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Original article

3-Benzylamino-β-carboline derivatives induce apoptosis through G_2/M arrest in human carcinoma cells HeLa S-3

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ABSTRACT

β-Carboline derivatives are known as the lead compounds for anti-tumor agents. To examine an optimal structure for anti-tumor activity, we synthesized a variety of β-carboline derivatives, possessing a variety of substituents on the nitrogen atom of the amino group of 3-amino-β-carboline, and evaluated their anti-tumor activity for HeLa S-3 cell line. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that an optimal structure for anti-tumor activity was 3-cyclohexylmethylamino (**1e**) or 3-benzylamino-β-carboline (**1f**). An optimal counter anion of 2-methyl-3-benzylamino- β-carbolinium salts was a triflate anion **2c**. In addition, the introduction of a hydroxyl group on the *meta*-position of the benzyl group of 3-benzylamino-β-carboline (**3e**) enhanced its anti-tumor activity. Hoechst 33342 staining and DNA fragmentation assay suggested that **1f**, **2c** and **3e** induced cell death by apoptosis unlike **1e**. Flow cytometry analysis showed that **1f**, **2c** and **3e** induced cell apoptosis through arrest of the cell cycle in the G_2/M phase.

1. Introduction

Natural products have long proven to be a bountiful resource for the identification of bioactive compounds used in the treatment of a variety of ailments and diseases, including cancer. Chemotherapeutic drugs, such as etoposide and taxol, are widely used in clinical oncology. β-Carboline is made up of planar tricyclic ring structures, and its derivatives are widely distributed in nature in plants, marine life, human tissues and body fluids [1]. β-Carboline and its derivatives are endowed with anti-tumor and anti-cancer properties [2–5]. The best-known members of the class are harman and normarman. These are DNA intercalating molecules with benzodiazepine receptors, and their high DNA binding affinity is thought to be responsible, in part, for these pharmacological properties [6–9]. The main reason for the interest in β-carboline and its derivatives for clinical purposes are their multiple physiological effects, such as the inhibition of cyclin-dependent kinase (CDK) [10], IkappaB kinase (IKK) [11,12] and topoisomerase I [8]. We have reported the anti-tumor activity of 3-benzylamino-β-carboline [13]. However, the optimization of the structure of β-carboline derivatives has not been defined. In this study, a variety of β-carboline derivatives **1**–**3** were synthesized and their biological activities were evaluated.

2. Results and discussions

2.1. Chemistry

To determine an optimal structure for anti-tumor activity, a variety of substituent was introduced onto the amino- or pyridyl-nitrogen atom of 3-amino-β-carboline, or onto the *meta*- or *para*-position of the benzyl group of 3-benzylamino-β-carboline according to the known methods (Schemes 1–4). The construction of β-carboline framework was accomplished by Pictet–Spengler reaction of L-tryptophan (**4**) with formaldehyde. After esterification of the carboxyl group with thionyl chloride and methanol, the 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid methyl ester (**7**) was dehydrogenated by trichloroisocyanuric acid, TCCA, leading to

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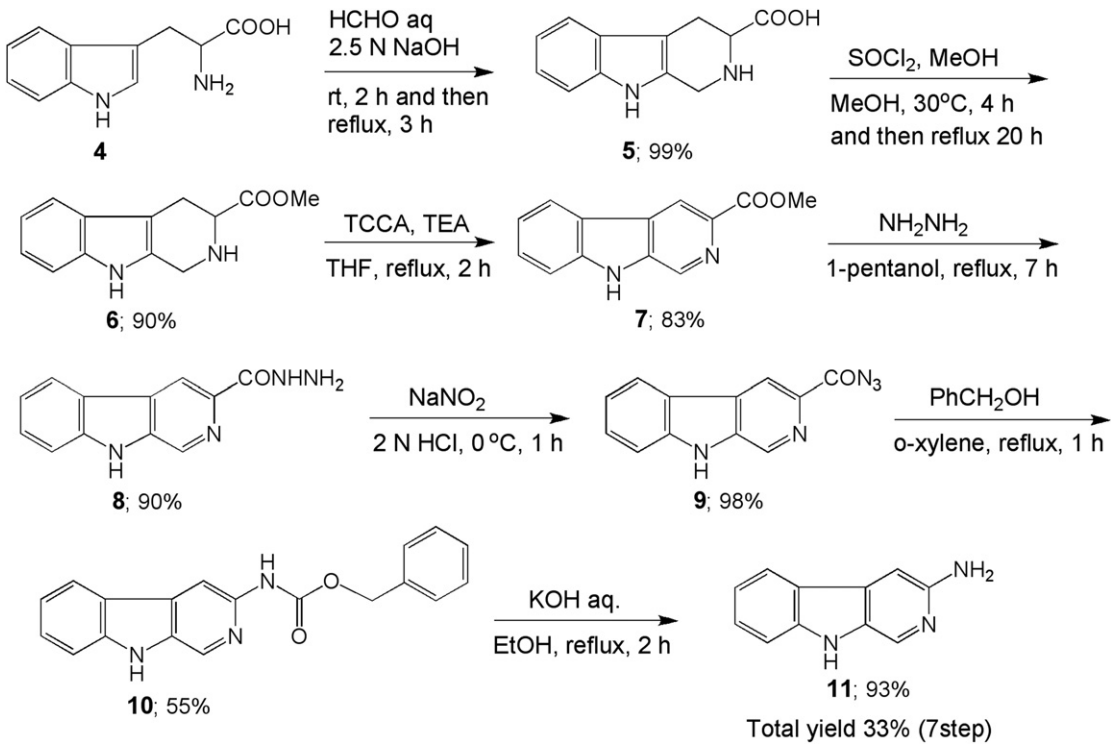
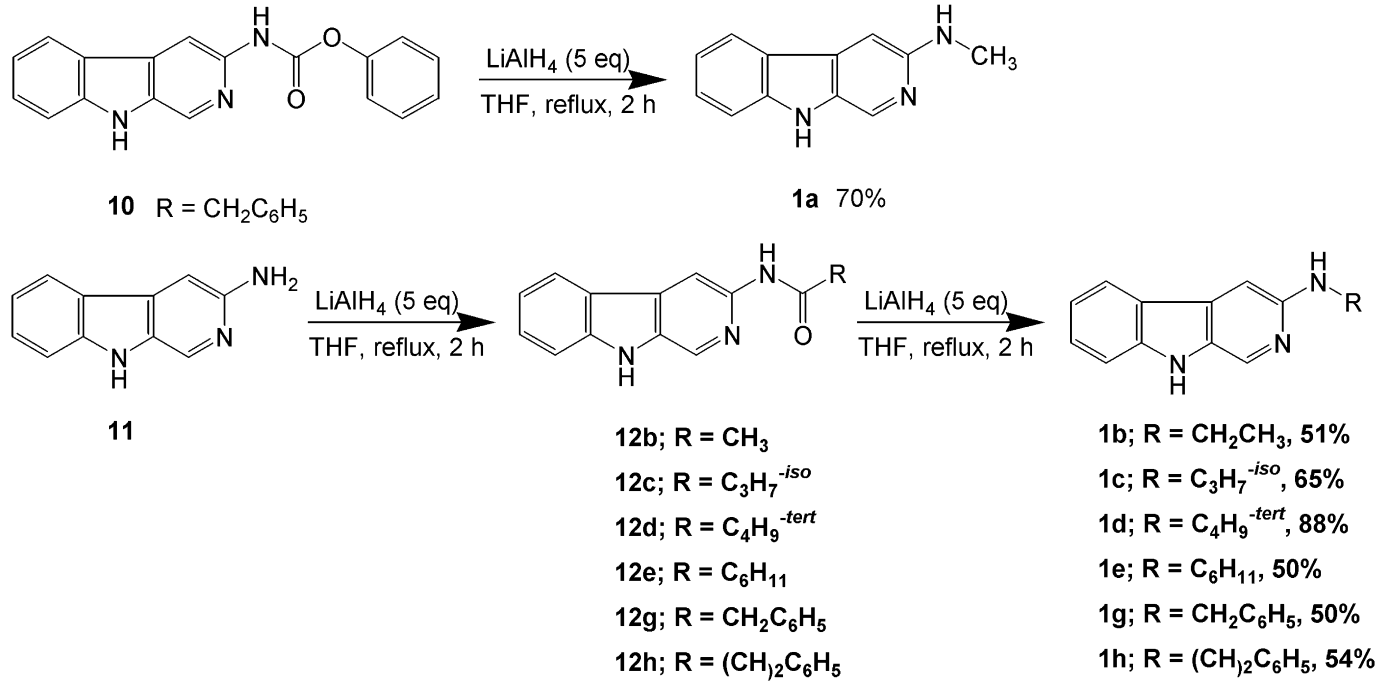


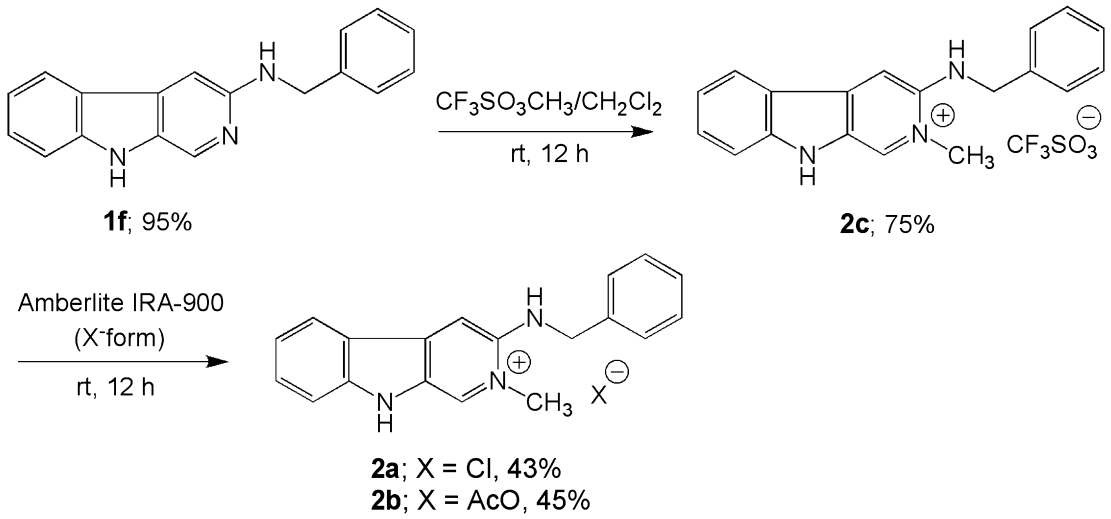
Scheme 1. Synthesis of **11**.

the formation of β-carboline-3-carboxylic acid methyl ester (**7**). The total synthesis of 3-amino-β-carboline (**11**) was completed in 7 steps and 33% overall yield, after Curtius rearrangement of the corresponding carbonylazide **9** followed by hydrolysis of the benzyl carbamate **10** (Scheme 1).

As shown in Scheme 2, 3-methylamino-β-carboline (**1a**) was prepared by LAH reduction of the benzyl carbamate **10** in 70% yield. In the same way, **1b**–**h** was synthesized by LAH reduction of the corresponding amides in moderate to high yields (Scheme 2). In addition, the synthesis of 2-methyl-3-benzylamino-β-carbolinium salts (**2a**–**c**) was illustrated in Scheme 3. The solution of **1f** in dichloromethane was treated with methyl trifluoromethanesulfonate to quanternize the N-2 atom of the pyridine ring to afford the trifluoromethanesulfonate salt **2c**. The counter anion of **2c** was changed



Scheme 2. Synthesis of **1a-h**.



Scheme 3. Synthesis of **2a-c**.

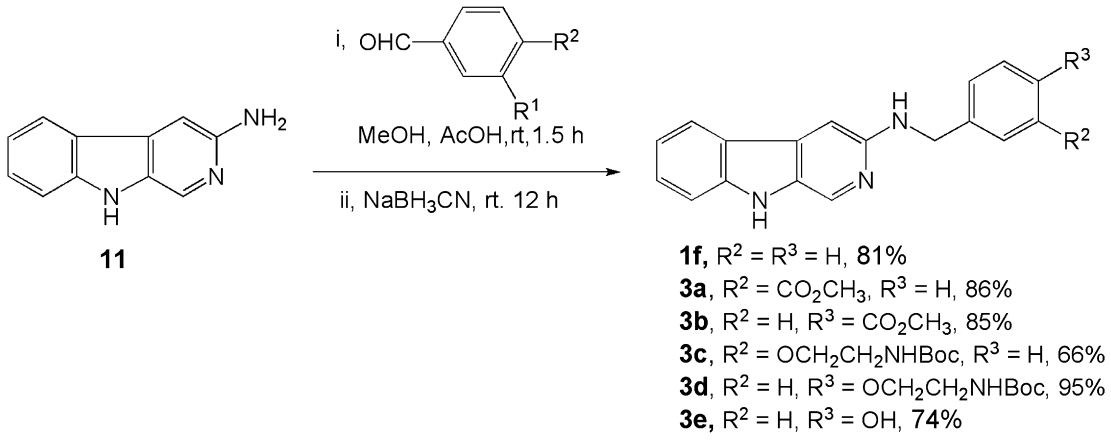
to chloride and acetate anions using Amberlite IRA-900 anion exchange resin to form **2a** and **2b** in 43, 45% yields, respectively (Scheme 3).

The *meta*- or *para*-substituted 3-benzylamino derivatives **3a-e** were synthesized from **11** by a convenient reductive amination with the corresponding *meta*- or *para*- substituted benzaldehydes and sodium cyanoborohydride in high yields, as shown in Scheme 4.

2.2. Anti-tumor activity

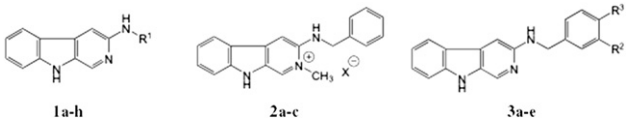
In order to determine the anti-tumor effects of synthesized β-carboline derivatives on the human cervical cancer cell line (HeLa S-3), anti-tumor evaluation was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Table 1). Initially, the 3-alkylamino-β-carboline derivatives were examined (**1a**–**h**). Most of their anti-tumor activities were higher than that of **1a**. Especially, 3-benzylamino-β-carboline (**1f**) showed the highest anti-tumor activity; the IC_{50} value is 0.12 μM [12], which is 10-fold higher than that of 3-isobutylamino-β-carboline (**1c**), 13 μM. It is notable that **1f** demonstrates high anti-tumor activity. In order to clarify the effect of the methylene group, we evaluated the anti-tumor activities of 3-(2-phenylethyl)amino-β-carboline (**1g**) and 3-(3-phenylpropyl)amino-β-carboline (**1h**); their IC_{50} values were 22 and 21 μM, respectively. These values are almost same as those of 3-ethylamino-β-carboline (**1b**), 19 μM, and 3-isopentyl-β-carboline (**1d**), 17 μM. That is to say, the extended carbon chain at the 3-position of **1f** had markedly decreased its anti-tumor activity. Surprisingly, the observed IC_{50} value of 3-(cyclohexylmethyl) amino-β-carboline (**1e**) is 5.2 μM, which is smaller than the expected from those of **1b**–**d**. Norharman without any substituent was used as a negative control. These results showed that introducing a substituent on the nitrogen atom of the amino group was important to increase anti-tumor activity, and that the benzyl group was appropriate for the substituent.

Next, the anti-tumor activities of 2-methyl-3-benzylamino-β-carbolinium salts (**2a**–**c**) with a different counter anion were examined. Surprisingly, these salts showed different anti-tumor activities. The compound **2a** with a chloride counter anion is less active against tumor cells; the value of IC_{50} was 99 μM. In contrast, the anti-tumor activity of **2b** with an acetate anion is 10-fold higher than that of **2a**. Furthermore, **2c** with a triflate counter anion showed the highest anti-tumor activity among the three 2-methyl-3-benzylamino-β-carbolinium salts; the value of IC_{50} of **2c** is 0.54 μM. Generally, it is said that such phenomena should be observed in pharmacokinetic studies. Although the reason is not clear, it is a sufficiently interesting and valuable fact that 2-methyl-



Scheme 4. Synthesis of 1f and **3a-e**.

Table 1
Anti-tumor activity of 3-alkylamino- and 3-benzylamino-b-carboline derivatives in HeLa S-3 cells.



Compd.	R^1	X	R^2	R^3	IC_{50} (μM)[a]
1a	CH_3				101 ± 11
1b	CH_2CH_3				19 ± 2
1c	$C_4H_9^{iso}$				13 ± 1
1d	$CH_2C_4H_9^{tert}$				17 ± 1
1e	$CH_2C_6H_{11}$				5.2 ± 0.3
1f	$CH_2C_6H_5$				0.12 ± 0.06
1g	$(CH_2)_2C_6H_5$				22 ± 2
1h	$(CH_2)_3C_6H_5$				21 ± 1
2a		Cl			99 ± 12
2b		CH_3COO			9.9 ± 0.4
2c		CF_3SO_3			0.54 ± 0.07
3a			H	$COOCH_3$	45 ± 3
3b			$COOCH_3$	H	17 ± 2
3c			H	OC_2H_4NHBoc	42 ± 3
3d			OC_2H_4NHBoc	H	16 ± 1
3e			OH	H	0.92 ± 0.1
	norharman				>100

[a] Drug toxicity was determined by 3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (see Experimental). IC_{50} is drug concentration inhibited the 50% cell growth. Data represent the mean ± S.D. from four independent experiments performed in triplicate experiments.

3-benzylamino-β-carbolinium chloride, acetate, and triflate (**2a**–**c**) showed different anti-tumor activities each other. Johansson et al. have reported that human antibacterial protein LL-37, a natively unfolded protein with extremely basic net charge, was shown to be essentially folded in the presence of several anions [14]. Schulman has also reported the influence of surface forces by various kinds of salts in membrane permeability [15]. In addition, Sauvant et al. (2003) have reported that stimulator of organic anion excretion was important mediator of inflammation, and uptake of organic anion was very important in cell maintenance and repair [16]. Thus, counter anions or cations affect on the function of biomolecules to a greater or lesser degree. Finally, the anti-tumor activity of the compounds **3a**–**e**, with a substituent at the *para*- or *meta*-position of the benzyl group of 3-benzylamino-β-carboline, was examined. Both **3b** and **3d** with a substituent at the *meta*-position showed higher anti-tumor activity than either **3a** or **3c** with a substituent at the *para*-position. In addition, **3e** with a hydroxyl group at the *meta*-position showed the highest anti-tumor activity among **3a**–**e**; the value of IC_{50} of **3e** was 0.92 μM.

2.3. Pharmacology

2.3.1. Hoechst 33258 staining

After the determination, using MTT assay, that **1e**, **1f**, **2c** and **3e** had high anti-tumor activity, the induced cell-death type was investigated. Chromatin condensation and fragmented nuclei are known as the classic characteristics of apoptosis. Hoechst 33342 staining showed significant morphological changes in the nuclear chromatin. After treatment with or without 5- or 10-fold concentration of IC_{50} for each compound, the nuclear chromatin was observed (Fig. 1). In the untreated group, the nuclei were stained less bright (Fig. 1A). With 5-fold concentration of IC_{50}, **1f**-, **2c**- and **3e**-treated cells exhibited numerous cells with fragmented nuclei (Fig. 1C–E, left panel), which was not the case with **1e**-treated cells (Fig. 1B, left panel). However, the cells with a 10-fold concentration of IC_{50} had detectable fragmented nuclei in cells treated with any compound (Fig. 1C–E, right panel).

2.3.2. DNA fragmentation

To verify induced cell death types, a DNA fragmentation assay was performed. DNA fragmentation is well known as the typical biochemical index of cell apoptosis. After exposure to **1e**, **1f**, **2c** and **3e** for 12–48 h, the genomic DNA was extracted and detected by electrophoresis (Fig. 2). The DNA fragmentation was readily detected in the cells treated with **1f** and **3e** for 24 h, and was enhanced with time. The DNA fragmentation in the **2c**-treated cells was undetectable at 24 h, but was detectable at 48 h. The **1e**-treated cells did not have detectable DNA fragmentation, even after treatment for 48 h. The DNA fragmentation assay demonstrated that **1f**, **2c** and **3e**, but not **1e**, induced DNA fragmentation in a time-dependent manner. Together with fluorescence microscopy analysis, these observations demonstrated that **1f**, **2c** and **3e** caused apoptotic cell death, whereas **1e** had a cell death pathway other than apoptosis.

2.3.3. Activated caspase-3 of execution factor for apoptosis

After determining that **1f**, **2c** and **3e** induced the chromatin condensation and the DNA fragmentation, we further investigated the activation of caspase-3 in these cells. It is well known that caspase-3 is the execution factor of apoptosis. Therefore, if caspase-3 is activated, these cells were induced apoptosis. As shown in Fig. 3, activated caspase-3 was detected in **1f**-, **2c**- and **3e**-treated cells, but not **1e**. In addition, activation levels of caspase-3 were dependent on IC_{50} value in anti-tumor activity. These observations are closely connected with results of the chromatin condensation and the DNA fragmentation, and are suggesting that **1f**, **2c** and **3e** induced apoptosis.

2.3.4. Flow cytometry

To examine the effects on cell cycle progression, the cells were exposed to either 1 μM or 5 μM of **1e**, **1f**, **2c**, and **3e** for 6–24 h. As shown in Fig. 4, the G_2/M phase populations in 1 μM of **1f**- and **2c**-treated cells were enhanced with time, but not those in **1e**-treated cells. After treatment with each 3-amino-β-carboline derivative for 12 h, the percentages of average G_2/M population in the control cells and in **1e**-, **1f**-, **2c**- and **3e**-treated cells were 23.1, 28.9, 84.3, 73.5 and 31.3%, respectively. In addition, the populations in **1f**-, **2c**- and **3e**-treated cells for 24 h, had a tendency to shift from the G_2/M phase to the mean apoptotic population sub-G_1 phase. These results may suggest that **1f**, **2c** and **3e**, but not **1e**, induced cell death through G_2/M cell cycle arrest.

2.3.5. Analyses of expression of proteins during progression of the cell cycle

Entry and exit of mitosis are regulated by the cyclin-B-cdc2 complex, also known as the M-phase-promoting factor. To obtain insight into the molecular mechanisms underlying the cell-cycle profiles in **1f**-, **2c**- and **3e**-treated cells, expression patterns of cdc2 and cdk2 protein were detected using western blot assay. The conditions of the experimental groups (Fig. 5) were identical to those in cell-cycle distribution assays (Fig. 4) but with one time point (24 h). The protein levels of cdc2 were considerably increased in **1f**-, **2c**- and **3e**-treated cells, however, the expression of cdk2 proteins remained unchanged. The changes were according to flow cytometric results showing stationary S-phase and increased G_2/M phase arrest, as the expression of cdc2 was raised and the protein levels of cdk2 were constant.

The anti-tumor activity of many anti-cancer drugs results mostly from interference with cell cycle progression, by inhibition of activation of cell cycle regulation, inhibition of DNA replication,

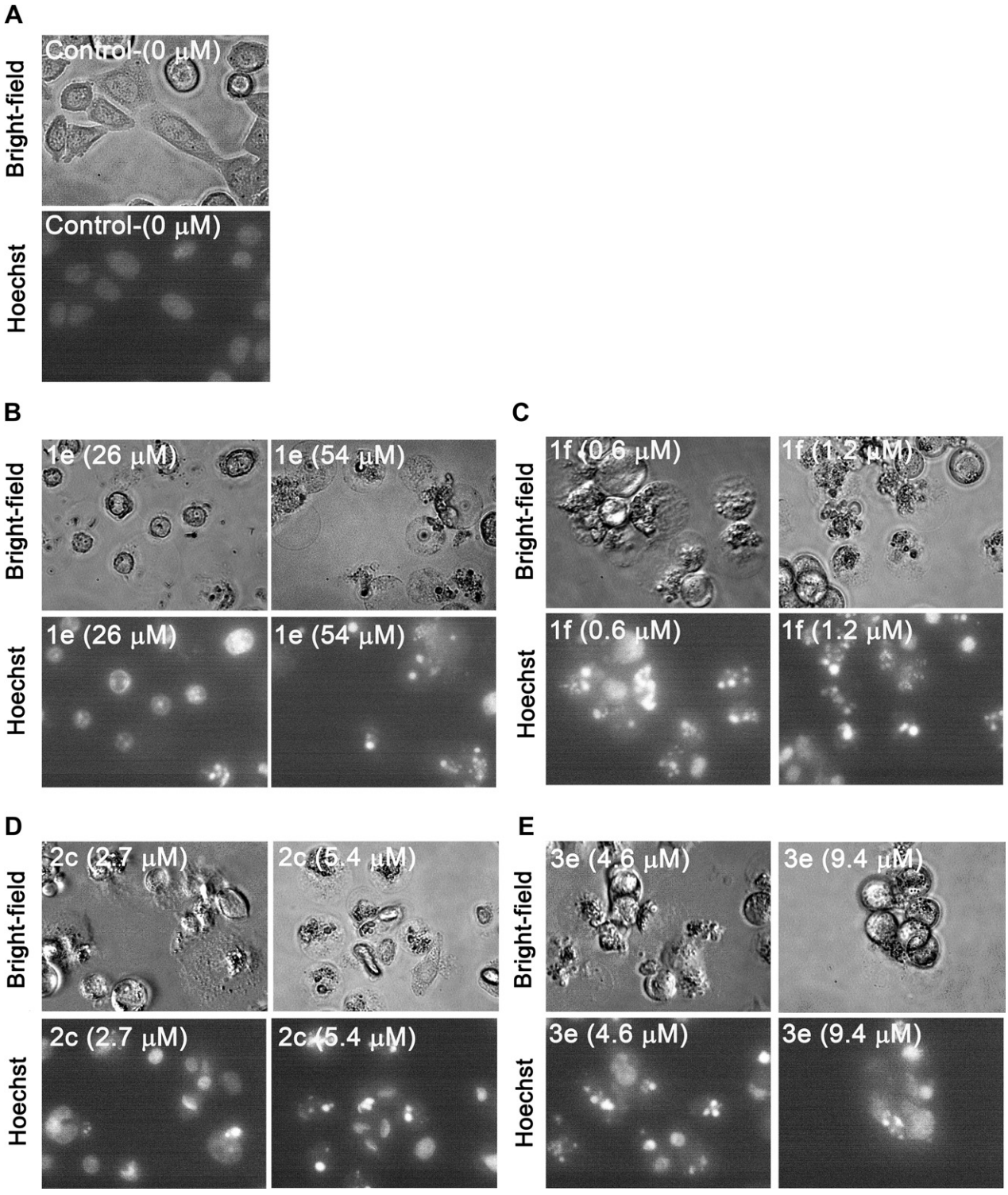


Fig. 1. Identification of **1e**-, **1f**-, **2c**- or **3e**-induced an anti-proliferative effect and apoptosis. HeLa S-3 cells were treated with **1e** (B), **1f** (C), **2c** (D) and **3e** (E) for 48 h. The concentration were either 26 μM (B, left panel) or 52 μM (B, right panel) **1e**, 0.6 μM (C, left panel) or 1.6 μM (C, right panel) **1f**, 2.7 μM (D, left panel) or 5.4 μM (D, right panel) **2c**, 4.6 μM (E, left panel) or 9.2 μM (E, right panel) **3e**, and non-treated group (A). Apoptosis was detected by Hoechst 33342 staining.

induction of DNA damage, or disruption of mitotic spindle formation [17–19]. In particular, since the discovery of taxol, spindle microtubules have been popular for use in the discovery of new anti-cancer drugs [20,21]. These drugs inhibit cell proliferation either by increasing microtubule polymerization, as taxol does, or by promoting microtubule depolymerization, as vinca alkaloids do, thus impeding cell division and inducing cell death at relatively high concentrations [19]. These reports indicate that a delay in progression through mitosis by the induction of aberrant spindle formation might be another mechanism for anti-cancer drugs to alter cancer cell growth. In this study, flow cytometric analysis demonstrated G_2/M-arrest of the cell cycle with **1f**, **2c** and **3e**.

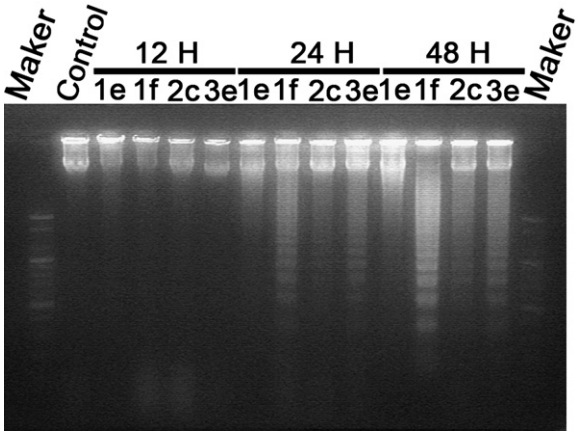


Fig. 2. Effect of **1e**, **1f**, **2c** or **3e** on apoptosis induction in HeLa S-3 cells by DNA fragment assay. Cells were treated with or without 10-fold concentrations of IC_{50} in each compound for 12 h, 24 h or 48h. DNA fragmentation was detected by electrophoresing on a 2% agarose gel. M: 100 bp ladder marker; C: non-treated cells.

3. Conclusions

We concluded that **1f**, **2c** and **3e** are new class of drugs for cell cycle arrest and induction of apoptosis. This study explored the molecular mechanisms of action for a β-carboline derivative and further characterized its potential as an anti-cancer agent.

4. Experimental

4.1. Chemistry

4.1.1. 1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid (**5**)

A mixture of L-tryprophan (**4**) (817 mg, 4 mmol) and 2.5 M NaOH (1.6 ml) was stirred until the mixture became clear at room temperature, and then 37% formalin (4.87 ml) was added. The mixture was stirred for 2 h at this temperature, refluxed for 3 h and then neutralized (pH 5) with 2 M HCl. The precipitate was filtered, washed with H_2O, MeOH and dried *in vacuo* to give the title compound [22]. Yield 99%; colorless solid, mp 302.2–304.6 °C. MS (FAB) *m*/*z* 217 (M + 1, 100%).

4.1.2. 1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid methyl ester (**6**)

To a methanol solution (500 ml) of **5** (43 mg, 0.2 mmol), $SOCl_2$ (16 ml) was added slowly at −10 °C, and the reaction mixture was stirred for 4 h at 30 °C, refluxed for 20 h and concentrated under reduce pressure. After adding water, the residue was neutralized (pH 8.0) with saturated Na_2CO_3, extracted with ethyl acetate, and the extract was dried over Na_2SO_4, and concentrated under reduced pressure to give the title compound [23]. Yield 91%; colorless solid, mp 187.2–188.8 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.72 (dd, *J* = 9.0, 14.5 Hz, 1H), 2.75–2.76 (br, 1H), 2.91 (dd, *J* = 5.0, 14.5Hz, 1H), 3.33 (s, 3H), 3.71 (dd, *J* = 5.0, 9.0 Hz, 1H), 3.90 (d, *J* = 16.5 Hz, 1H), 4.00 (d, *J* = 16.5 Hz, 1H), 6.92 (dd, *J* = 6.5, 7.5 Hz, 1H), 7.00 (dd, *J* = 6.5, 8.0 Hz, 1H), 7.25 (d, *J* = 7.5 Hz, 1H), 7.36 (d, *J* = 8.0 Hz, 1H), 10.71 (br s, 1H); MS (FAB) *m*/*z* 231 (M + 1, 55%), 169 (100%).

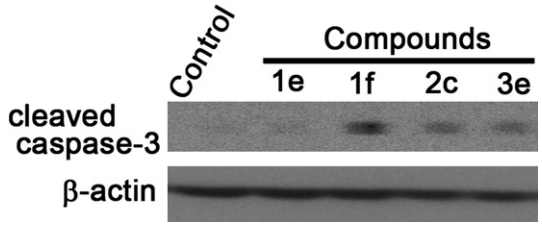


Fig. 3. Western blot analysis of activated caspase-3 in **1e**-, **1f**-, **2c**- and **3e**-treated cells. Cells were treated with or without 5-fold concentrations of IC_{50} in each compound for 24 h. The proteins were detected by western blot analysis. β-actin was used as an internal control. Control, non-treated cells.

4.1.3. β-Carboline-3-carboxylic acid methyl ester (**7**)

To *N*,*N*-dimethylformamide (DMF) (50 ml) solution of **6** (7.95 g, 34.5 mmol) and triethylamine (9.09 g, 89.8 mmol), a DMF solution of trichloroisocyanuric acid (8.0 g, 34.5 mmol) was added slowly at −20 °C. After the addition was completed, the reaction mixture was allowed to warm slowly up to 0 °C with stirring for 2 h. The resulting product was precipitated from ice water, filtrated, washed with ice water and dried *in vacuo*. The crude product was purified by silica gel column chromatography (AcOEt as the eluent), affording the title compound [24]. Yield 77%; colorless solid, mp 242.3–243.9 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.90 (s, 3H), 7.31 (dd, *J* = 8.1, 9.0 Hz, 1H), 7.60 (dd, *J* = 8.1, 9.0 Hz, 1H), 7.67 (d, *J* = 8.1 Hz, 1H), 8.38 (d, *J* = 8.1 Hz, 1H), 8.91 (s, 1H), 8.96 (s, 1H), 11.1–11.2 (br, 1H); MS (FAB) *m*/*z* 227 (M + 1, 80%), 195 (100%).

4.1.4. β-Carboline-3-carboxylic acid hydrazide (**8**)

Hydrazine monohydrate (5.76 g, 115 mmol) was added to the suspension of **7** (1.05 g, 4.64 mmol) in 1-pentanol (42.7 ml) and the mixture was stirred for 7 h at 150 °C. Then, the solution was evaporated and the crude compound was washed with hexane, dried *in vacuo* [24]. Yield 89%; yellow solid, mp >350 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.9–5.0 (br, 2H), 7.28 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.51 (dd, *J* = 7.5, 8.0 Hz, 1H), 7.64 (d, *J* = 7.5 Hz, 1H), 8.39 (d, *J* = 8.0 Hz, 1H), 8.81 (s, 1 H), 8.87 (s, 1H), 9.6–9.8 (br s, 1H), 11.5–12.0 (br, 1H); MS (FAB) *m*/*z* 227 (M + 1, 100%).

4.1.5. β-Carboline-3-carbonyl azide (**9**)

The acid hydrazide **8** (5.0 g, 22.1 mmol) was dissolved in 0.2 M HCl aq. solution (65.4 ml) and the mixture was cooled to 0 °C and then neutralized with Na_2CO_3 solution. The precipitate was filtrated, washed with ice water and dried *in vacuo*. Crude yield 90%; yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.33 (dd, *J* = 7.5, 8.0 Hz, 1H), 7.61 (dd, *J* = 8.0, 8.0 Hz, 1H), 7.67 (d, *J* = 8.0 Hz, 1H), 8.42 (d, *J* = 7.5 Hz, 1H), 8.96 (s, 1H), 8.98 (s, 1H), 11.17 (br s, 1H); MS (FAB) *m*/*z* 238 (M + 1, 100%).

4.1.6. (β-Carboline-3-yl)carbamic acid benzyl ester (**10**)

The carbonyl azide **9** (154.9 mg, 0.653 mmol), benzyl alcohol (141.7 mg, 1.31 mmol) were added to o-xylene (3.6 ml) and the suspension was stirred for 1 h at 160 °C. The hot solution was filtered. Hexane was added to the filtrate and the solution was cooled to 5 °C for 24 h. The deposited crystal was filtered, recrystallized from ethanol. Yield 55%; brown solid; mp 210–211 °C (dec.). IR (KBr/cm^{-1}) 3416, 1720; ^{1}H NMR (500 MHz, DMSO-d_6) δ 5.21 (s, 2H), 7.19 (t, 1H), 7.32 (dd, *J* = 6.0, 8.0 Hz, 1H), 7.39 (dd, *J* = 7.5, 8.0 Hz, 2H), 7.45 (d, *J* = 8.0 Hz, 2H), 7.51 (dd, *J* = 6.8, 8.0 Hz, 1H), 7.55 (d, *J* = 8.0 Hz, 1H), 8.16 (d, *J* = 8.0 Hz, 1H), 8.45 (s, 1H), 8.60 (s, 1H), 10.08 (br s, 1H), 11.42 (br s, 1H); ^{13}C NMR (500 MHz, DMSO-d_6) δ 65.5, 103.1, 111.9, 119.0, 120.7, 121.7, 127.8, 127.9, 128.3, 128.4, 129.9, 131.2, 133.2, 136.9, 141.6, 143.1, 153.8; MS (FAB) *m*/*z* 317 (M^+, 100%), HRMS (FAB): Calcd for $C_{19}H_{15}N_3O_2$: 317.1164, Found: 317.1162.

4.1.7. 3-Amino-β-carboline (**11**)

A mixture of **10** (99.9 mg, 0.315 mmol) and 6 M potassium hydroxide in aqueous alcohol (3.7 ml 6 M KOH aq and 4.5 ml EtOH)

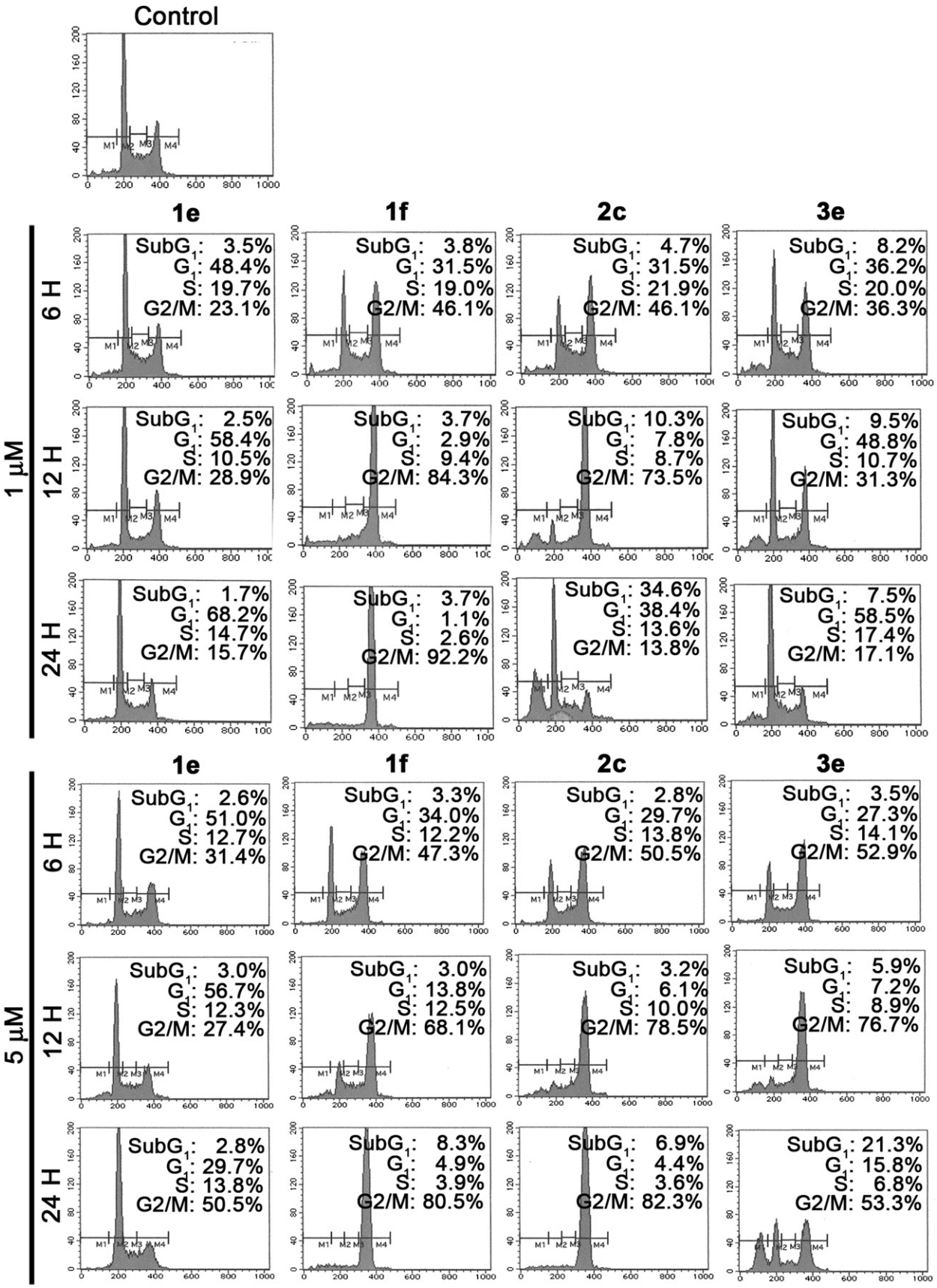


Fig. 4. Effect of **1e**, **1f**, **2c** or **3e** on the distribution of cell-cycle phase in HeLa S-3 cells. Cells were exposed to 1 μM or 5 μM of each 3-benzylamino-β-carboline derivatives for 6 h, 12 h or 24 h. The cells were then fixed and stained with propidium iodide to analyze DNA content by flow cytometric analysis. M1: subG$_1$-phase; M2: G$_1$-phase; M3: S-phase; M4: G$_2$/M-phase.

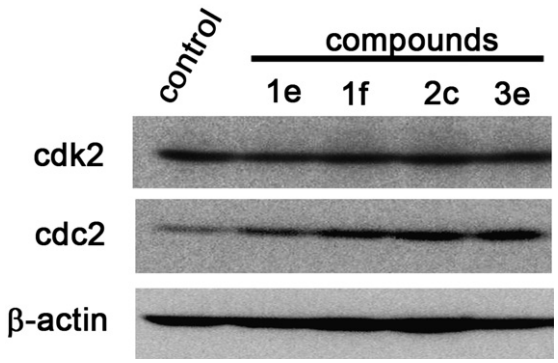


Fig. 5. Cell cycle-related expression of proteins in HeLa S-3 cells with **1e**, **1f**, **2c** or **3e**. Cells were treated with or without 5-fold concentrations of IC_{50} in each compound for 24 h. The proteins were detected by western blot analysis. β-actin was used as an internal control. Control, non-treated cells..

was stirred for 2 h under reflux. After the solvent was removed, the resulting residure was cooled to 5 °C for 24 h. The deposited crystal was filtered, washed with water, and dried *in vacuo*. The product was recrystallized from EtOH/hexane. Yield 85%; light-brownish solid; mp 287–292 °C ^{1}H NMR (500 MHz, DMSO-d_6) δ 5.30 (s, 2H), 7.06 (d, J = 8.0 Hz, 1H), 7.07 (s, 1H), 7.41 (s, 2H), 7.99 (d, J = 8.0 Hz, 1H), 8.29 (s, 1H), 10.87 (br s, 1H) [24].

*4.1.8. 3-Methylamino-β-carboline (**1a**)*

To a rapidly stirred suspension of the **10** (200 mg, 0.63 mmol) in anhydrous THF (12 ml) was added LAH (119 mg, 3.15 mmol) and the mixture was refluxed for 2 h. A piece of ice (1 g) was added, and the mixture was stirred at room temperature for 2 h and then filtered. The filtrate was concentrated *in vacuo* and the residue was purified by column chromatography on silica gel. Yield: 70%, colorless solid, mp 219.5–220.2 °C ^{1}H NMR (DMSO-d_6): δ 2.82 (d, 3H, J = 4.5 Hz), 5.80 (d, 1H, J = 4.5 Hz), 7.03–7.07 (m, 2H), 7.40–7.42 (m, 2H), 8.05 (d, 1H, J = 7.8 Hz), 8.35 (s, 1H), 10.85 (s, 1H). ^{13}C NMR (DMSO-d_6): δ 29.6, 94.4, 111.5, 118.0, 120.6, 121.7, 127.9, 130.4, 130.7, 131.3, 141.9, 153.8. MS (FAB): m/z 198 (M + H). HRMS (FAB): Calcd for $C_{12}H_{12}N_3$ (M + H) 198.1031, Found 198.1035.

*4.1.9. General procedure for the synthesis of 3-alkylamino-β-carboline (**1b**–**e**)*

A solution of **11** (50 mg, 0.27 mmol) and carbxylic anhydride (0.54 mmol) in anhydrous pyridine (5 ml) was stirred at room temperature for 5 h. Saturated aqueous Na_2CO_3 solution was added and the mixture was stirred at room temperature for 1 h and extracted twice with ethyl acetate. The extracts were dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel to afford (β-carbolin-3-yl)alkanecarboxamide (**12b**–**e**). To **12** in anhydrous THF (5 ml) was added $LiAlH_4$ (25 mg, 0.66 mmol), and the mixture was refluxed for 2 h. A piece of ice (1 ml) was added, and the mixture was stirred at room temperature for 2 h and then filtered. The filtrate was concentrated *in vacuo* and the residue was purified by column chromatography on silica gel to afford the title compounds (**1b**–**e**).

*4.1.9.1. 3-Ethylamino-β-carboline (**1b**).* Yield: 51%; yellow solid, mp 138.2–139.5 °C ^{1}H NMR ($CDCl_3$): δ 1.32 (t, 3H, J = 7.0 Hz), 3.36 (q, 2H, J = 7.0 Hz), 4.33 (br s, 1H), 7.16 (s, 1H), 7.18 (t, 1H, J = 7.5 Hz), 7.37 (d, 1H, J = 8.0 Hz), 7.46 (t, 1H, J = 7.5 Hz), 8.03 (d, 1H, J = 8.0 Hz), 8.26 (s, 1H), 8.38 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 15.0, 38.4, 95.0, 111.3, 119.1, 121.6, 121.8, 128.4, 130.8, 130.9, 132.7, 141.9, 153.4. MS (FAB): m/z 212 (M + H). HRMS (FAB): $C_{13}H_{14}N_3$ (M + H) 212.1188, Found 212.1163. HRMS (ESI): $C_{15}H_9N_3$ (M + H) 212.1187, Found 212.1189.

*4.1.9.2. (β-Carbolin-3-yl)isobutanecarboxamide (**12c**).* Yield: 92%; colorless solid, mp 192.8–193.6 °C ^{1}H NMR ($CDCl_3$): δ 1.31 (d, 6H, J = 7.0 Hz), 2.61 (septet, 1H, J = 7.0 Hz), 7.23 (t, 1H, J = 8.0 Hz), 8.07 (d, 1H, J = 8.0 Hz), 8.30 (s, 1H), 8.51 (s, 1H), 8.57 (s, 1H), 8.92 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 19.6, 36.8, 104.9, 111.4, 120.1, 121.8, 121.9, 122.3, 128.8, 130.5, 131.7, 133.6, 141.5, 143.5, 175.3. MS (FAB): m/z 254 (M + H). HRMS (FAB): Calcd for $C_{15}H_{16}N_3O$ (M + H) 254.1293, Found 254.1304.

*4.1.9.3. 3-Isobutylamino-β-carboline (**1c**).* Yield: 65%; yellow solid, mp 145.1–145.8 °C ^{1}H NMR ($CDCl_3$): δ 1.04 (t, 3H, J = 7.5 Hz), 1.72 (sex, 2H, J = 7.5 Hz), 3.35 (t, 2H, J = 7.5 Hz), 4.40 (br s, 1H), 7.00 (s, 1H), 7.18 (t, 1H, J = 7.5 Hz), 7.36 (d, 1H, J = 7.5 Hz), 7.46 (t, 1H, J = 7.5 Hz), 8.03 (d, 1H, J = 7.5 Hz), 8.33 (s, 1H), 9.16 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 20.4, 28.1, 51.5, 94.9, 111.4, 118.7, 121.2, 121.6, 128.2, 130.6, 130.9, 132.6, 142.1, 153.2. MS (FAB): m/z 240 (M + H). HRMS (FAB): Calcd for $C_{16}H_{20}N_3$ (M + H) 240.1501, Found 240.1521.

*4.1.9.4. 3-(2,2-Dimethylpropyl)amino-β-carboline (**1d**).* Yield: 88%; white solid, mp 169.5–170.4 °C ^{1}H NMR ($CDCl_3$): δ 1.02 (s, 9H), 3.10 (s, 2H), 7.02 (s, 1H), 7.16 (t, 1H, J = 7.5 Hz), 7.33–7.473 (m, 2H), 8.01 (d, 1H, J = 7.5 Hz), 8.34 (s, 1H), 8.52 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 27.6, 31.8, 55.6, 94.8, 111.4, 119.0, 121.5, 121.7, 128.4, 130.4, 130.7, 132.8, 142.1, 153.8. MS (FAB): m/z 268 (M + H). HRMS (FAB): $C_{16}H_{20}N_3$ (M + H) 254.1657, Found 254.1664.

*4.1.9.5. (β-Carbolin-3-yl)cyclohexanecarboxamide (**12e**).* Yield: 83%; colorless solid, mp 220.3–220.8 °C ^{1}H NMR ($CDCl_3$): δ 1.26–1.37 (m, 4H), 1.54–1.62 (m, 2H), 1.84–2.36 (m, 5H), 7.23–7.28 (m, 1H), 7.44–7.56 (m, 2H), 8.07–8.13 (m, 2H), 8.25 (s, 1H), 8.51 (s, 1H), 8.92 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 25.7, 25.7, 29.7, 46.6, 104.8, 111.4, 120.1, 121.9, 122.3, 128.8, 130.5, 131.7, 133.4, 141.5, 143.6, 174.4. MS (FAB): m/z 294 (M + H). HRMS (FAB): Calcd for $C_{18}H_{20}N_3O$ (M + H) 294.1606, Found 294.1587.

*4.1.9.6. 3-Cyclohexylmethylamino-β-carboline (**1e**).* Yield: 50%; yellow solid, mp 137.0–137.8 °C ^{1}H NMR ($CDCl_3$): δ 0.99–1.26 (m, 5H), 1.70–1.87 (m, 6H), 3.14 (d, 2H, J = 6.9 Hz), 4.49 (br s, 1H), 6.98 (s, 1H), 7.16 (t, 1H, J = 7.5 Hz), 7.32–7.47 (m, 2H), 8.03 (d, 1H, J = 7.5 Hz), 8.35 (s, 1H), 8.52 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 25.9, 26.5, 31.3, 37.7, 50.4, 94.9, 111.4, 119.0, 121.5, 121.8, 128.3, 130.7, 130.8, 132.7, 142.0, 142.0, 153.5. MS (FAB): m/z 280 (M + H). HRMS (FAB): Calcd for $C_{18}H_{22}N_3$ (M + H) 280.1814, Found 280.1803.

*4.1.10. 3-Benzylamino-β-carboline (**1f**)*

Acetic acid (528 μl, 8.82 mmol) and benzaldehyde (289 mg, 2.73 mmol) were added to a solution of **11** (100 mg, 0.55 mmol) in 5 ml methanol and the mixture was stirred for 1.5 h at room temperature. $NaBH_3CN$ (343 mg, 5.46 mmol) was then added and the mixture was stirred for an additional 5 h. The reaction was quenched by 6 N HCl (5 ml) and the reaction mixture was cooled to 0 °C, neutralized with NH_3 aq, and extracted with ethyl acetate. The combined organic extract was washed with brine, dried with Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica gel chromatography to give pure **1f**. Yield: 81%; yellow solid, mp 199.9–200.5 °C ^{1}H NMR ($CDCl_3$): δ 4.51 (d, 2H, J = 6.0 Hz), 6.42 (d, 1H, J = 6.0 Hz), 7.06 (t, 1H, J = 7.5 Hz), 7.10 (s, 1H), 7.18 (t, 1H, J = 7.5 Hz), 7.28 (t, 2H, J = 7.5 Hz), 7.39–7.43 (m, 4H), 7.99 (d, 1H, J = 7.5 Hz), 8.33 (s, 1H), 10.84 (s, 1H). ^{13}C NMR (DMSO-d_6): δ 45.9, 95.5, 111.4, 117.9, 120.5, 121.5, 126.2, 127.2, 127.8, 128.6, 130.5, 130.6, 131.2, 141.3, 141.9, 152.5. MS (FAB): m/z 274 (M + H). HRMS (ESI): Calcd for $C_{18}H_{16}N_3$ (M + H) 274.13442, Found 274.13555.

*4.1.11. 3-(2-Phenylethyl)amino-β-carboline (**1g**)*

(β-Carbolin-3-yl)-2-phenylacetamide (**12g**) was obtained from **11** and phenylacetyl chloride using a procedure similar to that of **12b**. Yield: 93%; colorless solid, mp 137.8–139.0 °C ^{1}H NMR ($CDCl_3$): δ 3.95 (s, 2H), 7.29 (t, 1H, *J* = 7.5 Hz), 7.40–7.55 (m, 5H), 7.67 (d, 1H, *J* = 7.5 Hz), 7.78 (t, 1H, *J* = 7.5 Hz), 8.15 (s, 1H), 8.33 (d, 1H, *J* = 7.5 Hz), 8.69 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 45.0, 104.8, 111.4, 120.1, 122.3, 127.6, 128.8, 129.2, 130.4, 131.5, 133.5, 134.3, 141.4, 143.1, 169.1. MS (FAB): *m*/*z* 320 (M + H). HRMS (FAB): Calcd for $C_{19}H_{16}N_3O$ (M + H) 302.1293, Found 302.1300. From **12g** thus obtained, the title compound **1g** was obtained using a procedure similar to that of **1b**. Yield: 50%; yellow solid, mp 164.3–165.1 °C ^{1}H NMR ($CDCl_3$): δ 3.00 (t, 2H, *J* = 6.5 Hz), 3.61 (t, 2H, *J* = 6.5 Hz), 4.43 (s, 1H), 7.00 (s, 1H), 7.17–7.46 (m, 7H), 7.47 (t, 1H, *J* = 7.5 Hz), 8.00–8.02 (m, 2H), 8.39 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 35.8, 44.9, 95.4, 111.4, 119.2, 121.6, 126.4, 128.4, 128.6, 128.9, 130.8, 130.9, 132.7, 139.5, 141.8, 153.0. MS (FAB): *m*/*z* 288 (M + H). HRMS (FAB): Calcd for $C_{19}H_{17}N_3$ (M + H) 288.1501, Found 288.1520.Anal. Calcd for $C_{19}H_{17}N_3$: C, 79.41; H, 5.96; N, 14.61; Found C, 79.33; H, 6.21; N, 14.81.

*4.1.12. 3-(3-Phenylpropyl)amino-β-carboline (**1h**)*

(β-carbolin-3-yl)-3-phenylpropionamide (**12h**) was obtained from 3-amino-β-carboline and phenylpropionyl chloride using a procedure similar to that of **12b**. Yield: 83%; colorless solid, mp 112.4–113.3 °C ^{1}H NMR ($CDCl_3$): δ 2.69 (t, 2H, *J* = 7.5 Hz), 3.07 (t, 2H, *J* = 7.5 Hz), 7.16–7.28 (m, 6H), 7.38–7.50 (m, 2H), 8.00 (d, 1H, *J* = 7.8 Hz), 8.60 (s, 1H), 8.75 (s, 1H), 8.80–8.85 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 31.4, 39.2, 105.1, 111.4, 120.7, 122.7, 128.8, 128.5, 128.8, 130.4, 131.5, 133.5, 140.6, 141.5, 143.1, 170.5. MS (FAB): *m*/*z* 316 (M + H). HRMS (FAB): Calcd for $C_{20}H_{18}N_3O$ (M + H) 316.1450, Found 316.1455. From the (β-carbolin-3-yl)-3-phenylpropionamide thus obtained, the title compound was obtained using a procedure similar to that of **1b**. Yield: 54%; yellow solid, mp 137.8–138.4 °C ^{1}H NMR ($CDCl_3$): δ 1.96 (quin, 2H, *J* = 6.9 Hz), 2.70 (t, 2H, *J* = 6.9 Hz), 3.30 (t, 2H, *J* = 6.9 Hz), 4.43 (br s, 1H), 6.92 (s, 1H), 7.11–7.30 (m, 6H), 7.41 (t, 1H, *J* = 7.5 Hz), 7.70 (t, 1H, *J* = 7.5 Hz), 8.31 (s, 1H), 8.78 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 31.1, 33.2, 43.1, 95.0, 111.3, 118.9, 121.3, 121.7, 125.8, 128.3, 128.4, 130.7, 130.9, 132.6, 141.6, 141.7, 142.0, 153.0. MS (FAB): *m*/*z* 302 (M + H). HRMS (FAB): $C_{20}H_{20}N_3$ (M + H) 302.1657, Found 302.1658.

*4.1.13. 2-Methyl-3-benzylamino-β-carbolinium chloride (**2a**)*

To the solution of 2-methyl-3-benzylamino-β-carbolinium trifluoromethanesulfonate **(2c)** (50 mg, 0.183 mmol) in methanol (10 ml), Amberlite IRA-900 (Cl^- form) was added and then stirred for 24 h at room temperature. The solution was filtrated, concentrated and dried *in vacuo*. Yield: 95%; ^{19}F NMR (CD_3OD, 470 MHz), no peak was detected.

*4.1.14. 2-Methyl-3-benzylamino-β-carbolinium acetate (**2b**)*

To the solution of 2-methyl-3-benzylamino-β-carbolinium trifluoromethanesulfonate (**2c**) (50 mg, 0.183 mmol) in methanol (10 ml), Amberlite IRA-900 (CH_3COO^- form) was added and then stirred for 24 h at room temperature. The solution was filtrated, concentrated and dried *in vacuo*. Yield: 90%; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.93 (s, 3H), 4.07 (s, 3H), 7.20 (t, 1H, *J* = 7.5 Hz), 7.31 (d, 1H, *J* = 7.5 Hz), 7.37–7.51 (m, 6H), 7.57 (t, 1H, *J* = 7.5 Hz), 7.99 (s, 1H), 8.37 (s, 1H). ^{13}C NMR (CD_3OD, 75 MHz): δ 24.1, 43.3, 47.7, 99.8, 113.0, 120.4, 121.4, 124.2, 125.5, 128.2, 129.9, 131.2, 133.3, 138.6, 139.1, 147.3, 148.8, 180.1.

*4.1.15. 2-Methyl-3-benzylamino-β-carbolinium trifluoromethanesulfonate (**2c**)*

The title compound **2c** was obtained from **1f**. To the stirred solution of **1f** (50 mg, 0.18 mmol) in dichloromethane (10 ml), methyl trifluoromethanesulfonate (4.8 μl, 0.22 mmol) was added and then stirred for 12 h. The precipitated product was filtrated, washed with cold dichloromethane, and dried *in vacuo*. Yield: 83%; yellow solid. ^{1}H NMR (CD_3OD, 500 MHz): δ 4.09 (s, 3H), 4.73 (s, 2H), 7.19 (t, 1H, *J* = 7.5 Hz), 7.28 (t, 1H, *J* = 7.5 Hz), 7.38 (t, 2H, *J* = 7.5 Hz), 7.45 (d, 1H, *J* = 7.5 Hz), 7.49 (t, 3H, *J* = 7.5 Hz), 7.60 (t, 1H, *J* = 7.5 Hz), 8.02 (d, 1H, *J* = 7.5 Hz), 8.39 (s, 1H). ^{13}C NMR (CD_3OD, 125 MHz): δ 43.3, 47.8, 100.0, 113.0, 120.5, 121.5, 124.3, 125.5, 128.2, 128.7, 129.9, 131.2, 133.4, 138.5, 139.3, 147.3, 148.9. ^{19}F NMR (CD_3OD, 470 MHz), A single peak was detected; MS (FAB): *m*/*z* 288 (M − CF_3SO_3); HRMS (FAB): Calcd for M − CF_3SO_3: $C_{19}H_{18}N_3$ 288.1501, Found 288.1511.

*4.1.16. 4-Methoxycarbonyl-3-benzylamino-β-carboline (**3a**)*

Methyl 4-formylbenzoate was synthesized from 4-formylbenzoic acid. The solution of dry 4-formylbenzoic acid (3 g, 20.00 mmol) in methanol (80 ml) was cooled to 0 °C under argon atmosphere, and thionyl chloride (2.1 ml, 30.00 mmol) was added drop-by-drop to the solution. The mixture was stirred for 1.5 h, and then heated under reflux for 20 h. The reaction mixture was concentrated *in vacuo*, dissolved in water, neutralized with sodium carbonate aqueous solution, and extracted with chloroform. The combined organic phase was dried with Na_2SO_4 to afford methyl 4-formylbenzoate. To **11** (50 mg, 0.27 mmol) in 35 ml methanol was added acetic acid (4.41 mmol) under argon atmosphere. The mixture was then treated with methyl 4-formylbenzoate (134 mg, 0.82 mmol), obtained above, and stirred for 1.5 h at room temperature. Subsequently, $NaBH_3CN$ (172 mg, 2.73 mmol) was added and the mixture was stirred for 12 h. The reaction was quenched by 6 N HCl (33 ml) and the reaction mixture was cooled to 0 °C. NH_3 aq was added to adjust the reaction mixture to pH 10, and then extracted with chloroform. The combined organic fraction was washed with brine, dried with Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica gel chromatography to afford pure **3a**. Yield: 85%; yellow solid. Mp 171.2–173.8 °C ^{1}H NMR ($CDCl_3$, TMS, 500 MHz): δ 3.88 (s, 3H), 4.60 (s, 2H), 6.94 (s, 1H), 7.14 (t, 1H, *J* = 7.5 Hz), 7.34 (d, 1H, *J* = 8.5 Hz), 7.45 (m, 3H), 7.92 (d, 1H, *J* = 7.5 Hz), 7.97 (d, 1H, *J* = 8.5 Hz), 8.37 (s, 1H), 8.53 (br, 1H). ^{13}C NMR ($CDCl_3$, TMS, 125 MHz): δ 47.3, 52.0, 95.6, 111.4, 119.2, 121.2, 121.8, 127.0, 127.1, 128.7, 128.9, 129.9, 130.1, 131.0, 133.0, 135.5, 142.1, 145.1, 152.2, 167.0. MS (FAB): *m*/*z* 332 (M + H); HRMS (FAB): Calcd for $C_{20}H_{18}N_3O_2$:333.1399, Found 332.1404.

*4.1.17. 3-Methoxycarbonyl-3-benzylamino-β-carboline (**3b**)*

Methyl 3-formylbenzoate was synthesized as described previously [24]. The title compound **3b** was synthesized in the same way as that of **3a**. Yield: 86%; yellow solid. Mp 170–173 °C ^{1}H NMR (CD_3OD, TMS, 500 MHz): δ 3.82 (s, 3H, $-OCH_3$), 4.53 (s, 2H, $-OCH_2-$), 4.82 (b, 1H, −NH−), 6.91 (s, 1H, −NH−), 7.10 (dd, 1H, 6-H, *J* = 7.5, 8 Hz), 7.31–7.40 (m, 3H, −Ph-4-H and 7-H, *J* = 8.5, 8, 7.5, 14, 7, 7.4 Hz), 7.58 (d, 1H, −Ph−, *J* = 8 Hz), 7.82 (b, 1H, NH indole), 7.89 (dd, 2H, −Ph− and 8-H, *J* = 8.75 Hz), 8.06 (s, 1H, 5-H), 8.35 (s, 1H, 1-H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 45.2, 52.0, 79.1, 95.7, 111.4, 118.0, 120.4, 121.6, 127.1, 127.9, 128.5, 129.5, 130.5, 131.3, 132.2, 141.9, 142.4, 152.3, 166.3. ^{19}F NMR (CD_3OD, 470 MHz), MS (FAB): *m*/*z* 288 (M − CF_3SO_3); HRMS (FAB): Calcd for M − CF_3SO_3: $C_{19}H_{18}N_3$ 288.1501, Found 288.1511. MS (FAB): *m*/*z* 331 (M + H); Anal. Calcd for $C_{20}H_{17}N_3O_2$: C, 72.49; H, 5.17; N, 12.68; Found C, 72.26; H, 5.26; N, 12.63.

*4.1.18. 3-[4-[2-(N-tert-Butoxycarbonyl)amino]ethoxybenzyl] amino-β-carboline (**3c**)*

4-[2-(*N*-*tert*-Butoxycarbonyl)amino]ethoxybenzaldehyde was synthesized as described previously [13]. The title compound **3c** was synthesized in the same way as that of **3a**. Yield: 66%; yellow solid. Mp 167.8–168.0 °C ^{1}H NMR (CD_3OD, TMS, 500 MHz): δ 1.41

(s, 9H), 3.38 (s, 2H), 3.96 (s, 2H), 4.44 (s, 2H), 6.88 (d, 2H, $J = 8.5$ Hz), 7.09 (t, 1H, $J = 7.5$ Hz), 7.13 (s, 1H), 7.32 (d, 2H, $J = 8.0$ Hz), 7.38 (d, 1H, $J = 8.5$ Hz), 7.41 (d, 1H, $J = 7.5$ Hz), 7.97 (d, 1H, $J = 8.0$ Hz), 8.26 (s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 28.7, 41.0, 47.9, 67.9, 80.2, 97.6, 112.4, 115.6, 119.6, 122.1, 122.7, 129.5, 129.7, 130.7, 132.7, 133.6, 134.1, 144.1, 154.0, 158.5, 159.3. MS (FAB): m/z 433 (M + H); Anal Calcd for $C_{25}H_{28}N_4O_3$: C, 69.42; H, 6.53; N, 12.95; Found C, 68.84; H, 6.76; N, 12.90.

4.1.19. 3-[3-[2-(N-tert-Butoxycarbonyl)amino]ethoxybenzyl] amino-β-carboline (**3d**)

3-[2-(*N-tert*-Butoxycarbonyl)amino]ethoxybenzaldehyde was synthesized as described previously [13]. The title compound **3d** was synthesized in the same way as that of **3a**. Yield: 95%; yellow solid. Mp 163.8–164.2 °C ^{1}H NMR (CD_3OD, TMS, 500 MHz): δ 1.28 (s, 9H), 3.26 (s, 2H), 3.81 (s, 2H), 4.36 (s, 2H), 6.64 (d, 2H, $J = 8.0$ Hz), 6.88 (s, 2H), 6.96 (m, 2H), 7.08 (t, 1H, $J = 8.0$ Hz), 7.26 (d, 1H, $J = 8.0$ Hz), 7.30 (d, 1H, $J = 7.0$ Hz), 7.81 (d, 1H, $J = 8.0$ Hz), 8.15 (s, 1H). ^{13}C NMR ($CDCl_3$, TMS, 125 MHz): δ 28.9, 41.1, 43.9, 61.7, 62.2, 68.0, 80.3, 97.6, 112.6, 114.8, 119.8, 121.1, 122.3, 129.7, 130.6, 131.0, 132.8, 134.3, 143.4, 144.3, 154.1, 158.6, 160.6. MS (FAB): m/z 433 (M + H); Anal Calcd for $C_{25}H_{29}N_3O_5$: C, 68.42; H, 6.53; N, 12.95; Found C, 68.22; H, 6.76; N, 12.77.

4.1.20. 3-Hydroxy-3-benzylamino-β-carboline (**3e**)

The title compound **3e** was synthesized in the same way as that of **3a**. Yield: 74%; brown solid. Mp 204.2–206.8 °C ^{1}H NMR (CD_3OD, TMS, 500 MHz): δ 4.37 (s, 2H), 6.56 (s, 1H), 6.80 (s, 2H), 7.00–7.05 (m, 3H), 7.29 (d, 1H, $J = 8.0$ Hz), 7.33 (d, 1H, $J = 7.0$ Hz), 7.87 (d, 1H, $J = 8.0$ Hz), 8.17 (s, 1H). ^{13}C NMR (CD_3OD, TMS, 125 MHz): δ 97.4, 112.4, 114.8, 115.2, 119.6, 122.2, 129.6, 130.5, 130.7, 132.7, 134.3, 143.1, 144.2, 151.4, 154.0, 158.7. MS (FAB): m/z 290 (M + H); HRMS (FAB): Calcd for M + H $C_{18}H_{16}N_3O_1$; 290.1215, Found 290.1302.

4.2. Anti-tumor activity

4.2.1. Cell lines and culture

HeLa S-3 cells were provided by Dr. Okada (Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology). The cells were maintained in MEM (Nissui) supplemented with 10% FBS (GIBCO), 2% HEPES, 3% NEAA and 2 mM L-glutamine in a water-saturated atmosphere of 5% CO_2 at 37 °C.

4.2.2. Cell viability assay

Cell viability was determined by 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide (MTT) assay, a method for determining cell viability by measuring the mitochondrial dehydrogenase action. Cells were seeded in a 96-well cell culture cluster (Becton Dickinson) at a density of 2×10^4 cells/ml and cultured 3 h prior to drug treatment. Cells were exposed to 3-benzylamino-β-carboline derivatives at 37 °C for 24–72 h. The MTT reagent (Nacalai Tesuque) was prepared at a concentration of 2 mg/ml in Dulbecco's PBS without calcium and magnesium, and stored at 4 °C. After treatment for the indicated times, cells were incubated with MTT reagent for 4 h at 37 °C. The plate was centrifuged at 3000 rpm for 10 min, and the medium was removed. To solubilize the resulting MTT-formazan, 200 μL/well of dimethyl sulfoxide (DMSO) was added to each well, followed by thorough mixing with a mechanical plate mixer. Absorbance at 540 nm was measured on a microplate reader (MTP-500, CORONA), and the percentage of cell viability was taken as the percentage absorbance at 540 nm of β-carboline-treated cells against control cells.

4.3. Pharmacology

4.3.1. Hoechst 33342 staining

The cells were treated with the vehicle alone (0.1% DMSO) and with various concentrations of either compounds for 48 h and stained with Hoechst 33342 (1 μg/ml) for 40 min at 37 °C. The cells were examined for apoptosis under a confocal laser microscopic system (IX71, Olympus), and evaluated by morphological changes of the hallmarks of apoptotic cells. The cells were treated with the vehicle alone (0.1% DMSO) and with each 3-aminobenzyl-β-carboline derivative for 48 h. The concentrations of either **1e**, **1f**, **2c** or **3e** were 26 or 52 μM, 0.6 or 1.2 μM, 2.7 or 5.4 μM and 4.6 or 9.2 μM, respectively.

4.3.2. DNA extraction and agarose-gel electrophoresis

The cells were treated with the vehicle alone (0.1% DMSO) and either compounds for various incubation times of 10, 24 or 48 h, and were then collected by centrifugation. The pellets were washed with PBS, lysed with DNA fragmentation lysis buffer (0.6% Sarcosine, 20 mM Tris–HCl pH7.4 and 20 mM EDTA pH 8.0) and incubated with RNase A (2 mg/ml) for 2 h at 50 °C. Proteinase K (1.2 mg/ml) was subsequently added to the samples overnight at 50 °C. DNA was isolated with neutral-phenol, phenol/chloroform (1:1), and chloroform/isoamyl alcohol (24:1), and subsequently centrifuged at 14,000 rpm for 30 min at 4 °C. The supernatants were electrophoresed on a 2% agarose gel in TAE buffer (40 mM Tris base, 20 mM EDTA and 20 mM acetic acid) at 50 V. The gel was stained with ethidium bromide and photographed with UV illumination.

4.3.3. Western blot analysis

The cells were treated with either **1e**, **1f**, **2c** or **3e**. After treatment, the cells were rinsed in ice-cold PBS and lysed in RIPA buffer consisting of 50 mM Tris–HCl (pH8.0), 150 mM NaCl, 1%NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) and Protease Inhibitor Cocktail (Sigma). The lysate was centrifuged at 12,000 rpm for 5 min to obtain whole cell extract. Subsequently, the whole cell extract were applied to electrophoresis on a polyacrylamide gel containing 0.1% SDS. After electrophoresis, polypeptides were transferred onto the polyviniylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) at 200 mA for 1 h. The membrane was blocked with 5% skim milk in Tris-buffer saline containing 0.05% Tween 20 (TTBS) and then incubated with specific primary polyclonal/monoclonal antibodies, including β-actin (1:5000; AC-15, Abcam), cdc2 (1:1000; C12720, Transduction Laboratories), cdk2 (1:1000; SC-163, Santa Cruz) and cleaved caspase-3 antibodies (1:1000; Asp175, Cell Signaling). The membrane was washed with TTBS and then incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories, Tokyo, Japan). After washing with TTBS, the antigen–antibody complex was visualized with the ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, England).

4.3.4. Cell cycle analysis

The cells were treated with either **1e**, **1f**, **2c** or **3e**. After treatment, the cells were washed twice with ice-cold PBS, collected by centrifugation, and fixed in ice-cold 70% (v/v) ethanol, washed with PBS, re-suspended with 0.1 mg/ml RNase, stained with 40 mg/ml PI, and analyzed by flow cytometry using FACScalibur (Becton Dickinson). The cell cycle distributions were calculated using Cell Quest software (Becton Dickinson).

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