Design of Antineoplastic Agents on the Basis of the "2-Phenylnaphthalene-Type" Structural Pattern. 2. Synthesis and Biological Activity Studies of Benzo[*b*]naphtho[2,3-*d*]furan-6,11-dione Derivatives

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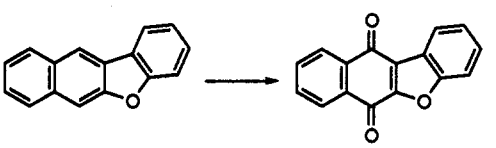
Based on the "2-phenylnaphthalene-type" structural pattern hypothesis developed in our laboratory, a number of benzo[*b*]naphtho[2,3-*d*]furan-6,11-diones were designed, synthesized, and evaluated *in vitro* for their inhibitory action against the growth of human promyelocytic leukemia cells (HL-60), small-cell lung cancer (SCLC), SCLC cells resistant to cisplatin (SCLC/CDDP), National Cancer Institute's disease-oriented primary antitumor 60 cell-line panel, and drug-stimulated topoisomerase II-mediated DNA cleavages. Many compounds designed were found to possess potent activity in one or more of the biological tests. In general, activity found in one of the cell lines tested is often echoed in other cell lines and many also expressed substantial inhibitory activity against topoisomerase II-mediated cleavage activities. One of these compounds, 3-[2-(dimethylamino)ethoxy]-1-hydroxybenzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (**8j**), exhibited strong inhibitory activity throughout the entire series of test panel. Thus, it appears that the proposed structural pattern hypothesis has received substantial support through experimental verification.

Introduction

A tricyclic chemical structural pattern, consisting of a phenyl ring attached to position 2 of a naphthalene nucleus, or composed of various heterocyclic ring units with similar molecular structural arrangements, has been observed among a large number of biologically and pharmacologically active compounds.[1] Examples include benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene (carcinogenic); coralyne and nitidine (antileukemic); chartreusin and rabelomycin (antibacterial, cytotoxic); camptothecin, streptonigrin, and ellipticine (antineoplastic); WS-5995A (anticoccidial); genistein (estrogenic); methaqualone (sedative, hypnotic, anticonvulsive); and gossypol (antioxidant, male contraceptive), among others.[1] The structural pattern *per se* may not be sufficient for attaining biological activity. Nevertheless, with proper groups or substituents attaching to specific positions on both ring units, it is believed that compounds having desired biological actions could eventually be designed.

Among compounds possessing this structural pattern, it is noticed that many antineoplastic agents assume a coplanar conformation. The coplanarity linking the two ring systems may either be assured through hydrogen-bond formation between the two ring units, such as streptonigrin, or through a condensed structure, such as camptothecin or ellipticine.

On the basis of the preceding concept, a search of suitable chemical structures to fulfill the requirements for our drug design was conducted. It was noted that a tetracyclic compound, brasan (β-brazan, **1**), originally isolated from coal tar distillate,[2] its derivatives being obtained from brazilin (isolated from *Caesalpinia echinata* Lam., Brazil wood),[3] would be a proper candidate for our study. The compound forms a reddish yellow quinone, **2**, on oxidation.[4]

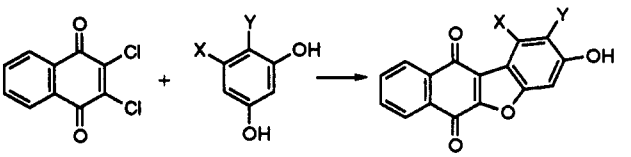


1 **2**

Both compounds **1** and **2** possess the characteristic "2-phenylnaphthalene-type" structural pattern. With the presence of the ethereal linkage, all connecting rings in these structures become planar. Since the quinone function has often appeared in a large number of agents of medicinal interest, compound **2** was selected as one of the suitable starting structures for our present drug design study.

Chemistry

Almost 100 years ago, Liebermann[5] synthesized 3-hydroxybenzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (**5a**) by the



3 **4a**: X, Y = H; **b**: X = OH; Y = H; **c**: X = H; Y = Cl; **d**: X = CH_3; Y = H **5a**: X, Y = H; **b**: X = OH; Y = H; **c**: X = H; Y = Cl; **d**: X = CH_3; Y = H

base-catalyzed condensation of 2,3-dichloro-1,4-naphthoquinone (**3**) with resorcinol (**4a**). Compound **5a** has since been resynthesized and studied by many investigators,[6-8]

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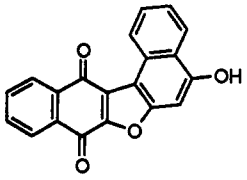
but in spite of our exhaustive search, the closely related 1,3-dihydroxy analog **5b** could not be located in the literature. This is indeed surprising because the other required starting material for the preparation of **5b**, phloroglucinol (**4b**), was equally available as resorcinol a century ago.

Condensation of **3** and **4a** was repeated in our laboratory under the reported reaction conditions[5] and compound **5a** was obtained in high yield, as described in the literature. However, using the same reaction conditions ascribed for **5a** for the preparation of **5b** gave erratic results. Occasionally impure **5b** was obtained in low (less than 20% crude) yields and needed to be painstakingly purified, but in most cases only tarry substance was formed in the reaction mixture. This preparation was therefore carefully studied in our laboratory. Finally it was found that the success of this preparation depends on many factors: the nature of the base used, the rate and mode of addition of phloroglucinol, and the reaction temperature and the pH of the reaction mixture. This precise reaction condition can now be readily repeated and over 50% yield of analytically pure **5b** can be obtained. The criticalness of the reaction conditions thus explains the lack of information for compound **5b** in the literature.

The corresponding 2-chloro (**5c**) and 1-methyl (**5d**) derivatives of **5a**, as well as the naphtho (**6**) and the methylenedioxy (**7**) analogs, were prepared without difficulty by the condensation of **3** with the appropriate phenolic derivatives.



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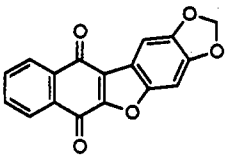

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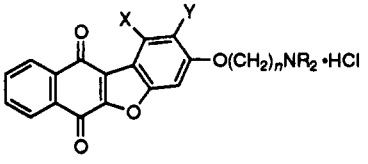
Treatment of compounds containing the 3-hydroxy function (**5a-d** and **6**) with dialkylaminoalkyl chloride in base yielded the following derivatives, which were evaluated biologically along with their parent compounds. The extra 1-hydroxy group in compound **5b** could not be easily derivatized because of its obvious hydrogen-bonding participation with the neighboring carbonyl oxygen atom.



8a: X, Y = H; $n = 2$; R = CH_3
b: X, Y = H; $n = 3$; R = CH_3
c: X = CH_3; Y = H; $n = 2$; R = CH_3
d: X = H; Y = Cl; $n = 2$; R = CH_3
e: X = H; Y = Cl; $n = 3$; R = CH_3
f: X + Y = CH=CHCH=CH; $n = 2$; R = CH_3 (free base)
g: X + Y = CH=CHCH=CH; $n = 3$; R = CH_3 (free base)
h: X = OH; Y = H; $n = 2$; R = CH_3
i: X = OH; Y = H; $n = 3$; R = CH_3
j: X = OH; Y = H; $n = 2$; R = C_2H_5
k: X = OH; Y = H; $n = 2$; R = $CH(CH_3)_2$

Results and Discussion

Compounds synthesized in this study were evaluated for their inhibitory action against the growth of human promyelocytic leukemia cells (HL-60), small-cell lung cancer (SCLC), SCLC cells resistant to cisplatin (SCLC/CDDP), National Cancer Institute's disease-oriented primary antitumor screen 60 cell-line panel, and drug-stimulated topoisomerase II-mediated DNA cleavages (see Table I). In general, activity found in one of the cell lines tested is often echoed in other tests and sometimes certain compounds, such as **5b** and **8j**, can exhibit excellent inhibitory activity throughout the entire *in vitro* tests.

Table I. Inhibitory Action of Benzo[*b*]naphtho[2,3-*d*]furan-6,11-diones

compd	IC_{50} (μM) HL-60[a]	SCLC[b]	SCLC/CDDP[b]	NCI screen[c]	topo II-mediated DNA cleavage activities[d]
5a	7.78	>3.8	>3.8	+	1 000
5b	0.22	1.03	1.28	++	>100 000
5c	6.09	>3.4	>3.4	+	1 000
5d	6.65	>3.5	>3.5	–	>100 000
6	80.6	>3.2	>3.2	–	20
7	>100	>40.3	>40.3	–	0
8a	0.30	0.73	0.94	++	10 000
8b	0.34	1.30	2.60	+	1 000
8c	1.80	>2.6	>2.6	–	20 000
8d	0.24	0.37	0.62	++	10 000
8e	0.28	0.59	0.59	+	>100 000
8f	0.87	1.30	1.95	–	10 000
8g	1.39	>2.5	>2.5	–	200
8h	0.046	2.13	2.56	+++	10 000
8i	0.045	0.68	0.68	+++	1 000
8j	0.098	0.24	0.48	+++	>100 000
8k	0.089	0.22	1.13	++	1 000

[a] Inhibitory concentration for established anticancer agent *m*-AMSA is 0.055 μM. [b] Cytotoxicity against SCLC (and SCLC/CDDP) for established anticancer agents (IC_{50}, μM) are as follows: cisplatin, 0.67 (6.7); VP-16, 0.5 (1.3); vinblastine, 0.004 (0.005); adriamycin, 0.04 (0.03); mitoxantrone, 0.02 (0.03); 5-FU, 5.4 (8.4). [c] National Cancer Institute preclinical 60 human tumor cell lines drug-discovery screen: complete inhibition of cell growth detected [log IC_{50} (M)] for at least one cell line at −7, +++; −6, ++; −5, +; inactive, −. [d] Relative activity to induce mammalian DNA topoisomerase II-mediated DNA cleavage is based on the effective doses which caused 50% of topoisomerase II-mediated DNA fragmentation of linearized 8.4 kb YEpG DNA. The cleavage activity of VM-26 (a potent topoisomerase II drug) to stimulate topoisomerase II-mediated DNA cleavage is taken as 100 000.

The hydroxyl function plays an important role with regard to biological activity. 1,3-Dihydroxybenzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (**5b**), which contains two hydroxyl functions, exhibited very good inhibitory activity against SCLC and SCLC/CDDP strains, and demonstrated higher topoisomerase II-mediated DNA cleavage than the anticancer agent teniposide (VM-26). However, the DNA cleavage pattern induced by compounds of this series is different from that induced by VM-26. Compounds containing only one hydroxyl function at position 3 are less active (the 1-methyl analog **5d** still possessed high topo II-DNA cleavage activity). The 2,3-(methylenedioxy) compound **7**, which does not contain a free hydroxyl function, is totally inactive.

Compounds containing a 3-dialkylamino alkylamino function (**8a-8k**) are uniformly more active than their parent compounds. Among these compounds, potent activities were noted for **8a**, **8d** and **8e**, but the most potent activities were found with compounds **8h-k**, which were derived from the parent 1,3-dihydroxy compound **5b**. The IC_{50} values of these compounds against HL-60 ranged from 0.05 to 0.1 μM, which are comparable to that for the antitumor agent *m*-AMSA. In a separate DNA relaxation inhibition assay, compounds **8d**, **8e**, **8i**, and **8j** were found to be more potent than *m*-AMSA.

For the cytotoxicity against the SCLC lines, quite a number of them possessed IC_{50} values lower than 1 μM, which are comparable to those of cisplatin and VP-16. It is of interest to note that for quite a number of these compounds, which demonstrated inhibitory activity against SCLC, similar activity was also observed against the SCLC/CDDP lines. This indicates that many compounds of this series are equally active against both the cisplatin-sensitive and cisplatin-resistant lines, which implies that these banzo[*b*]naphtho[2,3-*d*]furan-6,11-dione derivatives do not exhibit cross-resistance with cisplatin against the small-cell lung cancer lines.

In the National Cancer Institute 60-human tumor cell line screen, compounds **5a**, **5c**, **8b**, and **8e** showed complete inhibition of cell growth in at least one cell line at mean log IC_{50} (M) values of −5; compounds **5b**, **8a**, **8d** and **8k** are 10 times more active and compounds **8h**, **8i** and **8j** are 100 times more active.

We are pleased to acknowledge that the proposed "2-phenylnaphthalene-type" structural pattern hypothesis has been substantiated by the experimental results obtained from the previous[18] and the present paper.

We have also tested these compounds in CEM and CEM/MDR (multidrug resistant) cells. The latter possess multidrug-resistant phenotype and genotype (cell lines were a gift from Dr. Victor Ling) and exhibit 500-fold resistancy to doxorubicin. Our preliminary results indicated that these compounds were not recognized by this MDR efflux mechanism and thereby exhibited no cross resistance to other MDR drugs (the IC_{50} in CEM cells for compounds **5b**, **8a**, **8d**, and **8e** was 0.50, 0.20, 0.25, and 0.17 μM, respectively, whereas the IC_{50} in CEM/MDR cells were 0.60, 0.15, 0.30, and 0.15 μM, respectively). These findings may be of importance since MDR is one of the major mechanisms of resistance clinically, future design of cancer chemotherapeutic agents should aim at circumvence of this mechanism. Our series of compounds, unlike the presently available DNA topoisomerase II inhibitors such as VP-16 or mitoxantrone, are not recognized by MDR. This unique property should definitely be noted in future clinical trials.

Experimental Section

All melting points were taken on a Thomas-Hoover melting point apparatus. Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.3% of the theoretical values.

3-Hydroxybenzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (5a). To a stirred ethanolic NaOEt solution (prepared by dissolving 3.8 g (0.17 g-atom) of Na in 120 mL of EtOH) containing 6.9 g (0.03 mol) or 2,3-dichloro-1,4-naphthoquinone (**3**) was added dropwise, at 0 °C, 6.6 g (0.06 mol) of resorcinol (**4a**) in 80 mL of EtOH. After the addition, the mixture was stirred at room temperature overnight. The reaction mixture was acidified with 6 N HCl at 0 °C. The resulting solid was collected by filtration, washed successively with H_2O, MeOH, and Et_2O, and dried to give 7.8 g (97% yield) of **5a** as a deep yellow solid, mp > 310 °C (lit.[5] mp > 300 °C). Mass spec: *m/z* 264 (M^+). Anal. ($C_{16}H_8O_4$) C, H.

1,3-Dihydroxybenzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (5b). To a stirred solution of 315 mL of MeOH containing 12.6 g (0.225 mol) of KOH was added portionwise at 30 °C 10.2 g (0.045 mol) of powdered 2,3-dichloro-1,4-naphthoquinone (**3**). After 15 min the mixture appeared as a red crystalline suspension. To this was added dropwise with continuous stirring a 10% methanolic solution of 8.5 g (0.068 mol) of phloroglucinol (**4b**). The color of the reaction mixture gradually changed from red to blue black and then to dark brown. After stirring for 30 min at room temperature, the dark brown mixture became thickened. Stirring was continued for two additional hours whereupon the TLC examination indicated total consumption of the starting material **3**. The separated solids (the potassium salt of the product) were collected by filtration and washed thoroughly with MeOH. The solids were converted to the free phenolic compound **5b** by the treatment of more than 1 equiv of 0.2 N HCl at 0 °C. The resulting dark brown solid product was collected by filtration (the pH of the filtrate should be less than 3, an indication of total acidification), washed with EtOH, and dried *in vacuo* at 60 °C. It was recrystallized from dimethylformamide to give 6.9 g (54.8% yield) of analytically pure **5b** as dark brown needles, mp 340–342 °C dec. UV: λ_{max} (MeOH): 209 (ϵ 26 900), 238 (ϵ 18 920), 292 (ϵ 23 260), and 487 nm (ϵ 2240). Mass spec: *m/z* 280 (M^+). Anal. ($C_{16}H_8O_5$) C, H.

2-Chloro-3-hydroxybenzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (5c). This compound was prepared from 6.8 g (0.03 mol) of **3** and 6.5 g (0.045 mol) of 4-chlororesorcinol (**4c**) in a manner similar to that for the preparation of **5a** in 65% yield as red needles, mp 303–304 °C. Mass spec: *m/z* 278 (M^+). Anal. ($C_{16}H_7ClO_4$) C, H.

1-Methyl-3-hydroxybenzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (5d). This compound was prepared from 6.8 g of **3** and 6.4 g of orcinol monohydrate (**4d**) in a manner similar to that for the preparation of **5a** in 60% yield, mp 299–300 °C. Mass spec: *m/z* 278 (M^+). Anal. ($C_{17}H_{10}O_4$) C, H.

5-Hydroxydinaphtho[2,1-*b*:2′,3′-*d*]furan-8,13-dione (6). This compound was prepared from 5.3 g of **3** and 5.0 g of 1,3-dihydroxynaphthalene in a manner similar to that for the preparation of **5a** in 85% yield, mp > 300 °C. Mass spec: *m/z* 314 (M^+). Anal. ($C_{20}H_{10}O_4$) C, H.

2,3-(Methylenedioxy)benzo[*b*]naphtho[2,3-*d*]furan-7,11-dione (7). A mixture of 2.3 g (0.01 mol) of **3** and 1.5 g (0.015 mol) of 98% sesamol was dissolved in 120 mL of pyridine. To this solution was added, with stirring, 6.5 g of KOH pellets. The solution was stirred at room temperature for 30 min and then gently heated in an oil bath at 90 °C overnight with continuous stirring. The reaction mixture was poured onto crushed ice containing 100 mL of 15% HCl with vigorous stirring. The resulting solid was collected by filtration and washed successively with H_2O, dilute KOH, H_2O, and Et_2O to give 3 g (quantitative yield) of **7** as red crystals, mp 309–310 °C, which were recrystallized from a mixture of dimethylformamide and H_2O to yield golden crystals, mp 310 °C. Mass spec: *m/z* 292 (M^+). Anal. ($C_{17}H_8O_5$) C, H.

3-[2-(Dimethylamino)ethoxy]benzo[*b*]naphtho[2,3-*d*]furan-6,11-dione Hydrochloride (8a). A mixture of 2.5 g (9.5 mmol) of **5b**, 100 mL of $CHCl_3$, and 60 mL of H_2O was stirred at room temperature for 1 h. To the mixture was added, with vigorous stirring, 2.7 g (19 mmol) of 2-(dimethylamino)ethyl-chloride hydrochloride. The resulting mixture was refluxed for 5 h with continuous stirring. It was cooled and the organic layer was separated. The aqueous portion was extracted with $CHCl_3$ (2 × 50 mL). The combined organic solution was washed successively with 5% NaOH, brine, and H_2O and then dried. Evaporation of the solvent yielded the free base as a brown solid. This was acidified in EtOH–HCl to yield the hydrochloride as a yellow solid, mp 288–290 °C dec. Recrystallization from 95% EtOH gave 1.8 g (51.2% yield) of **8a** as yellow crystals, mp 298–299 °C dec. Anal. ($C_{20}H_{17}NO_4$·HCl) C, H, N.

3-[3-(Dimethylamino)propoxy]benzo[*b*]naphtho[2,3-*d*]furan-6,11-dione Hydrochloride (8b). This compound was prepared from 2.7 g (10 mmol) of **5b**, 2 g of NaOH, 20 mL of H_2O, 150 mL of $CHCl_3$, and 3.2 g (20 mmol) of 3-(dimethylamino)-propyl chloride hydrochloride in a manner similar to that for the preparation of **8a** as yellow crystals in 42% yield, mp 286–287 °C dec. Mass spec: *m/z* 349 (M^+, free base). Anal. ($C_{21}H_{19}NO_4$·HCl) C, H, N.

In a similar manner, the following compounds were prepared.

3-[2-(Dimethylamino)ethoxy]-1-methylbenzo[*b*]naphtho-[2,3-*d*]furan-6,11-dione hydrochloride (8c): yellow crystals from BuOH–$HCONMe_2$–H_2O, mp 280–281 °C, 9% yield. Anal. ($C_{21}H_{19}NO_4$·HCl) C, H, N.

2-Chloro-3-[2-(dimethylamino)ethoxy]benzo[*b*]naphtho-[2,3-*d*]furan-6,11-dione hydrochloride (8d): yellow crystals from EtOH, mp 277–278 °C dec, 52% yield. Anal. ($C_{20}H_{16}ClNO_4$·HCl) C, H, N.

2-Chloro-3-[3-(dimethylamino)propoxy]benzo[*b*]naphtho-[2,3-*d*]furan-6,11-dione hydrochloride (8e): orange red crystals from $HCONMe_2$–BuOH, mp 274 °C dec, 27.2% yield. Mass spec: *m/z* 383 (M^+, free base). Anal. ($C_{21}H_{18}ClNO_4$·HCl) C, H, N.

5-[2-(Dimethylamino)ethoxy]dinaphtho[2,1-*b*:2′,3′-*d*]furan-8,13-dione (8f): dark brown crystals from BuOH, mp 194–196 °C, 85.6% yield. Mass spec: *m/z* 385 (M^+). Anal. ($C_{24}H_{19}NO_4$) C, H, N.

5-[3-(Dimethylamino)propoxy]dinaphtho[2,1-b:2′,3′-d]furan-8,13-dione (8g): brick red crystals from BuOH, mp 190–191 °C, 41% yield. Mass spec: *m/z* 399 (M^+). Anal. ($C_{25}H_{21}NO_4$) C, H, N.

3-[2-(Dimethylamino)ethoxy]-1-hydroxybenzo[*b*]naphtho-[2,3-*d*]furan-6,11-dione (8h): black crystals from BuOH, mp 205–207 °C dec, 30.3% yield. Mass spec: *m/z* 351 (M^+). Anal. ($C_{20}H_{17}NO_5$) C, H, N.

The hydrochloride salt was also prepared. Dark brown crystals from BuOH–$HCONMe_2$, mp 286 °C dec. Anal. ($C_{20}H_{17}NO_5$·HCl) C, H, N.

3-[3-(Dimethylamino)propoxy]-1-hydroxybenzo[*b*]naphtho[2,3-*d*]furan-6,11-dione (8i): black crystals from BuOH, mp 197–199 °C dec, 30% yield. UV: λ_{max} (MeOH): 232 (ϵ 19 700), 270 (ϵ 21 000), and 287 nm (ϵ 25 100). Mass spec: *m/z* 365 (M^+). Anal. ($C_{21}H_{19}NO_5$) C, H, N.

For the synthesis of compounds **8j** and **8k**, the base K_2CO_3 rather than NaOH was used since products obtained with NaOH were contaminated by other impurities.

3-[2-(Diethylamino)ethoxy]-1-hydroxybenzo[*b*]naphtho-[2,3-*d*]furan-6,11-dione (8j). To a suspension of 1.4 g (5 mmol) of **5b** in 100 mL of $CHCl_3$ was added a solution of 2.1 g (15 mmol) of K_2CO_3 in 10 mL of H_2O followed by 1.7 g (10 mmol) of 2-(diethylamino)ethyl chloride hydrochloride in 10 mL of H_2O. The mixture was refluxed for 6 h with vigorous stirring. It was cooled and the organic phase was separated. The aqueous portion was extracted with $CHCl_3$ (2 × 50 mL). The combined organic phase was washed with brine and H_2O and dried (Na_2SO_4). Evaporation of the solvent gave 1.5 g of solid, which gave 1.25 g (66% yield) of pure **8j** as brick red crystals upon recrystallization from BuOH–$CHCl_3$, mp 199–200 °C. IR: max 3230 (br), 1650, 1625, 1575, 1550 cm^{-1}. Mass spec: *m/z* 379 (M^+). Anal. ($C_{22}H_{21}NO_5$) C, H, N.

The hydrochloride salt was obtained as black crystals after recrystallization from BuOH–$HCONMe_2$, mp 261–262 °C dec. Anal. ($C_{22}H_{21}NO_5$·HCl) C, H, N.

3-[2-[Bis(1-methylethyl)amino]ethoxy]-1-hydroxybenzo-[*b*]naphtho[2,3-*d*]furan-6,11-dione (8k). This compound was prepared in a manner similar to that for the preparation of **8j**. The free base was obtained in 59% yield as brick red crystals, mp 187-189 °C (from BuOH–$CHCl_3$). Mass spec: *m/z* 407 (M^+). Anal. ($C_{24}H_{25}NO_6$) C, H, N.

The hydrochloride salt was obtained as dark brown crystals after being recrystallized from BuOH–$HCONMe_2$, mp 254–255 °C dec. Anal. ($C_{24}H_{25}NO_5$·HCl) C, H, N.

Materials and Methods

1. HL-60 Cytotoxic Assay. The synthesized compounds were evaluated for their cytotoxic effects on HL-60 (human promyelocytic leukemia) cells. The assay was conducted in 96-well microplates. The compounds were serially diluted (in four to six steps) with DMSO and added to cell incubation medium (RPMI 1640 containing 10% fetal cell serum) at the final concentration of 1.0% DMSO in the medium. The cytotoxicity of the compounds were determined by a XTT-microculture tetrazolium assay[9]: 2′,3′-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) was prepared at 1 mg/mL in prewarmed (37 °C) medium without serum. Phenazine methosulfate (PMS) and fresh XTT were mixed together to obtain a 0.025 mM PMS–XTT solution (25 μL of the stock 5 mM PMS was added per 5 mL of 1 mg/mL XTT). Fifty microliters of this mixture was added to each well of the cell culture after 72-h exposure to the testing compound. After incubation at 37 °C for 6 h, absorbance at 450 and 630 nm was measured with a microplate reader (EL 340, Bio-Tek Instruments, Inc., Winooski, VT). The median-effect inhibitory concentration (IC_{50}) was determined by the median-effect plot[10] using a computer software program.[11]

2. SCLC (Small-Cell Lung Cancer) and SCLC/CDDP (SCLC Cells Resistant to Cisplatin) Cytotoxic Assay. Cells were seeded at a density of 2×10^5 cells/mL and allowed to attach overnight.[12] Various concentrations of each compound were added to the culture. At 72 h, cells were harvested and the viable cells (dye exclusion) were counted after mixing with 0.2% trypan blue. The IC_{50} for each testing compound was determined by plotting the number of viable cells as a percentage of control against drug concentration. All cytotoxicity assays were repeated three times. Cisplatin and other antineoplastic agents were used as controls to compare the activity with compounds studied presently.

3. NCI Antitumor Screen Assay. The *in vitro* disease-oriented antitumor screen cell panel operated by the National Cancer Institute consisted of 60 cell lines. A description of the rationale, background, and other details was published.[13] Testing results, dose–response curves, and IC_{50} mean graphs are provided for each compound at a minimum of five concentrations at 10-fold dilutions.

4. Topoisomerase II-Mediated DNA Cleavage Assay. (a) **Enzymes and Nucleic Acids.** DNA topoisomerase II was purified from calf thymus gland by a modification of the published procedures.[14] Plasmid YEpG, which is a derivative of YEp24, was purified by the alkali lysis method followed by phenol deproteination and by CsCl/ethidium isopycnic centrifugation.[15]

(b) **Preparation of End-Labeled DNA Fragments.** The procedure for end-labeling of plasmid DNA has been described previously.[16] Briefly, 10 μg of YEpG plasmid DNA was digested with *Bam*H1 restriction endonuclease and then labeled at its 3′ ends with the large fragment of *Escherichia coli* DNA polymerase I and [α-^{32}P]dATP. Unincorporated triphosphates were removed by two cycles of EtOH precipitation in the presence of 2.5 M NH_4OAc. In the DNA cleavage assay,[16,17] the 3′ end-labeled DNA was further digested with *NHe* 1 to remove one labeled end.

(c) **Topoisomerase II-Mediated DNA Cleavage Assay.** Reactions were carried out as described previously.[17] Briefly, reaction mixtures (20 μL each) containing 40 mM Tris, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/mL bovine serum albumin, 20 ng of ^{32}P-labeled DNA, 10 ng of calf thymus DNA topoisomerase II, and compounds were incubated at 37 °C for 30 min. The reactions were terminated by the addition of 2 μL of 5% SDS and then treated with 150 μg/mL proteinase K for another hour at 37 °C. Reaction products were analyzed by electrophoresis in 1% agarose gel in TBE (89 mM Tris–borate, pH 8.3, and 2 mM EDTA) electrophoresis buffer. The autoradiography cleavage pattern was quantitatively assessed.

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