Benzoylurea Derivatives as a Novel Class of Antimitotic Agents: Synthesis, Anticancer Activity, and Structure−Activity Relationships

Dan-Qing Song,[†,‡] Yan Wang,[†,‡] Lian-Zong Wu,[†] Peng Yang,[†] Yue-Ming Wang,[†] Li-Mei Gao,[†] Yan Li,[§] Jing-Rong Qu,[§] Yong-Hong Wang,[†] Ying-Hong Li,[†] Na-Na Du,[†] Yan-Xing Han,[†] Zhi-Ping Zhang,[†] and Jian-Dong Jiang*[,†]

Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China, and Division of Hematology and Oncology, Box 1131, Mount Sinai School of Medicine, New York, New York 10029

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Forty-six new compounds were synthesized on the basis of our knowledge of the 3-haloacylamino benzoylurea (HBU) series. Structure−activity relationship (SAR) analysis indicates that (i) the configuration of the chiral center in **1** (JIMB01) is not indispensable for the activity, (ii) the phenyl ring is essential, and (iii) a substitution at the 6-position of the phenyl ring with a halogen enhances the activity. Among the analogues, **11e** and **14b** bearing 6-fluoro substitution showed potent activities against nine human tumor cell lines, including CEM (leukemia), Daudi (lymphoma), MCF-7 (breast cancer), Bel-7402 (hepatoma), DU-145 (prostate cancer), PC-3 (prostate cancer), DND-1A(melanoma), LOVO (colon cancer), and MIA Paca (pancreatic cancer) with IC_{50} values between 0.01 and 0.30 μM. **14b** inhibited human hepatocarcinoma by 86% in volume in nude mice. The mechanism of **14b** is to inhibit microtubule assembly, followed by the M-phase arrest, bcl-2 inactivation, and then apoptosis. We consider **14b** promising for further anticancer investigation.

Introduction

Microtubule (MT[a]) dynamics have been identified as a rational site to interfere with the dividing of cancer cells. Drug agents that target cellular MT dynamics arrest cancer cell cycle at the M-phase followed by apoptosis. Drugs in this class have been widely used for cancer patients with great clinical success and are continuing to be important chemotherapeutic agents.[1–3] The commonly used tubulin-active anticancer drugs are paclitaxel and vinca alkaloids. These drugs, however, have three major limitations.[4,5] First of all, these drugs cause neurotoxic side effects in patients.[6] Second, they are difficult for de novo synthesis because the compounds are natural products existing as large molecular structures. Third, they are all substrates of P-glycoprotein and induce multidrug resistance (MDR), which is the main cause for the failure of clinical cancer chemotherapy.[7] Therefore, searching for drugable small tubulin ligands with novel chemical structures or antimitotic agents with novel modes of action has become an active field of investigation in medicinal chemistry.[8,9] For instance, combretastain A-4 (CA4), a new antimitotic agent binding at colchicine pocket on tubulin with a smaller molecule, has entered into phase II clinical trail.[1b]

The 3-haloacylamino benzoylureas (HBUs) analogues were designed and synthesized in our group.[10–13] Several compounds have been proved to be tubulin ligands that inhibit the polymerization of tubulin. The primary mechanism of these compounds is to bind at the region of the colchicine binding site on β-tubulin.[11a] The compounds have shown anticancer activity in vitro and in vivo and become a new family of tubulin ligand leads.[11–13] The representative compounds (Figure 1) are

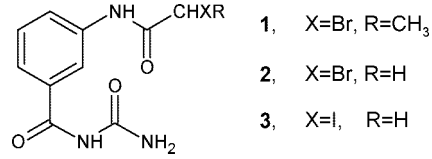

Figure 1. Structure of the lead compounds.

1 (JIMB01),[13] **2** (BAABU),[12] and **3** (IAABU),[11] which interfere with the assembly of mitotic spindle microtubules from the free tubulin pool in tumor cells, block the cell cycle at the M-phase, cause apoptotic cell death through promoting bcl-2 phosphorylation, and show therapeutic efficacy in nude mice bearing human hepatocarcinoma.[10–13] In contrast to the tubulin-active anticancer drugs that have been used in the clinic, these compounds have simple and small chemical structures, and that provides advantages in chemical synthesis and opportunities of formulation for oral administration. In preliminary SAR analysis, we have identified the essential structural and functional requirements for the anticancer activity.[10] The results indicated that the benzoylurea group is a key for good anticancer activity; the iodoacetylamino, bromoacetylamino, or bromopropionylamino chain at the 3-position (meta position in relationship to the benzoylurea group) plays a significant role in regulating the action. Their cytotoxicity in tumor cells was ranked in an order of the nature of halogens: I > Br > Cl > F.[10]

Benzoylurea derivatives constitute a class of antiproliferative agents, among which the N-substituted benzoylurea derivatives have been reported to have antitumor activities.[14–16] Therefore, the chemical and biological features of the 3-HBU derivatives provoked our strong interest for a further SAR study. In this study, we retain the benzoylurea group at the 1-position of the aromatic ring and focus the study on the configuration of the asymmetric center in the compound **1** structure, alteration of the aromatic ring, and substitutions on the positions of aromatic ring. On the basis of this strategy, new 3-HBU derivatives were designed and synthesized.

Here, we describe the synthesis, in vitro evaluation, SAR analysis, in vivo anticancer efficacy, and the primary mode of

* To whom correspondence should be addressed. Phone: 86-10-63165290. Fax: 86-10-63017302. E-mail: jiang.jdong@163.com.

† Chinese Academy of Medical Sciences and Peking Union Medical College.

‡ These authors made equal contribution to this work.

§ Mount Sinai School of Medicine.

[a] Abbreviations: HBU, haloacylamino benzoylurea; MT, microtubule; MDR, multidrug resistance; SAR, structure−activity relationship; DMA, *N*,*N*-dimethylacetamide; DMF, *N*,*N*-dimethylformamide; DTT, dithiolthreitol; ECL, enhanced chemiluminescence; PBS, phosphate buffered saline.

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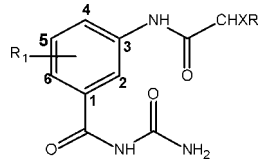
Table 1. Antiproliferative Activity of 3-HBUs in CEM Leukemia Cells



compd	R_1	X	R	method	formula	IC_{50}[a] (μM)
1	H	Br	CH_3	A	$C_{11}H_{12}BrN_3O_3$	1.47 ± 0.09
10a	H	Br	CH_3	A	$C_{11}H_{12}BrN_3O_3$	1.47 ± 0.12
10b	H	Br	CH_3	A	$C_{11}H_{12}BrN_3O_3$	1.47 ± 0.16
10c	2-CH_3	Br	CH_3	A	$C_{12}H_{14}BrN_3O_3$	0.48 ± 0.08
10d	4-CH_3	Br	CH_3	A	$C_{12}H_{14}BrN_3O_3$	3.36 ± 0.13
10e	6-CH_3	Br	CH_3	B	$C_{12}H_{14}BrN_3O_3$	>20
10f	4-F	Br	CH_3	B	$C_{11}H_{11}BrFN_3O_3$	3.13 ± 0.15
10g	6-F	Br	CH_3	B	$C_{11}H_{11}BrFN_3O_3$	0.52 ± 0.04
10h	4-Cl	Br	CH_3	A	$C_{11}H_{11}BrClN_3O_3$	9.80 ± 0.68
10i	6- Cl	Br	CH_3	B	$C_{11}H_{11}BrClN_3O_3$	2.55 ± 0.12
10j	6-Br	Br	CH_3	B	$C_{11}H_{11}Br_2N_3O_3$	7.63 ± 0.52
10k	4-OCH_3	Br	CH_3	A	$C_{12}H_{14}BrN_3O_4$	>20
10l	5-$COOCH_3$	Br	CH_3	B	$C_{13}H_{14}BrN_3O_5$	2.7 ± 1.50
10m	5-NO_2	Br	CH_3	A	$C_{11}H_{11}BrN_4O_5$	1.58 ± 0.38
2	H	Br	H	A	$C_{10}H_{10}BrN_3O_3$	0.725 ± 0.06
11a	2-CH_3	Br	H	A	$C_{11}H_{12}BrN_3O_3$	1.71 ± 0.032
11b	4-CH_3	Br	H	A	$C_{11}H_{12}BrN_3O_3$	4.28 ± 0.16
11c	6-CH_3	Br	H	B	$C_{11}H_{12}BrN_3O_3$	4.76 ± 0.19
11d	4-F	Br	H	B	$C_{10}H_9BrFN_3O_3$	2.29 ± 0.09
11e	6-F	Br	H	B	$C_{10}H_9BrFN_3O_3$	<0.01
11f	4-Cl	Br	H	A	$C_{10}H_9BrClN_3O_3$	2.91 ± 0.72
11g	6-Cl	Br	H	B	$C_{10}H_9BrClN_3O_3$	0.54 ± 0.04
11h	6-Br	Br	H	B	$C_{10}H_9Br_2N_3O_3$	1.16 ± 0.14
11i	4-OCH_3	Br	H	A	$C_{11}H_{12}BrN_3O_4$	1.88 ± 0.06
11j	5-CO_2CH_3	Br	H	B	$C_{12}H_{12}BrN_3O_5$	1.3 ± 0.20
11k	5-NO_2	Br	H	A	$C_{10}H_9BrN_4O_5$	1.53 ± 0.23
11l	6-OCH_3	Br	H	B	$C_{11}H_{12}BrN_3O_4$	1.57 ± 0.32
11m	4-OH	Br	H	B	$C_{10}H_{10}BrN_3O_4$	>20
12a	2-CH_3	Cl	CH_3	A	$C_{12}H_{14}ClN_3O_3$	>20
12b	4-CH_3	Cl	CH_3	A	$C_{12}H_{14}ClN_3O_3$	>20
12c	4-Cl	Cl	CH_3	A	$C_{11}H_{11}Cl_2N_3O_3$	>20
12d	4-OCH_3	Cl	CH_3	A	$C_{12}H_{14}ClN_3O_4$	>20
13a	2- CH_3	Cl	H	A	$C_{11}H_{12}ClN_3O_3$	2.70 ± 0.21
13b	4-CH_3	Cl	H	A	$C_{11}H_{12}ClN_3O_3$	3.36 ± 0.11
13c	4-F	Cl	H	B	$C_{10}H_9ClFN_3O_3$	>20
13d	6-F	Cl	H	B	$C_{10}H_9ClFN_3O_3$	7.68 ± 0.97
13e	4-Cl	Cl	H	A	$C_{10}H_9Cl_2N_3O_3$	15.9 ± 1.38
13f	6-Cl	Cl	H	B	$C_{10}H_9Cl_2N_3O_3$	2.44 ± 0.11
13g	4-OCH_3	Cl	H	A	$C_{11}H_{12}ClN_3O_4$	>20
13h	4-OH	Cl	H	B	$C_{10}H_{10}ClN_3O_4$	>20
3	H	I	H	A	$C_{10}H_{10}IN_3O_3$	<0.01
14a	6-CH_3	I	H	B	$C_{11}H_{12}IN_3O_3$	20.0 ± 0.76
14b	6-F	I	H	B	$C_{10}H_9FIN_3O_3$	<0.01
14c	5- CO_2CH_3	I	H	B	$C_{12}H_{12}IN_3O_5$	1.91 ± 0.21

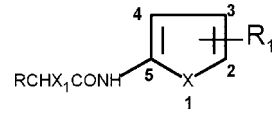
[a] IC_{50}: drug concentration required to inhibit 50% of cell proliferation after 72 h of treatment.

action of these new derivatives. Among the 3-HBUs, compound **14b** was selected as a representative agent for anticancer and mechanism investigation.

Chemistry

The 46 new 3-HBU derivatives were grouped as phenyl ring compounds (Table 1) and heterocyclic ring compounds (Table 2) and synthesized as described in Schemes 1 and 2, respectively. Scheme 1 includes two synthetic methods. The first one (route A) uses commercially available derivatives of *m*-aminobenzoic acid as starting materials, with the method reported previously.[10] In this reaction intermediate **7** reacted with the (*R*)- or (*S*)-2-bromopropionyl chloride to produce the final enantiomerically pure (*R*)-**1** (**10a**) or (*S*)-**1** (**10b**) with a good yield. The two isomers exhibited identical NMR spectra with opposite optical rotation, in which the (*R*)-form exhibiting dextrorotation was the (*R*)-(+)-enantiomer and the antipodal was

Table 2. Antiproliferative Activity of 3-HBUs in CEM Leukemia Cells: Heterocyclic Ring



compd	X	X_1	R	R_1	formula	IC_{50}[a] (μM)
18a	O	Br	CH_3	2-$CONHCONH_2$	$C_9H_{10}BrN_3O_4$	9.44 ± 2.63
18b	O	Br	H	2-$CONHCONH_2$	$C_8H_8BrN_3O_4$	4.05 ± 0.33
18c	O	Cl	H	2-$CONHCONH_2$	$C_8H_8ClN_3O_4$	16.62 ± 4.44
19a	S	Br	CH_3	3-$CONHCONH_2$	$C_9H_{10}BrSN_3O_3$	3.94 ± 0.18
19b	S	Br	H	3-$CONHCONH_2$	$C_8H_8BrSN_3O_3$	1.13 ± 0.08

[a] IC_{50}: compound concentration required to inhibit CEM leukemia cell proliferation by 50% after 72 h of treatment.

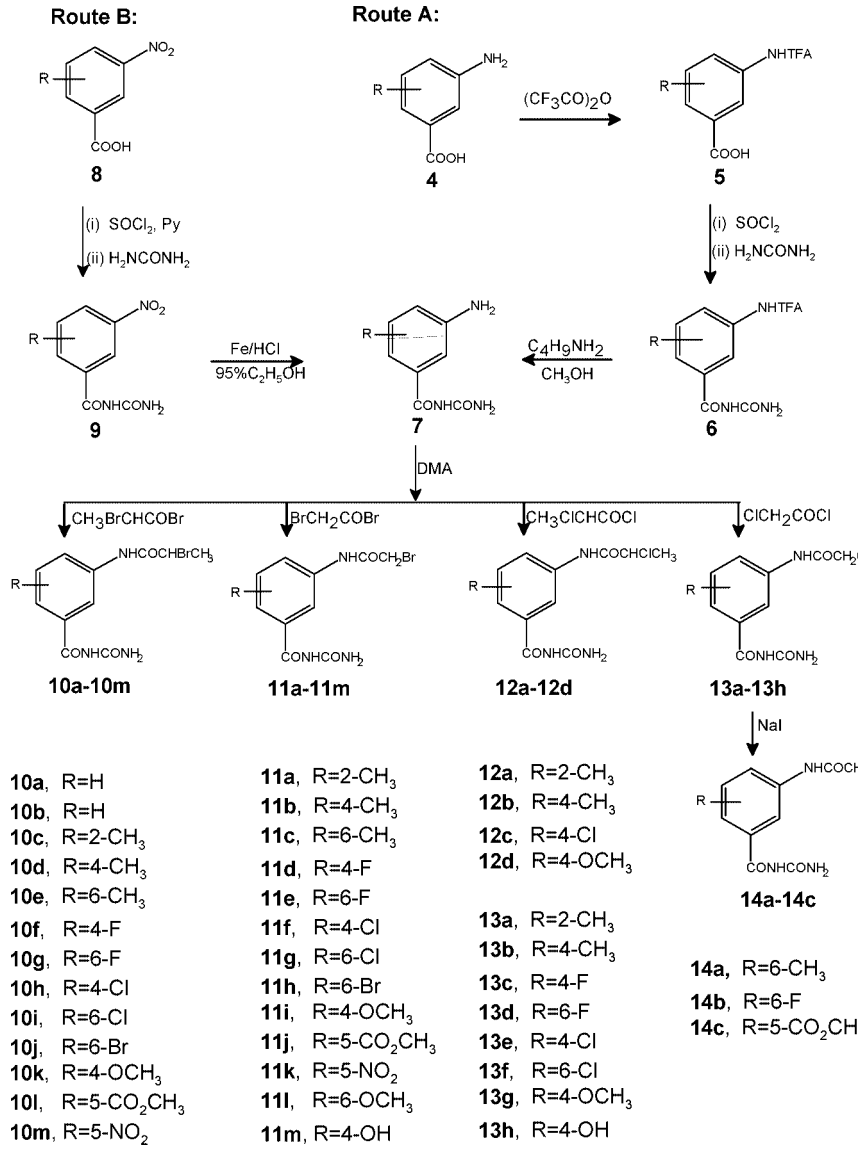
the (*S*)-(−)-enantiomer. Route A produced the compounds of **10a**−**d**, **10h**, **10k**, **10m**, **11a**, **11b**, **11f**, **11i**, **11k**, **12a**−**d**, **13a**, **13b**, **13e**, **13g**. The second one (route B) is the synthetic route using commercially available derivatives of *m*-nitrobenzoic acid for an acylation reaction. In this chemical reaction $SOCl_2$ was used as an acylating reagent as well as solvent, and pyridine was used as a catalyst. Then the resultant intermediate directly reacted with urea to yield key intermediate **9**. The nitro compounds **9** were then reduced to anilines analogues **7** after treatment in the conventional condition of Fe/HCl.[17] Finally, relevant anilines reacted with chloroacetyl chloride, bromoacetyl bromide, and 2-bromopropionyl bromide using *N*,*N*-dimethylacetamide (DMA) as solvent to yield desired products (**10e**−**g**, **10i**, **10j**, **10l**, **11c**–**e**, **11g**, **11h**, **11j**, **11l**, **11m**, **13c**, **13d**, **13f**, **13h**). The iodo compounds (**14a**−**c**) were obtained from the chloro intermediates, through an exchange reaction with NaI. The synthetic method of heterocyclic ring compounds was similar to that of route B (Scheme 2). The starting materials 5-nitrothiophene- 3-carboxylic acid or 5-nitro-2-furoic acid (**15**) were commercially available. The desired products (**18a**−**c**, **19a**, **19b**) were obtained after four step reactions including acylation, amidation, reduction, and amination.

Results and Discussion

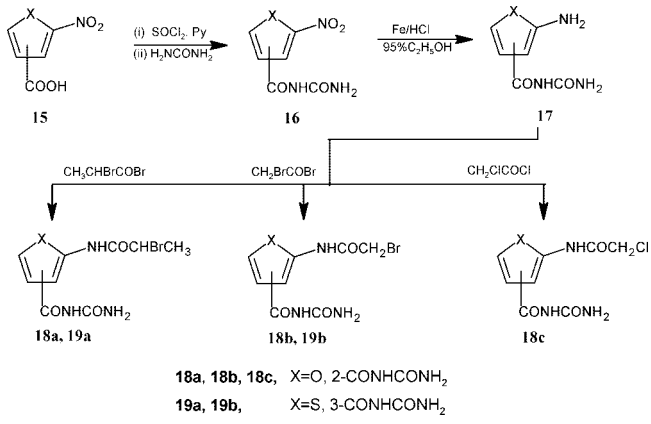
In Vitro Antiproliferative Activity and SAR. The antiproliferative activity of the 46 compounds in CEM leukemia cells was closely associated with their structure, as shown in Tables 1 and 2. CEM leukemia cells were used for initial biological screening because of their known rapid proliferation and high sensitivity to standard anticancer agents. Out of the 46 compounds, 22 exhibited good activity with IC_{50} values of <3.0 μM, among which 6 compounds (**10i**, **10l**, **11d**, **11f**, **13a**, **13f**) demonstrated IC_{50} values of 2.0−3.0 μM, 11 compounds (**10a**, **10b**, **10m**, **11a**, **11h**, **11i**, **11j**, **11k**, **11l**, **14c**, **19b**) of 1.0−2.0 μM, 3 compounds (**10c**, **10g**, **11g**) of 0.1−1.0 μM, and 2 compounds (**11e**, **14b**) of <0.01 μM. The last five compounds (**10c**, **10g**, **11e**, **11g**, **14b**) showed an anticancer activity in CEM cells equal to or greater than the lead compounds did.

The lead compound **1** was a racemic mixture; the SAR study was first focused on the effect of (*R*)- or (*S*)-configuration of the asymmetric center on the anticancer activity of **1** series. In a comparison of the cytotoxic activities of the isomers (**10a**, **10b**) of **1**, we found that introduction of the chiral center maintained the IC_{50} values at the 1.47 μM level, hinting that the configuration of chiral center was not indispensable for the activities of **1**. Therefore, the derivatives with a propionylamino chain were subsequently examined in the racemate but not the (*R*)- or (*S*)-enantiomer. It was also found that the orders of anticancer activity in this cell line appeared to be I > Br > Cl, and iodoacetyl > bromoacetyl > bromopropionyl ≫ chloro-

Scheme 1. Synthesis of the Phenyl Ring Compounds



Scheme 2. Synthesis of the Heterocyclic Ring Compounds



acetyl > chloropropionyl, consistent with the previous observations.[10] The alkyl halides of the agents are strongly electrophilic[18] and are reactive groups that interact with nucleophiles, the sulfhydryl (−SH) group present on the β-tubulin. We have shown before that the inhibition of microtubule assembly by such types of compounds could be significantly reduced in the presence of the reducing agent DTT (containing two −SH groups per molecule).[11b] Therefore, we believe that the −SH group displaces the halide ion and then results in the formation of a covalent bond between the β-tubulin and these types of agents. This is consistent with the previous reports.[19a,b,20]

Next, the aromatic ring of 3-HBUs was modified with substituents that varied in their size, electronic character, and position. The obtained IC_{50} values showed that the position of the substituent on the phenyl ring greatly affected the anticancer activity. Compounds methylated at the 6-position (e.g., **10e**, **14a**) were inactive. Compounds methylated at the 4-position were tolerated (**10d**, **11b**), and those at the 2-position were optimal (**10c**). The antitumor activities of methylated compounds decreased in the order of 2-CH_3 > 4-CH_3 > 6-CH_3. In addition, compounds **11i** and **11l** bearing OCH_3 substituent at the 4- and 6-position, respectively, had a similar activity (i.e., 4-OCH_3 ~ 6-OCH_3), which was, however, lower than that of their lead compound. In contrast, compounds bearing a halogen substituent at the 6-position showed an enhanced activity compared to the parent compounds. The introduction of an atom of fluorine at the 6-position afforded the most potent derivatives of **11e** and **14b** with IC_{50} less than 0.01 μM for both. However, moving the fluorine from the 6- to 4-position (**11d**) or replacing the

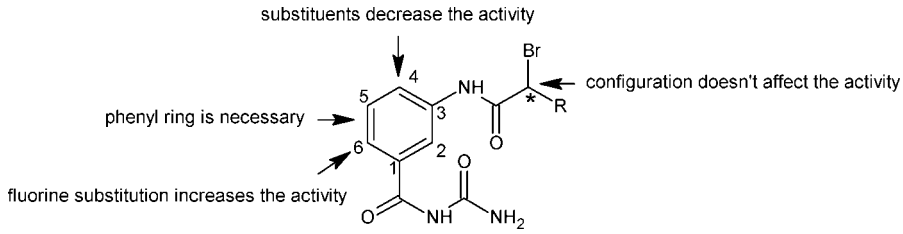


Figure 2. SAR of 3-HBUs.

fluorine with chlorine (**11g**) or bromine (**11h**) decreased the anticancer activity. The anticancer activities of the halogenated compounds decreased in the order of 6-F > 4-F, 6-Cl > 4-Cl and 6-F > 6-Cl > 6-Br. From these results, we deduce that substitution at the 6-position of the phenyl ring plays an important role in the anticancer activity; a small substituent like an atom of fluorine, an isosteric to the hydrogen atom, seemed to have the highest activity among the substituents of the aromatic ring moiety. Furthermore, compounds **10l**, **10m**, **11j**, and **11k** bearing electron-withdrawing groups at the 5-position (NO_2, CO_2CH_3) had lower activity than that of the corresponding parent compounds. The introduction of a hydroxyl group at the 4-position of compounds **11m** and **13h** completely abolished the activity. Therefore, with the exception of fluorine the substitution on the aromatic ring with electron-donating and electron-withdrawing groups resulted in partial or complete loss of the activity regardless of the position.

Furthermore, the modifications of the phenyl ring moiety were explored, as shown in Table 2. In the evaluation of the activities of heterocyclic ring compounds, we found that the thiophene derivatives (**19a**, **19b**) are anticancer agents with IC_{50} values ranging from 1.13 to 3.94 μM in CEM cells. In contrast, introduction of a furan ring, as that for compounds **18a**−**c**, resulted in a loss of cytotoxicity in cancer cells with elevated IC_{50} values between 4.05 and 16.62 μM. Moreover, as previously noticed, compounds **18a**−**c** in which the 4-position was substituted were inactive. These data demonstrated that analogues replaced by thiophene ring, a bioisosteric group to the phenyl ring, possess a considerable potency although not as high as that of the phenyl ring derivatives.

Thus, we have identified the essential structural and functional requirements of 3-HBUs for the anticancer activity and summarized the results in Figure 2. On the basis of the SAR analysis, replacement of hydrogen with fluorine at the 6-position could enhance the potency of the compounds; in contrast, the activity was lost completely when the substituent was methyl group. Compounds **11e** and **14b** bearing fluorine substituent at the 6-position showed the highest antitumor activity, and therefore, both of them were selected for further evaluation.

In Vitro Anticancer Activity of 11e and 14b in Human Cancer Cell Lines. The antiproliferative activity of compounds **11e** and **14b** was examined in nine human tumor cell lines (Table 3). The IC_{50} values of **11e** were in the range of 0.08−0.3 μM for solid tumor lines and less than 0.01 and 0.015 μM for leukemia or lymphoma cells, respectively. Among the solid tumors, the least sensitive cell line was DU-145 prostate cancer (IC_{50} = 0.30 μM), and the most sensitive cell line was MCF-7 breast cancer cells (IC_{50} = 0.08 μM). Compound **11e** showed an enhanced activity compared to the lead compound **2**. The IC_{50} values of **14b** were in the range 0.01−0.22 μM for solid tumor lines and less than 0.01 μM for leukemia or lymphoma cell lines. The least sensitive cell line was the DU-145 cells (IC_{50} = 0.22 μM), and the most sensitive cell lines was MCF-7 (IC_{50} < 0.01 μM), similar to that of **11e**.

Table 3. Antiproliferative Activities of **11e** and **14b** in Human Tumor Cell Lines

		IC_{50}[a] (μM)	
cell line	humantumor	**11e**	**14b**
CEM	T-cell leukemia	<0.01	<0.01
Daudi	B-cell lymphoma	0.015 ± 0.0058	<0.01
MCF-7	breast cancer	0.08 ± 0.01	<0.01
Bel-7402	hepatoma	0.16 ± 0.01	0.16 ± 0.02
DU-145	prostate cancer	0.30 ± 0.05	0.22 ± 0.05
PC-3	prostate cancer	0.19 ± 0.023	0.19 ± 0.028
DND-1A	melanoma	0.11 ± 0.021	0.02 ± 0.005
LOVO	colon cancer	0.25 ± 0.04	0.19 ± 0.03
MIA	pancreas cancer	0.19 ± 0.038	0.054 ± 0.013

[a] IC_{50}: drug concentration required to inhibit human tumor cells proliferation by 50% after 72 h of treatment.

Since fluorine has a size and electronic properties similar to those of hydrogen, it is often introduced as an isosteric to the hydrogen atom. **11e** and **14b** bears fluorine substituent at the 6-position and might have anticancer bioavailability in vivo superior to that of **2** and **3**. Therefore, **11e** and **14b** entered into animal experiments using human tumor-bearing nude mice.

Inhibition of Human Hepatocarcinoma by 11e and 14b in Nude Mice. The anticancer efficacy of **11e** and **14b** was shown in Figure 3. The drugs were given to the tumor-bearing nude mice when the hepatoma (Bel-7402) tumor size on their back was between 160 and 200 mm^3. When the tumor volume in the solvent-treated mice (control group) grew to 4300 mm^3 on day 25 post-treatment, it was 2700 mm^3 in the mice treated with 5 mg/kg of **11e** intraperitoneously (ip) every other day (q2d), and 2050 mm^3 in the group that received 10 mg/kg of **11e** (ip, q2d) (Figure 3A). The tumor growth inhibition rate was 37% and 52%, respectively. The average body weight of the mice in the **11e** treatment group (10 mg/kg) was 18.7 ± 0.9 g before and 18.6 ± 0.9 g after the treatment.

As demonstrated in Figure 3B, when the average tumor volume of untreated controls grew to 3000 mm^3 on day 28 post-treatment, the tumor volume of the mice treated with **14b** at 5 mg/kg (ip, q2d) was 1150 mm^3, showing 62% growth inhibition in tumor volume. **14b** at 10 mg/kg (ip, q2d) controlled the tumor volume within 420 mm^3, indicating an 86% inhibition of tumor growth. Toxic effects of this regimen were not observed during the treatment. The average body weight of the mice treated with **14b** (10 mg/kg group) was 19.6 ± 0.7 g before and 21.6 ± 0.9 g after the therapy. With respect to the parent compound **3**,**14b** at equal molar concentration showed a higher tumor inhibitory effect in vivo (57% inhibition by **3** vs 86% by **14b**). Since compound **14b** contains a fluorine substituent at the 6-position, it could be at least part of the explanation that **14b** possesses a longer half-time in vivo with respect to their parent compounds. In addition, the LD_{50} of **14b** (ip) in the nude mice was 38.8 mg/kg, higher than that of compound **3** (LD_{50}, 31 mg/kg).[10]

Mode of Action of 14b. We then turned our attention to the mechanism of **14b** to learn whether the chemical modification

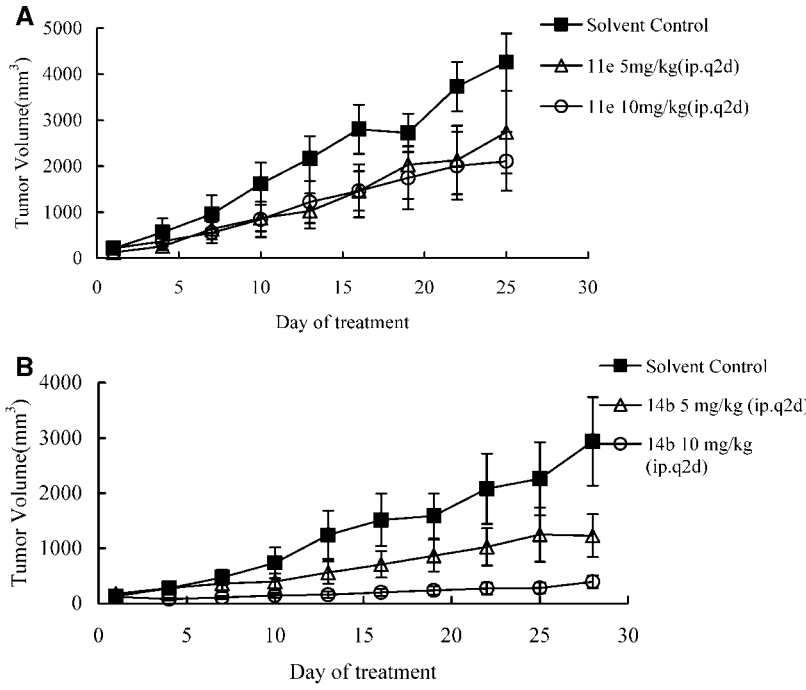


Figure 3. Anticancer efficacy of **11e** (A) and**14b** (B) in human hepatocarcinoma (Bel-7402) in nude mice. The nude mice were sc implanted with Bel-7402 cells, and the treatments were initiated when the average tumor volume was about 200 mm^3 (see Experimental Section). Presented in each time point is the mean of tumor volume ($n = 5$).

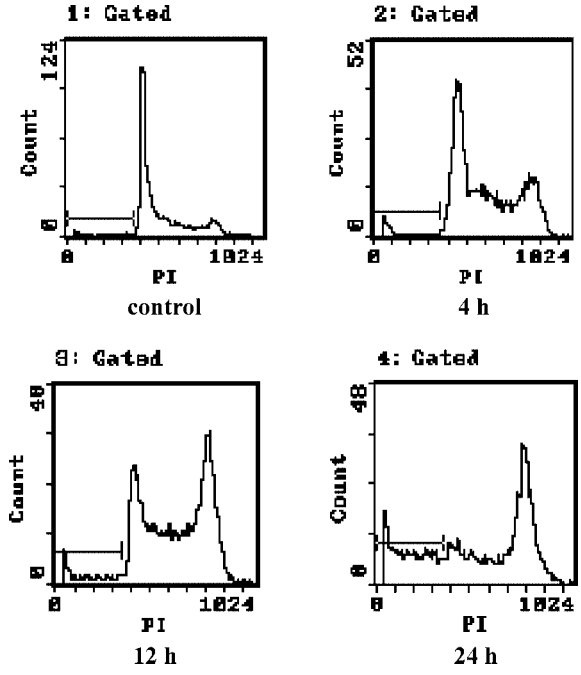


Figure 4. Cell cycle arrest at the M-phase by **14b**. CEM cells were incubated without (control) or with **14b** (0.22 μM) for 4, 12, or 24 h. Cells were then analyzed for their cell cycle distribution using flow cytometry. A significant M-phase accumulation appeared between 12 and 24 h post-treatment.

retains the mode of action of its parent compound. Flow cytometric analysis of the DNA profile in the CEM cells (Figure 4) showed that **14b** treatment (0.22 μM) produced a major shift of the cell population from G_0/G_1 to G_2/M, revealing a significant accumulation of cells in the G2/M-phase. CEM cells treated with **11e** exhibited a similar cell cycle distribution (data not shown). To further define the cell cycle arrest, morphology examination was done. CEM leukemic cells treated with **14b** (0.014 μM) for 24 h displayed the characteristic features of

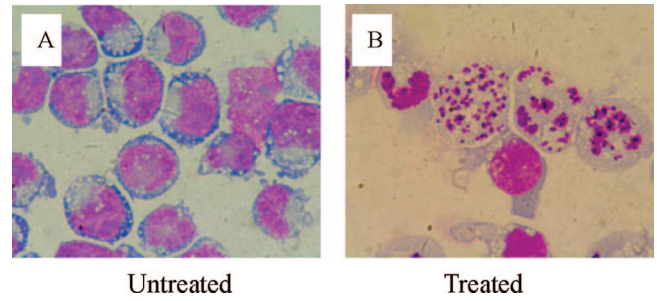


Figure 5. Metaphase block by **14b**. CEM cells were treated with **14b** at 0.014 μM for 24 h. Cells were then harvested on slides for morphology observation: (A) untreated; (B) **14b** treated (Giemsa, X400).

mitotic arrest, i.e., the disappearance of nuclear membrane and disorientation and loose dispersion of metaphase chromosomes in the cytoplasm (Figure 5), indicating that the cell cycle arrest by **14b** occurred at M but not G_2 phase. Bcl-2 expression was also examined because it is a cellular factor to inhibit apoptosis. As shown in Figure 6, bcl-2 protein was inactivated by phosphorylation in the cells treated with **14b**. These results suggest that compound **14b** can arrest the tumor cell cycle at the M-phase and induce apoptotic cell death by promoting bcl-2 phosphorylation.

Next, we accessed the direct effect of **14b** on microtubule assembly in a cell-free system using purified tubulin. **14b** significantly inhibited the process of microtubule assembly (Figure 7) but had no effect on the disassembly process (not shown). The result was consistent with the activity of the lead compounds **3**, **2**, and **1**. A significant inhibition of microtubule assembly was observed even when the concentration of **14b** was as low as 1 μM. The IC_{50} value of **14b** in the assay was 0.71 μM. Well-known tubulin-active drugs were used as references in the experiment system. An inhibitory effect on microtubule assembly was detected in vincristine but not in

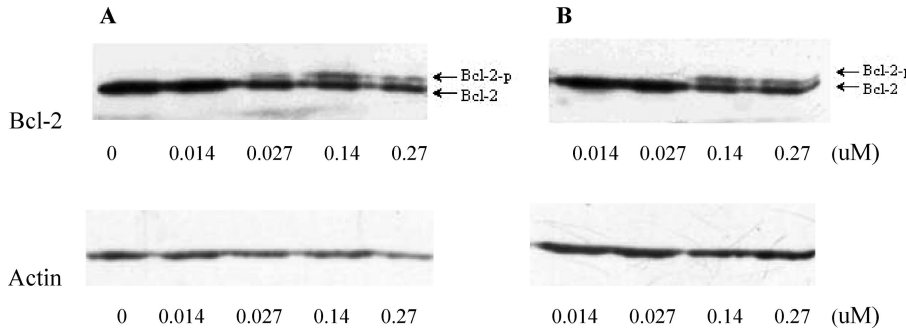


Figure 6. Bcl-2 phosphorylation by **14b**. CEM cells were treated with **14b** at different concentrations (0.014−0.27 μM) for 12 h (A) and 24 h (B). Cells were harvested, and the cellular proteins were extracted for Western blot analysis, Bcl-2-p, phosphorylated bcl-2.

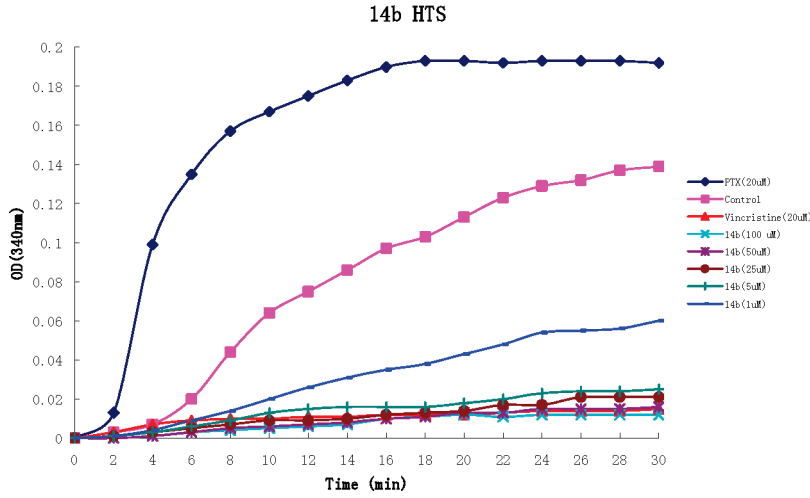


Figure 7. Effect of **14b** on the microtubule assembly process. Free β-tubulin in reaction buffer was incubated with GTP and Mg^{2+} at room temperature for the polymerization in the absence (control) or presence of **14b** (1, 5, 25, 50, or 100 μM) or paclitaxel (20 μM) or vinblastine (20 μM). Microtubule assembly was determined every 1.5 min by OD at 340 nm. Each point represents the mean of two independent experiments.

paclitaxel, suggesting that the mode of action of **14b** is similar to that of vincristine, as well as colchicine.

As M-phase arrest is a phenotype of tubulin disruption, it was examined to learn the relationship between chemical structure and anti-tubulin activity. As shown in Figure 8, compounds **14b** and **11e** with 6-F substitution caused M-phase arrest by 37.6% and 31.3%, respectively, after 12 h of treatment; however, replacing the fluorine with chlorine (**11g**) or bromine (**11h**) or moving the fluorine from the 6- to 4-position (**11d**) greatly decreased M-phase accumulation. The results are consistent with the results from the IC_{50} assay.

Conclusions

In continuation of our study on 3-HBU derivatives, we have synthesized 46 new analogues defined through variations of the three regions of the core structure. The SAR information revealed that (i) the configuration of asymmetric center present in **1** has no influence the antiproliferative activity and the configuration of **1** does not appear to be essential, (ii) the anticancer activity of the HBU largely depends on how active the halide ion is as a leaving group and therefore is ranked in the order of $-CH_2I > -CH_2Br > (\pm)$-$CHBrCH_3 = (R)$-$CHBrCH_3 = (S)$-$CHBrCH_3 \gg -CH_2Cl > -CHClCH_3$, (iii) a phenyl ring system is essential for the anticancer activity and replacing phenyl ring with thiophene or furan reduced the activity, and (iv) a substitution at the 6-position of the phenyl ring with an atom of fluorine enhances the anticancer activity.

Mechanism studies show that compound **14b** interferes with the microtubule dynamics selectively at the assembly phase, prevents the formation of mitotic spindles, suspends the cell cycle at M-phase, and eventually leads the tumor cells to apoptosis by promoting bcl-2 phosphorylation. Monotherapy with compound **14b** showed a strong anticancer effect against human hepatocarcinoma in nude mice with over 86% tumor growth inhibition. The activity was higher than that of the parent compound **3**. Taken together, the 3-HBU derivatives constitute a class of the anticancer tubulin ligands, and particularly, compound **14b** merits further investigation.

Experimental Section

Chemistry. Melting points (mp) were obtained with YRT-3 melting point apparatus and are uncorrected. ^1H nuclear magnetic resonance spectra were performed on a Varian Inova 400 MHz spectrometer (Varian, San Francisco, CA) in either DMSO-d_6 or CD_3OD (as internal standard on a δ scale). Infrared (IR) spectra were recorded on a Nicolet IMPACT-400 spectrometer (Thermo Electron Corporation, Madison WI). FAB and EI mass spectra were recorded on an Autospec UItima-TOF mass spectrometer (Micromass UK Ltd., Manchester, U.K.). For the sake of consistency, the positions of the substituents on the phenyl ring of the study compounds are designated according to the orientation presented in Figure 2.

Method A (for 10a−d, 10h, 10k, 10m, 11a, 11b, 11f, 11i, 11k, 12a−d, 13a, 13b, 13e, 13g). The parent intermediates (**5**−**7**) were synthesized according to the procedure previously described.[10]

2-Methyl-3-(2′-chloropropionamido)benzoylurea (12a). To a stirred solution of the 2-methyl-3-aminobenzoylurea (386 mg, 2.0 mmol) in 5 mL of DMA, 2-chloropropionyl chloride (1.0 mL, 10.2 mmol) was added dropwise. The reaction mixture was stirred at

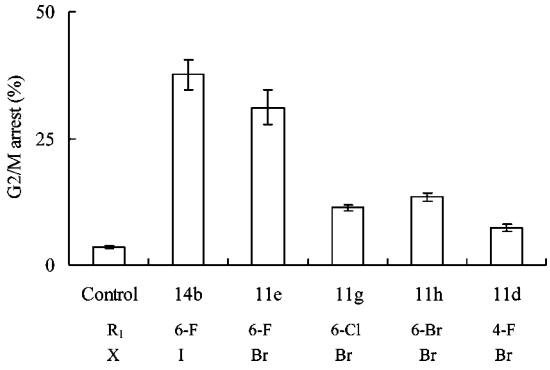


Figure 8. Relationship between chemical structure and anti-tubulin activity of the study compounds. CEM cells were treated with the compounds at their IC_{50} for 12 h. The cells were then collected for cell cycle analysis as described in Biological Methods.

room temperature for 2 h and poured into ice−water and extracted with ethyl acetate. The organic layer was washed with saturated brine, dried over Na_2SO_4, and evaporated. The crude product was purified by flash chromatography over silica gel, affording the title compound (256 mg, 60%) as a white solid, mp 244−246 °C. MS *m/z* 283 (M^+). ^{1}H NMR (CD_3OD): δ 1.68 (d, J = 6.6 Hz, 3H), 2.23 (s, 3H), 4.63 (q, J = 6.6 Hz, 1H), 7.23−7.30 (m, 2H), 7.37−7.40 (m, 1H). IR (KBr): γ 3383, 3311, 3242 (NH), 1712, 1664 (C=O) cm^{-1}. Anal. ($C_{12}H_{14}ClN_3O_3$) C, H, N.

(*R*)-(+)-3-(2′-Bromopropionamido)benzoylurea (10a). The title compound was obtained from 3-aminobenzoylurea and (*R*)-(+)-2-bromopropionyl chloride with a procedure similar to that for compound **12a**. Yield: 62%. White solid, mp 221−223 °C. $[\alpha]_D$ +20.87 (*c* 0.2, DMF); MS *m/z* 314 (M + H). ^{1}H NMR (DMSO-d_6): δ 1.75 (d, J = 6.8 Hz, 3H), 4.69 (q, J = 6.8 Hz, 1H), 7.44 (t, 1H), 7.67 (d, J = 7.6 Hz, 1H), 7.80 (d, J = 8.4 Hz, 1H), 7.38, 7.98 (s, s, 2H), 8.13 (s, 1H), 10.50 (s, 1H), 10.54 (s, 1H). IR (KBr): γ 3383, 3360, 3280 (NH), 1720, 1666 (C=O) cm^{-1}. Anal. ($C_{11}H_{12}BrN_3O_3$) C, H, N.

(*S*)-(−)3-(2′-Bromopropionamido)benzoylurea (10b). The title compound was obtained from 3-aminobenzoylurea and (*S*)-(−)-2-bromopropionyl chloride, using a procedure similar to that for compound **12a**. Yield: 62%. White solid, mp 221−223 °C. $[\alpha]_D$ −22.46 (*c* 0.2, DMF). MS *m/z* 314 (M + H). ^{1}H NMR (DMSO-d_6): δ 1.75 (d, J = 6.8 Hz, 3H), 4.69 (q, J = 6.8 Hz, 1H), 7.44 (m, 1H), 7.67 (d, J = 7.6 Hz, 1H), 7.80 (d, J = 8.4 Hz, 1H), 7.38, 7.98 (s, s, 2H), 8.13 (s, 1H), 10.50 (s, 1H), 10.54 (s, 1H). IR (KBr): γ 3384, 3370, 3287 (NH), 1712, 1674 (C=O) cm^{-1}. Anal. ($C_{11}H_{12}BrN_3O_3$) C, H, N.

2-Methyl-3-(2′-bromopropionamido)benzoylurea (10c). The title compound was obtained from 2-methyl-3-aminobenzoylurea and 2-bromopropionyl bromide using a procedure similar to that for compound **12a**. Yield: 60%. White solid, mp 240−242 °C. MS *m/z* 327 (M^+). ^{1}H NMR (CD_3OD): δ 1.80 (d, J = 6.6 Hz, 3H), 2.24 (s, 3H), 4.67 (q, J = 6.6 Hz, 1H), 7.24−7.31 (m, 2H), 7.38−7.41 (m, 1H). IR (KBr): γ 3383, 3311, 3242 (NH), 1712, 1664, 1626 (C=O) cm^{-1}. Anal. ($C_{12}H_{14}BrN_3O_3$) C, H, N.

4-Methyl-3-(2′-bromopropionamido)benzoylurea (10d). The title compound was obtained from 4-methyl-3-aminobenzoylurea and 2-bromopropionyl bromide using a procedure similar to that of compound **12a**. Yield: 56%. White solid, mp 217−218 °C. MS *m/z* 327 (M^+). ^{1}H NMR (DMSO-d_6): δ 1.75 (d, J = 6.8 Hz, 3H), 2.18 (s, 3H, CH_3), 4.80 (q, J = 6.8 Hz, 1H,), 7.23−7.28 (m, 2H), 7.40−7.43 (m, 1H), 7.37, 7.86 (s, s, 2H), 9.82 (s, 1H), 10.57 (s, 1H). IR (KBr): γ 3386, 3278 (NH), 1766, 1687 (C=O) cm^{-1}. Anal. ($C_{12}H_{14}BrN_3O_3$) C, H, N.

4-Chloro-3-(2′-bromopropionamido)benzoylurea (10h). The title compound was obtained from 4-chloro-3-aminobenzoylurea and 2-bromopropionyl bromide using a procedure similar to that of compound **12a**. Yield: 47%. White solid, mp 228−230 °C. MS *m/z* 348 (M + 1). ^{1}H NMR (CD_3OD): δ 1.81 (d, J = 6.6 Hz, 3H), 4.78 (q, J = 6.6 Hz, 1H), 7.56 (d, J = 8.4 Hz, 1H), 7.64 (dd, J = 2.4, 8.4 Hz, 1H), 8.22 (d, J = 2.4 Hz, 1H). IR (KBr): γ 3390, 3310, 3232 (NH), 1716, 1680, 1664 (C=O) cm^{-1}. Anal. ($C_{11}H_{11}BrClN_3O_3$) C, H, N.

4-Methoxy-3-(2′-bromopropionamido)benzoylurea (10k). The title compound was obtained from 4-methoxy-3-aminobenzoylurea and 2-bromopropionyl bromide using a procedure similar to that of compound **12a**. Yield: 54%. White solid, mp 222−224 °C. MS *m/z* 344 (M + 1). ^{1}H NMR (CD_3OD): δ 1.79 (d, J = 6.0 Hz, 3H), 3.93 (s, 3H), 4.79 (q, J = 6.0 Hz, 1H), 7.10 (d, J = 8.7 Hz, 1H), 7.71 (dd, J = 1.8, 8.7 Hz, 1H), 8.48 (d, J = 1.8 Hz, 1H). IR (KBr): γ 3377, 3215 (NH), 1709, 1674 (C=O) cm^{-1}. Anal. ($C_{12}H_{14}BrN_3O_4$) C, H, N.

5-Nitro-3-(2′-bromopropionamido)benzoylurea (10m). The title compound was obtained from 5-nitro-3-aminobenzoylurea and 2-bromopropionyl bromide using a procedure similar to that of compound **12a**. Yield: 43%. White solid, mp 190−193 °C. MS *m/z* 359 (M + 1). ^{1}H NMR (DMSO-d_6): δ 1.78 (d, J = 6.8 Hz, 3H), 4.70 (q, J = 6.8 Hz, 1H), 7.48, 7.89 (s, s, 2H), 8.44 (s, 1H), 8.53 (s, 1H), 8.77 (s, 1H), 10.97 (s, 1H), 10.99 (s, 1H). IR (KBr): γ 3471, 3354, 3327 (NH), 1768, 1707, 1684 (C=O) cm^{-1}. Anal. ($C_{11}H_{11}BrN_4O_5$) C, H, N.

2-Methyl-3-(bromoacetamido)benzoylurea (11a). The title compound was obtained from 2-methyl-3-aminobenzoylurea and bromoacetyl bromide using a procedure similar to that for compound **12a.** Yield: 52%. White solid, mp 242−244 °C. MS *m/z* 313 (M^+). ^{1}H NMR (CD_3OD): δ 2.25 (s, 3H), 4.00 (s, 2H), 7.26−7.30 (m, 2H), 7.40−7.44 (m, 1H). IR (KBr): γ 3411, 3309 (NH), 1697, 1682, 1662 (C=O) cm^{-1}. Anal. ($C_{11}H_{12}BrN_3O_3$) C, H, N.

4-Methyl-3-(bromoacetamido)benzoylurea (11b). The title compound was obtained from 4-methyl-3-aminobenzoylurea and bromoacetyl bromide using a procedure similar to that for compound **12a**. Yield: 54%. White solid, mp 220−222 °C. MS *m/z* 313 (M^+). ^{1}H NMR (CD_3OD): δ 2.29 (s, 3H), 4.01 (s, 2H), 7.35 (d, J = 7.5 Hz, 1H), 7.66 (dd, J = 1.5, 7.5 Hz, 1H), 7.85 (d, J = 1.5 Hz, 1H). IR (KBr): γ 3367, 3263 (NH), 1732, 1680, 1664 (C=O) cm^{-1}. Anal. ($C_{11}H_{12}BrN_3O_3$) C, H, N.

4-Chloro-3-(bromoacetamido)benzoylurea (11f). The title compound was obtained from 4-chloro-3-aminobenzoylurea and bromoacetyl bromide using a procedure similar to that for compound **12a**. Yield: 59%. White solid, mp 230−232 °C. MS *m/z* 333.5 (M^+). ^{1}H NMR (CD_3OD): δ 4.07 (s, 2H), 7.57 (d, J = 8.4 Hz, 1H), 7.66−7.70 (m, 1H), 8.27 (d, J = 2.1 Hz, 1H). IR (KBr): γ 3369, 3346, 3319 (NH), 1705, 1676 (C=O) cm $^{-1}$. Anal. ($C_{10}H_9BrClN_3O_3$) C, H, N.

4-Methoxy-3-(bromoacetamido)benzoylurea (11i). The title compound was obtained from 4-methoxy-3-aminobenzoylurea and bromoacetyl bromide using a procedure similar to that for compound **12a**. Yield: 46%. White solid, mp 224−226 °C. MS *m/z* 330 (M + 1). ^{1}H NMR (CD_3OD): δ 3.94 (s, 3H), 4.07 (s, 2H), 7.10 (d, J = 8.4 Hz, 1H), 7.70 (dd, J = 2.1, 8.4 Hz, 1H), 8.50 (d, J = 2.1 Hz, 1H). IR (KBr): γ 3383, 3228 (NH), 1745, 1684, 1670 (C=O) cm $^{-1}$. Anal. ($C_{11}H_{12}BrN_3O_4$) C, H, N.

5-Nitro-3-(bromoacetamido)benzoylurea (11k). The title compound was obtained from 5-nitro-3-aminobenzoylurea and bromoacetyl bromide using a procedure similar to that for compound **12a**. Yield: 42%. White solid, mp 178−182 °C. MS *m/z* 345 (M + 1). ^{1}H NMR (DMSO-d_6): δ 4.10 (s, 2H), 7.48, 7.89 (s, s, 2H), 8.41 (s, 1H), 8.53 (s, 1H), 8.75 (s, 1H), 10.97 (s, 1H), 11.04 (s, 1H). IR (KBr): γ 3491, 3352, 3325 (NH), 1716, 1670, 1624 (C=O) cm $^{-1}$. Anal. ($C_{10}H_9BrN_4O_5$) C, H, N.

4-Methyl-3-(2′-chloropropionamido)benzoylurea (12b). The title compound was obtained from 4-methyl-3-aminobenzoylurea and 2-chloropropionyl chloride using a procedure similar to that for compound **12a**. Yield: 52%. White solid, mp 232−234 °C. MS *m/z* 284 (M + 1). ^{1}H NMR (CD_3OD): δ 1.68 (d, J = 6.6 Hz, 3H), 2.23 (s, 3H), 4.63 (q, J = 6.6 Hz, 1H), 7.26 (d, J = 7.8 Hz, 2H), 7.38 (dd, J = 2.1, 7.8 Hz, 1H), 7.83 (d, J = 2.1 Hz, 1H). IR (KBr): γ 3458, 3278, 2983 (NH), 1705, 1670, 1665 (C=O) cm^{-1}. Anal. ($C_{12}H_{14}ClN_3O_3$) C, H, N.

4-Chloro-3-(2′-chloropropionamido)benzoylurea (12c). The title compound was obtained from 4-chloro-3-aminobenzoylurea and 2-chloropropionyl chloride using a procedure similar to that for compound **12a**. Yield: 52%. White solid, mp 230−231 °C. MS *m*/*z* 304 (M + 1). ^{1}H NMR (CD_3OD): δ 1.69 (d, J = 6.6 Hz, 3H), 4.73 (q, J = 6.6 Hz, 1H), 7.58 (d, J = 8.4 Hz, 1H), 7.70 (dd, J = 2.1, 8.4 Hz, 1H), 8.26 (d, J = 2.1 Hz, 1H). IR (KBr): γ 3375, 3350, 3236 (NH), 1710, 1690, 1666 (C═O) cm $^{-1}$. Anal. ($C_{11}H_{11}Cl_2N_3O_3$) C, H, N.

4-Methoxy-3-(2′-chloropropionamido)benzoylurea (12d). The title compound was obtained from 4-methoxy-3-aminobenzoylurea and 2-chloropropionyl chloride using a procedure similar to that for compound **12a**. Yield: 54%. White solid, mp 226−227 °C. MS *m*/*z* 300 (M + 1). ^{1}H NMR (CD_3OD): δ 1.66 (d, J = 6.6 Hz, 3H), 3.95 (s, 3H), 4.75 (q, J = 6.6 Hz, 1H), 7.11 (d, J = 8.7 Hz, 1H), 7.72 (dd, J = 2.4, 8.7 Hz, 1H), 8.50 (d, J = 2.4 Hz, 1H). IR (KBr): γ 3377, 3215 (NH), 1709, 1674 (C═O) cm $^{-1}$. Anal. ($C_{12}H_{14}ClN_3O_4$) C, H, N.

2-Methyl-3-(chloroacetamido)benzoylurea (13a). The title compound was obtained from 2-methyl-5-aminobenzoylurea and chloroacetyl chloride using a procedure similar to that for compound **12a**. Yield: 55%. Mp 246−248. MS *m*/*z* 269.5 (M^+). ^{1}H NMR (CD_3OD): δ 2.26 (s, 3H), 4.21 (s, 2H), 7.26−7.31 (m, 2H), 7.42−7.45 (m, 1H). IR (KBr): γ 3386, 3303, 3250 (NH), 1700, 1675, 1670 (C═O) cm $^{-1}$. Anal. ($C_{11}H_{12}ClN_3O_3$) C, H, N.

4-Methyl-3-(chloroacetamido)benzoylurea (13b). The title compound was obtained from 4-methyl-3-aminobenzoylurea and chloroacetyl chloride using a procedure similar to that for compound **12a**. Yield: 55%. White solid, mp 222−223 °C. MS *m*/*z* 270 (M + 1). ^{1}H NMR (CD_3OD): δ 2.29 (s, 3H), 4.30 (s, 2H), 7.34 (d, J = 8.1 Hz, 1H), 7.66 (dd, J = 1.8, 8.1 Hz, 1H), 7.87 (d, J = 1.8 Hz, 1H). IR (KBr): γ 3361, 3317 (NH), 1730, 1705, 1676 (C═O) cm $^{-1}$. Anal. ($C_{11}H_{12}ClN_3O_3$) C, H, N.

4-Chloro-3-(chloroacetamido)benzoylurea (13e). The title compound was obtained from 4-chloro-3-aminobenzoylurea and chloroacetyl chloride using a procedure similar to that for compound **12a**. Yield: 55%. White solid, mp 232−234 °C. MS *m*/*z* 290 (M + 1). ^{1}H NMR (CD_3OD): δ 4.28 (s, 2H), 7.58 (m, 1H), 7.66−7.77 (m, 1H), 8.32 (d, J = 1.8 Hz, 1H). IR (KBr): γ 3367, 3317, 3207 (NH), 1701, 1676 (C═O) cm $^{-1}$. Anal. ($C_{10}H_9Cl_2N_3O_3$) C, H, N.

4-Methoxy-3-(chloroacetamido)benzoylurea (13g). The title compound was obtained from 4-methoxy-3-aminobenzoylurea and chloroacetyl chloride using a procedure similar to that for compound **12a**. Yield: 50%. White solid, mp 230−231 °C. MS *m*/*z* 285 (M^+). ^{1}H NMR (CD_3OD): δ 3.81 (s, 3H), 4.29 (s, 2H), 7.35 (d, J = 8.1 Hz, 1H), 7.65−7.68 (m, 1H), 7.81 (d, J = 1.8 Hz, 1H). IR (KBr): γ 3458, 3388, 3271 (NH), 1705, 1670, 1650 (C═O) cm $^{-1}$. Anal. ($C_{11}H_{12}ClN_3O_4$) C, H, N.

Method B (for 10e−g, 10i, 10j, 10l, 11c−e, 11g, 11h, 11j, 11l, 11m, 13c, 13d, 13f, 13h, 14a−c, 18a−c, 19a, 19b). A solution of relevant *m*-nitrobenzoic acid (**8**, **15**, 0.01 mol, commercially available) in $SOCl_2$ (10 mL) was heated with reflux for 1.5 h. The reaction mixture was concentrated under vacuum to give an oil. To a stirred solution of the oil in toluene (50 mL) was added urea (0.05 mol). The reaction mixture was stirred at 90−100 °C for 2 h and concentrated under vacuum. The residue was stirred in water (60 mL) at room temperature for 0.5 h. The reaction mixture was filtered, and the solid was washed in water and dried to give the nitro compound (**9**, **16**),which was used for the next step without purification. The nitro compound (2.2 mmol) was dissolved in a mixture of 95% ethanol (20 mL) and 4 N HCl (0.5 mL), and to the solution was added iron powder (6.6 mmol) portionwise over 20 min at 70−80 °C. After continued stirring at the same temperature for 4 h, the reaction mixture was filtered immediately, and the filtrate was concentrated under reduced pressure, soaked in ether, filtered, and dried to give solid **7**, **17**, which was used for the next step without purification.

By use of a procedure similar to method A, the title compound was obtained from **7** or **17** after its reaction respectively with chloroacetyl chloride, bromoacetyl bromide, or 2-bromopropionyl bromide in DMA.

6-Methyl-3-(2′-bromopropionamido)benzoylurea (10e). Yield: 38%. White solid, mp 210−212 °C. MS *m*/*z* 328 (M + 1). ^{1}H NMR (DMSO-d_6): δ 1.74 (d, J = 6.8 Hz, 3H), 2.28 (s, 3H), 4.68 (q, J = 6.8 Hz, 1H), 7.22 (d, J = 8.4 Hz, 1H), 7.36, 7.84 (s, s, 2H), 7.57 (dd, J = 2.0, 8.4 Hz, 1H), 7.68 (d, J = 2.0 Hz, 1H), 10.58 (s, 1H), 10.69 (s, 1H). IR (KBr): γ 3375, 3325, 3219 (NH), 1703, 1682, 1666 (C═O) cm $^{-1}$. Anal. ($C_{12}H_{14}BrN_3O_3$) C, H, N.

4-Fluoro-3-(2′-bromopropionamido)benzoylurea (10f). Yield: 42%. White solid, mp 226−228 °C. MS *m*/*z* 331 (M^+). ^{1}H NMR (DMSO-d_6): δ 1.75 (d, J = 6.4 Hz, 3H), 4.90 (q, J = 6.4 Hz, 1H), 7.40−7.42 (m, 1H), 7.38, 7.98 (s, s, 2H), 7.82 (m, 1H), 8.49 (m, 1H), 10.28 (s, 1H), 10.61 (s, 1H). IR (KBr): γ 3392, 3359, 3282 (NH), 1718, 1689, 1664 (C═O) cm $^{-1}$. Anal. ($C_{11}H_{11}BrFN_3O_3$) C, H, N.

6-Fluoro-3-(2′-bromopropionamido)benzoylurea (10g). Yield: 43%. White solid, mp 167−168 °C. MS *m*/*z* 332 (M + 1). ^{1}H NMR (DMSO-d_6): δ 1.74 (d, J = 6.4 Hz, 3H), 4.67 (q, J = 6.4 Hz, 1H), 7.30 (t, 1H), 7.44 (s, 1H), 7.71−7.75 (m, 2H), 7.88 (dd, J = 2.4, 2.8 Hz, 1H), 10.50 (s, 1H), 10.52 (s, 1H). IR (KBr): γ 3375, 3228, 3084 (NH), 1697, 1674, 1628 (C═O) cm $^{-1}$. Anal. ($C_{11}H_{11}BrFN_3O_3$) C, H, N.

6-Chloro-3-(2′-bromopropionamido)benzoylurea (10i). Yield: 42%. White solid, mp 202−204 °C. MS *m*/*z* 348 (M + 1). ^{1}H NMR (DMSO-d_6): δ 1.75 (d, J = 6.8 Hz, 3H), 4.68 (q, J = 6.8 Hz, 1H), 7.44, 7.70 (s, s, 2H), 7.47 (d, J= 2.4 Hz, 1H), 7.66 (dd, J = 2.4, 8.8 Hz, 1H), 7.78 (d, J = 8.8 Hz, 1H), 10.59 (s, 1H), 10.70 (s, 1H). IR (KBr): γ 3373, 3309, 3230, 3134 (NH), 1709, 1685 (C═O) cm $^{-1}$. Anal. ($C_{11}H_{11}BrClN_3O_3$) C, H, N.

6-Bromo-3-(2′-bromopropionamido)benzoylurea (10j). Yield: 36%. White solid, mp 185.5−187 °C. MS *m*/*z* 414 (M + Na). ^{1}H NMR (DMSO-d_6): δ 1.74 (d, J = 6.8 Hz, 3H), 4.67 (q, J = 6.8 Hz, 1H), 7.43, 7.69 (s, s, 2H), 7.57−7.62 (m, 2H), 7.74 (d, J= 2.4 Hz, 1H), 10.58 (s, 1H), 10.69 (s, 1H). IR (KBr): γ 3375, 3309 (NH), 1709, 1687 (C═O) cm $^{-1}$. Anal. ($C_{11}H_{11}Br_2N_3O_3$) C, H, N.

5-Methyl Formate 3-(2′-Bromopropionamido)benzoylurea (10l). Yield: 52%. White solid, mp 193−195 °C. MS *m*/*z* 372 (M + 1). ^{1}H NMR (DMSO-d_6): δ 1.76 (d, J = 6.8 Hz, 3H), 3.88 (s, 3H), 4.68 (q, J = 6.8 Hz, 1H), 7.41, 7.91 (s, s, 2H), 8.21 (s, 1H), 8.33 (s, 1H), 8.44 (s, 1H), 10.73 (s, 1H), 10.82 (s, 1H). IR (KBr): γ 3464, 3259 (NH), 1768, 1703 (C═O) cm $^{-1}$. Anal. ($C_{13}H_{14}BrN_3O_5$) C, H, N.

6-Methyl-3-(bromoacetamido)benzoylurea (11c). Yield: 41%. White solid, mp 226.5−227.5 °C. MS *m*/*z* 314 (M + 1). ^{1}H NMR (DMSO-d_6): δ 2.29 (s, 3H), 4.02 (s, 2H), 7.22 (d, J = 8.4 Hz, 1H), 7.36, 7.84 (s, s, 2H), 7.55 (dd, J = 2.4 Hz, 1H), 7.65 (d, J = 2.4 Hz, 1H), 10.45 (s, 1H), 10.51 (s, 1H). IR (KBr): γ 3379, 3303, 3222 (NH), 1703, 1685, 1662 (C═O) cm $^{-1}$. Anal. ($C_{11}H_{12}BrN_3O_3$) C, H, N.

4-Fluoro-3-(bromoacetamido)benzoylurea (11d). Yield: 47%. White solid, mp 256−258 °C. MS *m*/*z* 317 (M^+). ^{1}H NMR (DMSO-d_6): δ 4.16 (s, 2H), 7.38−7.43 (m, 2H), 7.43, 7.80 (s, s, 2H), 8.51 (d, J = 6.0 Hz, 1H), 10.35 (s, 1H), 10.62 (s, 1H). IR (KBr): γ 3363, 3315, 3205, 3032 (NH), 1705, 1682 (C═O) cm $^{-1}$. Anal. ($C_{10}H_9BrFN_3O_3$) C, H, N.

6-Fluoro-3-(bromoacetamido)benzoylurea (11e). Yield: 38%. White solid. mp 214−215 °C. MS *m*/*z* 318 (M + 1). ^{1}HNMR (DMSO-d_6): δ 4.03 (s, 2H), 7.29 (t, 1H), 7.44 (s, 1H), 7.68−7.72 (m, 2H), 7.85 (dd, J = 2.8 Hz, 1H), 10.50 (s, 1H), 10.58 (s, 1H). IR (KBr): γ 3352, 3290, 3222 (NH), 1711, 1685, 1672 (C═O) cm $^{-1}$. Anal. ($C_{10}H_9BrFN_3O_3$) C, H, N.

6-Chloro-3-(bromoacetamido)benzoylurea (11g). Yield: 46%. White solid, mp 224−226 °C. MS *m*/*z* 334 (M + 1). ^{1}H NMR (DMSO-d_6): δ 4.04 (s, 2H), 7.46−7.49 (m, 2H), 7.62−7.65 (m, 1H), 7.70 (s, 1H), 7.74 (d, J =3.2 Hz, 1H), 10.66 (s, 1H), 10.72 (s, 1H). IR (KBr): γ 3383, 3317, 3251, 3134 (NH), 1711, 1678 (C═O) cm $^{-1}$. Anal. ($C_{10}H_9BrClN_3O_3$) C, H, N.

6-Bromo-3-(bromoacetamido)benzoylurea (11h). Yield: 46%. Off-white solid, mp 213.5−215.5 °C. MS *m*/*z* 400 (M + Na). ^{1}H NMR (DMSO-d_6): δ 4.04 (s, 2H), 7.43, 7.63 (s, s, 2H), 7.56 (dd, J = 2.4, 2.8 Hz, 1H), 7.61 (s, 1H), 7.72 (d, J = 2.4 Hz, 1H), 10.64 (s, 1H), 10.69 (s, 1H). IR (KBr): γ 3379, 3296 (NH), 1702, 1689, 1678 (C═O) cm $^{-1}$. Anal. ($C_{10}H_9Br_2N_3O_3$) C, H, N.

5-Methyl Formate 3-(Bromoacetamido)benzoylurea (11j). Yield: 48%. White solid, mp 136−139 °C. MS *m/z* 358 (M + 1). ^{1}H NMR (DMSO-d_6): δ 3.89 (s, 3H), 4.07 (s, 2H), 7.42, 7.92 (s,s, 2H), 8.22 (s, 1H), 8.32 (s, 1H), 8.42 (s, 1H), 10.79 (s, 1H), 10.82 (s, 1H). IR (KBr): γ 3429, 3329 (NH), 1718, 1685 (C═O) cm $^{-1}$. Anal. ($C_{12}H_{12}BrN_3O_5$) C, H, N.

6-Methoxy-3-(bromoacetamido)benzoylurea (11l). Yield: 36%. White solid, mp 203−205 °C. MS *m/z* 330 (M + 1). ^{1}H NMR (DMSO-d_6): δ 3.86 (s, 3H), 4.01 (s, 2H), 7.19 (d, J = 8.8 Hz, 1H), 7.43, 7.88 (s, s, 2H), 7.76 (d, J = 8.8 Hz, 1H), 7.97 (s, 1H), 9.96 (s, 1H), 10.43 (s, 1H). IR (KBr): γ 3454, 3329, 3307 (NH), 1707, 1680 (C═O) cm $^{-1}$. Anal. ($C_{11}H_{12}BrN_3O_4$) C, H, N.

4-Hydroxy-3-(bromoacetamido)benzoylurea (11m). Yield: 51%. Pale-yellow solid, mp 212−214 °C. MS *m/z* 316 (M + 1)$^+$. ^{1}H NMR (DMSO-d_6): δ 4.20 (s, 2H), 6.90−6.92 (m, 1H), 7.28, 8.06 (s, s, 2H), 7.66−7.68 (m, 1H), 8.51 (m, 1H), 9.66 (s, 1H), 10.29 (s, 1H), 10.86 (s, 1H). IR (KBr): γ 3373, 3329, 3220 (NH), 1709, 1670 (C═O) cm $^{-1}$. Anal. ($C_{10}H_{10}BrN_3O_4$) C, H, N.

4-Fluoro-3-(chloroacetamido)benzoylurea (13c). Yield: 48%. White solid, mp 248−250 °C. MS *m/z* 273 (M$^+$). ^{1}H NMR (DMSO-d_6): δ 4.37 (s, 2H), 7.39−7.44 (m, 2H), 8.0 (s, 1H), 7.80−7.84 (m, 1H), 8.48−8.50 (m, 1H), 10.27 (s, 1H), 10.62 (s, 1H). IR (KBr): γ 3361, 3311, 3207, 3150 (NH), 1703, 1684 (C═O) cm $^{-1}$. Anal. ($C_{10}H_9ClFN_3O_3$) C, H, N.

6-Fluoro-3-(chloroacetamido)benzoylurea (13d). Yield: 56%. White solid, mp 228.1−229 °C. MS *m/z* 274 (M + 1). ^{1}H NMR (DMSO-d_6): δ 4.25 (s, 2H), 7.30 (t, 1H), 7.44 (s, 1H), 7.70−7.74 (m, 2H), 7.84−7.86 (m, 1H), 10.49 (s, 2H). IR (KBr): γ 3365, 3327, 3222 (NH), 1711, 1685, 1672 (C═O) cm $^{-1}$. Anal. ($C_{10}H_9ClFN_3O_3$) C, H, N.

6-Chloro-3-(chloroacetamido)benzoylurea (13f). Yield: 47%. White solid, mp 234−236 °C. MS *m/z* 290 (M + 1). ^{1}H NMR (DMSO-d$_6$): δ 4.27 (s, 2H), 7.46, 7.70 (s, s, 2H), 7.48 (d, J = 8.7 Hz, 1H), 7.66 (dd, J = 1.8, 8.7 Hz, 1H), 7.74 (d, J = 1.8 Hz, 1H), 10.58 (s, 1H), 10.72 (s, 1H). IR (KBr): γ 3386, 3311, 3255, 3136 (NH), 1711, 1678 (C═O) cm $^{-1}$. Anal. ($C_{10}H_9Cl_2N_3O_3$) C, H, N.

4-Hydroxy-3-(chloroacetamido)benzoylurea (13h). Yield: 54%. Pale-yellow solid, mp 220−223 °C. MS *m/z* 272 (M + H)$^+$. ^{1}H NMR (DMSO-d_6): δ 4.38 (s, 2H), 6.89−6.91 (m, 1H), 7.28, 8.00 (s, s, 2H$_2$), 7.62−7.64 (m, 1H), 8.52 (m, 1H), 9.56 (s, 1H), 10.34 (s, 1H), 10.90 (s, 1H). IR (KBr): γ 3375, 3334, 3215 (NH), 1711, 1668 (C═O) cm $^{-1}$. Anal. ($C_{10}H_{10}ClN_3O_4$) C, H, N.

6-Fluoro-3-(iodoacetamido)benzoylurea (14b). A mixture of **13d** (300 mg, 1.1 mmol) and sodium iodine in 5 mL of DMA was stirred at room temperature for 2 h. The reaction mixture was slowly poured into the ice−water and extracted with ethyl acetate. The organic layer was washed with saturated brine, dried over Na_2SO_4, and evaporated. The crude product was purified by flash chromatography over silica gel, affording the title compound as a white solid. Yield: 45%. Mp 207−210 °C. MS *m/z* 388 (M + Na). ^{1}H NMR (DMSO-d_6): δ 3.81, 4.25 (s, s 2H), 7.25−7.32 (m, 1H), 7.43 (s, 1H), 7.65−7.74 (m, 2H), 7.82−7.86 (m, 1H), 10.49 (s, 1H), 10.52 (s, 1H). IR (KBr): γ 3437, 3315, 3267 (NH), 1711, 1684, 1676 (C═O) cm $^{-1}$. Anal. ($C_{10}H_9FIN_3O_3$) C, H, N.

6-Methyl-3-(iodoacetamido)benzoylurea (14a). The title compound was obtained from the corresponding chloro intermediate and NaI using a procedure similar to that for compound **14b**. Yield: 40%. White solid, mp 227.1−228.4 °C. MS *m/z* 362 (M + 1). ^{1}H NMR (DMSO-d_6): δ 2.27 (s, 3H), 3.30 (s, 2H), 7.20−7.23 (m, 1H), 7.36, 7.84 (s, s, 2H), 7.51−7.58 (m, 1H), 7.63−7.66 (m, 1H), 10.39 (s, 1H), 10.51 (s, 1H). IR (KBr): γ 3383, 3303, 3251 (NH), 1705, 1670 (C═O) cm $^{-1}$. Anal. ($C_{11}H_{12}IN_3O_3$) C, H, N.

5-Methyl Formate 3-(Iodoacetamido)benzoylurea (14c). The title compound was obtained from the corresponding chloro intermediate and NaI using a procedure similar to that for compound **14b**. Yield: 43%. White solid, mp 133−137 °C. MS *m/z* 406 (M + 1). ^{1}H NMR (DMSO-d_6): δ 3.88 (s, 3H), 4.29 (s, 1H), 7.41, 7.91 (s, s, 2H), 8.22 (s, 1H), 8.32 (s, 1H), 8.42 (s, 1H), 10.69 (s, 1H), 10.81 (s, 1H). IR (KBr): γ 3406, 3259 (NH), 1718, 1685 (C═O) cm $^{-1}$. Anal. ($C_{12}H_{12}IN_3O_5$) C, H, N.

5-(2′-Bromopropionamido)-2-furoylurea (18a). Yield: 43%. White solid, mp 184.3−185 °C. MS *m/z* 304 (M + 1). ^{1}H NMR (DMSO-d_6): δ 1.72 (d, J =6.8 Hz, 3H), 4.65 (q, J = 6.8 Hz, 1H), 6.41 (d, J = 3.6 Hz, 1H), 7.28, 7.80 (s, s, 2H), 7.73 (d, J = 3.6 Hz, 1H), 10.28 (s, 1H), 11.98 (s, 1H). IR (KBr): γ 3427, 3307, 3224 (NH), 1699, 1676 (C═O) cm $^{-1}$. Anal. ($C_9H_{10}BrN_3O_4\cdot 0.5H_2O$) C, H, N.

5-Bromoacetamido-2-furoylurea (18b). Yield: 43%. White solid, mp 148−149 °C. MS *m/z* 290 (M + 1). ^{1}H NMR (DMSO-d_6): δ 4.08 (s, 2H), 6.40 (d, J = 3.6 Hz, 1H), 7.29, 7.80 (s, s, 2H), 7.73 (d, J = 3.6 Hz, 1H), 10.28 (s, 1H), 12.00 (s, 1H). IR (KBr): γ 3321, 3248 (NH), 1715, 1670 (C═O) cm $^{-1}$. Anal. ($C_8H_8BrN_3O_4\cdot 0.5H_2O$) C, H, N.

5-Chloroacetamido-2-furoylurea (18c). Yield: 48%. White solid, mp 192.8−194 °C. MS *m/z* 246 (M + H)$^+$. ^{1}H NMR (DMSO-d_6): δ 4.31 (s, 2H), 6.40 (d, J = 3.6 Hz, 1H), 7.29, 7.80 (s, s, 2H), 7.73 (d, J = 3.6 Hz, 1H), 10.28 (s, 1H), 11.93 (s, 1H). IR (KBr): γ 3381, 3321, 3244 (NH), 1730, 1672 (C═O) cm $^{-1}$. Anal. ($C_8H_8ClN_3O_4\cdot H_2O$) C, H, N.

5-(2′-Bromopropionamido)thiophene-3-carbonylurea (19a). Yield: 51%. White solid, mp 216−220 °C. MS *m/z* 320 (M + 1). ^{1}H NMR (DMSO-d_6): δ 1.76 (d, J = 6.8 Hz, 3H), 4.70 (q, J = 6.8 Hz, 1H), 7.32, 7.97 (s, s, 2H), 7.14 (d, J = 1.2 Hz, 1H), 8.10 (s, 1H), 10.37 (s, 1H), 11.73 (s, 1H). IR (KBr): γ 3381, 3319, 3228 (NH), 1705, 1668 (C═O) cm $^{-1}$. Anal. ($C_9H_{10}BrSN_3O_3$) C, H, N.

5-(Bromoacetamido)thiophene-3-carbonylurea (19b). Yield: 48%. White solid, mp 227−230 °C. MS *m/z* 306 (M + 1). ^{1}H NMR (DMSO-d_6): δ 4.08 (s, 2H), 7.32, 7.97 (s, s, 2H), 7.13 (s, 1H), 8.09 (s, 1H), 10.37 (s, 1H), 11.77 (s, 1H). IR (KBr): γ 3491, 3367, 3213 (NH), 1709, 1684,1633 (C═O) cm $^{-1}$. Anal. ($C_8H_8BrSN_3O_3$) C, H, N.

Biological Methods. Tumor Cell Lines. The human tumor cell lines CEM (leukemia), MCF-7 (breast cancer), DU-145 (prostate cancer), PC-3 (prostate cancer), Lovo (colon cancer), Daudi (lymphoma), and MIA Paca (pancreatic cancer) were from the American Tissue Culture Collection (ATCC, Rockville, MD). DND-1 melanoma cells were from Dr. Ohnuma (Mount Sinai School of Medicine, New York, NY). Bel-7402 hepatoma cell originating from Chinese human hepatocarcinoma patients were provided by Cancer Institute, Chinese Academy of Medical Sciences. All cells were cultivated in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum, penicillin (150 μg/mL), and streptomycin (150 μg/mL) with 5% CO_2 at 37 °C. Cells in exponential growth were used for experiments.

Anticancer Activity in Vitro. Cells were distributed into 96-well plates (Falcon, Oxnard, CA) with 1×10^5 cells and a total volume of 250 μL per well, followed by treatment with study compounds at concentrations between 0.001 and 10 μM at 37 °C for 48 h. Cell viability was assessed by Trypan blue. The IC_{50} value was defined as a drug concentration killing 50% cells in comparison with untreated controls and calculated by nonlinear regression analysis. The IC_{50} values were determined in duplicate, and each experiment was repeated three times under identical conditions.

Morphology. Cell samples on slides were prepared in a Cytospin centrifuge (LTP-C, Tianjin,China) at 700*g* for 5 min. Slides were air-dried, fixed in methanol, and stained with Giemsa at room temperature for 15 min. Cells in the mitotic arrest were recognized by the disappearance of the nuclear membrane and the appearance of chromosomes throughout the cytoplasm. The percentage of mitotic cells was counted after 24 h of incubation using optical microscopy. Apoptotic cells were identified using common criteria, i.e., cell shrinkage, chromatin condensation, and fragmentation of the nucleus into discrete masses.

Bcl-2 Phosphorylation. Tumor cells were treated with different concentrations of **14b** for 12 h. Aliquots of cells were taken and lysed with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 1% SDS, 250 mM NaCl, 15 mM $MgCl_2$, 1 mM DTT, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 1 mM PMSF, 10 mg/mL leupeptin, and 10 mg/mL aprotinin. Equal amounts of lysate were subjected to electrophoresis using 10% SDS−polyacrylamide gels. The gels were blotted onto PVDF membranes (Millipore, CA) using a semidry electrophoretic transfer system (Bio-Rad, CA). After blocking with 5% nonfat milk in TBST buffer (20 mM Tris-HCl, 137 mM NaCl,

0.05% Tween-20) at 37 °C for 1 h, Bcl-2 protein was probed with anti-Bcl-2 monoclonal antibody (Calbiochem, CA), followed by goat antimouse HRP. The signals were detected by enhanced chemiluminescence (ECL) and exposure to X-film.

Cell Cycle Analysis. The cell cycle was analyzed by a method reported previously.[11] Briefly, suspensions of the CEM cells treated with **14b** were incubated at 37 °C for 48 h and were resuspended in 70% ethanol for at least 12 h at 4 °C. After being washed with PBS, the cells were treated with RNase A (50 μg/mL, Sigma, Mo) at 37 °C for 30 min and then exposed to propidium iodide at a final concentration of 50 μg/mL at 4 °C for 30 min. At least 10^4 cell events per sample were analyzed in a flow cytometer (Beckman Coulter EPICS XL, San Jose, CA) with EPICS software (version 2.0).

Microtubule Polymerization Assays. The HTS-tubulin polymerization assay kit (Cytoskeleton Inc., Denver, CO) was used to measure polymerization, following the process recommended by the vendor. G-PEM buffer (80 mM PIPES, pH 6.9, 1 mM EGTA, 5% glycerol, 1 mM GTP) was used as the diluent, and **14b** was added into the ice-cold 96-well plate. Each well contain G-PEM buffer, **14b** at different concentrations, and β-tubulin at a concentration of 1 mg/mL. The plate was shaken for 20 s and warmed to 37 °C, and the absorbance was read at 340 nm every 1.5 min for 30 min.

Anticancer Effect in Vivo. Balb/c nude mice (male, 16−20 g, 6−7 weeks old) were from the Experimental Animal Center, Chinese Academy of Medical Sciences, and used for the human hepatocarcinoma (Bel-7402) xenograft. Experimental mice received subcutaneous injection of Bel-7402 at 5×10^6 cells per mouse on their backs. Twelve days later, tumor-bearing nude mice were divided randomly into solvent control and treatment cages with 5 mice per group. **11e** or **14b** at 5 or 10 mg/kg were given ip on day 12 postimplantation. The regimens were continued with six injections at 2-day intervals (q2d). Therapeutic response was monitored by measuring tumor volume every week until the tumor volume of the controls reached a size greater than 2500 mm^3, the criterion for euthanatizing the animals.[21] Two perpendicular tumor measurements, width and length, were obtained with calipers, followed by calculation using the following formula: tumor volume = (length)(width)^2(0.52).[22]

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Supporting Information Available: Element analysis data of new 3-haloacylaminobenzoylurea compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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