Dependence of Antitumor Activity on the Electrophilicity of 2-Substituted 1,4-Naphthoquinone Derivatives

Yongseog Chung,[†] Jikang Yoo, Si-Hyun Park,[‡] Bok Hee Kim,[§] Xi Chen,[#] Chang-Guo Zhan,[#,*] and Hoon Cho[*]

Department of Polymer Science & Engineering, Chosun University, Gwangju 501-759, Korea. [*]E-mail: hcho@chosun.ac.kr
[†]Department of Chemistry, BITRC, Institute for Basic Science, Chungbuk National University, Chungbuk 361-763, Korea
[‡]Department of Photonic Engineering, Chosun University, Gwangju 501-759, Korea
[§]Department of Food Science and Nutrition, Chosun University, Gwangju 501-759, Korea
[#]Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY 40536-0082, U.S.A.
[*]E-mail: zhan@uky.edu
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Naphthoquinones are widely distributed in nature and have been used for centuries in home remedies as well as in cosmetics. Many clinically important antitumor drugs containing quinone nucleus, such as anthracyclines, mitoxantrones and saintopin, show excellent anticancer activity. These anticancer agents are effective inhibitors of DNA topoisomerase, and it is understood that the cytotoxicity of quinone analogues results from the inhibition of DNA topoisomerase II.[1-4] 1,4-Naphthoquinone derivatives have also been shown to inhibit human DNA topoisomerase I.[5-10] Furthermore, they can induce the formation of the semiquinone radical, which can transfer an electron to oxygen to produce superoxide. This process is catalyzed by flavoenzymes such as NADPH-cytochrome-*P*-450 reductase. Both superoxide and semiquinone radical anions of naphthoquinone analogues can generate the hydroxyl radical, which is known to cause DNA strand breaks.[11-14] In addition, a number of 1,4-naphthoquinone derivatives have been found to possess powerful pharmacological effects such as antibacterial,[15,16] antifungal,[15-18] anti-inflammatory,[18-22] antithrombotic,[23,24] antiplatelet,[19-25] antiviral,[23-25] antiallergic,[26] apoptotic,[27-29] lipoxygenase inhibiting,[30,31] radical scavenging[6] and antiringworm[15] activities. Previously we reported that 6-substituted 5,8-dimethoxy-1,4-naphthoquinone derivatives exhibited higher antitumor activity than 2-substituted 5,8-dimethoxy-1,4-naphthoquinone. It was suggested that the C2 or C3 of 6-substituted compounds are better Michael-type acceptor than the C3 of 2-substituted compounds and are attacked more easily by nucleophiles such as amine or thiol functional groups in the cells.[32-36] We also reported that a compound having a higher ^{1}H-NMR chemical shift at the C3-H (δ_H) usually should exhibit a lower ED_{50} value.[35] In the present paper, a series of new 5,8-dimethoxy-1,4-naphthoquinone (DMNQ) and 5,8-dihydroxy-1,4-naphthoquinone (DHNQ) derivatives were synthesized, their net atomic charge of C3 in the quinoid moiety was obtained from the natural population analysis of the molecular orbitals calculated at the HF/6-31G* level, and their cytotoxicity against L1210 and P388 cancer cells was examined. The antitumor action was also assessed in mice bearing S-180 cells in the peritoneal cavity.

Results and Discussion

We synthesized new 2-substituted DMNQ and DHNQ derivatives and measured the net atomic charge of C-3 in the

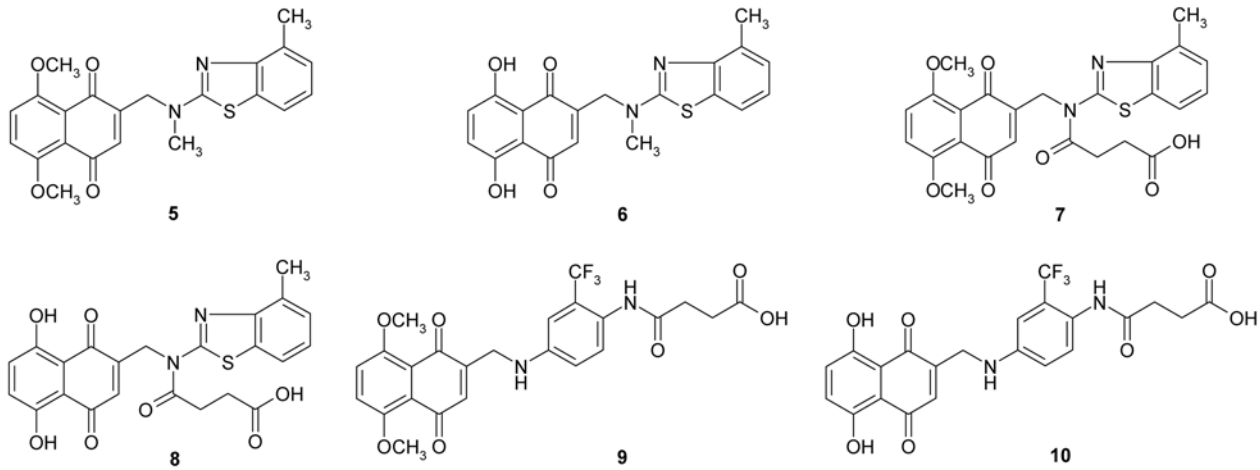
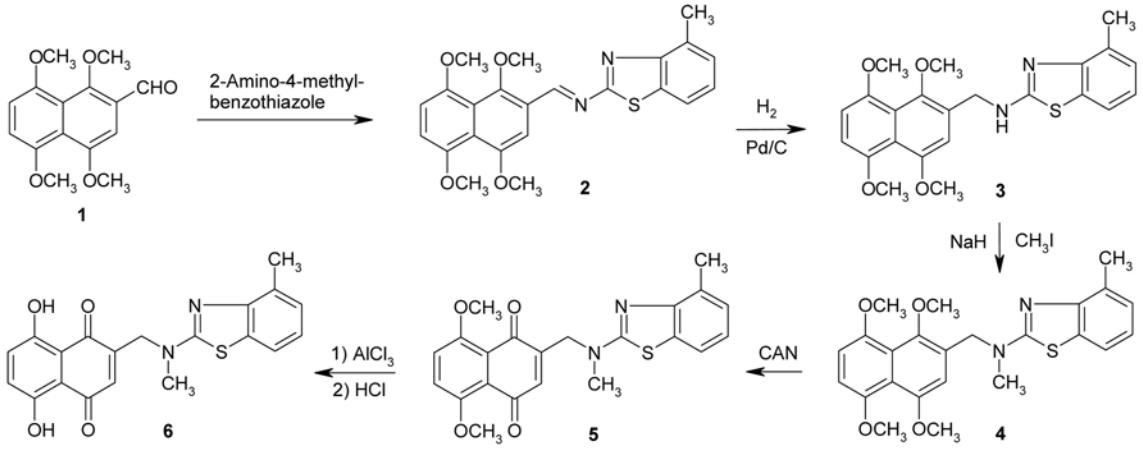


Figure 1. Structures of 5,8-dimethoxy-1,4-naphthoquinone analogs and 5,8-dihydroxy-1,4-naphthoquinone analogs.



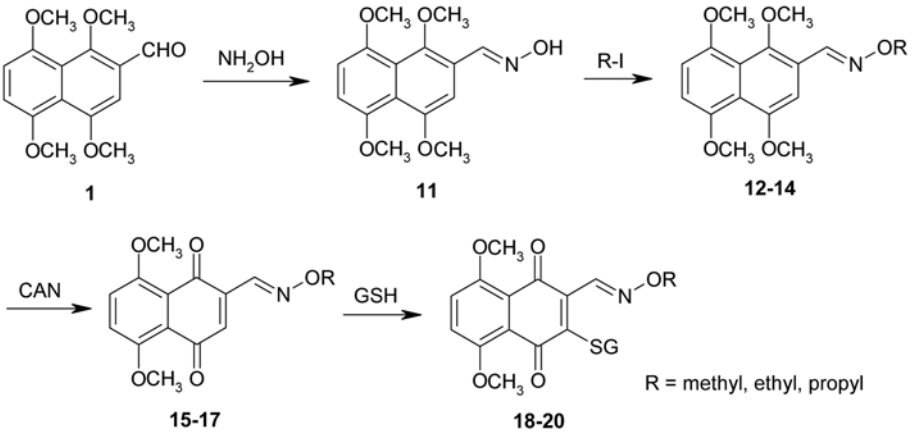
Scheme 1

quinoid moiety from the natural population analysis of the molecular orbitals calculated at the HF/6-31G* level and the chemical shift (δ_H) of 3-H from NMR analysis. DMNQ and DHNQ derivatives, shown in Figure 1, were synthesized as described in a previous report.[36] The starting compound, 2-formyltetramethoxynaphthalene (**1**) was prepared from 1,5-dihydroxynaphthalene through 4-step reactions of methylation (86%), bromination (85%), methoxylation (61%), and formylation (96%).[37] The reaction of 2-formyltetramethoxynaphthalene (**1**) with hydroxylamine afforded 2-(hydroxyiminomethyl)-1,4,5,8-tetramethoxynaphthalene (**11**), which was then alkylated to obtain the desired 2-(alkoxyiminomethyl)-1,4,5,8-tetramethoxynaphthalene derivatives (**12-14**). The derivatives of 2-(alkoxyiminomethyl)-5,8-dimethoxy-1,4-naphthoquinone (**15-17**) were prepared from compounds **12-14** by oxidation with ammonium cerium (IV) nitrate and the resulting compounds were conjugated with glutathione (GSH), to produce 2-(alkoxyiminomethyl)-3-gluthathionyl-5,8-dimethoxy-1,4-naphthoquinone derivatives (**18-20**), respectively. Compounds **5**, **7** and **9** were obtained by the oxidation of the corresponding 2-substituted 1,4,5,8-tetramethoxynaphthalenes with cerium (IV) ammonium nitrate (CAN), followed by demethylation with $AlCl_3$ and HCl to provide compounds **6**, **8** and **10** (Scheme 1). Overall yields ranged from 60% to 80%.

Table 1. Correlation of cytotoxicity and antitumor activity with the atomic charge and δ_H of the C-3 position of DMNQ and DHNQ derivatives

No. of Compd.	ED_{50} (μg/mL)		T/C (%)	atomic charge of C3	δ_H of 3-H
	L1210	P388			
5	1.21	0.56	214	–0.275	6.75
6	0.45	0.26	257	–0.252	6.99
7	4.76	4.19	105	–0.328	6.34
8	0.34	0.18	331	–0.243	7.37
9	8.92	40.41	101	–0.250	6.66
10	1.57	1.64	240	–0.238	7.01
Adriamycin	0.07	0.14	234		

The cytotoxicity of naphthoquinone derivatives was measured against L1210 (Lymphocytic leukemia) and P388 (Lymphoid neoplasma) cancer cell lines using the MTT colorimetric method.[38] The ED_{50} value (μg/mL) was defined as the concentration of compound which produced a 50% reduction in viability relative to the control in three independent experiments. In comparison with DMNQ derivatives (**5**, **7**, **9**), interestingly, DHNQ derivatives (**6**, **8**, **10**) exhibited higher cytotoxic activity against L1210 and P388. The DHNQ



Scheme 2

Table 2. Cytotoxicity of DMNQ derivatives with and without glutathione at the C-3 position

No. of Compd.	R	ED_{50} (μg/mL)					
		L1210	P388	HL-60	A549	SNU-1	Vero
15	Methyl	4.97	2.24	1.26	56.42	11.82	41.37
16	Ethyl	5.10	2.29	2.17	61.18	10.76	45.04
17	Propyl	5.28	2.76	2.03	48.06	19.14	51.29
18	Methyl	–	–	–	–	–	–
19	Ethyl	–	–	–	–	–	–
20	Propyl	–	–	–	–	–	–

–, no activity

showed better antitumor activity than DMNQ in mice bearing S-180 cells in the peritoneal cavity (Table 1).

From a toxicological perspective, quinones possess two principal chemical properties that confer their reactivity in biological systems: they are electrophiles and oxidants. Quinones are Michael acceptors, and cellular damage can occur through alkylation of crucial cellular proteins and/or DNA. Alternatively, quinones are highly redox active molecules which can redox cycle with their semiquinone radicals, leading to the formation of reactive oxygen species, including superoxide, hydrogen peroxide, and ultimately the hydroxyl radical.[39] In an attempt to elucidate the electrophilic effect of DMNQ, the C3 position of DMNQ was protected by alkylation of glutathione (GSH) (Scheme 2) and the cytotoxicity was measured against L1210, P388, HL-60, A549, SNU-1 cancer cell lines and normal Vero cells. Although, the GSH-bound DMNQ (compounds **18**-**20**) may still retain the ability to redox cycle, alkylation of the C3 position of DMNQ by GSH rendered the compounds totally inactive (Table 2). Thus the biological activity of naphthoquinone derivatives is believed to be dependent upon the electrophilicity of the quinone moiety. We also demethylated the 5,8-dimethoxy groups to produce DHNQ and cytotoxic activity against L1210 and P388 cancer cells was examined. The antitumor action was also assessed in mice bearing S-180 cells in the peritoneal cavity. Interestingly, removal of the methyl groups of the 5,8-dimethoxy groups significantly increased both bioactivities.

As shown in Table 1, removal of the methyl groups of 5,8-dimethoxy-1,4-naphthoquinone (DMNQ), which produced 5,8-dihydroxy-1,4-naphthoquinone (DHNQ), increased the chemical shift (δ_H) of 3-H from 6.75, 6.34 and 6.66 to 6.99, 7.37 and 7.07, respectively, suggesting that the antitumor activity of DHNQ is greater than that of DMNQ. The natural population analysis of the molecular orbitals calculated at the HF/6-31G* level also showed that the electron charge of C-3 in DHNQ was decreased more than that in DMNQ, suggesting that the antitumor activity of DHNQ is greater than that of DMNQ. As anticipated, Compound **8**, which had a higher electrophilicity in the C3 position, demonstrated better antitumor activity than the others did. The lower antitumor activity of compound **7** could be also ascribed to the lower electrophilicity at the C3 position of the compound.

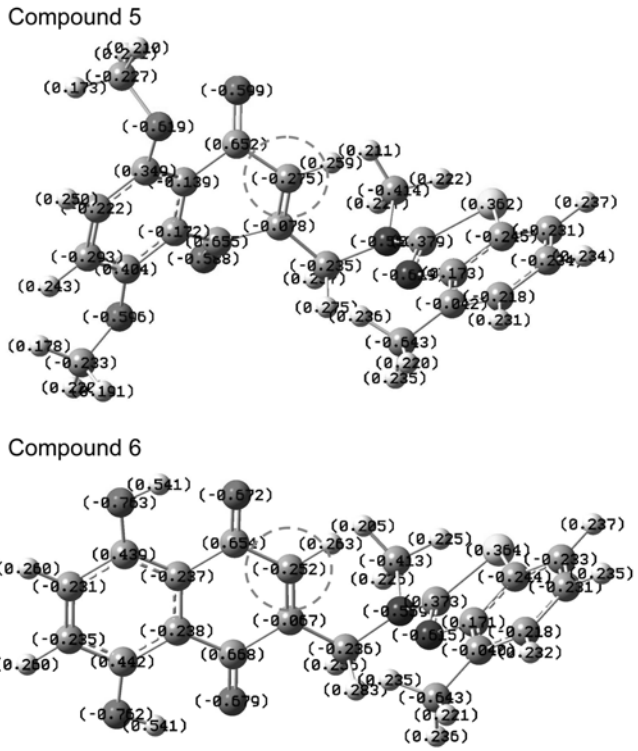


Figure 2. Optimized geometries of compounds **5** and **6**, in which the net atomic charges were obtained from the natural population analysis of the molecular orbitals calculated at the HF/6-31G* level.

Experimental Section

***In vitro* cytotoxicity (MTT Assay).** Target cancer cells were suspended at 2×10^5 cells/mL in medium (10% fetal bovine serum) containing various concentration of synthesized naphthoquinone derivatives and vigorously vortexed, after which 100 μL aliquots were dispensed into 96-well, flat-bottomed microtiter plates using a multichannel pipette. Plates were then incubated at 37 °C for 72 h in a 5% CO_2 incubator. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was dissolved in PBS at 5 mg/mL and filtered to sterilize and remove a small amount of insoluble residue present in MTT batches. An aliquot of 10 μL of MTT stock solution was added to each well using a multichannel pipette and the plate was incubated at 37 °C for 4 h. To each well, 150 μL of 0.01N HCl solution containing 10% sodium dodecyl sulfate was added to solubilize the MTT formazan. Plates were gently shaken until all formazan crystals were dissolved, and the absorbance at 540 nm was determined with a Microplate Reader (SPECTRA MAX 340). All results were corrected for background absorbance detected in wells without added MTT. Preliminary experiments showed a linear relationship between the cell numbers and the absorbance at 540 nm, when cells in the range of 4×10^2 to 4×10^5 per well were examined.

***In vivo* antitumor activity in ICR mice bearing S-180 cells.** The test samples dissolved in saline, including 2% DMSO and 4% Tween 80, were stored at 4 °C. S-180 cells

(0.1 mL per mouse) suspended in saline (1×10^7 cells/mL) were inoculated intraperitoneally to male ICR mice. Twenty-four hours after the transplantation, the mice were divided into groups of 8 mice. The test compounds were administered daily into the intraperitoneal cavity of the mice for 5 days. The rate of growth inhibition (T/C, %) was calculated by the following equation:

$$\text{T/C (\%)} = \frac{\text{Average survival period in the test group}}{\text{Average survival period in the control group}} \times 100$$

Computational methods. The molecular geometries were fully optimized by performing PM3 calculations. The optimized geometries were used to carry out *ab initio* molecular orbital calculations by using the Hartree-Fock (HF) theory with 6-31G* basis set.[40] All of these electronic structure calculations were performed by using the Gaussian03 program[41] on a 34-processors IBM Linux computer cluster in our lab. The electronic wave functions calculated at the HF/6-31G* level were used to derive possibly useful electronic structure-based molecular descriptors, such as molecular dipole moments, net atomic charges, and energies of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO).

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