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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 1651–1658
Bioorganic & Medicinal Chemistry

Synthesis, cytotoxicity, and DNA topoisomerase II inhibitory activity of benzofuroquinolinediones

Hee-Kyung Rhee,[a] Hyen Joo Park,[a] Sang Kook Lee,[a] Chong-Ock Lee[b] and Hea-Young Park Choo[a,*]

[a]*School of Pharmacy, Ewha Womans University, Seoul 120-750, Republic of Korea*
[b]*Pharmaceutical Screening Division, Korea Research Institute of Chemical Technology, Daejeon 305-606, Republic of Korea*

Received 24 October 2006; revised 8 December 2006; accepted 9 December 2006
Available online 13 December 2006

Abstract—Benzofuroquinolinediones (**7c** and **7d**) were synthesized by base-catalyzed condensation of dichloroquinolinediones with phenolic derivatives. Their dialkylaminoalkoxy derivatives (**8i–8p**) were prepared by reaction with various dialkylaminoalkyl chlorides. The cytotoxicity of the synthesized compounds was evaluated against eight types of human cancer cell lines, and their topoisomerase II inhibition was assessed. In general, the cytotoxicity of benzofuroquinolinediones (**8i–8p**) was similar or superior to that of doxorubicin and showed more potent inhibitory activity than naphthofurandiones (**8a–8h**). Also, most of the compounds exhibited excellent topoisomerase II inhibitory activity at a concentration of 5 μM and two compounds, **8d** and **8i**, showed IC_{50} values of 1.19 and 0.68 μM, respectively, and were much more potent than etoposide (IC_{50} = 78.4 μM), but similar to doxorubicin (IC_{50} = 2.67 μM). However their inhibitory activity on topoisomerase I was lower, and **8d** and **8i** showed IC_{50} values of 42.0 and 64.3 μM, respectively.

1. Introduction

Many of the important chemotherapeutic drugs presently in clinical use have been recognized to exert their activity by interfering with the DNA. Compounds containing a planar chromophore with two to four fused aromatic rings act as DNA intercalator, which causes enzyme blockade and reading errors during the replication process. A tetracyclic structural pattern consisting of a benzene ring attached to the 2-position of a naphthalene nucleus or heterocyclic ring moiety was observed among these compounds, and known as 2-phenylnaphthalene-type structural pattern hypothesis. For example, a pentacyclic alkaloid, camptothecin **1**, is a potent antitumor agent against numerous types of cancers.[1] WS-5995A **2** is active against L1210 leukemic cells,[2] and a natural plant alkaloid, ellipticine **3**, is an anticancer drug.[3] Compound **4** was reported to be active against human leukemia cells (HL-60) and small-cell lung cancer (SCLC)[4] (Fig. 1).

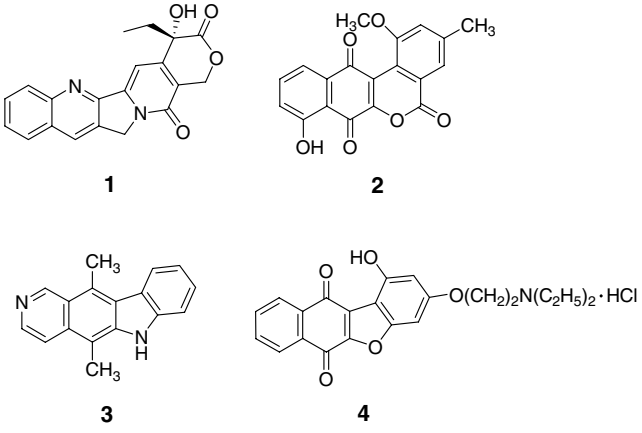

Figure 1. The '2-phenylnaphthalene type' structures as anticancer agents.

The cytotoxic mechanisms of coplanar annulated polycyclic compounds have been shown to consist of intercalation[5] in human DNA and further, modification of topoisomerases. Topoisomerase I and II are essential enzymes in the regulation of DNA topology and supercoiling, which are crucial for transcription and replication.

Keywords: Benzofuroquinolinediones; Human cancer cell lines; Cytotoxicity; Topoisomerase II.
* Corresponding author. E-mail: hypark@ewha.ac.kr

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doi:10.1016/j.bmc.2006.12.012

Topoisomerase I (topo I) breaks one of the DNA strands and the DNA can then unwind by revolving about the remaining phosphodiester bond to remove supercoils. The enzyme then relegates the break to restore the DNA with an altered linking number. The only topo I inhibitors to have undergone clinical trials as antitumor drugs are camptothecin **1** and its structural derivatives.

Topoisomerase II (topo II) is an enzyme that catalyzes changes in the topology of DNA via a mechanism that involves the transient double-strand breaking and rejoining of phosphodiester bonds. This enzyme plays several key roles in DNA metabolism and chromosome structure, and it is the primary molecular target for a number of potent anticancer drugs such as etoposide.[6] Topo II inhibitors display great structural diversity and are widely used as antitumor agents, and six topo II cleavable complex inhibitors (Etoposide, Teniposide, Doxorubicin, Daunorubicin, Idarubicin, and Mitoxantrone) have been approved for clinical use. Recently, topo II inhibitors have created a great deal of interest in the design of new antitumor agents.[7–10]

The derivatives of benzonaphthoquinolindione **4** have a suitable structure possessing the coplanar conformation and were reported to have cytotoxicity and topoisomerase II inhibitory activity.[4] In our continuous effort in development of novel DNA intercalators based on nitrogen-containing heterocyclic ring with a *para*-conjugated quinone structure, we synthesized the benzofuroquinolinedione analogues and their cytotoxicity against the eight human tumor cell lines and inhibitory activity against topo I and II are reported in this study. Also some benzonaphthofurandiones were prepared to compare the cytotoxic activity.

2. Results and discussion

2.1. Chemistry

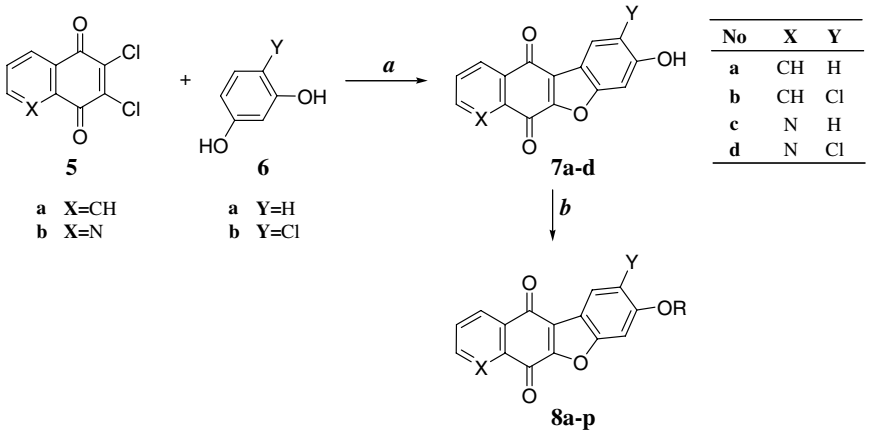
Condensation of **5** (2,3-dichloronaphthalene-1,4-dione **5a** or 6,7-dichloroquinoline-5,8-dione **5b**) and **6** (resorcinol **6a** or 4-chlororesorcinol **6b**) was achieved with modification of the reported reaction conditions.[4,11] Treatment of compounds containing the hydroxyl group with dialkylaminoalkyl chloride in base and phase transfer catalysis (PTC, benzyltriethylammonium chloride) gave the corresponding dialkylaminoalkoxy derivatives, as shown the Scheme 1. Benzonaphthofurandiones (**7a** and **7b**) were synthesized by base-catalyzed condensation of the compounds (**5a** and **5b**) with phenolic derivatives (**6a** and **6b**) in alcohol. Also, benzofuroquinolinediones (**7c** and **7d**) were designed and synthesized because the introduction of nitrogen could increase the reductive potential of the quinolinediones and their cytotoxicity.[12] However, the preparation of the compounds required elevated temperature in the presence of toluene as a solvent and pyridine as a base-catalyst/solvent. The purified compounds were obtained by recrystallization in dimethylformamide. Their dialkylaminoalkoxy derivatives (**8a**–**8p**) were prepared by base-catalyzed condensation of the prepared compounds **7a**–**7d** with dialkylaminoalkyl chlorides.[4] The products were purified by recrystallization in co-solvent of chloroform and methanol or flash column chromatography (chloroform/methanol 2:1) with 13–89% yields. The yields for compounds with bis(1-methylethylamino) group were higher than the others. Introduction of (dimethylamino)isopropyl group to compounds **7a**–**7d** gave two isomers for each compound (**8c** and **8d**, **8g** and **8h**, **8k** and **8l**, **8o** and **8p**, respectively). Two isomers were isolated by column chromatography and rearrangement of methyl group was identified on the ^{1}H NMR spectra. These configurations can be elucidated by aziridinium ion (**9**) pathway in the alkylation step as proposed for methadone synthesis[13] (Scheme 2).

2.2. Cytotoxic activity

The in vitro cytotoxic activities of the synthesized compounds were evaluated against various human tumor cell lines (lung; A549, stomach; SNU-638, colon; HCT116, fibro sarcoma; HT1080, myeloid leukemic; HL-60, ovarian; SK-OV-3, melanoma; SK-MEL-2 and CNS; XF-498) by SRB assay method.[14] The inhibitory activities were presented as micromolar concentrations of the compounds that cause 50% inhibition per unit of enzyme (IC_{50}) under the assay conditions and compared with that of doxorubicin, clinically used agents for the treatment of solid tumors (Table 1). In general, cytotoxicity of benzofuroquinolinediones (**8i**–**8p**) was similar or superior to that of doxorubicin and exhibited more potent inhibitory activity against all kinds of cancer cell lines than the corresponding benzonaphthofurandiones (**8a**–**8h**). The higher cytotoxic activity of benzofuroquinolinediones demonstrates that the presence of the nitrogen atom plays an important role in determining the activity of the compound.[12] Especially compound **8l** showed significantly high cytotoxicity among all compounds. All the methyl branch side-chain containing compounds such as **8k**, **8l**, **8o**, and **8p** showed high activity. However, the largest molecule **8f**, containing bulky bis(1-methylethylamino) group and adjacent chlorine atom, showed no cytotoxic activity against all the tested human cancer cell lines. To investigate the cytotoxic mechanism of tested compounds, inhibitory activity for topo I and II, essential enzymes for DNA metabolism, was evaluated.

2.3. Inhibitory activity for topo I and II

DNA topoisomerases are nuclear enzymes regulating the topological state of DNA by breaking and rejoining of DNA strands. While DNA-topo I catalyzes changes in the topological state of duplex DNA by performing transient single-strand breakage-union cycles, topo II alters the topology of DNA by causing a transient enzyme bridged, double-strand break, followed by strand passing and resealing. They are extensively involved in DNA metabolism including replication and transcription. The antitumor activity is associated with the ability of the topoisomerase poisons to stabilize the enzyme–DNA cleavable complex. The precise mechanism of ellipticine has not yet been fully understood but it is suggested including DNA intercalation and inhibition

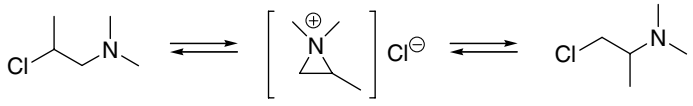


No	X	Y
a	CH	H
b	CH	Cl
c	N	H
d	N	Cl

a. *Na in EtOH or KOH in MeOH (X=CH) or pyridine (X=N)*
b. *RCl·HCl, K_2CO_3, benzyltriethylammonium chloride in $CHCl_3$ and H_2O*

No	X	Y	R
8a	CH	H	$(CH_2)_2N(C_2H_5)_2$
8b	CH	H	$(CH_2)_2N\{CH(CH_3)_2\}_2$
8c	CH	H	$CH(CH_3)CH_2N(CH_3)_2$
8d	CH	H	$CH_2CH(CH_3)N(CH_3)_2$
8e	CH	Cl	$(CH_2)_2N(C_2H_5)_2$
8f	CH	Cl	$(CH_2)_2N\{CH(CH_3)_2\}_2$
8g	CH	Cl	$CH(CH_3)CH_2N(CH_3)_2$
8h	CH	Cl	$CH_2CH(CH_3)N(CH_3)_2$
8i	N	H	$(CH_2)_2N(C_2H_5)_2$
8j	N	H	$(CH_2)_2N\{CH(CH_3)_2\}_2$
8k	N	H	$CH(CH_3)CH_2N(CH_3)_2$
8l	N	H	$CH_2CH(CH_3)N(CH_3)_2$
8m	N	Cl	$(CH_2)_2N(C_2H_5)_2$
8n	N	Cl	$(CH_2)_2N\{CH(CH_3)_2\}_2$
8o	N	Cl	$CH(CH_3)CH_2N(CH_3)_2$
8p	N	Cl	$CH_2CH(CH_3)N(CH_3)_2$

Scheme 1. Synthesis of benzonaphthofurandiones and benzofuroquinolinediones.



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Scheme 2. Aziridinium ion pathway.

of topo II activity. Also, it is a general tendency that the antitumor activity of anthracyclines such as doxorubicin mainly results from an inhibition of mammalian topo II.[15,16] The decatenation assay[17,18] is specific for measuring 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 these KDNA by the enzyme can be seen in agarose gels. Based on this information, effects of the synthesized compounds on the catalytic activity of topo II were evaluated. The decatenation of KDNA was induced by topo II. The results of topo II inhibition of the tested compounds and the standard drug etoposide[6] (VP-16, 200 μM as a positive control) are shown in Table 2. Also, the electrophotogram on the inhibitory activity of test compounds is presented in Figure 2. All synthesized compounds were found to be capable of inhibiting

Table 1. Cytotoxicity against human cancer cells of benzonaphthofurandiones and benzofuroquinolinediones

Compound	IC_{50} (μM)							
	A549	SNU-638	HCT 116	HT 1080	HL-60	SK-OV-3	SK-MEL-2	XF498
8a	1.09	3.11	1.68	2.19	0.65	>20.0	>20.0	>20.0
8b	2.01	2.57	1.39	1.02	1.11	3.55	1.53	3.17
8c	0.37	0.88	0.27	0.27	0.25	2.26	1.52	1.75
8d	0.21	0.84	2.20	0.34	0.39	19.03	2.15	4.69
8e	1.59	2.77	1.52	2.73	2.27	>20.0	5.73	>20.0
8f	>20.0	>20.0	>20.0	>20.0	>20.0	>20.0	>20.0	>20.0
8g	0.62	0.10	0.42	0.49	0.67	0.55	0.34	0.55
8h	0.45	1.14	0.34	0.65	0.63	0.65	0.50	0.55
8i	0.05	0.14	0.06	0.07	0.25	0.27	0.16	0.14
8j	0.37	0.45	0.18	0.18	0.08	1.02	0.94	0.94
8k	0.06	0.14	0.08	0.17	0.09	0.15	0.15	0.12
8l	0.02	0.11	0.07	0.06	0.18	0.08	0.09	0.10
8m	0.09	0.14	0.13	0.15	0.31	0.25	0.24	0.40
8n	0.18	0.53	0.41	0.22	0.70	0.75	0.73	0.91
8o	0.10	0.10	0.13	0.10	0.06	0.15	0.15	0.17
8p	0.09	0.18	0.14	0.11	0.22	0.15	0.14	0.14
Etoposide	1.16	0.23	1.87	0.25	0.37	4.28	5.72	3.48
Doxorubicin	0.15	0.06	0.62	0.06	0.04	0.29	0.07	0.04

A549, human lung tumor cell line; SNU-638, human stomach tumor cell line; HCT116, human colon tumor cell line; HT1080, human fibrosarcoma tumor cell line; HL-60, human myeloid leukemic tumor cell line; SK-OV-3, human ovarian tumor cell line; SK-MEL-2, human melanoma tumor cell line; XF-498, human CNS tumor cell line.

Table 2. Inhibitory activity of naphthofurans and furoquinolines on topoisomerase

Compound	Decatenation activity (for Topo II) % inhibition	Relaxation activity (for Topo I) % inhibition
8a	**91**	12
8b	36	10
8c	**84**	12
8d	**100**	14
8e	50	19
8f	59	14
8g	**88**	6
8h	68	0
8i	**100**	0
8j	71	0
8k	24	0
8l	67	0
8m	47	0
8n	35	0
8o	11	0
8p	59	0
Etoposide (200 μM)	89	—
Camptothecin (100 μM)	—	77
Doxorubicin (5 μM)	78	—

Decatenation assay for topo II and relaxation assay for topo I were done at a concentration of 5 μM of samples, respectively.

topo II completely at an initial concentration of 200 μM. When these compounds were tested at a concentration of 5 μM, most of the test compounds showed overmoderate activity. Especially, two compounds **8d** and **8i** showed the IC_{50} values to be 1.19 and 0.68 μM, respectively, and were 66 and 115 times more potent, respectively, than that of etoposide (IC_{50} = 78.4 μM), but similar to doxorubicin (IC_{50} = 2.67 μM). To further study the mechanism of the synthesized compounds, topo I-mediated DNA relaxation activity was also evaluated by comparison with camptothecin inhibiting DNA synthesis via strand scission and causing cell death during the S-phase of the cell cycle.[1] The tested samples did not show inhibitory activity at concentration of 5 μM, the same concentration in topo II assay. (Table 2) Topo I inhibitory assay for five compounds (**8a**, **8c**, **8d**, **8g**, and **8i**) with over 80% inhibition against the topo II was carried out at concentration of 200 μM and the electrophotogram on the result is presented in Figure 3. IC_{50} values of **8c**, **8d**, and **8i** among these compounds were 59.5, 42.0, and 64.3 μM, respectively. As a result of these tests, all tested benzonaphthofurandiones (**8a**–**8h**) and benzofuroquinolinediones (**8i**–**8p**) were considered as potent positive topo II inhibitors. Although the correlation of the cytotoxicity and the inhibitory activity of DNA decatenation by topo II is not perfectly clear, at least, these results suggest a possible mechanism for the cytotoxicity against tumor cells and provide advanced opportunities to design and develop new chemotherapeutic agents because DNA topoisomerase based on the information of

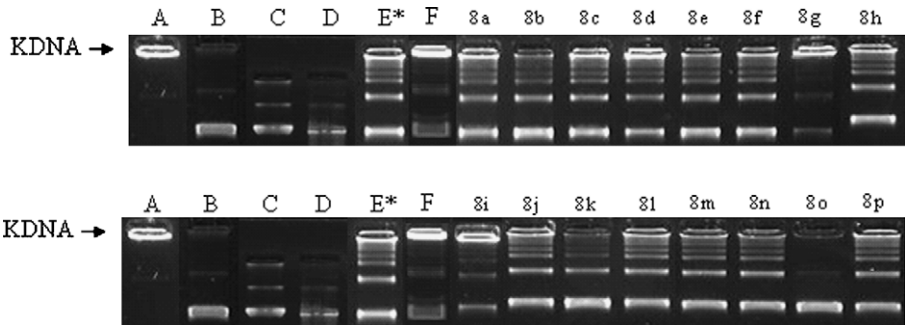

Figure 2. Effects of the prepared compounds on the decatenation of KDNA by topo II. Lane A, KDNA without enzyme (catenated form); lane B, KDNA with 1 U of topo II (decatenated form); lane C, decatenated KDNA marker, lane D; linear KDNA marker; lane E*, etoposide, 200 μM; lane F, doxorubicin, 5 μM; lanes **8a**–**8p**, KDNA with 1 U of topo II in the presence of compounds **8a**–**8p** at a concentration of 5 μM, respectively.

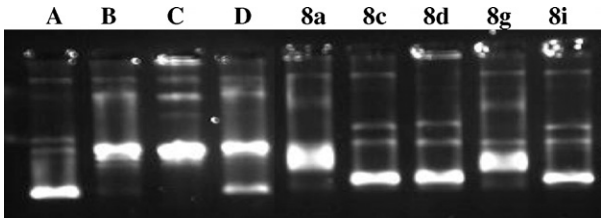

Figure 3. Effects of the selected compounds on the relaxation of supercoiled DNA by topo I Plasmid DNA (pHOT1, 0.25 mg) was incubated with topo I (1 U) in the presence of test agents and then seen on an agarose gel. Lane A, supercoiled DNA without enzyme; lane B, supercoiled DNA with topo I (relaxed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

DNA intercalating potential is considered as important targets for cancer chemotherapy.[19]

3. Conclusion

The benzonaphthofurandione and benzofuroquinolinedione derivatives were synthesized by base-catalyzed condensation. The cytotoxicity of the benzofuroquinolinedione derivatives was as high as that of doxorubicin and higher than that of the corresponding naphthoquinone analogues. The compounds with dimethylaminoisopropoxy group especially showed excellent inhibitory activity throughout the entire in vitro test. Most of the test compounds showed high activity on topo II at a concentration of 5 μM, especially, **8d** and **8i** showed IC_{50} values of 1.19 and 0.68 μM, respectively. However, their inhibitory activity on topo I was low and IC_{50} values of **8d** and **8i** were 42.0 and 64.3 μM, respectively.

4. Experimental

4.1. Materials

4.1.1. Synthesis. All melting points were taken in Pyrex capillaries using electrothermal digital melting point apparatus (Büchi). ^{1}H NMR spectra were recorded on a 400 MHz Varian FT-NMR spectrometer using tetramethylsilane as an internal standard. Samples were dissolved in $CDCl_3$ or DMSO-d_6. Mass spectra were obtained on the Mass spectrometer JMS-700 (Jeol, Japan) at the Korea Basic Science Institute (Seoul). Most of the reagents were purchased from Sigma–Aldrich Chemical 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 were from Invitrogen (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. Methods

4.2.1. Synthesis. General procedure for the preparation of benzonaphthofurandiones followed Cheng et al.'s method.[4] To a stirred ethanolic NaOEt solution or solution of MeOH containing KOH was added portionwise at rt powdered 2,3-dichloro-1,4-naphthoquinone. After stirring for a minute, the alcoholic solutions of resorcinol, phloroglucinol, and 4-chlororesorcinol were added dropwise with continuous stirring, respectively. The mixture was stirred at rt overnight. The reaction mixture was acidified with 6 N and 0.2 N HCl at 0 °C. The resulting solid was collected by filtration, washed successively with water, methanol, and diethyl ether, and dried in vacuo at 60 °C. Benzofuroquinolinediones were prepared by refluxing for 15 h the mixture of 6,7-dichloroquinoline-5,8-dione and resorcinol or 4-chlororesorcinol in toluene/pyridine (1:1). The reaction mixture was then cooled and the organic solvent was evaporated, and the residue was mixed with water and filtered. The prepared solid was dried in vacuo at 60 °C. These were recrystallized from dimethylformamide (DMF).

4.2.2. 3-Hydroxybenzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (7a). Deep yellow solid (24%); mp >310 °C; ^{1}H NMR (DMSO-d_6) δ 10.53 (s, 1H), 8.10–8.13 (m, 2H), 8.00

(d, J = 8.8 Hz, 1H), 7.88–7.90 (m, 1H), 7.19 (s, 1H), 7.08 (d, J = 8.8 Hz, 1H); HR-FABMS calcd for $C_{16}H_8O_4$ $(M+H)^+$: 265.0501, found: 265.0505.

4.2.3. 2-Chloro-3-hydroxybenzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (7b). Brick red solid (31%); mp 309–310 °C; ^{1}H NMR (DMSO-d_6) δ 11.37 (s, 1H), 8.11–8.13 (m, 2H), 8.08 (s, 1H), 7.89–7.92 (m, 2H), 7.41 (s, 1H); HR-FABMS calcd for $C_{16}H_7ClO_4$ $(M+H)^+$: 299.0111, found: 299.0112.

4.2.4. 8-Hydroxybenzofuro[3,2-*g*]quinoline-5,11-dione (7c). Dark brown solid (28%); mp >310 °C; ^{1}H NMR (DMSO-d_6) δ 10.59 (s, 1H), 9.03 (d, J = 4.8 Hz, 1H), 8.46 (d, J = 7.6 Hz, 1H), 8.00 (d, J = 8.8 Hz, 1H), 7.86 (dd, J = 4.8 Hz; J = 4.4 Hz, 1H), 7.21 (s, 1H), 7.09 (d, J = 8.8 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 180.44, 172.20, 160.23, 157.60, 153.68, 152.91, 148.32, 134.00, 129.89, 127.58, 123.68, 123.43, 116.66, 113.71, 98.35; HR-FABMS calcd for $C_{15}H_7NO_4$ $(M+H)^+$: 266.0453, found: 266.0449.

4.2.5. 7-Chloro-8-hydroxybenzofuro[3,2-*g*]quinoline-5,11-dione (7d). Dark brown solid (30%); mp >310 °C; ^{1}H NMR (DMSO-d_6) δ 9.03 (d, J = 4.4 Hz, 1H), 8.49 (d, J = 7.6 Hz, 1H), 8.10 (s, 1H), 7.95 (s, 1H), 7.88 (dd, J = 4.8 Hz; J = 4.8 Hz, 1H), 7.35 (s, 1H); HR-FABMS calcd for $C_{15}H_6ClNO_4$ $(M+H)^+$: 300.0064, found: 300.0065.

4.2.6. General procedure for the preparation of (dialkylamino)alkoxy-benzo[*b*]naphtho[2,3-*d*]furan-6,11-diones and (dialkylamino)alkoxy-benzofuro[3,2-*g*]quinoline-5,11-diones. To a suspension of 0.15 mmol of benzo[*b*]naphtho[2,3-*d*]furan-6,11-diones or benzofuro[3,2-*g*]quinoline-5,11-diones in 10 mL $CHCl_3$, a solution of 1.28 mmol K_2CO_3 and 0.02 mmol benzyltriethylammonium chloride in 2 mL H_2O was added followed by 0.3 mmol of (dialkylamino)alkyl chloride hydrochloride in 2 mL H_2O. The mixture was stirred at rt for 1 h and refluxed overnight with vigorous stirring. It was cooled and the organic layer was separated. The aqueous portion was extracted with $CHCl_3$ (210 mL). The combined organic phase was washed with brine and water, and dried (Na_2SO_4). The solids were acquired by evaporation of the solvent, which gave pure products upon recrystallization (from EtOAc) or flash column chromatography ($CHCl_3$/MeOH 2:1).

4.2.7. 3-[2-(Diethylamino)ethoxy]benzo[*b*]naphtho[2,3-*d*]-furan-6,11-dione (8a). Orange pearl solid (35.4%); mp 212–213 °C; ^{1}H NMR ($CDCl_3$) δ 8.19–8.26 (m, 2H), 8.20 (d, J = 7.2 Hz, 1H), 7.77–7.80 (m, 2H), 7.19 (s, 1H), 7.12 (d, J = 8.4 Hz, 1H), 4.69 (t, J = 4.6 Hz, 2H), 3.52 (t, J = 7.2 Hz, 2H), 3.29 (q, J = 7.0 Hz, 4H), 1.50 (t, J = 7.4 Hz, 6H); HR-FABMS calcd for $C_{22}H_{21}NO_4$ $(M+H)^+$: 364.1549, found: 364.1547.

4.2.8. 3-[2-[Bis(1-methylethylamino)]ethoxy]benzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (8b). Orange red solid (84.3%); mp 183–184 °C; ^{1}H NMR ($CDCl_3$) δ 8.20–8.25 (m, 2H), 8.17 (d, J = 8.8 Hz, 1H), 7.76–7.78 (m, 2H), 7.22 (s, 1H), 7.12 (d, J = 8.8 Hz, 1H), 4.80 (t, J = 6.0 Hz, 2H), 3.77–3.86 (m, 2H), 3.46 (q, J = 5.2 Hz, 2H), 1.61 (d, J = 6.4 Hz, 6H), 1.53 (d, J = 6.8 Hz, 6H); HR-FABMS calcd for $C_{24}H_{25}NO_4$ $(M+H)^+$: 392.1862, found: 392.1883.

4.2.9. 3-[2-(Dimethylamino)isopropoxy]benzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (8c). Orange red solid (32.7%); mp 259–260 °C; ^{1}H NMR ($CDCl_3$) δ 8.19–8.27 (m, 2H), 8.15 (d, J = 8.8 Hz, 1H), 7.75–7.79 (m, 2H), 7.20 (s, 1H), 7.12 (d, J = 8.8 Hz, 1H), 4.57–4.70 (m, 1H), 2.68–2.76 (m, 1H), 2.45–2.55 (m, 1H), 2.35 (s, 6H), 1.38 (d, J = 6.0 Hz, 3H); HR-FABMS calcd for $C_{21}H_{19}NO_4$ $(M+H)^+$: 350.1392, found: 350.1404.

4.2.10. 3-[2-(Dimethylamino)propoxy]benzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (8d). Orange solid (21.2%); mp 260–261 °C; ^{1}H NMR ($CDCl_3$) δ 8.19–8.27 (m, 2H), 8.16 (d, J = 9.2 Hz, 1H), 7.75–7.80 (m, 2H), 7.14–7.17 (m, 2H), 4.12–4.17 (m, 1H), 3.94–4.01 (m, 1H), 3.03–3.14 (m, 1H), 2.42 (s, 6H), 1.21 (d, J = 6.8 Hz, 3H); HR-FABMS calcd for $C_{21}H_{19}NO_4$ $(M+H)^+$: 350.1392, found: 350.1397.

4.2.11. 2-Chloro-3-[2-(diethylamino)ethoxy]benzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (8e). Dark yellow pearl solid (14.2%); mp: 211–212 °C; ^{1}H NMR ($CDCl_3$) δ 8.32 (s, 1H), 8.22–8.27 (m, 2H), 7.79–7.81(m, 2H), 7.28 (s, 1H), 4.77 (t, J = 6.8 Hz, 2H), 3.59 (t, J = 6.8 Hz, 2H), 3.37 (q, J = 5.8 Hz, 4H), 1.52 (t, J = 7.6 Hz, 6H); HR-FABMS calcd for $C_{22}H_{20}ClNO_4$ $(M+H)^+$: 398.1159, found: 398.1169.

4.2.12. 2-Chloro-3-[2-[bis(1-methylethylamino)]ethoxy]-benzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (8f). Orange pearl solid (24.8%); mp 246–247 °C; ^{1}H NMR ($CDCl_3$) δ 8.30 (s, 1H), 8.20–8.27 (m, 2H), 7.77–7.81 (m, 2H), 7.38 (s, 1H), 4.92 (t, J = 5.2 Hz, 2H), 3.84–3.95 (m, 2H), 3.56 (q, J = 5.1 Hz, 2H), 1.62 (d, J = 6.8 Hz, 6H), 1.55 (d, J = 6.8 Hz, 6H); HR-FABMS calcd for $C_{24}H_{24}ClNO_4$ $(M+H)^+$: 426.1472, found: 426.1444.

4.2.13. 2-Chloro-3-[2-(dimethylamino)isopropoxy]benzo-[*b*]naphtho[2,3-*d*]furan-6,11-dione (8g). Orange solid (25.9%); mp 211–212 °C; ^{1}H NMR ($CDCl_3$) δ 8.29 (s, 1H), 8.21–8.26 (m, 2H), 7.76–7.81 (m, 2H), 7.32 (s, 1H), 4.60–4.70 (m, 1H), 2.73–2.82 (m, 1H), 2.55–2.63 (m, 1H), 2.37 (s, 6H), 1.43 (d, J = 6.0 Hz, 3H); HR-FABMS calcd for $C_{21}H_{18}ClNO_4$ $(M+H)^+$: 384.1003, found: 384.1016.

4.2.14. 2-Chloro-3-[2-(dimethylamino)propoxy]benzo-[*b*]-naphtho[2,3-*d*]furan-6,11-dione (8h). Dark orange solid (15.0%); mp 196–197 °C; ^{1}H NMR ($CDCl_3$) δ 8.30 (s, 1H), 8.21–8.26 (m, 2H), 7.76–7.81 (m, 2H), 7.19 (s, 1H), 4.18–4.22 (m, 1H), 3.97–4.02 (m, 1H), 3.10–3.18 (m, 1H), 2.42 (s, 6H), 1.25 (d, J = 6.8 Hz, 3H); HR-FABMS calcd for $C_{21}H_{18}ClNO_4$ $(M+H)^+$: 384.1003, found: 384.1023.

4.2.15. 8-(2-(Diethylamino)ethoxy)benzofuro[3,2-*g*]quinoline-5,11-dione (8i). Yellow brown solid (24.2%); mp 189–190 °C; ^{1}H NMR ($CDCl_3$) δ 9.08 (d, J = 4.4 Hz, 1H), 8.56 (d, J = 7.6 Hz, 1H), 8.21 (d, J = 8.8 Hz, 1H),

7.73 (t, J = 6.2 Hz, 1H), 7.20 (s, 1H), 7.17 (d, J = 8.0 Hz, 1H), 4.72 (t, J = 7.2 Hz, 2H), 3.52 (t, J = 4.0 Hz, 2H), 3.31 (q, J = 4.0 Hz, 4H), 1.51 (t, J = 7.2 Hz, 6H); HR-FABMS calcd for $C_{21}H_{20}N_2O_4$ $(M+H)^+$: 365.1501, found: 365.1504.

4.2.16. 8-(2-(Diisopropylamino)ethoxy)benzofuro[3,2-*g*]-quinoline-5,11-dione (8j). Red brown solid (55.9%); mp 234–235 °C; ^{1}H NMR ($CDCl_3$) δ 9.07 (d, J = 4.8 Hz, 1H), 8.55 (d, J = 8.0 Hz, 1H), 8.18 (d, J = 9.2 Hz, 1H), 7.72 (t, J = 6.4 Hz, 1H), 7.23 (s, 1H), 7.18 (d, J = 8.8 Hz, 1H), 4.82 (t, J = 6.0 Hz, 2H), 3.75–3.85 (m, 2H), 3.45 (t, J = 5.2 Hz, 2H), 1.62 (d, J = 6.8 Hz, 6H), 1.53 (d, J = 6.8 Hz, 6H); HR-FABMS calcd for $C_{23}H_{24}N_2O_4$ $(M+H)^+$: 393.1814, found: 393.1818.

4.2.17. 8-(1-(Dimethylamino)propan-2-yloxy)benzofuro-[3,2-*g*]quinoline-5,11-dione (8k). Orange red solid (15.7%); mp 134–135 °C; ^{1}H NMR ($CDCl_3$) δ 9.06 (d, J = 4.4 Hz, 1H), 8.54 (d, J = 8.0 Hz, 1H), 8.14 (d, J = 8.8 Hz, 1H), 7.71 (d, J = 7.6 Hz, 1H), 7.22 (s, 1H), 7.16 (d, J = 8.8 Hz, 1H), 4.70–4.81 (m, 1H), 2.77–2.86 (m, 1H), 2.58–2.67 (m, 1H), 2.43 (s, 6H), 1.39 (d, J = 6.0 Hz, 3H); HR-FABMS calcd for $C_{20}H_{18}N_2O_4$ $(M+H)^+$: 351.1345, Found: 351.1351.

4.2.18. 8-(2-(Dimethylamino)propoxy)benzofuro[3,2-*g*]-quinoline-5,11-dione (8l). Brick brown solid (12.9%); mp 195–196 °C; ^{1}H NMR ($CDCl_3$) δ 9.07 (d, J = 4.8 Hz, 1H), 8.55 (d, J = 8.0 Hz, 1H), 8.16 (d, J = 8.4 Hz, 1H), 7.71 (d, J = 7.6 Hz, 1H), 7.20 (s, 1H), 7.16–7.18 (m, 1H), 4.14–4.19 (m, 1H), 3.97–4.06 (m, 1H), 3.07–3.17 (m, 1H), 2.44 (s, 6H), 1.23 (d, J = 6.8 Hz, 3H); HR-FABMS calcd for $C_{20}H_{18}N_2O_4$ $(M+H)^+$: 351.1345, found: 351.1338.

4.2.19. 8-(2-(Diethylamino)ethoxy)-7-chlorobenzofuro[3,2-*g*]quinoline-5,11-dione (8m). Yellow brown solid (48.5%); mp 198–199 °C; ^{1}H NMR ($CDCl_3$) δ 9.09 (d, J = 4.8 Hz, 1H), 8.57 (d, J = 8.0 Hz, 1H), 8.30 (s, 1H), 7.74 (t, J = 6.2 Hz, 1H), 7.31 (s, 1H), 4.76–4.84 (m, 2H), 3.56–3.64 (m, 2H), 3.27–3.48 (m, 4H), 1.51 (t, J = 7.2 Hz, 6H); HR-FABMS calcd for $C_{21}H_{19}ClN_2O_4$ $(M+H)^+$: 399.1112, found: 399.1148.

4.2.20. 8-(2-(Diisopropylamino)ethoxy)-7-chlorobenzofuro[3,2-*g*]quinoline-5,11-dione (8n). Red brown solid (89.2%); mp 215–216 °C; ^{1}H NMR ($CDCl_3$) δ 9.08 (d, J = 4.0 Hz, 1H), 8.56 (d, J = 8.0 Hz, 1H), 8.26 (s, 1H), 7.73 (t, J = 6.0 Hz, 1H), 7.40 (s, 1H), 4.96 (t, J = 4.4 Hz, 2H), 3.85–3.96 (m, 2H), 3.56 (t, J = 5.2 Hz, 2H), 1.63 (d, J = 6.4 Hz, 6H), 1.56 (d, J = 6.4 Hz, 6H); HR-FABMS calcd for $C_{23}H_{23}ClN_2O_4$ $(M+H)^+$: 427.1425, Found: 427.1397.

4.2.21. 8-(1-(Dimethylamino)propan-2-yloxy)-7-chlorobenzofuro[3,2-*g*]quinoline-5,11-dione (8o). Orange red solid (31.0%); mp 194–195 °C; ^{1}H NMR ($CDCl_3$) δ 9.07 (d, J = 4.4 Hz, 1H), 8.56 (d, J = 8.0 Hz, 1H), 8.28 (s, 1H), 7.72 (d, J = 8.0 Hz, 1H), 7.35 (s, 1H), 4.66–4.79 (m, 1H), 2.78–2.88 (m, 1H), 2.60–2.70 (m, 1H), 2.41 (s, 6H), 1.44 (d, J = 6.0 Hz, 3H); HR-FABMS calcd for $C_{20}H_{17}ClN_2O_4$ $(M+H)^+$: 385.0955, found: 385.0984.

4.2.22. 8-(2-(Dimethylamino)propoxy)-7-chlorobenzofuro[3,2-*g*]quinoline-5,11-dione (8p). Brick brown solid (22.4%); mp: 189–190 °C; ^{1}H NMR ($CDCl_3$) δ 9.07 (d, J = 4.4 Hz, 1H), 8.55 (d, J = 8.0 Hz, 1H), 8.27 (s, 1H), 7.73 (d, J = 8.0 Hz, 1H), 7.20 (s, 1H), 4.20–4.24 (m, 1H), 3.99–4.09 (m, 1H), 3.13–3.23 (m, 1H), 2.45 (s, 6H), 1.28 (d, J = 6.4 Hz, 3H); HR-FABMS calcd for $C_{20}H_{17}ClN_2O_4$ $(M+H)^+$: 385.0955, found: 385.0937.

4.2.23. In vitro antitumor activity evaluation by SRB assay[14]. The in vitro cytotoxic activities were evaluated by SRB method. Human tumor cell lines: lung (A549), stomach (SNU-638), colon (HCT116), fibro sarcoma (HT1080), myeloid leukemic (HL-60), ovarian (SK-OV-3), melanoma (SK-MEL-2), and CNS (XF-498) (5×10^4 cells/mL) were treated with different concentrations of the test agents for 3 days. After treatment, cells were fixed with TCA and cell viability was determined with sulforhodamine B (SRB) protein staining method. The result was expressed as a percentage, relative to solvent-treated control incubations, and the IC_{50} values were calculated using non-linear regression analysis (percent survival versus concentration).

4.3. Decatenation assay for topoisomerase II activity[17]

The assay was done according to the protocol provided by TopoGen, Inc. The total reaction volume of the topoisomerase II-mediated cleavage reaction was fixed at 20 μL. Briefly, assay buffer (50 mM Tris–HCl, pH 8, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 30 μg/mL bovine serum albumin (BSA)) containing 200 ng KDNA (TopoGEN) and a solution of the test drugs were added to one unit of the human recombinant topoisomerase II (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 stained with SYBR Green I (Molecular Probes, Eugene, OR) and observed under UV illumination. 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.

4.4. Relaxation assay for topoisomerase I activity[17]

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 ethylene diamine tetraacetic 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.

Acknowledgment

This work was supported from the Korea Research Foundation (KRF-2005-005-J01501).

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