AR

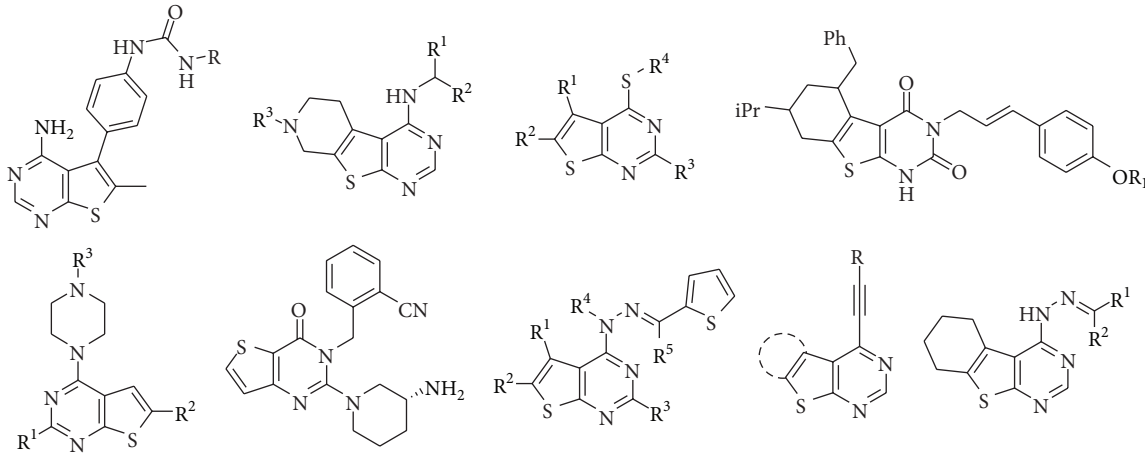


Figure 1: Representative active compounds containing thieno[2,3-*d*]pyrimidinone core.

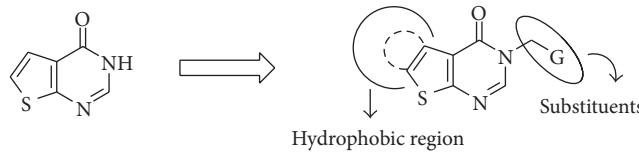


Figure 2: Design strategy for cycloalkylthieno[2,3-*d*]pyrimidinone derivatives.

grade, and were used as received. Anhydrous CH_2Cl_2 and CH_3CN were dried according to standard methods [18]. All other solvents and reagents were analytical reagents and used directly without purification.

*2.2. General Synthetic Procedure for Cycloalkylthiophene Amino Acid Derivatives **4a–c**.* To a solution of methyl cyanoacetate (10 mmol), sulfur (10 mmol), and cycloketone (10 mmol) in EtOH (20 mL) was added diethylamine (20 mmol) dropwise under ice bath. After that, the reaction mixture was allowed to room temperature, which was stirred and detected by TLC. Then the mixture was poured into ice water, and the formed precipitate was filtered and washed with water (30 mL) and dried in vacuo to give the yellow powder. Their physicochemical properties and the spectra data are as follows.

*2.2.1. Methyl 2-Amino-5,6-dihydro-4H-cyclopenta[b]thiophene-3-carboxylate **4a**.* This compound was obtained as yellowish powder following the previously mentioned method. Yield 55%, m.p. 152.6–154.1°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.10 (bs, 2H, NH_2), 3.81 (s, 3H, CH_3), 2.82–2.69 (m, 4H, CH_2), 2.34–2.27 (m, 2H, CH_2); MS (ESI) m/z 196.7 (M^+), calcd. for $C_9H_{11}NO_2S$ m/z = 197.1.

*2.2.2. Methyl 2-Amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate **4b**.* This compound was obtained as yellowish powder following the previously mentioned method. Yield 67%, m.p. 125.2–126.8°C; MS (ESI) m/z 210.7 (M^+), calcd. for $C_{10}H_{13}NO_2S$ m/z = 211.1.

*2.2.3. Methyl2-Amino-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxylate **4c**.* This compound was obtained as yellowish powder following the previously mentioned method. Yield 46%, m.p. 75.8–77.5°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.26 (bs, 2H, NH_2), 3.81 (s, 3H, CH_3), 2.99–2.94 (m, 2H, CH_2), 2.69–2.57 (m, 2H, CH_2), 1.84–1.51 (m, 6H, CH_2).

*2.3. General Synthetic Procedure for Cycloalkylthieno[2,3-d]pyrimidinone **5a–c**.* The solution of the appropriate cycloalkylthiophene amino acid derivatives **4a–c** (2 mmol) and formamide (10 mL) was heated at about 90–100°C for 4–6 h; then the mixture was left to cool to room temperature. The solid formed was filtered, washed with water, dried, and recrystallized from ethanol. Their basic physicochemical properties are as follows.

Cyclopenta[*b*]thieno[2,3-*d*]pyrimidin-4(3*H*)-one **5a**: fuscous powder, yield 90%, m.p. 247.6–249.1°C, Lit. m.p. 240°C [19].

Cyclohexyl[*b*]thieno[2,3-*d*]pyrimidin-4(3*H*)-one **5b**: brown needle crystal, yield 95%, m.p. 260.1–260.9°C, Lit. m.p. 255–257°C [20].

Cycloheptyl[*b*]thieno[2,3-*d*]pyrimidin-4(3*H*)-one **5c**: khaki powder, yield 92%, m.p. 210.8–213.5°C, Lit. m.p. 209–211°C [20].

*2.4. General Synthetic Procedure for the Target Compounds **6a–i**.* To a stirred solution of sodium hydride (2.5 mmol) in 10 mL dry THF at 0°C, the corresponding cycloalkylthieno[2,3-*d*]pyrimidinone **5a–c** (2 mmol) was added slowly.

After the hydrogen gas ceased, the mixture was kept at room temperature for 0.5 h. Then the halide (2.2 mmol) in THF was added dropwise slowly. The reaction mixture was stirred at room temperature, which was detected by TLC. Then it was quenched with 1% HCl aqueous and extracted three times with ethyl acetate. The organic layer is washed to neutral with water and dried via Na_2SO_4. After filtered and concentrated, the organic residue is purified by silica gel column-chromatography (ethyl acetate/petroleum ether) to give white solid or crystal. Their physicochemical properties and the spectra data are as follows.

2.4.1. 3-(4-Bromobenzyl)cyclopenta[b]thieno[2,3-d]pyrimidin-4(3H)-one ***6a***. This compound was obtained as white powder following the previously mentioned method. Yield 75%, m.p. 159.2–160.0°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.96 (s, 1H), 7.48 (d, J = 8 Hz, 2H), 7.23 (d, J = 8 Hz, 2H), 5.13 (s, 2H), 3.08 (t, J = 6.8 Hz, 2H), 2.97 (t, J = 7.0 Hz, 2H), 2.52–2.40 (m, 2H); MS (ESI) m/z 361.6 $(M+H)^+$, calcd. for $C_{16}H_{13}BrN_2OS$ m/z = 360.0.

2.4.2. 3-(4-Bromobenzyl)cyclohexyl[b]thieno[2,3-d]pyrimidin-4(3H)-one ***6b***. This compound was obtained as light yellow solid following the previously mentioned method. Yield 80%, m.p. 111.6–112.0°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.52 (s, 1H), 7.54 (d, J = 8.4 Hz, 2H), 7.30 (d, J = 8.4 Hz, 2H), 5.14 (s, 2H), 2.89 (d, J = 4.8 Hz, 5H), 2.36 (t, J = 7.6 Hz, 3H); MS (ESI) m/z 375.6 $(M + H)^+$, calcd. for $C_{17}H_{15}BrN_2OS$ m/z = 374.0.

2.4.3. 3-(4-Bromobenzyl)cycloheptyl[b]thieno[2,3-d]pyrimidin-4(3H)-one ***6c***. This compound was obtained as colorless crystal following the previously mentioned method. Yield 72%, m.p. 140.5–141.6°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.97 (s, 1H), 7.47 (d, J = 8 Hz, 2H), 7.22 (d, J = 8 Hz, 2H), 5.10 (s, 2H), 3.40–3.27 (m, 2H), 2.92–2.78 (m, 2H), 1.99–1.83 (m, 3H), 1.25 (bs, 3H); MS (ESI) m/z 389.5 $(M + H)^+$, calcd. for $C_{18}H_{17}BrN_2OS$ m/z = 388.0.

2.4.4. 3-(4-Fluorobenzyl)cyclopenta[b]thieno[2,3-d]pyrimidin-4(3H)-one ***6d***. This compound was obtained as white powder following the previously mentioned method. Yield 58%, m.p. 122.6–124.2°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.97 (s, 1H), 7.34 (t, 2H), 7.03 (t, 2H), 5.14 (s, 2H), 3.08 (s, 2H), 2.96 (s, 2H), 2.57–2.40 (m, 2H); MS (ESI) m/z 301.6 $(M + H)^+$, calcd. for $C_{16}H_{13}FN_2OS$ m/z = 300.1.

2.4.5. 3-(4-Fluorobenzyl)cyclohexyl[b]thieno[2,3-d]pyrimidin-4(3H)-one ***6e***. This compound was obtained as colorless needle crystal following the previously mentioned method. Yield 78%, m.p. 133.2–136.8°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.97 (s, 1H), 7.34 (q, J = 8 Hz, 2H), 7.04 (t, J = 10 Hz, 2H), 5.12 (s, 2H), 3.02 (t, J = 6 Hz, 2H), 2.78 (t, J = 6 Hz, 2H), 1.93–1.80 (m, 4H); MS (ESI) m/z 315.6 $(M + H)^+$, calcd. for $C_{17}H_{15}FN_2OS$ m/z = 314.1.

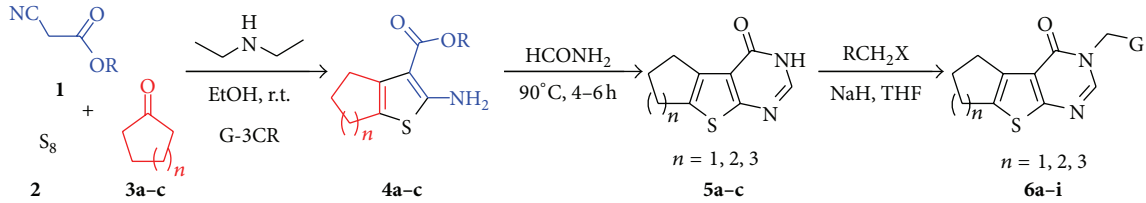
2.4.6. 3-(4-Fluorobenzyl)cycloheptyl[b]thieno[2,3-d]pyrimidin-4(3H)-one ***6f***. This compound was obtained as colorless crystal following the previously mentioned method. Yield 74%, m.p. 112.6–114.2°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.97 (s, 1H), 7.34 (q, J = 8 Hz, 2H), 7.04 (t, J = 8 Hz, 2H), 5.12 (s, 2H), 3.34 (t, J = 6 Hz, 2H), 2.85 (t, J = 6 Hz, 2H), 1.94–1.85 (m, 2H), 1.75–1.65 (m, 4H); MS (ESI) m/z 329.5 $(M+H)^+$, calcd. for $C_{18}H_{17}FN_2OS$ m/z = 328.1.

2.4.7. 3-(2-Acetate Ethyl)cyclopenta[b]thieno[2,3-d]pyrimidin-4(3H)-one ***6g***. This compound was obtained as yellowish solid following the previously mentioned method. Yield 54%, m.p. 105.3–106.5°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.85 (s, 1H), 4.69 (s, 2H), 4.27 (q, J = 8 Hz, 2H), 3.07 (t, J = 8 Hz, 2H), 2.97 (t, J = 8 Hz, 2H), 2.51–2.43 (m, 2H), 1.31 (t, J = 7.2 Hz, 3H); MS (ESI) m/z 279.6 $(M + H)^+$, calcd. for $C_{13}H_{14}N_2O_3S$ m/z = 278.1.

2.4.8. 3-(2-Acetate Ethyl)cyclohexyl[b]thieno[2,3-d]pyrimidin-4(3H)-one ***6h***. This compound was obtained as colorless needle crystal following the previously mentioned method. Yield 92%, m.p. 104.2–105.0°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.85 (s, 1H), 4.66 (s, 2H), 4.27 (q, J = 7.2 Hz, 2H), 3.07–2.97 (m, 2H), 2.86–2.75 (m, 2H), 1.94–1.80 (m, 4H), 1.31 (t, J = 7.2 Hz, 3H); MS (ESI) m/z 293.6 $(M + H)^+$, calcd. for $C_{14}H_{16}N_2O_3S$ m/z = 292.1.

2.4.9. 3-(2-Acetate Ethyl)cycloheptyl[b]thieno[2,3-d]pyrimidin-4(3H)-one ***6i***. This compound was obtained as colorless needle crystal following the previously mentioned method. Yield 68%, m.p. 145.7–147.1°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.85 (s, 1H), 4.66 (s, 2H), 4.27 (q, J = 8 Hz, 2H), 3.43–3.28 (m, 2H), 2.95–2.81 (m, 2H), 1.96–1.85 (m, 2H), 1.78–1.66 (m, 4H), 1.31 (t, J = 7.0 Hz, 3H); MS (ESI) m/z 307.6 $(M + H)^+$, calcd. for $C_{15}H_{18}N_2O_3S$ m/z = 306.1.

2.5. Biological Assay. The in vitro cytotoxicity of the synthesized compounds **6a–i** against various human cancer cell lines was measured by the MTT colorimetric method [21]. Generally, the tested compounds were initially dissolved in DMSO and were further diluted with Dulbecco's modified Eagle's medium DMEM HyClone containing 2% fetal calf serum (FCS) for testing. The final maximum DMSO concentration was 0.05%, and the same amount of DMSO was added to control cells. HepG2 (hepatocellular liver carcinoma), MCF-7 (breast epithelial adenocarcinoma), and BCG-823 (gastric cancer) cells were seeded at 2×10^4 cells per well in 96-well plates (Corning Costar) and grown to subconfluence. After removal of the growth medium, cells were incubated with various concentrations of each compound with 4 wells per dilution. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 72 h of exposure, the culture medium was removed, and 30 μL of the MTT solution (5 mg/mL in PBS) was added to each well. The plate was further incubated for 4 h to allow MTT formazan formation. To dissolve the resulting MTT formazan, 50 μL of DMSO was added to each well, followed by thorough mixing with a microplate shaker. Absorbance at 570 nm was measured on a microplate reader (Thermo Scientific, MK3). All the data of the experiment were analyzed according to SPSS



Scheme 1: General synthetic route for target compounds **6a–i**.

software, and the 50% inhibitory concentrations (IC_{50}) of each compound for the different cell lines were determined. All assays were performed in triplicate on three independent experiments, and measurement data were expressed as the mean ± S.D.

3. Results and Discussion

3.1. Synthesis of Cycloalkylthieno[2,3-d]pyrimidinone Derivatives. In the present study, a series of novel cycloalkylthieno[2,3-*d*]pyrimidinone derivatives were constructed by integrating functionalized cycloalkylthiophene derivatives **4a–c** with pyrimidinone heterocycle. The general method for the preparation of cycloalkylthieno[2,3-*d*]pyrimidinone derivatives **6a–i** is outlined in Scheme 1.

The low-cost cycloalkyl-ketone, element sulfur, and alkyl cyanoacetate were selected as starting materials, which were routinely transferred to the corresponding cycloalkylthiophene amino acid derivatives **4a–c** by general Gewald three-component reaction [22]. The following heterocyclization reaction of compounds **4a–c** was treated with $HCONH_2$ resulting in cycloalkylthieno[2,3-*d*]pyrimidinone derivatives **5a–c** via classical cyclocondensation reaction, and the key intermediate **5a–c** can be conveniently separated as needle crystals with high yields. Alkylation reaction of the cycloalkylthieno[2,3-*d*]pyrimidinone **5a–c** with appropriate halides provides the corresponding N-substituted cycloalkylthieno[2,3-*d*]pyrimidinone derivatives **6a–i** in the presence of sodium hydride. All the target N-substituted cycloalkylthieno[2,3-*d*]pyrimidinone derivatives **6a–i** gave satisfactory chemical analyses, and the chemical structures of the synthesized compounds were summarized in Table 1.

3.2. Spectroscopy. Structures of target compounds **6a–i** were confirmed by their ^{1}H NMR and ESI-MS spectra, and all the ^{1}H NMR and ESI-MS spectra analyses were consistent with the assigned structures. Their ^{1}H NMR spectra showed distinctive signals of methylene attached to nitrogen in pyrimidine ring, which presented an obvious singlet at about 4.66–5.14 ppm. The singlet at low field about 7.85–7.97 ppm in the ^{1}H NMR spectra of compounds **6a–i** was assigned to the methine proton in pyrimidine ring as shown in the typical spectra data. For compounds **6a–i**, the signals that appeared in the high field in the spectrum were attributed to the aliphatic protons of the cycloalkylthiophene scaffold. In particular, the typical protons for ethyl in compounds **6g–i** were also presented in their spectrum, respectively, which can confirm the target compounds.

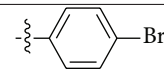
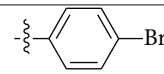
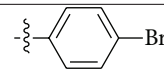
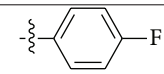
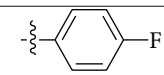
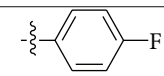
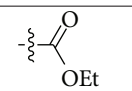
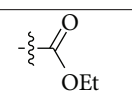
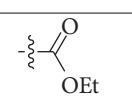
3.3. Antitumor Activity Evaluation. The N-substituted cycloalkylthieno[2,3-*d*]pyrimidinone derivatives **6a–i** were screened for their in vitro cytotoxicity effects against HepG2 (hepatocellular liver carcinoma), BCG-823 (gastric cancer), and MCF-7 (breast adenocarcinoma) cell lines by the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [21] using (5-Fluorouracil) 5-FU as a positive control. Some of the results are summarized in Figure 3 and Table 2. The IC_{50} value represents the drug concentration (μM) required to inhibit cell growth by 50%.

The initial experiment was conducted using HepG2 and MCF-7 cell lines, and the test concentration is 40 μg/mL. Generally, as shown in Figure 3, some the target compounds **6a–i** displayed moderate inhibition activities against these two human cancer cell lines. Notably, the compound **6b** exhibited significant inhibitory activities against these two tested cell lines with 66.8–69.3% growth inhibition at 40 μg/mL concentration compared to the positive control 5-FU (68.2–72.5%). Also, it is interesting to note that compounds **6c** and **6d** showed cytotoxic selectivity for a special human hepatocellular liver carcinoma cell.

Following, in order to further investigate the potential activities, the IC_{50} values were evaluated and compared to those control compounds. The inhibitory activities expressed as IC_{50} values for the target compounds are presented in Table 2. The results further testify that compound **6b** exhibited higher inhibition activity than the commercial 5-FU under the same conditions. As indicated in Table 2, compound **6c** also exhibited obviously selective inhibition activity against HepG2 cell line. Additionally, based on the data from Figure 3 and Table 2, we can find that compound **6b** exert better activities than compound **6c**, which indicated that the six-member ring in molecule **6b** might increase the potential activities. These results indicated that the heterocyclic molecule containing 4-bromobenzyl group might be an active scaffold, which further confirmed that the compound **6b** should be a potential lead molecule for discovery of cycloalkylthieno[2,3-*d*]pyrimidinone derivatives as potential drugs.

Furthermore, for comparison, the selective intermediates **4a–c** and **5a–c** have also been tested for their in vitro cytotoxic effects. However, all these intermediates exhibited almost no activities against all tested cell lines. By comprehensive

Table 1: The chemical structure of salicylamide derivatives **6a–i**.

Entry	Compd. no.	Substituents		Appearance	Mp (°C)	Yield (%)[a]
		n	G			
1	**6a**	1		White powder	159.2–160.0	75
2	**6b**	2		Yellowish solid	111.6–112.0	80
3	**6c**	3		Colorless crystal	140.5–141.6	72
4	**6d**	1		White powder	122.6–124.2	58
5	**6e**	2		Colorless needle crystal	133.2–136.8	78
6	**6f**	3		Colorless crystal	112.6–114.2	74
7	**6g**	1		Yellowish solid	105.3–106.5	54
8	**6h**	2		Colorless needle crystal	104.2–105.0	92
9	**6i**	3		Colorless needle crystal	145.7–147.1	68

[a]Isolated yield.

Table 2: Cytotoxicities of target compounds **6a–i** against various cancer cell lines.

Entry	Compd. no.	In vitro cytotoxicity IC_{50}[a] (μM)		
		HepG2[b]	MCF-7[b]	BCG-823[b]
1	**6a**	>200	>200	>200
2	**6b**	14.96 ± 0.9	78.88 ± 2.7	45.01 ± 1.3
3	**6c**	45.19 ± 1.8	>200	>200
4	**6d**	108.9 ± 1.7	164 ± 3.5	146.7 ± 3.2
5	**6e**	>200	>200	244.17
6	**6f**	>200	>200	>200
7	**6g**	>200	>200	>200
8	**6h**	>200	>200	>200
9	**6i**	>200	>200	>200
10	5-FU[c]	86.09 ± 1.6	100.08 ± 2.4	77.33 ± 1.1

[a]IC_{50}: Compound concentration required to inhibit tumor cell proliferation by 50%.
[b]Abbreviations: HepG2: human hepatocellular liver carcinoma cell line; BCG-823: human gastric cancer cell line; MCF-7: human breast adenocarcinoma cell line.
[c]Used as a positive control.

consideration of these results, it can be figured out that the compound **6b** has obviously better activities than the control, which may be used as potential lead compound for optimization of novel anticancer agents.

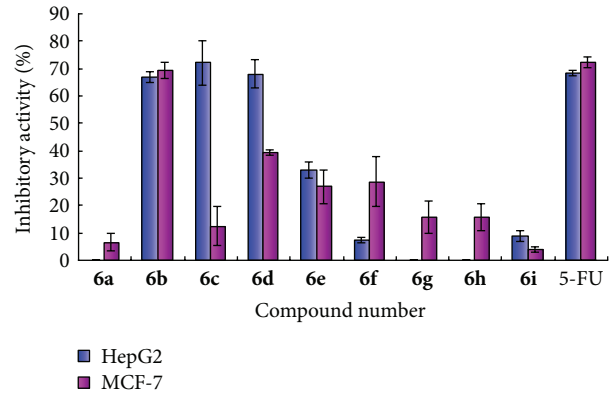


Figure 3: Inhibition activities against cell proliferation for compounds **6a–i** at 40 μg/mL. Abbreviations: HepG2: human hepatocellular liver carcinoma cell line; MCF-7: human breast adenocarcinoma cell line; 5-FU: 5-fluorouracil, used as a positive control.

4. Conclusion

In summary, we have described the convenient synthesis and biological evaluation of a series of novel thieno[2,3-*d*]pyrimidinone derivatives as potential tumor cell growth inhibitors. The preliminary bioassay results indicated that some of target compounds exhibited obviously inhibition activities against human tumor cell compared to 5-FU.

Further structural optimization and activity profiles about the designed novel cycloalkylthieno[2,3-*d*]pyrimidinone derivatives are well ongoing in our laboratory.

Conflict of Interests

There is no conflict of interests.

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