Bioorganic & Medicinal Chemistry 17 (2009) 3873–3878

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Synthesis and activity evaluation of phenylurea derivatives as potent antitumor agents

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ARTICLE INFO

Article history:
Received 3 March 2009
Revised 11 April 2009
Accepted 14 April 2009
Available online 17 April 2009

Keywords:
Phenylurea
Anticancer
Structure–activity relationship
Tubulin
Mitotic arrest

ABSTRACT

We have discovered several tubulin-active compounds in our previous studies. In the establishment of a compound library of small molecule weight tubulin ligands, 14 new *N*-3-haloacylaminophenyl-*N*′-(alkyl/aryl) urea analogs were designed and synthesized. The structure–activity relationship (SAR) analysis revealed that (i) the order of anticancer potency for the 3-haloacylamino chain was following –CH_2Br > –$CHBrCH_3$; (ii) the *N*′-substituent moiety was not essential for the anticancer activity, and a proper alkyl substitution might enhance the anticancer activity. Among these analogs, the compounds **16j** bearing bromoacetyl at the *N*′-end exhibited a potent activity against eight human tumor cell lines, including CEM (leukemia), Daudi (lymphoma), MCF-7 (breast cancer), Bel-7402 (hepatoma), DU-145 (prostate cancer), DND-1A (melanoma), LOVO (colon cancer) and MIA Paca (pancreatic cancer), with the IC_{50} values between 0.38 and 4.07 μM. Interestingly, compound **16j** killed cancer cells with a mechanism independent of the tubulin-based mechanism, indicating a significant change of the action mode after the structure modification.

1. Introduction

3-Haloacylamino benzoylurea (3-HBU) analogs are a novel class of antimitotic agents described in our previous reports. The structure–activity relationship (SAR) was elucidated in CEM leukemic cells for their antiproliferative activity.[1,2] Among these analogs, JIMB01 (**1**), BAABu (**2**) and IAABu (**3**) are the representative compounds (Fig. 1) that inhibit tubulin polymerization, block cell cycle at the M-phase, cause apoptotic cell death through promoting bcl-2 phosphorylation and show promising therapeutic efficacy in nude mice bearing human tumors.[2–5] According to the previous studies, the haloacylamino chain at the 3-position of the aromatic ring is considered to be of significant importance in regulating the action, and its cytotoxicity in tumor cells was ranked in an order of –CH_2I > –CH_2Br > –$CHBrCH_3$ > –CH_2Cl.[1,2,6] Furthermore, introduction of suitable aryl at the N′-end of the formylurea chain at the 1-position could retain or improve the anticancer activity, but change the mechanism from mitotic arrest to that different from the parent compounds.[6]

N-Aryl-*N*′-2-(chloroethyl)urea (CEU) analogs are another new class of antimitotic agents.[7–10] Among the CEU analogs, *N*-(4-tert-butylphenyl)-*N*′-(2-chloroethyl)urea (**4**, Fig. 1) disrupts the microtubule assembly through alkylation of Cys239 residue of β-tubulin near the colchicine binding site, and shows potent anticancer activity.[7,8] SAR analysis showed that an exocyclic urea and a 2-chloroethyl moiety were required to ensure significant cytotoxicity. Especially, the *N*′-2-chloroethyl moiety is prerequisite for the alkylation of β-tubulin.[7,8]

In the establishment of a chemical library of small molecule tubulin ligands, we have designed a group of compounds based upon the principle of conjugating the two pharmacophores of the HBUs and CEUs (**5**, Fig. 1), with an anticipation that the obtained hybrids could possess powerful antimitotic activity. In this structure design, we retain the haloacylamino chain at the 3-position of the HBUs, and replace the formylurea chain with the 2-chloroethylurea chain of the CEUs. Therefore, a new class of *N*-3-haloacylaminophenyl-*N*′-2-chloroethyl urea analogs (**16a–c**) were designed and synthesized.

In addition, as the trimethoxyphenyl group (**6**, Fig. 2) is essential for the antiproliferative activity of colchicine which is a well-known antimitotic agent,[11] this active group was used to replace the 2-chloroethyl moiety at the N′-end, from which two analogs (**16m**, **16n**) were then created. In addition, a phenyl group was also employed as substituent at the same position (**16l**). Accordingly, three new diarylurea derivatives (**16l–n**) were generated.

Next, the structure–activity relationship (SAR) study for the anticancer effect of the substituents at the N′-end was carried

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doi:10.1016/j.bmc.2009.04.022

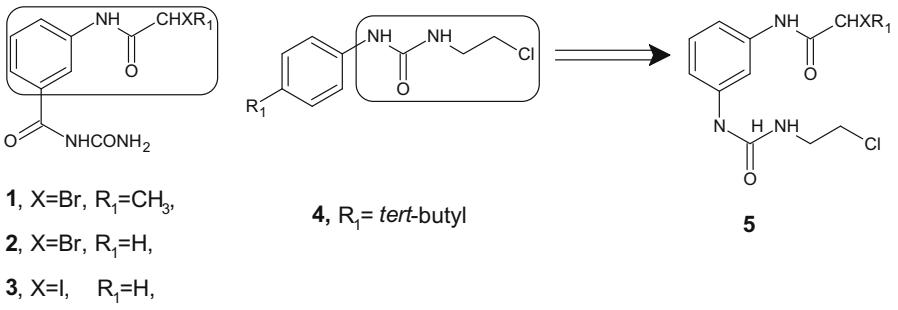


Figure 1. Chemical structures of compounds **1**–**5**.

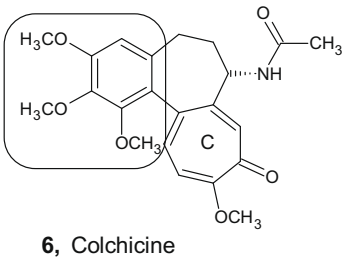


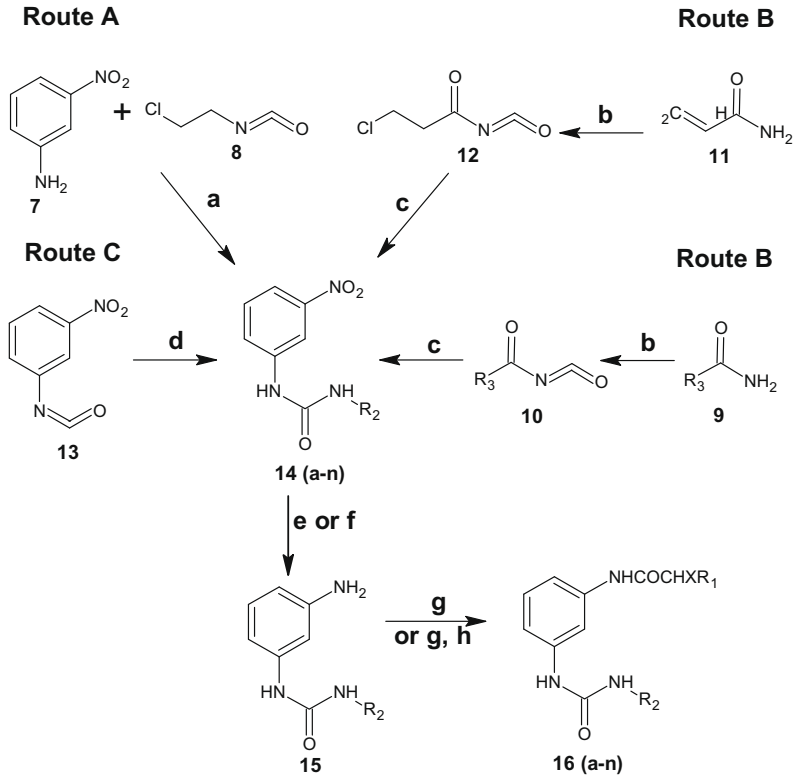
Figure 2. Chemical structure of chochicine.

out. Instead of the 2-chloroethyl moiety, different aliphatic acyl substituents such as formyl (**16h**, **16i**), acetyl (**16f**, **16g**), bromoacetyl (**16j**), bromopropionyl (**16k**) and 3-chloropropionyl (**16d**, **16e**) with sizes bigger or smaller than that of 2-chloroethyl were used, and accordingly eight *N*-3-haloacylamino phenyl-*N*′-acylurea analogs were synthesized.

2. Chemistry

The 14 new *N*′-alkyl/arylphenylurea analogs were synthesized as described in Scheme 1, that includes three synthetic methods (route A, B and C) according to the different starting materials. The preparation of nitro compound **14** is the key step for all of the methods. The route A for **16a**–**c** uses commercially available *m*-nitroaniline (**7**) and 2-chloroethyl isocyanate (**8**) as starting materials, with a standard preparation procedure of N,N-unsymmetrical ureas,[7] followed by a reduction and amidation reaction[1,2] for **16a–c**. In the reductive reaction, nitro compound **14** was reduced to aniline analogs **15** under conventional conditions using Pd/C-hydrogenation in a good yield.

The desired products **16d**–**i** (route B) were prepared according to the standard procedure reported previously.[1,6,12] The starting materials were the commercial available alkylamide (**9**, **11**), which reacted with oxalyl chloride in refluxing methylene chloride and converted into the acyl isocyanates (**10**, **12**). The phenylureas **16d**–**i** were obtained through condensation of **10** or **12** with



Scheme 1. Synthetic routes of phenylurea analogs **16a**–**n**. Reagents and conditions for the chemical synthesis: (a) DMF, 12 h; (b) $(COCl)_2$, CH_2ClCH_2Cl, reflux, 4 h; (c) *m*-nitroaniline, CH_3CN, 12 h; (d) NH_3/relevant aniline, DMF, 12 h; (e) Pd/C, EtOH, 4 h; (f) Fe/HCl, 95%EtOH, reflux, 4 h; (g) CH_3CHR_1COX, DMA, 2 h; (h) NaI, DMA, 2 h.

m-nitroaniline, then reduction with Fe/HCl as reducing agent and amidation using similar conditions to that reported previously.[6,7] In the course of condensation of **11** with oxalyl chloride, the hydrochloride produced in the reaction underwent 1,4-conjugate addition, and gave the product 3-chloropropionyl isocyanate (**12**).

The desired products **16j–n** were prepared as shown in route C with the commercial available *m*-nitrophenyl isocyanate (**13**) as starting material. Condensation of **13** with 3,4,5-trimethoxylaniline, aniline or NH_3 in DMF provided the nitro compounds **14**, which were converted into **16j–n** by reduction and amidation reactions as previously reported.[6,7]

3. Results and discussion

The human CEM leukemic cells were used for initial biological screening because of their rapid proliferation and high sensitivity to anticancer agents. The antiproliferative activity of the 14 compounds was closely associated with their structures, as shown in Table 1. Out of the 14 compounds, 6 compounds (**16a**, **16c**, **16h**, **16j**, **16l** and **16m**) exhibited potent activity with IC_{50} values of $\leqslant$1.0 μM. The anticancer potency of the 3-bromoacylamino chain followed the order of bromoacetyl > bromopropionyl, consistent with our previous observation on 3-HBUs.[1,2,6]

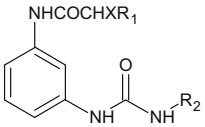
The compounds **16a** and **16c** bearing the active 2-chloroethyl at N′-end showed a potent activity with IC_{50} values of 0.77 and 0.10 μM, respectively. Replacing *N*′-2-chloroethyl with smaller substituents, we obtained compound **16h** bearing formyl and **16f** bearing acetyl at the N′-end; these two compounds showed an anticancer activity with IC_{50} values of 0.85 and 3.02 μM, respectively. Substitution with groups bigger than the size of chloroethyl afforded comparable activity. The compounds (**16d**, **16e**) bearing *N*′-3-chloropropionyl exhibited anticancer IC_{50} values of 1.90 and 2.05 μM, respectively. The compounds with bromoacetyl (**16j)** or bromopropionyl (**16k**) at the same position showed good activity with IC_{50} values of 0.56 μM for **16j** and 1.34 μM for **16k**. These data suggest that *N*′-2-chloroethyl was not essential for the anticancer activity, and proper *N*′-acyl such as *N*′-formyl (**16h**) and *N*′-bromoacetyl (**16j**) afforded promising activity against cancer.

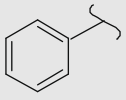
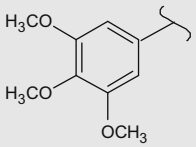
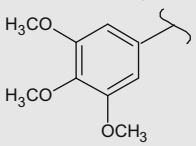
To confirm the observation, more modifications were made at this position. The compound (**16m**) possessing an active group of *N*′-trimethoxyphenyl provided good activity with IC_{50} value of 1.0 μM. Replacing trimethoxyphenyl with phenyl, compound **16l** showed an IC_{50} of 0.86 μM. Therefore, we concluded that the group at the N′-end is replaceable, and could be used to optimize the compounds.

As **16j** showed a potent activity in leukemia, the antiproliferative activity of this compound was further examined in eight solid or liquid human tumor cell lines (Table 2). It appears that **16j** was a potent killer for lymphoma (Daudi), breast cancer (MCF-7) and melanoma (DND-1) with IC_{50} values in the range of 0.38–0.59 μM. The least sensitive one was PC-3 prostate cancer cells with an IC_{50} > 100 μM. Human colon cancer (LOVO), prostate cancer (DU-145), pancreatic cancer (MIA paca) and hepatoma (Bel-7402) showed moderate degree of susceptibility to the compound. Figure 3 shows the killing effect on the cancer cells by **16j**. The kinetics of the cell death demonstrates a dose-dependent killing of the CEM leukemia cells in **16j** treatment.

After the activity evaluation we extend our work to the mechanism investigation. To learn whether or not these analogs would still block cell cycle progress at the M-phase as their parent compounds do, flow cytometric analysis was done with the individual compound **16c**, **16h** and **16j** (Fig. 4) using **1** as the reference. The cell population accumulated on the left hand side of the G1/G0 peak in the samples treated with compound **1**, **16c**, **16h** or **16j**

Table 1
Structures and anticancer activity of the phenylureas in the CEM cells



Compound	R_1	X	R_2	IC_{50}[a] (μM)
1[b]				1.47 ± 0.09
2[b]				0.725 ± 0.06
16a	H	Br	$-CH_2CH_2Cl$	0.77 ± 0.09
16b	CH_3	Br	$-CH_2CH_2Cl$	22.7 ± 5.15
16c	H	I	$-CH_2CH_2Cl$	0.10 ± 0.026
16d	H	Br	$-COCH_2CH_2Cl$	1.90 ± 0.14
16e	CH_3	Br	$-COCH_2CH_2Cl$	2.05 ± 0.06
16f	H	Br	$-COCH_3$	3.20 ± 0.68
16g	CH_3	Br	$-COCH_3$	10.56 ± 0.07
16h	H	Br	–CHO	0.85 ± 0.06
16i	CH_3	Br	–CHO	10.2 ± 0.13
16j	H	Br	$-COCH_2Br$	0.56 ± 0.06
16k	CH_3	Br	$-COCHBrCH_3$	1.34 ± 0.26
16l	H	Br		0.86 ± 0.29
16m	H	Br		1.00 ± 0.068
16n	CH_3	Br		3.99 ± 0.022

[a] IC_{50}: drug concentration required to inhibit 50% of cell proliferation after 72 h of treatment. Each experiment was repeated three times.
[b] See Figure 1.

Table 2
Antiproliferative activities of **16j** in human tumor cell lines

Cell line	Human tumor	IC_{50}[a] (μM)
Daudi	B-cell lymphoma	0.38 ± 0.05
MCF-7	Breast cancer	0.59 ± 0.06
Bel-7402	hepatoma	4.07 ± 0.20
DU-145	Prostate cancer	1.28 ± 0.08
PC-3	Prostate cancer	>100
DND-1A	Melanoma	0.51 ± 0.08
LOVO	Colon cancer	1.99 ± 0.18
MIA	Pancreas cancer	2.81 ± 0.36

[a] IC_{50}: drug concentration required to inhibit 50% of cell proliferation after 72 h of treatment. Each experiment was repeated three times.

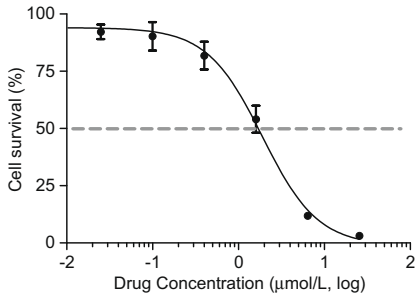


Figure 3. Dose-dependent killing of CEM cells by **16j**. CEM cells were treated with **16j** at different concentrations for 24 h and the cell death was measured using MTT assay as described in Section 5.

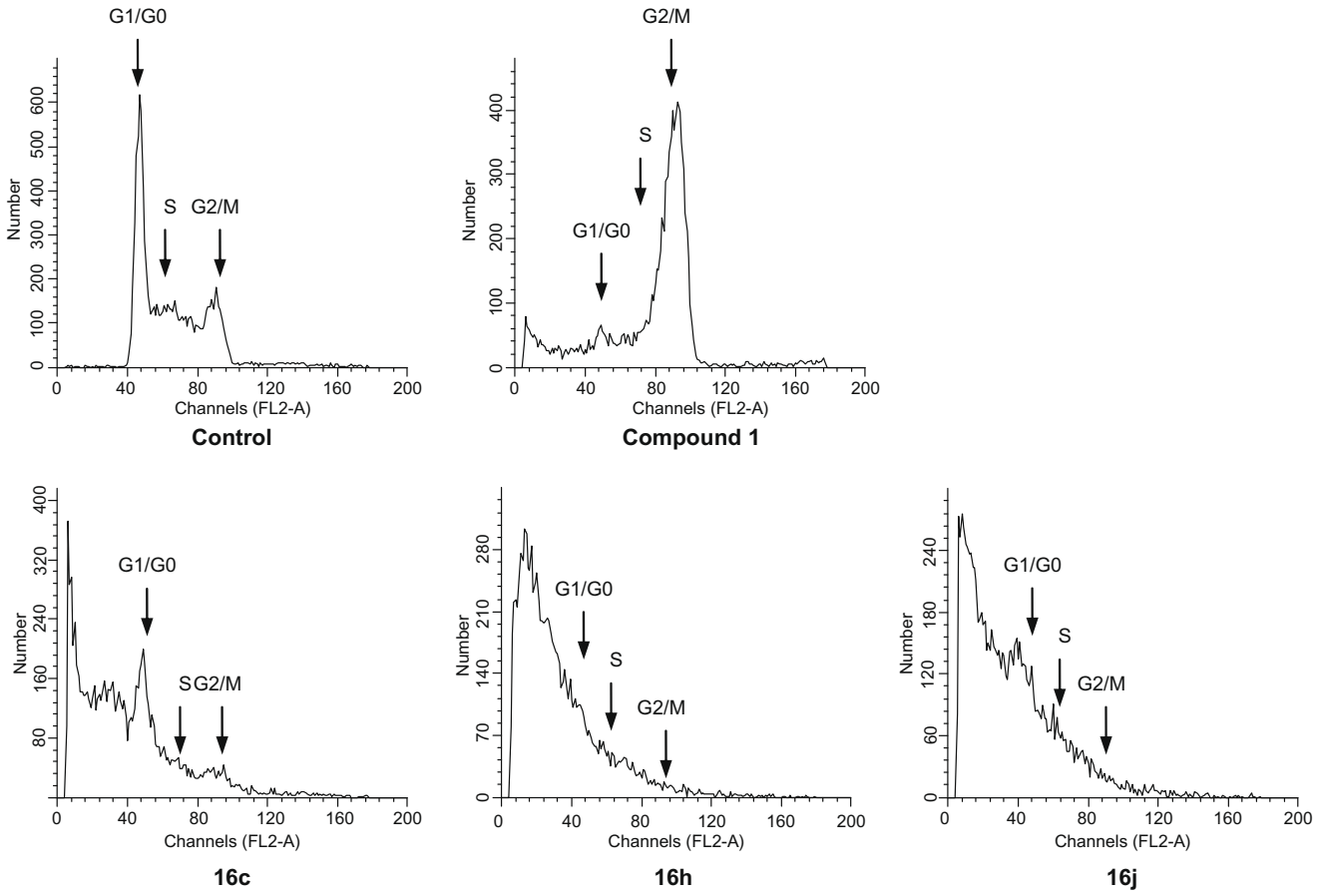


Figure 4. Cell cycle analysis. CEM cells were untreated or treated with **1** (3.2 μM), **16c** (10 μM), **16h** (3.3 μM) and **16j** (6.4 μM) respectively, for 18 h at 37 °C. Cell cycle was analyzed as described in Section 5.5.

represents the dead cells with DNA degradation, featuring apoptotic cells death, and the % of the dead cells are in Table 3. Compound **1** arrested CEM cells in the G2/M phase as expected, **16c**, **16h** and **16j** caused no G2/M phase arrest in CEM cells, and appeared to kill the CEM cells through G1/G0 suspension. To validate the observation, we accessed the direct effect of **16c**, **16h** and **16j** on microtubule assembly in a cell-free system. As shown in Figure 5, different from the parent compound **1**, none of three analog compounds inhibited microtubule assembly. The results indicate that the modifications made on **16c**, **16h** and **16j** compounds have changed the anticancer mechanism from mitotic arrest to the one different from their parent compounds.

4. Conclusions

Taken together, we consider that *N*-3-haloacylaminophenyl-*N*′-2-alkylureas are a class of promising antiproliferative agents with a mode of action independent of tubulin-based mechanism. The SAR analysis showed that the anticancer potency of the 3-haloacylamino chain followed the order of $-CH_2Br > -CHBrCH_3$. The substituents at the N′-end play a role for chemical regulation of the anticancer activity, and suitable aliphatic substituents might improve the activity. Of the study compounds, compound **16j** exhibited good anti-proliferative activity in a variety of in vitro cell culture experiments using cell lines derived from human liquid and solid tumors, and a mechanism different from its parent compounds. The in vivo anticancer activity and detailed mechanism pathway of the compound are currently under investigation in our laboratory.

Table 3
Apoptotic cell death by study compounds[a]

	G0/G1	S	G2/M	Apoptosis (%)
Control	34.5	54.6	9.4	1.5
1	2.0	25.3	63.2	9.5
16j	48.3	13.7	13.2	24.8
16h	33.5	12.9	2.0	52.6
16c	34.1	30.1	2.6	33.2

[a] The cell cycle distribution and apoptotic cell death was determined using flow cytometry (see Section 5).

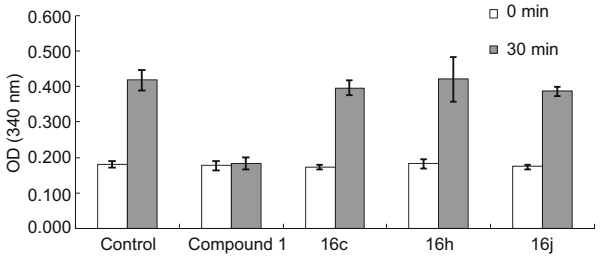


Figure 5. Compounds **16c**, **16h** and **16j** showed no inhibitory effect on microtubule polymerization. The OD at 340 nm was taken as a proxy measurement of the degree of microtubule polymerization. Microtubule polymerization was determined by measuring OD value (340 nm) at 0 min and 30 min after initiation of the assembly reaction. The sample was treated with the solvent (control), compound **1** (3.2 μM), **16c** (10 μM), **16h** (3.3 μM) and **16j** (6.4 μM), respectively. Each column represents the mean ± SD of three independent assays.

5. Experimental

5.1. Chemical methods

Melting point (mp) was obtained with YRT-3 melting point apparatus and uncorrected. ^{1}H NMR spectra was performed on a Varian Inova 400 MHz spectrometer (Varian, San Francisco, CA) in DMSO-d_6, with Me_4Si as the internal standard. ESI high-resolution mass spectra (HSMS) were recorded on an Autospec Ultima-TOF mass spectrometer (Micromass UK Ltd, Manchester, UK). Purity estimation was done with TLC (thin layer chromatography), and only those with purity around 95% were tested for biological activity.

Solvent and reagent abbreviations used are DMF = dimethylformamide, DMA = dimethylacetamide, DMSO = dimethylsulfoxide.

5.2. General procedure for the synthesis of compounds 16a–c (route A)

To a solution of *m*-nitroaniline (2.76 g, 20 mmol) in EtOAc (20 ml) and EtOH (20 ml) was added dropwise 2-chloroethyl isocyanate (2.5 ml, 29 mmol) with stirring. The mixture was stirred at room temperature (rt) for 12 h, and the precipitated product was filtered, washed with ether and dried to give the nitro compound (**14**). The nitro compound (2.0 g, 8.2 mmol) was dissolved in absolute ethanol (60 ml), and to the solution was added 10% Pd/C (0.2 g). After stirring at rt for 6 h under a hydrogen atmosphere, the catalyst was removed by filtration, and filtrate was concentrated under reduced pressure, soaked in ether, filtered and dried to give white solid **15**, which was used for the next step without purification.

By use of a procedure previously described,[5,6] the title compounds were obtained from **14** after its reaction respectively with chloroacetyl chloride, bromoacetyl bromide, or 2-bromopropionyl bromide in DMA. The residue is purified by recrystallization from ethanol.

5.2.1. *N*-(3-Bromoacetamido)phenyl-*N*′-2-chloroethylurea (16a)

Using the previous procedure, yield: 65%. White needle, mp 155–157 °C. ^{1}H NMR: δ 3.40 (q, J = 6.0 Hz, 2H), 3.65 (t, J = 6.0 Hz, 2H), 4.00 (s, 2H), 6.34 (t, J = 4.4, 4.8 Hz, 1H), 7.10–7.15 (m, 3H), 7.71 (s, 1H), 8.70 (s, 1H), 10.30 (s, 1H); IR (KBr) γ 3319, 3242 (NH), 1660, 1633 (C=O) cm^{-1}; HRMS: calcd for $C_{11}H_{13}BrClN_3O_2$ (M+Na)$^+$ 355.9777, found 355.9761.

5.2.2. *N*-(3-β-Bromopropionamido)phenyl-*N*′-2-chloroethylurea (16b)

Using the previous procedure, yield: 66%. White needle, mp 171–173 °C. ^{1}H NMR: δ 1.74 (d, J = 6.4 Hz, 3H), 3.43 (q, J = 6.0 Hz, 2H), 3.66 (t, J = 6.0 Hz, 2H), 4.70 (q, J = 6.8, 6.4 Hz, 1H), 6.37 (t, J = 5.6, 6.0 Hz, 1H), 7.09–7.21 (m, 3H), 7.75 (s, 1H), 8.70 (s, 1H), 10.26 (s, 1H); IR (KBr) γ 3317, 3240 (NH), 1660, 1633 (C=O) cm^{-1}; HRMS: calcd for $C_{12}H_{15}BrClN_3O_2$ (M+Na)$^+$ 369.9934, found 369.9903.

5.2.3. *N*-(3-Iodoacetamido)phenyl-*N*′-2-chloroethylurea (16c)

Using the previous procedure, yield: 65%. White needle, mp 164–166 °C (dec). ^{1}H NMR: δ 3.42 (q, J = 6.0 Hz, 2H), 3.66 (t, J = 6.0 Hz, 6.4 Hz, 2H), 3.81 (s, 2H), 6.35 (m, 1H), 7.11–7.15 (m, 3H), 7.70 (s, 1H), 8.70 (s, 1H), 10.25 (s, 1H); IR (KBr) γ 3319, 3240 (NH), 1651, 1640 (C=O) cm^{-1}; HRMS: calcd for $C_{11}H_{13}ClIN_3O_2$ (M+Na)$^+$ 403.9639, found 403.9611.

5.3. General procedure for the synthesis of compounds 16d–k

To a solution of relevant acylamide (**9**, **11**, 10 mmol) in anhydrous dichloroethane (40 ml) was added oxalyl chloride (1.3 ml, 15 mmol) dropwise. The reaction mixture was heated to reflux for 4 h and concentrated under vacuum to give an oil. To a stirring solution of the oil in CH_3CN (40 ml) was added *m*-nitroaniline (1.39 g, 10 mmol). The reaction mixture was stirred for 12 hrs at rt and concentrated under vacuum. The residue was stirred in water (60 ml) for 30 min. The mixture was filtered, and the solid was washed with water and dried to give nitro compound (**13**), which was used for the next step without purification. Using procedures previous reported,[5,6] the title compounds were obtained after two steps reactions including reduction (Fe/HCl as a reductive agent) and acylamidation.

5.3.1. *N*-(3-Bromoacetamido)phenyl-*N*′-3-chloropropionylurea (16d)

Using the previous procedure, yield: 8%. Mp 193.8–194.7 °C. ^{1}H NMR: δ 2.91 (t, J = 6.0, 6.4 Hz, 2H), 3.85 (t, J = 6.0, 6.4 Hz, 2H), 4.02 (s, 2H), 7.15 (d, J = 8.0, 1H), 7.27 (t, J = 8.0 Hz, 1H), 7.37 (d, J = 8.0 Hz, 1H), 7.87 (s, 1H), 10.42 (s, 1H), 10.46 (s, 1H), 10.82 (s, 1H); IR (KBr) γ 3297, 3133 (NH), 1719, 1671, 1608 (C=O) cm^{-1}; HRMS: calcd for $C_{12}H_{13}BrClN_3O_3$ (M+Na+H)$^+$ 384.9805, found 384.9794.

5.3.2. *N*-(3-β-Bromopropionamido)phenyl-*N*′-3-chloropropionylurea (16e)

Using the previous procedure, yield: 15%. Mp 198.7–199.4 °C. ^{1}H NMR: δ 1.74 (d, J = 6.4 Hz, 3H), 2.92 (t, J = 6.0, 6.4 Hz, 2H), 3.85 (t, J = 6.0, 6.4 Hz, 2H), 4.69 (q, J = 6.8, 6.4 Hz, 1H), 7.14 (d, J = 8.0 Hz, 1H), 7.27 (t, J = 8.0 Hz, 1H), 7.39 (d, J = 8.0 Hz, 1H), 7.90 (s, 1H), 10.37, 10.46, 10.83 (s, s, s, 3H); IR (KBr) γ 3291, 3132 (NH), 1720, 1690, 1666, 1607 (C=O) cm^{-1}; HRMS: calcd for $C_{13}H_{15}BrClN_3O_3$ (M+Na+H)$^+$ 398.9961, found 398.9968.

5.3.3. *N*-(3-Bromoacetamido)phenyl-*N*′-acetylurea (16f)

Using the previous procedure, yield: 28%. White solid, mp 199.7–201.4 °C. ^{1}H NMR: δ 2.49 (s, 3H), 3.31 (s, 2H), 7.14 (d, J = 8.0 Hz, 1H), 7.25 (t, J = 8.0 Hz, 1H), 7.35 (d, J = 8.4 Hz, 1H), 7.82 (s, 1H), 10.41, 10.53, 10.66 (s, s, s, 3H); IR (KBr) γ 3259, 3140 (NH), 1714, 1662, 1609 (C=O) cm^{-1}; HRMS: calcd for $C_{11}H_{12}BrN_3O_3$ (M+Na)$^+$ 335.9960, found 335.9955.

5.3.4. *N*-(3-β-Bromopropionamido)phenyl-*N*′-acetylurea (16g)

Using the previous procedure, yield: 29%. White solid, mp 214.6–216.3 °C. ^{1}H NMR: δ 1.73 (d, J = 6.8 Hz, 3H), 2.49 (s, 3H), 4.68 (q, J = 6.8 Hz, 1H), 7.14 (d, J = 8.0 Hz, 1H), 7.25 (t, J = 8.0 Hz, 1H), 7.38 (d, J = 8.4 Hz, 1H), 7.85 (s, 1H), 10.35, 10.54, 10.67 (s, s, s, 3H); IR (KBr) γ 3251, 3145 (NH), 1714, 1663, 1608 (C=O) cm^{-1}; HRMS: calcd for $C_{12}H_{14}BrN_3O_3$ (M+Na) 350.0116, found 350.0096.

5.3.5. *N*-(3-Bromoacetamido)phenyl-*N*′-formylurea (16h)

Using the previous procedure, yield: 16%. White solid, mp 214.6–216.7 °C. ^{1}H NMR: δ 4.02, 4.24 (s, s 2H), 7.27 (m, 1H), 7.43 (m, 2H), 7.97 (s, 1H), 8.17 (s, 1H), 8.26, 10.36, 10.56 (s, s, s, 3H); IR (KBr) γ 3296 (NH), 1714, 1667, 1609 (C=O) cm^{-1}; HRMS: calcd for $C_{10}H_{10}BrN_3O_3$ (M+Na)$^+$ 321.9803, found 321.9823.

5.3.6. *N*-(3-β-Bromopropionamido)phenyl-*N*′-formylurea (16i)

Using the previous procedure, yield: 16%. White solid, mp 191–193 °C. ^{1}H NMR: δ 1.73 (d, J = 6.4 Hz, 3H), 4.71 (q, J = 6.4 Hz, 1H), 7.27 (m, 1H), 7.43 (m, 2H), 7.97 (s, 1H), 8.13 (s, 1H), 8.25, 10.36, 10.56 (s, s, s, 3H); IR (KBr) γ 3390, 3302 (NH), 1668, 1605 (C=O) cm^{-1}; HRMS calcd for $C_{11}H_{12}BrN_3O_3$ (M+H)$^+$ 314.0140, found 314.0127.

5.4. General procedure for the synthesis of compounds 16j–n

To a solution of *m*-nitrophenyl isocyanate (0.66 g, 4.0 mmol) in DMF (10 ml) was added relevant aniline or ammonia (4.0 mmol)

with stirring at rt. The reaction mixture was stirred at rt for 6–8 h, then poured into ice water. The precipitated product was collected by filtration and washed with water, then dried to give nitro compound (**13**). Using procedures previous reported,[5,6] the title compounds were obtained after two steps reactions, including reduction (with Fe/HCl as reducing agent) and acylamidation.

5.4.1. *N*-(3-Bromoacetamido)phenyl-*N*′-bromoacetylurea (16j)

Using the previous procedure, yield: 54%. White solid, mp 186–187 °C. ^{1}H NMR: δ 4.02 (s, 2H), 4.14 (s, 2H), 7.16 (d, J = 8.8 Hz, 1H), 7.27 (t, J = 8.0 Hz, 1H), 7.37 (d, J = 8.8 Hz, 1H), 7.86–7.87 (m, 1H), 10.20 (s, 1H), 10.45 (s, 1H), 11.00 (s, 1H); IR (KBr) γ 3340, 3257 (NH), 1655 (C=O) cm^{-1}; HRMS: calcd for $C_{11}H_{11}Br_2N_3O_3$ (M+Na)$^+$ 413.9065, found 413.9094.

5.4.2. *N*-(3-β-Bromopropionamido)phenyl-*N*′-2-bromopropionylurea (16k)

Using the previous procedure, yield: 56%. White silod, mp 191–192 °C. ^{1}H NMR: δ 1.73 (d, J = 6.4 Hz, 6H), 4.69 (q, J = 6.8 Hz, 2H), 7.14–7.16 (m, 1H), 7.25–7.29 (m, 1H), 7.39–7.41 (m, 1H), 7.93 (s, 1H), 10.28 (s, 1H), 10.40 (s, 1H), 11.05 (s, 1H); IR (KBr) γ 3340, 3257 (NH), 1655, 1599 (C=O) cm^{-1}; HRMS: calcd for $C_{13}H_{15}Br_2N_3O_3$ (M+Na)$^+$ 441.9378, found 441.9419.

5.4.3. *N*-(3-Bromoacetamido)phenyl-*N*′-phenylurea (16l)

Using the previous procedure, yield: 72%. White solid, mp 184–186 °C (dec). ^{1}H NMR: δ 4.03 (s, 2H), 6.95–6.99 (m, 1H), 7.16–7.30 (m, 5H), 7.44–7.46 (m, 2H), 7.81 (s, 1H), 8.60 (s, 1H), 8.74 (s, 1H), 10.37 (s, 1H); IR (KBr) γ 3340, 3257 (NH), 1655 (C=O) cm^{-1}; HRMS: calcd for $C_{15}H_{14}BrN_3O_2$ (M+Na)$^+$ 370.0167, found 370.0158.

5.4.4. *N*-(3-Bromoacetamido)phenyl-*N*′-3′,4′,5′-trimethoxyphenylurea (16m)

Using the previous procedure, yield: 53%. White solid, mp 124–127 °C. ^{1}H NMR: δ 3.60 (s, 3H), 3.70 (s, 3H), 3.71 (s, 3H), 4.02 (s, 2H), 6.79 (s, 2H), 7.08 (d, J = 8.0 Hz, 1H), 7.20 (m, 1H), 7.26 (d, J = 8.0 Hz, 1H), 7.82 (s, 1H), 8.54 (s, 1H), 8.67 (s, 1H), 10.37 (s, 1H); IR (KBr) γ 3307, 3221 (NH), 1761, 1668 (C=O) cm^{-1}; HRMS: calcd for $C_{18}H_{20}BrN_3O_5$ (M+Na)$^+$ 460.0484, found 460.0488.

5.4.5. *N*-(3-β-Bromopropionamido)phenyl-*N*′-3,4,5-trimethoxyphenylurea (16n)

Using the previous procedure, yield: 52%. White solid, mp 187.2–191 °C. ^{1}H NMR: δ 1.74 (d, J = 6.8 Hz, 3H), 3.60 (s, 3H), 3.74 (s, 6H), 4.70 (q, J = 6.8 Hz, 1H), 6.79 (s, 2H), 7.05 (d, J = 8.8 Hz, 1H), 7.18–7.22 (m, 1H), 7.29 (d, J = 8.8 Hz, 1H), 7.86 (m, 1H), 8.55 (s, 1H), 8.66 (s, 1H), 10.31 (s, 1H); IR (KBr) γ 3365, 3317, 3280 (NH), 1704, 1684 (C=O) cm^{-1}; HRMS: calcd for $C_{19}H_{22}BrN_3O_5$ (M+Na)$^+$ 474.0640, found 474.0633.

5.5. Biological methods

5.5.1. 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 Type Culture Collection (ATCC, Manassas, VA, USA). DND-1 melanoma cells were from Dr. Ohnuma (Mount Sinai School of Medicine, New York, NY). Bel-7402 hepatoma cells were from the Cancer Institute, Chinese Academy of Medical Sciences. All of the cells were cultivated in the RPMI-1640 medium (Invitrogen) supplemented with 10% inactivated fetal calf serum, penicillin (150 μg/ml) and streptomycin (150 μg/ml), in the atmosphere of at 5% CO_2 at 37 °C.

5.5.2. Anticancer activity in vitro

Cells were seeded into 96-well plates (Falcon, CA) with 1×10^5 cells in 250 μL per well, followed by treatment with test compounds at concentrations between 0.001 and 100 μM for 72 h at 37 °C. Cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT). The IC_{50} value was defined as the drug concentration killing 50% of the cells in comparison with the untreated controls, and calculated with non-linear regression analysis. The IC_{50} values were determined in duplicates, and each experiment was repeated three times under identical conditions.

5.5.3. Cell cycle analysis

Cell cycle was analyzed using a method reported previously.[11] Briefly, the CEM cells were treated with **16c**, **16h** and **16j**, respectively, at 37 °C for 18 h. The cells were then re-suspended in 70% ethanol for 18 h at 4 °C. The cells were treated with RNase A (50 μg/ml, Sigma, MO) for 0.5 h at 37 °C, and exposed to propidium iodide at a final concentration of 50 μg/ml for 0.5 h at 4 °C. 10^4 cells per sample were analyzed in a flow cytometer (BD FACS Calibur System, San Jose, CA) and the cell cycle phase was calculated with Cell Quest Pro software (version 2.0).

5.5.4. Microtubule polymerization assays

Purified tubulin from calf brain (Sigma, St. Luis, MO) was used to determine polymerization reaction.[4] The OD at 340 nm was taken as a proxy measurement of the degree of microtubule polymerization. 100 μL of β-tubulin (1 mg/ml) was mixed gently with 400 μL of reaction buffer containing 0.1 M 2-[*N*-morpholino]ethane sulfonic acid (MES), 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, and 2.5 M glycerol. Then, the solvent (control), compound **1**, **16c**, **16h**, or **16j** was added to each sample cuvet. After adding GTP to each sample to a final concentration of 1 mM, the starting (0 min) OD was measured as the baseline by a spectrophotometer (UNIC UV-2100) at 340 nm. Assembly reaction was completed within 30 min at room temperature.

Acknowledgment

This study was supported by the National Natural Science Foundation of the PR China (Grant 30371673).

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