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Synthesis of 6-chloroisoquinoline-5,8-diones and pyrido[3,4-*b*]-phenazine-5,12-diones and evaluation of their cytotoxicity and DNA topoisomerase II inhibitory activity

Jin Sung Kim, Hee-Kyung Rhee, Hyen Joo Park, In-Kyoung Lee, Sang Kook Lee, Myung-Eun Suh, Hwa Jeong Lee, Chung-Kyu Ryu* and Hea-Young Park Choo*

School of Pharmacy, Ewha Womans University, Seoul 120-750, South Korea

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Abstract—The substituted chloroisoquinolinediones and pyrido[3,4-*b*]phenazinediones were synthesized, and the cytotoxic activity and topoisomerase II inhibitory activity of the prepared compounds were evaluated. Chloroisoquinolinediones have been prepared by the reported method employing 6,7-dichloroisoquinoline-5,8-dione. The cyclization to pyrido[3,4-*b*]phenazinediones was achieved by adding the aqueous sodium azide solution to the dimethylformamide solution of corresponding chloroisoquinoline-5,8-dione. The cytotoxicity of the synthesized compounds was evaluated by a SRB (Sulforhodamine B) assay against various cancer cell lines such as A549 (human lung cancer cell line), SNU-638 (human stomach cancer cell), Col2 (human colon cancer cell line), HT1080 (human fibrosarcoma cell line), and HL-60 (human leukemia cell line). Almost all the synthesized pyrido[3,4-*b*]phenazinediones showed greater cytotoxic potential than ellipticine (IC_{50} = 1.82–5.97 μM). In general, the cytotoxicity of the pyrido[3,4-*b*]phenazinediones was higher than that of the corresponding chloroisoquinolinediones. The caco-2 cell permeability of selected compounds was 0.62×10^{-6}–35.3×10^{-6} cm/s. The difference in cytotoxic activity among tested compounds was correlated with the difference in permeability to some degree. To further investigate the cytotoxic mechanism, the topoisomerase II inhibitory activity of the synthesized compounds was estimated by a plasmid cleavage assay. Most of compounds showed the topoisomerase II inhibitory activity (28–100%) at 200 μM. IC_{50} values for the most active compound **6a** were 0.082 μM. However, the compounds were inactive for DNA relaxation by topoisomerase I at 200 μM.

1. Introduction

Various biological properties including enzyme inhibition, antibacterial, antifungal, and anticancer activities have been reported for quinones and quinone derivatives.[1–4] The 7-amino-5,8-quinolinedione moiety of streptonigrin has been proposed to be important in determining its antitumor activity. The antitumor activity of the quinone moiety has been thoroughly studied and it is known that they act as topoisomerase inhibitors via the DNA-intercalation[5–7] and the reduction of the quinone moiety by DT-diaphorase (quinone oxidoreductase).[8–10] The intercalation of aromatic molecules which are required to have 3–4 coplanar rings to a DNA double helix has been known to be important for cytotoxicity.[11,12] The antitumor activity of nitrogen containing hetero-cyclic quinones such as benzophenazinediones **1**, pyridophenazinediones **2**, and pyridazinophenazinedione derivatives **3** was reported in our previous papers (Fig. 1).[13–15] In a continuous study to develop novel anticancer agents based on nitrogen containing heterocyclic quinones, a series of substituted 6-chloroisoquinoline-5,8-diones (**5a**–**i**) and pyrido[3,4-*b*]phenazine-5,12-diones (**6a**–**e**), polycyclic quinone analogues with three or four nitrogen atoms, were synthesized and their cytotoxicity against human cancer cell lines and inhibitory activity against topoisomerase were evaluated.

2. Results and discussion

2.1. Synthesis

6,7-Dichloro-5,8-isoquinolinedione **4** was refluxed with various aryl amines in ethanol to give 6-chloro-7-aryl-

Keywords: 6-Chloroisoquinoline-5,8-diones; Pyrido[3,4-*b*]phenazine-5,12-diones; Cytotoxicity; DNA topoisomerase inhibitory activity; Permeability.
* Corresponding authors. E-mail: hypark@ewha.ac.kr

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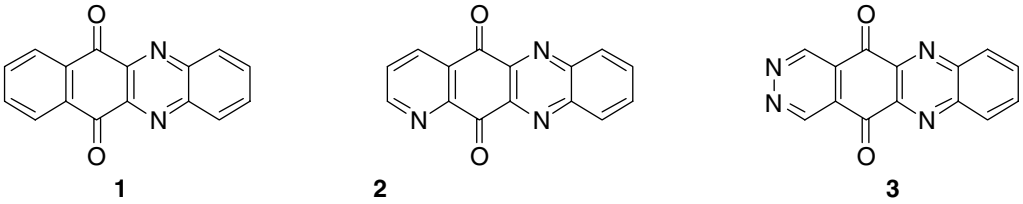
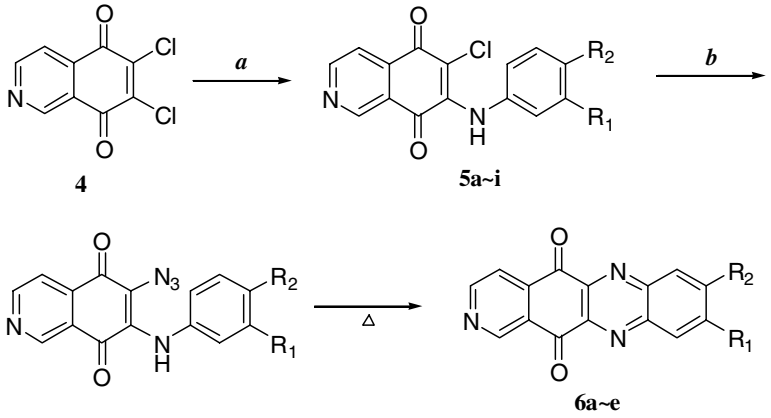


Figure 1. The structures of nitrogen containing heterocyclic quinones.



Scheme 1. The synthesis of 6-chloro-7-arylaminoisoquinoline-5,8-diones **5a**–**i** and substituted pyrido[3,4-*b*]phenazine-5,12-diones derivatives **6a**–**e**. Reagents and condition: (a) Arylamine/EtOH, 2–5 h, reflux; (b) NaN_3, DMF.

aminoisoquinoline-5,8-diones **5a**–**i** in accordance with the previously reported method.[16,17] This nucleophilic substitution was regioselective on the electron deficient position.[16] The chloro-arylaminoisoquinolinediones prepared with yields of 40–90% were reacted with sodium azide in *N*,*N*-dimethylformamide (DMF) at 90 °C, which occurred via substitution of azide for chlorine in situ and an intra-molecular cyclization, to yield the substituted pyrido[3,4-*b*]phenazine-5,12-diones **6a**–**e**[13,14] as presented in Scheme 1. In the preparation of the pyridophenazinedione derivatives **6a**–**e**, the intramolecular cyclization of chloro-isoquinolinediones **5a**–**e** with *p*-substituted arylamine was successful except **5f**, the compound with amide substituent. It is likely that nucleophilicity of the nitrogen affects the nucleophilic substitution for cyclization. Cyclization of chloro-isoquinolinediones with *m*-substituted arylamine **5g**–**i** was not able to be accomplished. These final products were identified by NMR, IR, and mass spectra. The exact position of substitution was able to be predicted from X-ray crystallographic structure of pyridophenazinedione obtained in the previous study.[13]

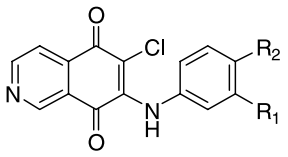
2.2. Cytotoxicity

The cytotoxic activity of the prepared substituted 6-chloro-7-arylaminoisoquinoline-5,8-diones **5a**–**i** and pyrido[3,4-*b*]phenazine-5,12-dione derivatives **6a**–**e** was evaluated against the five types of cancer cell lines; A549 (lung), SNU-638 (stomach), Col2 (colon), HT1080 (fibrosarcoma), and HL-60 (myeloid leukemic) by SRB assay method[18] (Table 1). All chloro-arylaminoisoquinolinediones **5a**–**i** exhibited higher cytotoxicity against human stomach cancer cell lines than the positive control, ellipticine (IC_{50} = 5.97 μM). Among the chloro-isoquinolinediones, cytotoxic activity of **5c**, compound with hydroxyethyl group at R_2 position, was superior (IC_{50} = 0.15–1.10 μM) to that of ellipticine (IC_{50} = 1.82–5.97 μM) against all cell lines. Introduction of highly electronegative fluorine at R_1 position (**5g**) reduced the cytotoxicity. In general, the cytotoxicity of pyrido[3,4-*b*]phenazine-5,12-diones **6a**–**e**, annulated coplanar tetracyclic heteroquinones with three nitrogen atoms, was much higher than that of the corresponding 6-chloro-7-arylaminoisoquinoline-5,8-diones **5a**–**e** for A549, SNU-638, and HT1080 cancer cell lines. However, pyrido[3,4-*b*]phenazine-5,12-diones **6a**–**e** and the corresponding 6-chloro-7-arylamino-isoquinoline-5,8-diones **5a**–**e** showed comparable activity for Col2, and HL-60 cancer cell lines with few exceptions.

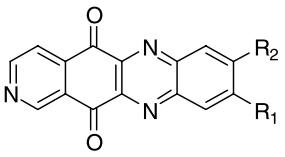
In previous studies, the other pyridophenazinediones and the substituted pyridazino[4,5-*b*]phenazine-5,12-diones with three or four nitrogen atoms showed much greater cytotoxicity than benzophenazinediones possessing two nitrogen atoms.[13,19] The reported cytotoxicity of pyridophenazinediones **2** (IC_{50} = 0.10–0.70 μM) for A549 was comparable to that (IC_{50} = 0.13–0.60 μM) of **6a**–**e** which have similar scaffold but were different from the nitrogen positions.[13,14] The four nitrogen congeners, pyridazinophenazinediones **3**, were reported to have similar cytotoxic activity (IC_{50} = 0.01–0.58 μM).[14,15]

Most of quinone compounds with two to four fused aromatic rings are known to exhibit cytostatic activity through DNA intercalation, which causes enzyme blockade and reading errors during the replication pro-

Table 1. In vitro cytotoxic potential of substituted pyrido[3,4-*b*]phenazine-5,12-dione derivatives



5a~5i



6a~6e

Compound	R_1	R_2	IC_{50} (μM)				
			A549	SNU-638	Col2	HT1080	HL-60
Ellipticine			2.07	5.97	1.82	5.01	2.60
5a	H	OCH_3	1.59[a]	1.35	7.23	1.56	0.78
5b	H	$CH(CH_3)_2$	13.97	1.96	0.14	10.38	2.64
5c	H	CH_2CH_2OH	1.86[a,b]	0.49	0.58	1.10	0.15
5d	H	$COOC_2H_5$	1.91[b]	1.07	7.07	9.54	0.51
5e	H	OCF_3	3.53[b]	0.93	5.49	4.36	0.53
5f	H	$CONH_2$	4.02	1.07	1.57	>20	6.22
5g	F	H	11.56	1.22	9.76	14.89	2.08
5h	$COCH_3$	H	1.04[b]	0.60	1.75	>20	1.15
5i	OH	H	1.62	0.35	1.83	7.01	0.84
6a	H	OCH_3	0.27	1.06	1.26	0.81	0.76
6b	H	$CH(CH_3)_2$	0.13	0.12	0.55	0.55	0.37
6c	H	CH_2CH_2OH	0.33	0.26	1.05	1.14	1.14
6d	H	$COOC_2H_5$	0.60	0.71	0.50	0.82	>20
6e	H	OCF_3	0.15	0.67	0.14	0.50	0.40

Human lung (A549), human stomach (SNU-638), human colon (Col2), human fibrosarcoma (HT1080), and human myeloid leukemic (HL-60) tumor cell lines. [a]Ref. 16, [b]Ref. 17.

cess. The primary nucleic acid intercalation may be further modulated by binding to other substrates such as topoisomerase I/II.

A positive charge, generally needed for cytotoxicity, is usually provided by nitrogen of the compounds.[20] Therefore, the number and positions of nitrogen atoms are important for cytotoxicity as reported by Shaikh et al.[21]

Also the redox activity of quinones is known to have a role in the cleavage of DNA mediated by oxygen or sulfur radicals and is related to side effects such as myelosuppression and cardiotoxicity.[20] They can easily accept one electron, forming the semiquinone radical, followed by a further electron to give the hydroquinone, the driving force being the formation of a fully aromatic system.[22] In biological systems quinones can undergo biochemical reductions either by one or two electrons. One-electron reducing enzymes are catalyzed by flavoenzymes such as NADPH: cytochrome b_5 reductase. The superoxide radicals formed in the quinone redox cycle generate the extremely harmful reactive oxygen species such as hydroxyl radicals. The most important two electron reducing enzyme is NAD(P)H: DT-diaphorase. The hydroquinone formed represents the biologically active form for the bioreductive alkylating agent mitomycin C.

Since the definite cytotoxic mechanism of the synthesized compound is not established yet, further study on DNA-binding and alkylating properties is necessary.

2.3. Permeability and stability evaluation

Caco-2 cell permeability coefficient values of eight representative compounds (**5a**, **5c**, **5e**, **5g**, **5i**, and **6b–d**) are shown in Table 2. Six compounds (**5a**, **5c**, **5i**, and **6b–d**) were highly permeable, while the permeability of **5e** and **5g** was quite low. In addition, the eight compounds were stable for 3 days at 37 °C in three different cell culture media, which were used for cytotoxicity assay (Table 2). Based on the results, highly permeable compounds showed greater cytotoxic effects against five different cancer cell lines (Tables 1 and 2). These results suggest that the difference in cytotoxic activity among tested compounds may be, at least in part, due to the difference in permeability among the compounds.

Table 2. In vitro permeability and stability of the compounds

Compound	P_{app} ($\times 10^{-6}$ cm/s)	Stability (%)		
		RPMI	MEM	DMEM
5a	21.9 ± 11.2	101.4 ± 1.33	93.8 ± 14.8	102.8 ± 2.19
5c	17.8 ± 2.22	83.0 ± 8.85	102.1 ± 7.96	96.2 ± 7.78
5e	2.24 ± 0.48	97.3 ± 2.22	92.0 ± 3.67	87.8 ± 4.16
5g	0.62 ± 2.33	112.6 ± 5.00	98.2 ± 24.1	118.9 ± 18.9
5i	35.3 ± 2.31	107.6 ± 1.47	94.5 ± 1.37	103.1 ± 1.58
6b	10.5 ± 1.67	103.5 ± 1.19	106.1 ± 4.00	99.8 ± 3.65
6c	20.2 ± 1.03	102.7 ± 1.15	100.6 ± 1.52	95.5 ± 4.55
6d	16.0 ± 0.86	94.3 ± 3.15	96.5 ± 2.94	99.3 ± 3.08

The data are presented as means ± SD (n = 3–4).

2.4. Effects of the synthesized compounds on assay for topoisomerase II-induced DNA decatenation[23]

The decatenation assay is specific for measuring topoisomerase II (topo II) activity because it is based on the conversion of catenated DNA to its decatenated form, which requires DNA double strand breakage followed by strand rotation and ligation activities uniquely done by topo II. The removal of this KDNA by the enzyme can be seen in agarose gels. In addition, with respect to topo II, the decatenation of KDNA was induced by topo II which was inhibited by treatment with etoposide (VP16, 200 μM). To further elucidate the mechanism of action of test compounds for cytotoxicity, topo II-mediated DNA decatenation activity was evaluated. Most of the synthesized compounds showed moderate or strong inhibitory activity for the conversion of KDNA to the decatenated form. In particular, compounds **5f** and **6a** were found to completely inhibit the catenated KDNA to the decatenated form by topo II-mediated catalytic activity (Table 3). The electrophotogram on the inhibitory activity of test compounds is presented in Figure 2. The IC_{50} values of compounds exhibited over 80% inhibition at 200 μM were determined by calculation using nonlinear regression analysis (percent survival versus concentration) at concentration of 0.25, 0.10, and 0.05 μM. IC_{50} values for **5b**, **5d**, **5f**, **5g**, **5i**, and **6a** were 1.00, 0.52, 0.02, 0.34, 0.60, and 0.082 μM, respectively, and were much more potent than etoposide (IC_{50} = 78.4 μM). Although correlation between the cytotoxicity and the topo II inhibitory activity of the tested compounds is not clear, the potential inhibitory activity against topo II of several compounds suggests potentiality of a mechanism for their cytotoxic activity against human cancer cell lines.

Table 3. Inhibitory activity on topoisomerase II of the compounds

Compound	Decatenation activity (for Topo II) % inhibition[a]
5a	53
5b	80
5c	65
5d	93
5e	28
5f	**100**
5g	94
5h	78
5i	87
6a	**100**
6b	56
6c	39
6d	46
6e	74
Etoposide	89

[a] Decatenation assay for topoisomerase II catalytic activity (200 μM) was done as described in Section 4.1.

2.5. Effects of the synthesized compounds on assay for topoisomerase I-induced DNA relaxation[23]

To further study the reaction mechanism for cytotoxicity of the tested compounds, topo I-mediated DNA relaxation activity was also evaluated. Selected samples of the synthesized compounds (**5c**, **5d**, **5g**, **6c**, and **6d**) showed no inhibitory activity at 200 μM (Table 4). The electrophotogram on the inhibitory activity of test compounds is presented in Figure 3.

3. Conclusion

In conclusion, a series of 6-chloroisoquinoline-5,8-diones and pyrido[3,4-*b*]phenazine-5,12-diones were

Table 4. Inhibitory activity on topoisomerase I of the compounds

Compound	Relaxation activity (for Topo I) % inhibition[a]
5c	3
5d	4
5g	5
6c	4
6d	7
Camptothecine (100 μM)	47

[a] Relaxation assay for topoisomerase I catalytic activity was done at a concentration of 200 μM for synthesized compounds.

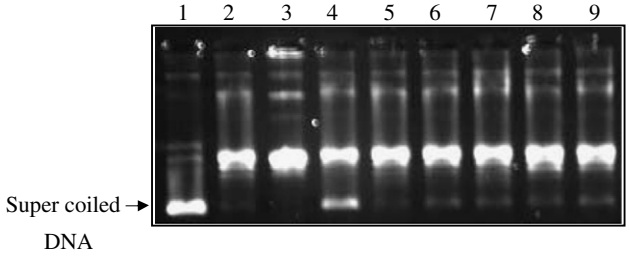


Figure 3. Effects of the prepared compounds on the relaxation of supercoiled DNA by topoisomerase I. Plasmid DNA (pHOT1, 0.25 mg) was incubated with topoisomerase I (1 U) in the presence of test agents and then seen on an agarose gel. Lane 1, supercoiled DNA without enzyme; lane 2, supercoiled DNA with topo I (relaxed form); lane 3, relaxed DNA marker; lane 4, supercoiled DNA with topo I in the presence of 100 μM camptothecin. Lanes 5–9 in the presence of compounds **5c**, **5d**, **5g**, **6c**, and **6d** at a concentration of 200 μM, respectively.

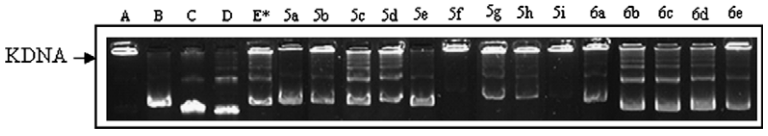


Figure 2. Effects of the prepared compounds on the decatenation of KDNA by topoisomerase II. Lane A, KDNA without enzyme (catenated form); lane B, KDNA with 1 U of topo II (decatenated form); lane C, decatenated KDNA marker; lane D, linear KDNA marker; lane E*, etoposide, 200 μM; lanes **5a**–**5i** and **6a**–**6e**, KDNA with 1 U of topo II in the presence of compounds at a concentration of 200 μM, respectively.

synthesized and their cytotoxicity was evaluated. In general, the cytotoxicity of the pyrido[3,4-*b*]phenazinediones was higher than that of the corresponding chloroisoquinolinediones.

Highly permeable compounds in a series showed greater cytotoxic effects against five different cancer cell lines. However, the cytotoxicity of these compounds was not clearly correlated with DNA topoisomerase inhibitory activity. Whether these compounds exert the cytotoxicity through binding by DNA intercalation or other mechanisms will be clarified by further study.

4. Experimental

4.1. Materials and methods

4.1.1. Synthesis. Melting points were taken in Pyrex capillaries using electrothermal digital melting point apparatus (Büchi) and are not corrected. The IR spectra were recorded on a FT-Infrared spectrometer (Bio-Rad Co., USA) using KBr pellet. ^{1}H NMR spectra were recorded on a 400 MHz Varian FT-NMR spectrometer facility by using tetramethylsilane as an internal standard. Mass spectra were obtained on a Tandem Mass spectrometer JMS-HX110/110A (Jeol). Most of the reagents were purchased from Aldrich Chemical Company and Merck Company.

4.1.2. Cytotoxicity and topoisomerase inhibitory assay. Trichloroacetic acid (TCA) and sulforhodamine B (SRB) were purchased from Sigma Chemical Co. (St. Louis, MO). Minimal essential medium with Eagles' salt (MEME), fetal bovine serum (FBS), non-essential amino acid solution (10 mM, 100×), trypsin–EDTA solution (1×), and antibiotic–antimycotic solution (PSF) were from Gibco-BRL (Grand Island, NY). Topo I, II and assay kits were purchased from Topogen, Inc. (Columbus, OH, USA). Stock solutions of all test agents were dissolved in dimethylsulfoxide (DMSO) for topo I relaxation assay and 10% DMSO in acetone for topo II decatenation assay.

4.2. Synthesis

4.2.1. General procedure for the preparation of 6-chloro-7-arylaminoisoquinoline-5,8-diones (5a–i). To a solution of 6,7-dichloro-5,8-isoquinolinedione, prepared according to the literature[16,17] (10 mmol) in 95% ethanol (100 mL), appropriate arylamine (11 mmol) was added and heated under reflux. The reaction mixture was cooled and then filtered. The filtered precipitate was washed with cold 95% ethanol. The physical data of the prepared compounds, **5a**, **5c**, **5d**, **5e**, and **5h**, were previously reported.[16,17]

4.2.1.1. 6-Chloro-7-[*N*-(4-isopropylphenyl)amino]isoquinoline-5,8-dione (5b). Yield: 84%; shining dark violet powder; mp 179–180 °C; ^{1}H NMR ($CDCl_3$) δ 9.33 (1H, d, J = 0.8 Hz), 9.06 (1H, d, J = 4.8 Hz), 8.00 (1H, dd, J = 4.8 and 0.8 Hz) 7.77 (1H, br s), 7.22 (2H, d, J = 8.4 Hz), 7.03 (2H, d, J = 8.4 Hz), 2.92 (1H, quin, J = 6.8 Hz), 1.27 (6H, d, J = 6.8 Hz); IR (CH_2Cl_2) 3208, 1681, 1585 cm^{-1}; HR-FABMS Calcd for $C_{18}H_{16}ClO_2N_2$ (M^++1): 327.0900. Found: 327.0896.

4.2.1.2. 4-(6-Chloro-5,8-dihydro-5,8-dioxoisoquinolin-7-ylamino)benzamide (5f). Yield: 42%; red powder; mp 297–299 °C; ^{1}H NMR (DMSO) δ 9.64 (2H, br s), 9.18 (1H, d, J = 0.8 Hz), 9.08 (1H, d, J = 5.2 Hz), 7.90 (1H, dd, J = 5.2 and 0.8 Hz), 7.82 (2H, d, J = 8.4 Hz), 7.29 (1H, br s), 7.18 (2H, d, J = 8.4 Hz); IR (KBr) 3417, 3263, 1679, 1658, 1588, 1556 cm^{-1}; HR-FABMS Calcd for $C_{16}H_{11}ClO_3N_3$ (M^++1): 328.0489. Found: 328.0491.

4.2.1.3. 6-Chloro-7-[*N*-(3-fluorophenyl)amino]isoquinoline-5,8-dione (5g). Yield: 67%; brick brown powder; mp 253–254 °C; ^{1}H NMR ($CDCl_3$) δ 9.36 (1H, d, J = 0.8 Hz), 9.09 (1H, d, J = 4.8 Hz), 8.00 (1H, dd, J = 4.8 and 0.8 Hz), 7.71 (1H, br s), 7.34 (1H, m), 6.97 (1H, m), 6.89 (1H, m), 6.82 (1H, m); IR (CH_2Cl_2) 3220, 1678, 1557 cm^{-1}; HR-FABMS Calcd for $C_{15}H_9ClFO_2N_2$ (M^++1): 303.0337. Found: 303.0333.

4.2.1.4. 6-Chloro-7-[*N*-(3-hydroxyphenyl)amino]isoquinoline-5,8-dione (5i). Yield: 56%; dark red powder; mp 286–287 °C; ^{1}H NMR (acetone) δ 9.22 (1H, d, J = 0.8 Hz), 9.08 (1H, d, J = 4.8 Hz), 8.65 (1H, br s), 8.48 (1H, br s), 7.94 (1H, dd, J = 4.8 and 0.8 Hz), 7.17 (1H, m), 6.69–6.74 (3H, m); IR (KBr) 3268, 1686, 1554 cm^{-1}; HR-FABMS Calcd for $C_{15}H_{10}ClO_3N_2$ (M^++1): 301.0380. Found: 301.0381.

4.2.2. General procedure for the preparation of pyrido[3,4-b]phenazinediones (6a–e).[14] To the solution of chloroisoquinoline-5,8-dione **5** (1 equiv) in DMF (0.02 M), sodium azide (1.5 equiv), which was suspended in little amount of distilled water, was added. The mixture was heated at 90 °C on a steam bath overnight. The reaction mixture was cooled, the filtered precipitate was extracted with ethyl acetate. The organic layer was washed with water, dried with anhydrous $MgSO_4$, concentrated, and then the residue was purified by recrystallization or column chromatography.

4.2.2.1. 8-Methoxypyrido[3,4-b]phenazine-5,12-dione (6a). The general procedure was followed with 6-chloro-7-[*N*-(4-methoxyphenyl)-amino]isoquinoline-5,8-dione **5a** (24.0 mg, 0.076 mmol), and the concentrated residue was yellow solid (15.0 mg, 68%). Mp >300 °C; ^{1}H NMR (DMSO) δ 9.45 (1H, s), 9.19 (1H d, J = 5.2 Hz), 8.32 (1H, d, J = 9.2 Hz), 8.14 (1H, d, J = 5.2 Hz), 7.76–7.82 (2H, m), 4.07 (3H, s); IR (CH_2Cl_2) 1683, 1613 cm^{-1}; HR-FABMS Calcd for $C_{16}H_{10}O_3N_3$ (M^++1): 292.0722. Found: 292.0720.

4.2.2.2. 8-Isopropylpyrido[3,4-b]phenazine-5,12-dione (6b). The general procedure was followed with 6-chloro-7-[*N*-(4-isopropylphenyl)-amino]isoquinoline-5,8-dione **5b** (40.0 mg, 0.12 mmol), and the concentrated residue was yellow solid (25.0 mg, 68%). Mp >300 °C; ^{1}H NMR (acetone) δ 9.58 (1H, d, J = 0.8 Hz), 9.22 (1H, d, J = 4.8 Hz), 8.33 (1H, d, J = 8.8 Hz), 8.20–8.22 (2H, m), 8.13 (1H, dd, J = 8.8 and 2.2 Hz), 3.33 (1H, quin, J = 6.8 Hz), 1.45 (6H, d, J = 6.8 Hz); IR (CH_2Cl_2)

1696, 1585 cm^{-1}; HR-FABMS Calcd for $C_{18}H_{14}O_2N_3$ (M^++1): 304.1086. Found: 304.1089.

4.2.2.3. 8-(2-Hydroxyethyl)pyrido[3,4-b]phenazine-5,12-dione (6c). The general procedure was followed with 6-chloro-7-{*N*-[4-(2-hydroxyethyl)- phenyl]amino}isoquinoline-5,8-dione **5c** (40.0 mg, 0.12 mmol), and the concentrated residue was black green solid (11.5 mg, 31%). Mp >300 °C; ^{1}H NMR (CD_3OD) δ 9.61 (1H, s), 9.18 (1H, d, J = 5.2 Hz), 8.36 (1H, d, J = 8.4 Hz), 8.23–8.25 (2H, m), 8.08 (1H, dd, J = 8.4 and 2.0 Hz), 3.98 (2H, t, J = 6.8 Hz), 3.19 (2H, t, J = 6.8 Hz); IR (CH_2Cl_2) 1693, 1585 cm^{-1}; HR-FABMS Calcd for $C_{17}H_{12}O_3N_3$ (M^++1): 306.0879. Found: 306.0875.

4.2.2.4. Ethyl 5,12-dihydro-5,12-dioxopyrido[3,4-*b*]phenazine-8-carboxylate (6d). The general procedure was followed with 6-chloro-7-[*N*-(4-ethylcarboxyphenyl)amino] isoquinoline-5,8-dione **5d** (40.0 mg, 0.11 mmol), and the concentrated residue was yellow solid (22.0 mg, 59%). Mp >300 °C; ^{1}H NMR (acetone) δ 9.61 (1H, d, J = 0.8 Hz), 9.25 (1H, d, J = 5.2 Hz), 8.97 (1H, dd, J = 1.6 and 0.4 Hz), 8.62 (1H, dd, J = 8.8 and 1.6 Hz), 8.51 (1H, dd, J = 8.8 and 0.4 Hz), 8.24 (1H, dd, J = 5.2 and 0.8 Hz), 4.52 (2H, q, J = 7.2 Hz), 1.49 (3H, t, J = 7.2 Hz); IR (CH_2Cl_2) 1716, 1689, 1585 cm^{-1}; HR-FABMS Calcd for $C_{18}H_{12}O_4N_3$ (M^++1): 334.0828. Found: 334.0824.

4.2.2.5. 8-(Trifluoromethoxy)pyrido[3,4-*b*]phenazine-5,12-dione (6e). The general procedure was followed with 6-chloro-7-[*N*-(4-(trifluoromethoxy-phenyl)amino)]isoquinoline-5,8-dione **5e** (40.0 mg, 0.11 mmol), and the concentrated residue was yellow solid (4.7 mg, 12.5%). Mp >300 °C; ^{1}H NMR (CD_3OD) δ 9.63 (1H, s), 9.19 (1H, d, J = 5.2 Hz), 8.55 (1H, d, J = 9.6 Hz), 8.27–8.31 (2H, m), 8.06 (1H, d, J = 5.2 Hz); IR (CH_2Cl_2) 1692, 1583 cm^{-1}; HR-FABMS Calcd for $C_{16}H_7F_3O_3N_3$ (M^++1): 346.0440. Found: 346.0435.

4.3. In vitro antitumor activity evaluation by SRB assay

The in vitro cytotoxic activities were evaluated by SRB method. Human colon carcinoma (Col2, University of Illinois at Chicago), stomach carcinoma (SNU-638, Korea Cell Bank at Seoul National University) cells, fibrosarcoma carcinoma (HT1080), and myeloid leukemic carcinoma (HL-60) (5×10^4 cells/mL) were treated with different concentrations of test agent for 3 days. After treatment, cells were fixed with TCA and cell viability was determined with sulforhodamine B (SRB) protein staining method.[19] The result was expressed as a percentage, relative to solvent-treated control incubations, and the IC_{50} values were calculated using nonlinear regression analysis (percent survival versus concentration).

4.4. Decatenation assay for topoisomerase II activity[23]

The total reaction volume of the topo II-mediated cleavage reaction was fixed at 20 μL. Briefly, assay buffer [0.5 mM Tris–HCl, pH 8, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, and 30 μg/mL bovine serum albumin (BSA)] containing 200 ng KDNA (TOPOgen; Columbus, USA), and a solution of the test drugs were added to 1 U of the human recombinant topo II (TOPOgen; the amount of enzyme, which resulted in the complete decatenation of 200 ng KDNA). After 10 min of incubation at 37 °C, the reaction was stopped by addition of 5 μL of stop buffer containing the loading dye (1% Sarkosyl, 0.025% bromophenol blue, and 5% glycerol), and then the reaction mixture was analyzed on a 1% agarose gel by running at 40 V for 3.5 h in TBE buffer (89 mM Tris, 89 mM borate, and 2 mM Na-EDTA, pH 8.3). Gels were made visible as described above for the relaxation assay. Gels were photographed, and remaining KDNA from photographic negatives was scanned using AlphaImager 2200 (AlphaEase version 5.5). The inhibition of topo II was calculated from the equation; % Inhibition = [Intensity of sample-treated DNA/Intensity of vehicle-treated control DNA] × 100.

4.5. Relaxation assay for topoisomerase I activity[23]

For the measurement of topoisomerase I catalytic activity, an assay was done using supercoiled pHOT1 DNA as a substrate according to the protocol provided by TopoGEN, Inc. (Columbus, USA). Supercoiled (Form I) plasmid substrate DNA was used in a reaction volume of 20 μL containing the following: 10 mM Tris–HCl, pH 7.9, 1 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol, and 1 U of purified human topoisomerase I (TopoGEN, Inc.). The appropriate inhibitor was added as indicated, and the reaction was started by the addition of the enzyme. After 10 min of incubation at 37 °C, the reaction was stopped by addition of 5 μL of stop buffer containing the loading dye (1% Sarkosyl, 0.025% bromophenol blue, and 5% glycerol), and then the reaction mixture was analyzed on a 1% agarose gel by running at 40 V for 3.5 h in TBE buffer (89 mM Tris, 89 mM borate, and 2 mM Na-EDTA, pH 8.3). Gels were stained with SYBR Green I (Molecular Probes, Eugene, OR) and observed under UV illumination. For the quantitative determination of topo I activity, photographic negatives were densitometrically scanned using AlphaImager 2200 (AlphaEase version 5.5). The inhibition of topo I was calculated from the equation: % Inhibition = [Intensity of sample-treated DNA/Intensity of vehicle-treated control DNA] × 100.

4.6. In vitro permeability and stability studies[24]

$$P_{app} = \frac{V_B * C_{B,\,2\,h}}{SA * 7200 * C_{A,\,0\,h}},$$

where V_B is the volume of basal side (1.5 mL), SA is the surface area of the filter (1.12 cm^2), $C_{B,2\,h}$ is the basal analyte concentration at 2 h, and $C_{A,0\,h}$ is the apical analyte concentration at 0 h.

For the stability study, the compounds at a final concentration of 30 μM were added into three different cell culture media (RPMI, MEM, and DMEM) and incubated for 3 days at 37 °C. Then, the samples were analyzed with UV spectrophotometer at a maximum wavelength.

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