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Synthesis of 6-substituted 9-methoxy-11*H*-indeno[1,2-*c*]quinoline-11-one derivatives as potential anticancer agents

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ABSTRACT

We have synthesized certain 6-substituted 9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-ones for antiproliferative evaluation. Results indicated that 6-alkylamine derivatives, 6-[2-(dimethylamino)ethylamino]-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (**5a**) and its 6-[3-(dimethylamino)propylamino] derivative, **5b**, were able to inhibit cells growth completely at a concentration of 100 μM while most of the 6-arylamine derivatives **6b**–**6h** were inactive at the same concentration. Comparable mean GI_{50} (drug molar concentration causing 50% cell growth inhibition) values for **5a** (3.47 μM) and **5b** (3.39 μM) indicated the cytotoxicity may not be affected by the length of alkyl substituents at C-6 position. Compound **5b**, with a mean GI_{50} value of 3.39 μM, was the most active and therefore was selected for further evaluation on its effects of H460 lung cancer cell cycle distribution. Results indicated that **5b** induced cell cycle arrest in G2/M phase after 24 h treatment, while the hypodiploid (sub-G0/G1 phase) cells increased. We found that H460 cell became shrinked after the treatment of **5b**, indicating that apoptosis may be a mechanism by which **5b** kills the cancer cells. DNA unwinding assay indicated that **5b** may bind to DNA through intercalation. Our results have also demonstrated that PARP was cleaved while caspase-3 and caspase-8 activities were induced after the treatment of **5b** at 10 μM for 24 h. Thus, compound **5b** intercalates DNA, induces cell cycle arrest at G2/M phase via cleavage of PARP, induces caspase-3 and caspase-8 activities, and consequently causes the cell death.

1. Introduction

Quinoline skeleton is present in a large number of biologically active compounds and is frequently condensed with various heterocycles.[1–7] For examples, camptothecin (CPT) is an anticancer alkaloid isolated from *Camptotheca acuminate* which bears a condensed quinoline skeletone.[1,2] The clinical use of CPT is hampered due to its poor water solubility. Subsequent structural optimization led to the discovery of highly water soluble topotecan which is currently used as an anticancer drug. TAS-103, which possesses the tetracyclic indeno[2,1-*c*]quinoline pharmacophore, has been proved to be a dual topoisomerases I and II targeting agent.[8–10] Recently, we have synthesized certain isomeric indeno[1,2-*c*]quinoline derivatives of TAS-103 for anticancer evaluation.[11–14] Among them, 9-methoxy-6-(piperazin-1-yl)-11*H*-indeno[1,2-*c*]quinolin-11-one *O*-3-aminopropyl oxime (**1**), *(E)*-6-hydroxy-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one *O*-2-(pyrrolidin-1-yl)ethyl oxime (**2**), and 2,9-bis[3-(dimethylamino)propoxy]-6-{4-[3-(dimethylamino)propoxy]phenyl}-11*H*-indeno[1,2-*c*]-quinolin-11-one (**3**) exhibited IC_{50} value of 0.64, 0.89, and 0.68 μM, respectively against the growth A549, which was more active than CPT and topotecan (Fig. 1). The present report intends to establish the antiproliferative structure–activity relationships (SAR) of indenoquinoline derivatives by the introduction of various substituents at C-6 position and to identify more potent anticancer drug candidates.

2. Chemistry

Reaction of 2-hydroxy-3-(4-methoxyphenyl)quinoline-4-carboxylic acid with $POCl_3$ afforded 6-chloro-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (**4**),[12] which was reacted with alkyl amines to give 6-[2-(dimethylamino)ethylamino]-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (**5a**) and its 6-[3-(dimethylamino)propylamino] derivative **5b**. Treatment of **4** with the respective arylamine in ethoxyethanol (50 mL) and was heated with stirring under microwave irradiation (150 W) afforded 6-arylamino-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-ones **6a**–**6j** as described in Scheme 1.

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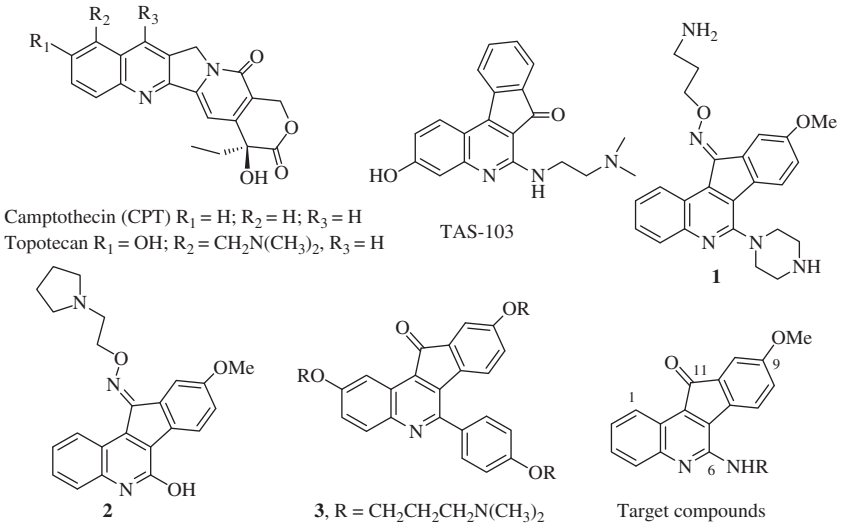
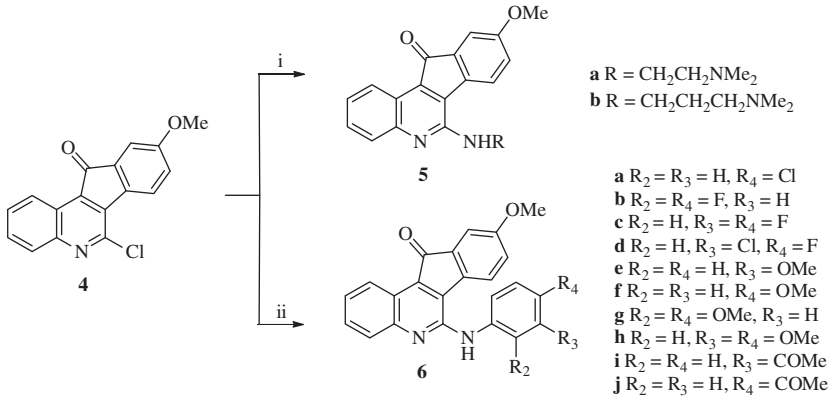
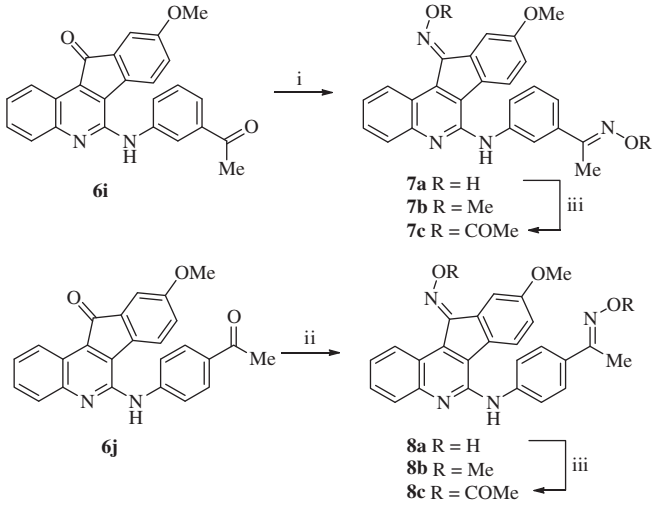


Figure 1. Structures of Camptothecin, Topotecan, 6-[2-(dimethylamino)ethylamino]-3-hydroxy-7*H*-indeno[2,1-*c*]quinolin-7-one (TAS-103), aminoalkyl substituted indenoquinolines **1–3**, and targeted compounds.



Scheme 1. Reagents and conditions: (i) RNH_2, reflux, 8 h (42% for 5**3a**, 35% for 5**b**); (ii) $ArNH_2$, MW, 1 h (32–87%).

The preparation of (*E*)-6-{{3-[(*E*)-1-(hydroxyimino)ethyl] phenyl}amino}-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one oxime (**7a**) and its positional isomer **8a** are described in Scheme 2.



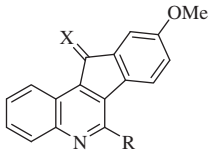
Scheme 2. Reagents and conditions: (i) NH_2OH, MW, 0.5 h (67–69%); (ii) NH_2OMe, MW, 0.5 h (78–81%); (iii) Ac_2O, pyridine, 1 h (82–86%).

Reaction of 6-(3-acetylphenylamino)-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (**6i**) with NH_2OH or NH_2OMe gave exclusively *E*-form isomer of oxime **7a** or *O*-methyloxime **7b**, respectively.[6,13] Acetylation of **7a** with acetic anhydride afforded (*E*)-6-{{3-[(*E*)-1-(acetoxyimino)ethyl]phenyl}amino}-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one *O*-acetyloxime (**7c**) in 86% yield. Accordingly, compounds **8a**–**8c** were prepared from **6j** under similar reaction conditions.

3. Results and discussion

All compounds were evaluated in vitro against a 3-cell line panel consisting of NCI-H460 (Lung), MCF7 (Breast), and SF-268 (CNS). In this protocol, each cell line is inoculated and preincubated on a microtiter plate. Test agents are then added at a single concentration (100 μM) and the culture incubated for 48 h. End-point determinations are made with alamar blue.[15] Results for each test agent are reported as the percent of growth of the treated cells when compared to the untreated control cells. Compounds which reduced the growth percentage of any one of the cell lines to 32% or less are passed on for evaluation in the full panel of 60 cell lines over a 5-log dose range. Results from Table 1 indicated 6-alkylamine derivatives **5a** and **5b** were able to inhibit cells growth completely while most of the 6-arylamine derivatives **6b**–**6g** were inactive at the same concentration. Compounds **6a**, **6i**, and **6j**

Table 1
Antiproliferative activity of 6-substituted 9-methoxyindeno[1,2-*c*]quinoline derivatives



Compd	R	X	Growth percentages[a]			Mean GI_{50}[b] (μM)
			NCI-H460 (lung)	MCF7 (breast)	SF-268 (CNS)	
5a	$NHCH_2CH_2NMe_2$	O	0	0	6	3.47
5b	$NHCH_2CH_2CH_2NMe_2$	O	0	0	0	3.39
6a	NHPh-4-Cl	O	10	96	42	15.14
6b	NHPh-2,4-F	O	96	99	68	Nd[c]
6c	NHPh-3,4-F	O	96	106	69	Nd
6d	NHPh-3-Cl-4-F	O	Nd	Nd	Nd	Nd
6e	NHPh-3-OMe	O	83	79	87	Nd
6f	NHPh-4-OMe	O	69	74	65	Nd
6g	NHPh-2,4-OMe	O	110	103	107	Nd
6h	NHPh-3,4-OMe	O	68	90	41	Nd
6i	NHPh-3-COMe	O	0	17	7	12.88
6j	NHPh-4-COMe	O	18	61	11	39.81
7a	NHPh-3-C(=NOH)Me	NOH	11	84	66	28.18
7b	NHPh-3-C(=NOMe)Me	NOMe	82	80	92	Nd
7c	NHPh-3-C(=NOCOMe)Me	NOAc	96	32	43	53.70
8a	NHPh-4-C(=NOH)Me	NOH	0	1	2	4.17
8b	NHPh-4-C(=NOMe)Me	NOMe	112	89	108	Nd
8c	NHPh-3-C(=NOCOMe)Me	NOAc	37	24	36	52.48

[a] Data obtained from NCI's in vitro disease-oriented tumor cell screen. In this protocol, each cell line is inoculated and preincubated on a microtiter plate. Test agents are added at a single concentration (100 μM) and the culture is incubated for 48 h. End-point determinations are made with alamar blue. Results for each test agent are reported as the percent of growth of the treated cells when compared to the untreated control cells. Compounds which reduced the growth percentage of any one of the cell lines to 32% or less are active.

[b] Mean values over all cell lines tested. Theses cell lines are: leukemia (CCRF-CEM, HL-60 (TB), K-562, MOLT-4, PRMI-8226, and SR); non-small cell lung cancer (A549/ATCC, EKVX, HOP-62, HOP-92, NCI-H226, NCI-H23, NCI-H322M, and NCI-H522); colon cancer (COLC 205, HCC-2998, HCT-116, HCT-15, HT29, KM12, and SW-620); CNS cancer (SF-268, SF-295, SF-539, SNB-19, SNB-75, and U251); melanoma (LOX IMVI, MALME-3M, M14, SK-MEL-2, SK-MEL-28, SK-MEL-5, and UACC-257); ovarian cancer (IGROV1, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, and SK-OV-3); renal cancer (786-0, A498, ACHN, CAKI-1, RXF 393, SN12C, TK-10, and UO-31); prostate cancer (PC-3 and DU-145); and breast cancer (MCF7, MCF7/ADR-RES, MDA-MB-231/ATCC, HS 578T, MDA-MB-435, MDA-N and T-47D); GI_{50}: Drug molar concentration causing 50% cell growth inhibition.

[c] Not determined.

exhibited only marginal antiproliferative activity. Therefore, an alkylamino side chain at C-6 position is crucial for cytotoxicity. Those active compounds were evaluated in the full panel of 60 human tumor cell lines derived from nine cancer cell types (leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer). For each compound, dose–response curves for each cell line were measured with five different drug concentration, the concentration causing 50% cell growth inhibition (GI_{50}) compared with the control were calculated.[16] Comparable mean GI_{50} values for **5a** (mean GI_{50} = 3.47 μM) and **5b** (mean GI_{50} = 3.39 μM) indicated the cytotoxicity may not be affected by the length of alkyl substituents at C-6 position. However, the C-6 arylamine derivatives of these 9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-ones demonstrated only weakly active or inactive in which **6a**, **6i**, and **6j** exhibited mean GI_{50} values of 15.14, 12.88, and 39.81 μM, respectively.

The antiproliferative activity of 6,11-disubstituted 9-methoxyindeno[1,2-*c*]quinoline derivatives is summarized in Table 1. Compound **7a** was weakly active with a mean GI_{50} value of 28.18 μM while its methyl derivative **7b** and acetoxy derivative **7c** were inactive indicated that a hydrogen-donating group at C-11 is essential for cytotoxicity. The same trend was observed for its positional isomers in which (*E*)-6-{{4-[(*E*)-1-(hydroxyimino)ethyl]phenyl}amino}-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one oxime (**8a**) was active against the growth of all three cell lines tested while its methyl derivative **8b** and acetoxy derivative **8c** were inactive. Compound **8a** (with a mean GI_{50} value of 4.17 μM) was more active than **7a** implied the position of substitution is crucial for cytotoxicity.

Cytotoxicity of selected 9-methoxy-11*H*-indeno[1,2-*c*]quinoline-11-one derivatives is summarized in Table 2. 6-[3-(Dimethylamino)propylamino]-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (**5b**) was the most active with a mean GI_{50} value of 3.39 μM and inhibited the growth of SR, A549, H460, HCT-116, and U251 with GI_{50} values of less than 1.86 μM in each case. Therefore, compound **5b** was selected for further evaluation on its effects of H460 cell cycle distribution by flow cytometric analysis. Compound **5b** induced cell cycle arrest in a concentration dependent manner as shown in Figure 2 and Table 3. The proportion of cells was decreased in the G1 and accumulated in G2/M phase after

Table 2
Cytotoxicity of selected 9-methoxy-11*H*-indeno[1,2-*c*]quinoline-11-one derivatives [GI_{50} (μM)][a]

Cell lines	GI_{50} (μM)		
	5b	**6i**	**8a**
SR (Leukemia)	1.10	21.38	1.48
A549 (Lung)	1.86	17.70	1.45
H460 (Lung)	1.33	23.50	29.50
HCT-116 (colon)	1.26	2.63	1.48
U251 (CNS)	1.10	3.09	2.29
SK-MEL-5 (Melanoma)	3.89	5.25	3.80
IGROV1 (Ovarian)	6.92	3.63	15.14
RXF 393 (Renal)	Nd[b]	9.77	<0.01
DU-145 (Prostate)	6.61	11.75	2.04
HS 578t (Breast)	3.72	3.16	7.08

[a] GI_{50}: Drug molar concentration causing 50% cell growth inhibition.

[b] Not determined.

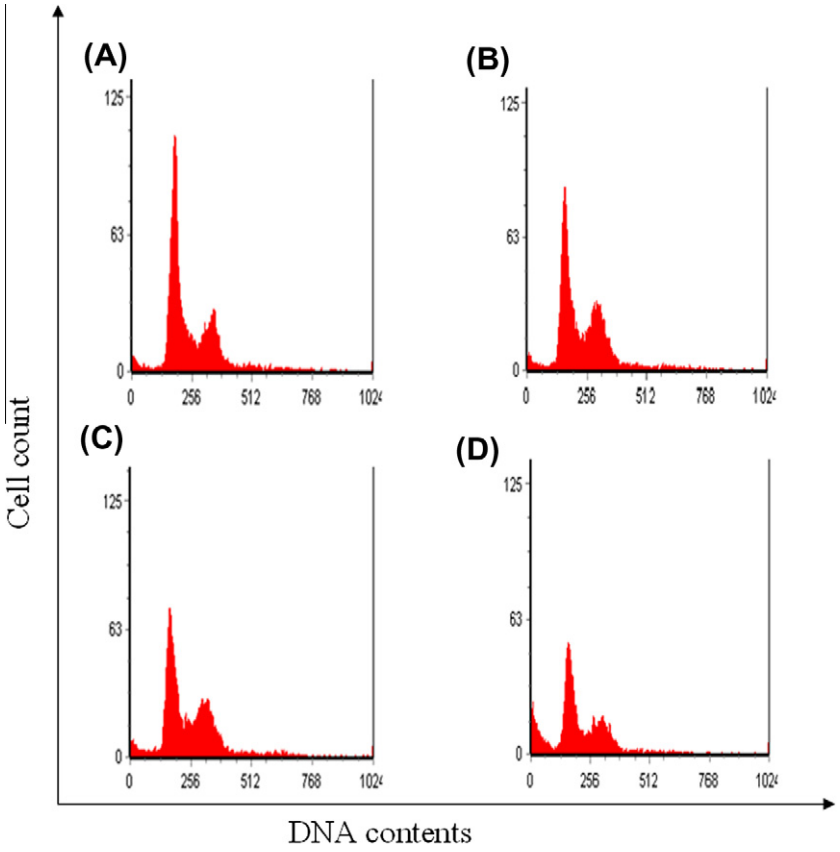


Figure 2. Flow cytometric analysis of H460 cells. Cells were treated with DMSO (A), compound **5b** at 1.0 μM (B), 5.0 μM (C) or 10.0 μM (D) in H460 cells; 24 h later the cells were harvested, fixed, and stained with propidium iodide as described in Section 5 prior to analysis by flow cytometry. The percentage of cells in each cell cycle phase was quantified (Table 3).

Table 3
Effects of **5b** on the cell cycle progression of lung cancer cell (H460)

Concentration (μM)	Cell cycle distribution[a] (%)			
	Sub G1	G1	S	G2/M
DMSO	0.9 ± 0.3	66.2 ± 2.1	14.3 ± 1.9	18.6 ± 1.7
1.0	3.7 ± 1.2	58.1 ± 3.5	10.1 ± 1.5	28.1 ± 2.2
5.0	8.2 ± 1.6	49.2 ± 1.7	9.1 ± 2.5	33.5 ± 2.3
10.0	25.3 ± 3.7	41.4 ± 2.2	11.8 ± 1.4	21.5 ± 1.8

[a] Values representative mean ± SD from three experiments.

24 h treatment of **5b**, while the hypodiploid (sub-G0/G1 phase) cells increased (Table 3). Apoptosis can be characterized by morphological and biochemical changes in the cell nucleus, including chromatin condensation and nuclear shrinking. Morphological changes of cells treated with **5b** can be visually observed with light microscopy (Fig. 3). We found that the H460 cell became shrinked after the treatment of **5b** at 10 μM for 24 h. Such morphological changes were not apparent in the control cells. These findings indicated that apoptosis may be a mechanism by which **5b** kills the cancer cells. In order to evaluate if the antiproliferative effect of

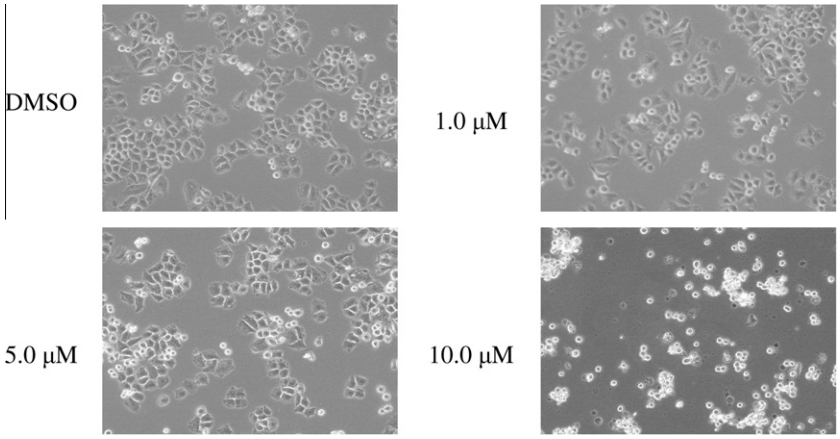


Figure 3. Induction of morphological change in H460 cells. Cells were treated with DMSO or compound **5b** (1.0–10.0 μM) for 24 h at 37 °C and photographed under a microscope.

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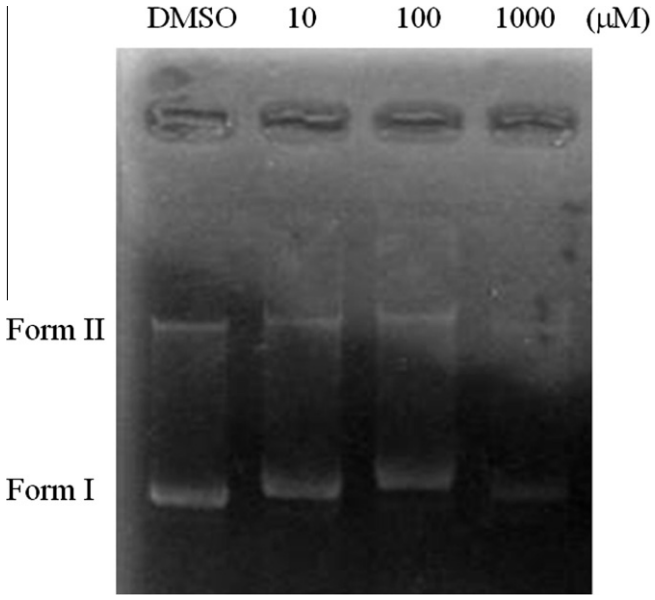

Figure 4. Compound **5b** unwind plasmid DNA. Increasing concentration of compound **5b** were incubated with negatively supercolied pBR322 (0.4 μg) for 12 h in TE buffer and then run for 2.5 h on a 1% gel.

5b was a consequence of DNA intercalation, we performed an in vitro DNA unwinding assay. DNA intercalators enhance the unwinding of negatively supercoiled DNA (form I), which leads to a decreased mobility in agarose gel during electrophoresis.[17] As shown in Figure 4, it seems that compound **5b** between 0 and 100 μM exhibited a dosage response, but not 1000 μM. The plasmid DNA at this concentration cannot be clearly seen. Further, the mechanism of shift in gel mobility can also be due to the formation of huge complexes. These data indicate that **5b** may bind to DNA through intercalation. To determine whether **5b** preferentially kills cancer cells by apoptosis, procaspase-3, procaspase-8, and PARP were evaluated in **5b**-treated H460 cells by western blotting. Caspases are the family of cysteine proteases and they mediate apoptotic pathway in mammalian.[18] PARP, a nuclear poly (ADP-ribose) polymerase, is involved in DNA repair predominantly in response to environmental stress, and is important for the maintenance of cell viability.[19] Our results have shown that PARP was cleaved while caspase-3 and caspase-8 activities were induced after the treatment of **5b** for 24 h in a concentration-dependent manner (Fig. 5). Thus, compound **5b** may intercalate DNA, induces cell cycle arrest at G2/M phase via cleavage of PARP, induces caspase-3 and caspase-8 activities, and consequently causes the cell death.

4. Conclusion

A number of 6-substituted 9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-ones were synthesized and evaluated for their antiproliferative activities. Among them, compound **5b** was the most active with a mean GI_{50} value of 3.39 μM and therefore was selected as a new lead for further mechanism studies. Results indicated that **5b** induced cell cycle arrest in G2/M phase after 24 h treatment, while the hypodiploid (sub-G0/G1 phase) cells increased. DNA unwinding assay indicated that **5b** may bind to DNA through intercalation. In addition, PARP was cleaved while caspase-3 and caspase-8 activities were induced after the treatment of **5b** for 24 h in a concentration-dependent manner. Thus, compound **5b** may intercalate DNA, induces cell cycle arrest at G2/M phase via cleavage of PARP, induces caspase-3 and caspase-8 activities and consequently to cause the cell death. Further study on the structure optimization of **5b** is ongoing.

5. Experimental

5.1. General

TLC: precoated (0.2 mm) Silica Gel 60 F_{254} plates from EM Laboratories, Inc.; detection by UV light (254 nm). All chromatographic separations were performed using silica gel (Merck 60 230–400 mesh). Mp: Yamato MP-21 melting-point apparatus; uncorrected. ^{1}H and ^{13}C NMR spectra: Varian-Unity-400 spectrometer at 400 and 100 MHz, chemical shifts in ppm with $SiMe_4$ as an internal standard (=0 ppm), coupling constants *J* in Hz. Mass spectra (HRMS) were recorded on Finnigan/Thermo Quest MAT 95XL. Elemental analyses were carried out on a Heraeus CHN-O-Rapid elemental analyzer, and results were within ±0.4% of calculated values. Microwave reactions were conducted using a CEM Discover Synthesis Unit (CEM Corp., Matthews, NC).

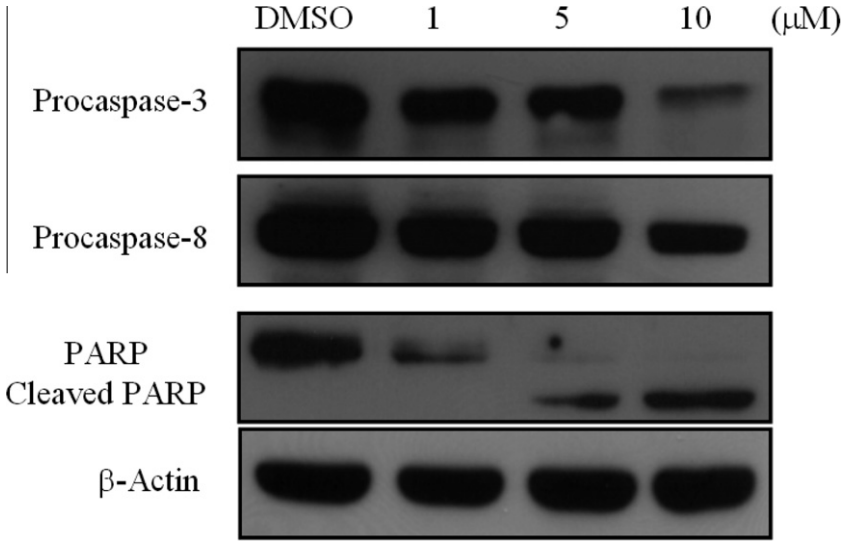

Figure 5. Expression of procapase-3, -8 and PARP in H460 cells by treatment with compound **5b** for 24 h. Equal amounts of cell lysate were resolved using SDS–PAGE and analyzed by western blot using anti-procaspase-3, -8, PARP and β-actin antibody to confirm equal protein loading.

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5.1.1. 6-[2-(Dimethylamino)ethylamino]-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (5a)

A mixture of 6-chloro-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (**4**, 0.30 g, 1 mmol) and *N,N*-dimethylethylenediamine (0.26 g, 3 mol) in 2-ethoxyethanol (30 mL) was refluxed for 8 h (by TLC monitoring). The mixture was then cooled and evaporated in vacuo to give a residue which was treated with H_2O (50 mL). The resulting precipitate was filtered and washed with H_2O. The crude product was chromatographed on a column of silica gel using CH_2Cl_2/MeOH = 10:1 to give 0.15 g (42%) of **5a** as a red solid. Mp: 168–169 °C (dec). ^{1}H NMR (400 MHz, $CDCl_3$): 2.36 (s, 6H, $N(CH_3)_2$), 2.67 (t, 2H, J = 6.0 Hz, $NHCH_2CH_2N$), 3.71 (q, 2H, J = 6.0 Hz, $NHCH_2CH_2N$), 3.85 (s, 3H, 9-OCH_3), 5.84 (br s, 1H, NH), 6.88 (dd, 1H, J = 8.4, 2.4 Hz, 8-H), 7.21 (d, 1H, J = 2.4 Hz, 10-H), 7.26–7.33 (m, 2H, 2- and 7-H), 7.46–7.50 (m, 1H, 3-H), 7.67 (d, 1H, J = 8.4 Hz, 4-H), 8.59 (dd, 1H, J = 8.0, 1.2 Hz, 1-H). ^{13}C NMR (100 MHz, $CDCl_3$): 38.69, 45.19 (2C), 55.79, 57.66, 111.61, 118.18, 118.96, 122.20, 123.83, 124.37, 126.40, 129.17, 129.47, 134.13, 134.44, 135.41, 149.34, 152.62, 160.44, 195.49. HRMS (ESI) calcd for $C_{21}H_{22}N_3O_2$ $[M+H]^+$ 348.1712; found 348.1709.

5.1.2. 6-[3-(Dimethylamino)propylamino]-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (5b)

Compound **5b** was obtained from **4** and 3-dimethylaminopropylamine as described for the preparation of **5a** in 72% yield. (0.13 g, 35%) as a red solid. Mp: 113–114 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 1.86 (m, 2H, $NHCH_2CH_2CH_2N$) 2.33 (s, 6H, $N(CH_3)_2$), 2.57 (t, 2H, J = 5.6 Hz, $NHCH_2CH_2CH_2N$), 3.77 (q, 2H, J = 5.6 Hz, $NHCH_2CH_2CH_2N$), 3.85 (s, 3H, 9-OCH_3), 6.81 (dd, 1H, J = 8.4, 2.8 Hz, 8-H), 7.17 (br s, 1H, 6-NH), 7.19 (d, 1H, J = 2.8 Hz, 10-H), 7.23–7.27 (m, 1H, 2-H), 7.43–7.48 (m, 2H, 3-H and 7-H), 7.67 (d, 1H, J = 8.4 Hz, 4-H) 8.58 (dd, 1H, J = 8.0, 1.6 Hz, 1-H). ^{13}C NMR (100 MHz, $CDCl_3$): 24.99, 42.98, 45.75 (2C), 55.69, 60.04, 111.09, 117.94, 118.69, 122.04, 123.71, 123.97, 126.26, 129.07, 129.26, 133.92, 134.67, 135.31, 149.39, 152.32, 160.24, 195.68. HRMS (EI) calcd for $C_{22}H_{23}N_3O_2$ $[M]^+$ 361.1790; found 361.1788.

5.2. General procedure for coupling of substituted-anilines derivatives 6a–6j

A mixture of **4** (1.0 mmol), substituted aniline (3.0 mmol), and K_2CO_3 (0.62 g, 4.5 mmol) in ethoxyethanol (50 mL) was heated with stirring under microwave irradiation (150 W) for 1 h (TLC monitoring). The mixture was cooled, concentrated**,** and triturated with water. The resulting precipitate was collected and crystallized from MeOH.

5.2.1. 6-(4-Chlorophenylamino)-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (6a)

Red solid (54%); mp: 241–242 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 3.83 (s, 3H, 9-OCH_3), 7.04 (dd, 1H, J = 8.4, 2.8 Hz, 8-H), 7.14 (d, 1H, J = 2.8 Hz, 10-H), 7.35–7.42 (m, 3H, 2- and Ar-H), 7.52–7.57 (m, 1H, 3-H), 7.62 (d, 1H, J = 8.4 Hz, 7-H), 7.73–7.76 (m, 2H, Ar-H), 7.78 (d, 1H, J = 8.4 Hz, 4-H), 8.48 (dd, 1H, J = 8.4, 1.6 Hz, 1-H), 8.62 (s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$): 55.82, 111.00, 118.54, 119.63, 121.87 (2C), 122.94, 125.08, 125.44, 126.05, 126.89, 128.25 (2C), 129.76, 130.40, 133.07, 134.37, 134.52, 139.93, 147.70, 149.20, 160.43 (2C), 194.52. Anal. Calcd for $C_{23}H_{15}ClN_2O_2 \cdot 0.1H_2O$: C, 71.06; H, 3.95; N, 7.21. Found: C, 70.82; H, 3.91; N, 7.09.

5.2.2. 6-(2,4-Diflourophenylamino)-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (6b)

Red solid (55%); mp: 225–226 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 3.87 (s, 3H, 9-OCH_3), 7.12–7.16 (m, 2H, 2- and 8-H), 7.21 (d, 1H, J = 2.4 Hz, 10-H), 7.33–7.42 (m, 2H, 3- and Ar-H), 7.49–7.55 (m, 2H, 7- and Ar-H), 7.71–7.77 (m, 1H, Ar-H), 7.93 (d, 1H, J = 8.0 Hz, 4-H), 8.33 (br s, 1H, NH), 8.52 (d, 1H, J = 8.4 Hz, 1-H). ^{13}C NMR (100 MHz, DMSO-d_6): 55.80, 103.86, 104.12, 104.37, 110.76, 111.01, 111.19, 118.58, 119.31, 122.87, 124.72, 125.73, 126.79, 129.52, 129.64, 133.12, 134.13, 134.49, 147.82, 149.82, 160.43, 194.57. Anal. Calcd for $C_{23}H_{14}F_2N_2O_2$: C, 71.13; H, 3.63; N, 7.21. Found: C, 70.75; H, 3.93; N, 7.20.

5.2.3. 6-(3,4-Difluorophenylamino)-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (6c)

Red solid (78%); mp: 240–241 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 3.85 (s, 3H, 9-OCH_3), 7.07 (dd, 1H, J = 8.4, 2.8 Hz, 8-H), 7.17 (d, 1H, J = 2.8 Hz, 10-H), 7.36–7.45 (m, 2H, 2- and Ar-H), 7.51–7.59 (m, 2H, 3- and Ar-H), 7.64 (dd, 1H, J = 8.4, 2.0 Hz, 4-H), 7.82 (d, 1H, J = 8.4 Hz, 7-H), 7.90 (ddd, 1H, J = 13.2, 7.8, 2.4 Hz, Ar-H), 8.50 (dd, 1H, J = 8.0, 1.2 Hz, 1-H), 8.67 (br s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 55.82, 109.19 (J = 21.2 Hz), 111.04, 116.49 (J = 9.1, 3.1 Hz), 116.90 (J = 17.4 Hz), 118.57, 119.65, 122.92, 125.07, 126.15, 126.91, 129.82, 130.23, 132.96, 134.43, 134.51, 137.97 (J = 8.3 Hz), 144.58 (J = 238.0, 12.9 Hz), 147.57, 149.05, 148.90 (J = 240.2, 12.8 Hz), 160.47, 194.46. Anal. Calcd for $C_{23}H_{14}F_2N_2O_2$: C, 71.13; H, 3.63; N, 7.21. Found: C, 70.77; H, 3.87; N, 7.18.

5.2.4. 6-(3-Chloro-4-fluorophenylamino)-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (6d)

Red solid (40%); mp: 235–236 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 3.85 (s, 3H, 9-OCH_3), 7.06 (dd, 1H, J = 8.0, 2.4 Hz, 8-H), 7.16 (d, 1H, J = 2.4 Hz, 10-H), 7.37 (d, 1H, J = 9.2 Hz, Ar-H), 7.40–7.44 (m, 1H, 2-H), 7.54–7.62 (m, 2H, 3- and 4-H), 7.74 (ddd, 1H, J = 8.8, 4.0, 2.4 Hz, Ar-H), 7.83 (d, 1H, J = 8.0 Hz, 7-H), 7.97 (dd, 1H, J = 6.8, 2.4 Hz, Ar-H), 8.49 (dd, 1H, J = 8.4, 1.6 Hz, 1-H), 8.62 (br s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 55.84, 111.06, 116.41 (J = 22.0 Hz), 118.58, 118.71 (J = 24.2 Hz), 119.66, 120.75 (J = 6.8 Hz), 120.78, 121.82, 122.95, 125.09, 126.13, 126.86, 129.85, 130.20, 132.96, 134.47 (J = 8.3 Hz), 138.13, 147.56, 149.10, 152.50 (J = 239.5 Hz), 160.49, 194.48. Anal. Calcd for $C_{23}H_{14}ClFN_2O_2$: C, 68.24; H, 3.49; N, 6.92. Found: C, 67.91; H, 3.68; N, 6.84.

5.2.5. 6-(3-Methoxyphenylamino)-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (6e)

Purple solid (32%); mp: 113–114 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 3.79 (s, 3H, Ar-OCH_3), 3.85 (s, 3H, 9-OCH_3), 6.61 (dd, 1H, J = 8.0, 2.4 Hz, Ar-H), 7.07 (dd, 1H, J = 8.0, 2.4 Hz, 8-H), 7.18 (d, 1H, J = 2.4 Hz, 10-H), 7.22–7.32 (m, 2H, Ar-H), 7.40–7.44 (m, 1H, 2-H), 7.47–7.48 (m, 1H, Ar-H), 7.55–7.60 (m, 1H, 3-H), 7.655 (d, 1H, J = 8.4 Hz, 4-H), 7.78 (d, 1H, J = 8.0 Hz, 7-H), 8.51 (d, 1H, J = 8.0 Hz, 1-H), 8.54 (br s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 54.97, 55.84, 105.73, 107.65, 111.03, 112.51, 118.58, 119.55, 122.96, 125.10, 126.00, 126.92, 129.19, 129.80, 130.57, 133.20, 134.34, 134.58, 142.19, 147.85, 149.45, 159.55, 160.43, 194.65. Anal. Calcd for $C_{24}H_{18}N_2O_3$: C, 75.38; H, 4.74; N, 7.33. Found: C, 75.28; H, 4.74; N, 7.32.

5.2.6. 6-(4-Methoxyphenylamino)-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (6f)

Purple solid (42%);mp: 225–226 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 3.78 (s, 3H, Ar-OCH_3), 3.91 (s, 3H, 9-OCH_3), 6.93–6.97 (m, 2H, Ar-H), 7.18 (dd, 1H, J = 8.4, 2.4 Hz, 8-H), 7.18 (d, 1H, J = 2.4 Hz, 10-H), 7.35–7.39 (m, 1H, 2-H), 7.50–7.57 (m, 2H, 3- and 4-H), 7.62–7.66 (m, 2H, Ar-H), 7.87 (d, 1H, J = 8.4 Hz, 7-H), 8.30 (br s, 1H, NH), 8.49 (d, 1H, J = 8.4 Hz, 1-H). ^{13}C NMR (100 MHz, DMSO-d_6): 55.22, 55.83, 111.08, 113.65 (2C), 118.43, 119.21, 122.91, 122.99 (2C), 124.92, 125.33, 126.63, 129.62, 129.99, 133.24, 133.68, 134.06, 134.56, 148.00, 150.08, 154.99, 160.36, 194.79. Anal. Calcd for $C_{24}H_{18}N_2O_3 \cdot 0.1H_2O$: C, 75.02; H, 4.78; N, 7.29. Found: C, 74.79; H, 4.78; N, 7.20.

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5.2.7. 6-(2,4-Dimethoxyphenylamino)-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (6g)

Purple solid (68%); mp: 240–241 °C. ^{1}H NMR (400 MHz, DMSO-d_6 + TFA-*d*): 3.85 (s, 3H, 9-OCH_3), 3.92 (s, 6H, Ar-OCH_3), 6.79 (d, 1H, *J* = 8.8 Hz, Ar-H), 6.88 (s, 1H, Ar-H), 7.16 (d, 1H, *J* = 6.8 Hz, 8-H), 7.35 (s, 1H, 10-H), 7.51 (d, 1H, *J* = 8.4 Hz, Ar-H), 7.57–7.60 (m, 1H, 2-H), 7.71–7.74 (m, 1H, 3-H), 8.09 (d, 1H, *J* = 8.0 Hz, 7-H), 8.26 (d, 1H, *J* = 8.0 Hz, 4-H), 8.75 (d, 1H, *J* = 7.8 Hz, 1-H). ^{13}C NMR (100 MHz, DMSO-d_6 + TFA): 55.97, 56.37, 56.41, 106.48, 114.48, 114.33, 117.17, 119.31, 120.02, 124.87, 125.23, 124.87, 126.05, 127.67, 130.12, 131.21, 132.41, 133.09, 134.49, 138.28, 138.49, 156.72, 162.26, 162.33, 193.15. Anal. Calcd for $C_{25}H_{20}N_2O_4$: C, 72.80; H, 4.89; N, 6.79. Found: C, 72.59; H, 4.94; N, 6.73.

5.2.8. 6-(3,4-Dimethoxyphenylamino)-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (6h)

Purple solid (32%). Mp: 166–167 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 3.78 (s, 3H, Ar-OCH_3), 3.80 (s, 3H, Ar-OCH_3), 3.88 (s, 3H, 9-OCH_3), 6.95 (d, 1H, *J* = 8.8 Hz, Ar-H), 7.07 (dd, 1H, *J* = 8.0, 2.4 Hz, 8-H), 7.19 (d, 1H, *J* = 2.4 Hz, 10-H), 7.28 (dd, 1H, *J* = 8.8, 2.4 Hz, Ar-H), 7.37–7.41 (m, 1H, 2-H), 7.51 (d, 1H, *J* = 2.4 Hz, Ar-H), 7.53–7.57 (m, 1H, 3-H), 7.61 (d, 1H, *J* = 8.0 Hz, 4-H), 7.88 (d, 1H, *J* = 8.0 Hz, 7-H), 8.42 (br s, 1H, NH), 8.50 (d, 1H, *J* = 8.4 Hz, 1-H). ^{13}C NMR (100 MHz, DMSO-d_6): 55.43, 55.83, 55.84, 106.71, 111.06, 112.06, 113.15, 118.47 (2C), 125.23, 126.72, 127.16, 129.83 (2C), 130.58, 132.99, 133.59, 134.23, 134.46, 144.68, 148.55, 149.72, 160.40 (2C), 194.57. Anal. Calcd for $C_{25}H_{20}N_2O_4 \cdot 0.4H_2O$: C, 71.54; H, 5.01; N, 6.67. Found: C, 71.30; H, 4.94; N, 6.60.

5.2.9. 6-(3-Acetylphenylamino)-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (6i)

Purple solid (82%); mp: 174–175 °C. ^{1}H NMR (400 MHz,$CDCl_3$): 2.66 (s, 3H, C(=O)CH_3), 3.85 (s, 3H, 9-OCH_3), 6.80 (br s, 1H, NH), 6.87 (dd, 1H, *J* = 8.0, 2.4 Hz, 8-H), 7.19 (d, 1H, *J* = 2.4 Hz 10-H), 7.28 (d, 1H, *J* = 8.4 Hz, Ar-H), 7.35–7.39 (m, 1H, 2-H), 7.46 (t, 1H, *J* = 8.0 Hz, Ar-H), 7.51–7.55 (m, 1H, 3-H), 7.64–7.66 (m, 1H, Ar-H), 7.72 (d, 1H, *J* = 8.0 Hz, 7-H), 8.07 (dd, 1H, *J* = 8.0, 1.2 Hz, 4-H), 8.25–8.26 (m, 1H, Ar-H), 8.60 (d, 1H, *J* = 8.4 Hz, 1-H). ^{13}C NMR (100 MHz,$CDCl_3$): 26.33, 55.82, 111.89, 118.47, 118.88, 120.20, 122.26, 122.68, 123.79, 123.97, 126.26, 127.26, 129.26, 129.97, 133.50, 135.19, 135.37, 137.83, 140.49, 148.35, 148.67, 148.75, 160.85, 194.49, 198.15. Anal. Calcd for $C_{25}H_{18}N_2O_3 \cdot 0.1H_2O$: C, 75.77; H, 4.64; N, 7.07. Found: C, 75.66; H, 4.67; N, 7.06.

5.2.10. 6-(4-Acetylphenylamino)-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one (6j)

Purple solid (84%); mp: 251–252 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 2.54 (s, 3H, C(=O)CH_3), 3.84 (s, 3H, 9-OCH_3), 7.07 (dd, 1H, *J* = 8.4, 2.8 Hz, 8-H), 7.18 (d, 1H, *J* = 2.8 Hz, 10-H), 7.45–7.49 (m, 1H, 2-H),, 7.58–7.62 (m, 1H, 3-H), 7.69 (d, 1H, *J* = 8.0 Hz, 4-H), 7.73 (d, 1H, *J* = 8.4 Hz, 7-H), 7.79 (d, 2H, *J* = 8.8 Hz, Ar-H), 7.94 (d, 2H, *J* = 8.8 Hz, Ar-H), 8.52 (dd, 1H, *J* = 8.0, 0.8 Hz, 1H), 9.09 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 26.33, 55.82, 110.97, 118.25 (2C), 118.72, 120.05, 122.96, 125.23, 126.72, 127.16, 129.37 (2C), 129.90, 130.06, 131.04, 133.05, 134.56, 134.69, 145.90, 147.69, 148.56, 160.52, 194.39, 195.16. Anal. Calcd for $C_{25}H_{18}N_2O_3 \cdot 0.7H_2O$: C, 73.76; H, 4.81; N, 6.88. Found: C, 73.49; H, 4.86; N, 6.86.

5.2.11. (*E*)-6-{{3-[(*E*)-1-(Hydroxyimino)ethyl]phenyl}amino}-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one oxime (7a)

To a suspension of **6i** (0.40 g, 1.0 mmol) in ethoxyethanol (30 mL) was added $NH_2OH \cdot HCl$ (0.20 g, 3.0 mmol). The reaction mixture was heated with stirring under microwave irradiation (100 W) for 30 min (TLC monitoring). The solvent was removed in vacuo and the residue was poured into H_2O (20 mL). The resulting precipitate was collected, washed with H_2O, and dried to give a crude solid, which was purified by crystallization from MeOH to give **7a** (1.39 g, 67%). Mp: 239–240 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 2.19 (s, 3H, CH_3), 3.85 (s, 3H, 9-OCH_3), 7.10 (dd, 1H, *J* = 8.4, 2.4 Hz, 8-H), 7.29–7.42 (m, 4H), 7.54–7.7.58 (m, 1H, 3-H), 7.66 (d, 1H, *J* = 8.0 Hz), 7.73 (d, 1H, *J* = 7.6 Hz), 7.98 (d, 1H, *J* = 8.4 Hz, 7-H), 8.06 (d, 1H, *J* = 2.0 Hz), 8.64 (s, 1H, NH), 8.75 (d, 1H, *J* = 8.4 Hz, 1-H), 11.18 (s, 1H, NOH), 13.54 (s, 1H, NOH). ^{13}C NMR (100 MHz, DMSO-d_6): 11.81, 55.68, 115.14, 115.25, 117.60, 119.15, 120.45, 120.54, 124.03, 124.70, 124.91, 125.17, 127.27, 128.54, 129.09, 129.96, 130.71, 137.38, 138.34, 141.50, 146.35, 149.41, 153.20, 153.83, 159.72. HRMS (EI) calc. for $C_{25}H_{20}N_3O_4$ $[M]^+$ 424.1535; found 424.1538.

5.2.12. (*E*)-9-Methoxy-6-{{3-[(*E*)-1-(methoxyimino)ethyl]phenyl}amino}-11*H*-indeno[1,2-*c*]quinolin-11-one *O*-methyloxime (7b)

Compound **7b** was obtained from **6i** and 40% *O*-methylhydroxylamine hydrochloride according to the preparation of **7a** in 69% yield. Mp: 143–144 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 2.21 (s, 3H, CH_3), 3.84 (s, 3H, 9-OCH_3), 3.92 (s, 3H, $NOCH_3$), 4.35 (s, 3H, $NOCH_3$), 7.09 (dd, 1H, *J* = 8.4, 2.4 Hz, 8-H), 7.29–7.41 (m, 5H), 7.53–7.57 (m, 1H, 3-H), 7.63 (d, 1H, *J* = 8.4 Hz), 7.76 (d, 1H, *J* = 7.6 Hz), 7.87 (d, 1H, *J* = 2.4 Hz), 7.97 (d, 1H, *J* = 8.4 Hz), 8.10 (s, 1H), 8.65 (*s*, 1H, NH), 8.52 (d, 1H, *J* = 8.0 Hz, 1-H). ^{13}C NMR (100 MHz, DMSO-d_6): 12.46, 55.63, 61.56, 64.54, 115.20, 115.88, 117.79, 119.34, 120.18, 121.13, 124.17, 124.96 (2C), 127.20, 128.42, 129.09, 130.27, 130.42, 136.18, 137.32, 141.37, 146.38, 149.19, 153.59, 154.18, 159.65. HRMS (EI) calc. for $C_{27}H_{24}N_3O_4$ $[M]^+$ 452.1848; found 452.1850.

5.2.13. (*E*)-6-{{3-[(*E*)-1-(Acetoxyimino)ethyl]phenyl}amino}-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one *O*-acetyloxime (7c)

To a suspension of **7a** (0.42 g, 1.0 mmol) in acetic anhydride (10 mL) was added pyridine (1.0 mL). The reaction mixture was stirred at room temperature for 1 h. The solvent was removed in vacuo, and the residue was triturated with H_2O (20 mL), filtered, and washed with H_2O. The crude product was crystallized from EtOH to give **7c** (0.44 g, 86%). Mp: 214–215 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 2.25 (s, 3H, CH_3), 2.41 (s, 3H, C(=O)CH_3), 2.46 (s, 3H, C(=O)CH_3), 3.86 (s, 3H, 9-OCH_3), 7.13 (dd, 1H, *J* = 8.4, 2.4 Hz, 8-H), 7.41–7.47 (m, 3H), 7.56–7.60 (m, 1H, 3-H), 7.66 (d, 1H, *J* = 8.4 Hz, 7-H), 7.86 (d, 1H, *J* = 2.4 Hz, 10-H), 7.88–7.89 (m, 1H), 7.95 (d, 1H, *J* = 8.8 Hz, 4-H), 8.24 (br s, 1H), 8.69 (dd, 1H, *J* = 8.4, 0.8 Hz, 1-H), 8.73 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 14.14, 19.34, 19.72, 55.64, 116.42, 117.44, 118.52, 120.05, 120.36, 122.51, 124.69, 124.92, 125.55, 126.60, 127.46, 128.71, 129.50, 130.02, 131.26, 134.82, 137.13, 141.41, 146.69, 148.97, 158.56, 159.74, 162.49, 167.63, 168.47. HRMS (ESI) calc. for $C_{29}H_{25}N_4O_5$ $[M+H]^+$ 509.1825; found 509.1823.

5.2.14. (*E*)-6-{{4-[(*E*)-1-(Hydroxyimino)ethyl]phenyl}amino}-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one oxime (8a)

Compound **8a** was obtained from **6j** as described for **7a** in 81% yield. Mp: 165–166 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 2.17 (s, 3H, CH_3), 3.86 (s, 3-H, 9-OCH_3), 7.10 (dd, 1H, *J* = 8.4, 2.4 Hz, 8-H), 7.40–7.44 (m, 1H, 2-H), 7.56–7.77 (m, 6H), 7.97 (d, 1H, *J* = 8.4 Hz, 7-H), 8.07 (d, 1H, *J* = 2.4 Hz, 10-H), 8.73 (s, 1H, NH), 8.75 (dd, 1H, *J* = 8.4, 0.8 Hz, 1-H), 11.00 (s, 1H, NOH), 13.52 (s, 1H, NOH). ^{13}C NMR (100 MHz, DMSO-d_6): 11.46, 55.60, 115.05, 115.17, 119.44 (2C), 120.46, 123.96, 124.75, 124.90, 125.08, 125.82 (2C), 127.33, 128.96, 129.87, 130.61, 130.65, 138.28, 142.00, 146.36, 149.09, 152.69, 153.72, 159.66. HRMS (EI) calc. for $C_{25}H_{20}N_4O_3$ $[M]^+$ 424.1535; found 424.1532.

5.2.15. (*E*)-9-Methoxy-6-{{4-[(*E*)-1-(methoxyimino)ethyl]phenyl}amino}-11*H*-indeno[1,2-*c*]quinolin-11-one *O*-methyloxime (8b)

Compound **8b** was obtained from **6j** as described for **7b** in 78% yield. Mp: 108–109 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 2.19 (s, 3H, CH_3), 3.85 (s, 3H, 9-OCH_3), 3.92 (s, 3H, $NOCH_3$), 4.37 (s, 3H, $NOCH_3$), 7.10 (dd, 1H, J = 8.4, 2.8 Hz, 8-H), 7.41–7.45 (m, 1H, 2-H), 7.56–7.60 (m, 1H, 3-H), 7.65–7.77 (m, 5H), 7.87 (d, 1H, J = 2.8 Hz, 10-H), 7.93 (d, 1H, J = 8.4 Hz, 7-H), 8.71 (dd, 1H, J = 8.4, 0.8 Hz, 1-H), 8.81 (br s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 12.13, 55.64, 61.41, 64.58, 115.26, 115.86, 119.52 (2C), 120.22, 123.87, 124.17, 124.98, 125.23, 126.17 (2C), 127.04, 128.99, 129.20, 130.14, 130.40, 137.56, 142.28, 146.32, 148.78, 153.52, 153.72, 159.71. HRMS (EI) calc. for $C_{27}H_{24}N_4O_3$ $[M]^+$ 452.1848; found 452.1847.

5.2.16. (*E*)-6-{{4-[(*E*)-1-(Acetoxyimino)ethyl]phenyl}amino}-9-methoxy-11*H*-indeno[1,2-*c*]quinolin-11-one *O*-acetyloxime (8c)

Compound **8c** was obtained from **6j** as described for **7c** in 82% yield. Mp: 188–189 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 2.23 (s, 3H, CH_3), 2.36 (s, 3H, C(=O)CH_3), 2.45 (s, 3H, C(=O)CH_3), 3.86 (s, 3H, 9-OCH_3), 7.09 (dd, 1H, J = 8.4, 2.4 Hz, 8-H), 7.40–7.44 (m, 1H, 2-H), 7.56–7.60 (m, 1H, 3-H), 7.69–7.88 (m, 7H), 8.67 (br s, 1H, NH), 8.69 (d, 1H, J = 8.4 Hz, 1-H). ^{13}C NMR (100 MHz, DMSO-d_6): 13.32, 18.94, 19.34, 55.38, 116.38, 117.15, 119.03 (2C), 119.99, 124.31, 124.68, 125.50, 126.66, 126.99 (2C), 127.27, 129.16, 129.84, 130.82, 131.06, 137.09, 143.48, 146.53, 148.28, 158.34, 159.63, 161.69, 167.30, 168.19. HRMS (ESI) calc. for $C_{29}H_{25}N_4O_5$ $[M+H]^+$ 509.1825; found 509.1823.

5.3. Pharmacological methods

5.3.1. Antiproliferative assay

The human tumor cell lines of the cancer screening panel are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cell suspensions that were diluted according to the particular cell type and the expected target cell density (5000–40,000 cells per well based on cell growth characteristics) were added by pipet (100 ml) into 96-well microtiter plates. Inoculates were allowed a preincubation period of 24 h at 37 °C for stabilization. Dilution at twice the intended test concentration was added at time zero in 100-μL aliquots to the microtiter plate wells. Usually, test compounds were evaluated at five 10-fold dilutions. In a routine testing, the highest well concentration is 10E-4M, but for the standard agents the highest well concentration used depended on the agent. Incubation lasted for 48 h in 5% CO_2 atmosphere and 100% humidity. The cells were assayed by using the sulforhodamine B assay.[20] A plate reader was used to read the optical densities.[21,22]

5.3.2. Cell cycle analysis

H460 cells were treated with DMSO, **5b** at different concentrations (1.0, 5.0, 10.0 μM) for 24 h. Cells were harvested, rinsed in PBS, resuspended, fixed in 70% ethanol, and stored at −20 °C in fixation buffer until ready for analysis. The pellets were suspended in 1 mL of propidium iodide (PI) solution containing 20 μg/μL of PI, 0.2 mg/mL RNase, and 0.1% (v/v) Trition X-100. Cell samples were incubated at room temperature in the dark for at least 30 min and analyzed by a flow cytometer (Coulter Epics). Data recording was made using Epics software and cell cycle data were analyzed using Multicycle software (coulter).

5.3.3. DNA mobility assay (DNA unwinding)[17]

Negative supercolied pBR322 (400 ng) was incubated in TE buffer, pH 8.0, with **5b** different indenoquinoline concentration for 12 h at room temperature. Following the addition of 2 μl of loading buffer (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol), the samples was loaded onto a 1% agarose gel. The gel was run at 6 V/cm for 2.5 h in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA-Na2, pH 8.5) and stained with ethidium bromide and photographed under UV illumination using Polaroid instant film.

5.3.4. Immunoblot analysis

After treatment of compound **5b**, cells were collected and washed twice with cold PBS and then lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 μg/mL aprotinin, and 25 μg/mL leupeptin) and kept on ice for 30 min. The lysates were centrifuged at 12,000g at 4 °C for 20 min and the supernatants were stored at −70 °C. The protein concentration was determined by the Bradford method. Protein (20 μg) were separated by 10% SDS–PAGE and transferred onto a PVDF membrane using a glycine transfer buffer (192 mM glycine, 25 mM Tris–HCl, pH 8.8, and 20% methanol [v/v]). After blocking with 5% non-fat dried milk, the membrane was incubated for 2 h with primary antibodies, followed by 30 min with secondary antibodies in milk containing Tris-buffered saline (TBS) and 0.5% Tween. The membrane was then exposed to X-ray film. Protein bands were detected using the enhanced chemiluminescence blotting detection system (Amersham, USA).

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