Pharmaceutical Research, Vol. 23, No. 1, January 2006 (© 2006)
DOI: 10.1007/s11095-005-8813-4

Research Paper

Evaluation of Cancer-Preventive Activity and Structure–Activity Relationships of 3-Demethylubiquinone Q_2, Isolated from the Ascidian *Aplidium glabrum*, and its Synthetic Analogs

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Received July 13, 2005; accepted September 28, 2005

Purpose. 3-Demethylubiquinone Q_2 (**1**) was isolated from the ascidian *Aplidium glabrum*. The cancer-preventive properties and the structure–activity relationship for 3-demethylubiquinone Q_2 (**1**) and 12 of its synthetic analogs (**3**–**14**) are reported.

Methods. Compounds **3**–**14**, having one or several di- or triprenyl substitutions and quinone moieties with methoxyls in different positions, were synthesized. The cancer-preventive properties of compounds **1** and **3**–**14** were tested in JB6 Cl41 mouse skin cells, using a variety of assessments, including the methanethiosulfonate (MTS) assay, flow cytometry, and soft agar assay. Statistical nonparametric methods were used to confirm statistical significance.

Results. All quinones tested were shown to inhibit JB6 Cl41 cell transformation, to induce apoptosis, AP-1, and NF-κB activity, and to inhibit p53 activity. The most promising effects were indicated for compounds containing two isoprene units in a side chain and a methoxyl group at the *para*-position to a polyprenyl substitution.

Conclusions. Quinones **1** and **3**–**14** demonstrated cancer-preventive activity in JB6 Cl41 cells, which may be attributed to the induction of p53-independent apoptosis. These activities depended on the length of side chains and on the positions of the methoxyl groups in the quinone part of the molecule.

KEY WORDS: apoptosis; cancer prevention; marine prenylated quinones; nuclear factor; structure–activity relationship.

INTRODUCTION

Interest in developing effective cancer therapies led to the discovery of new important technologies and biomolecules, including viral vaccines, microbial-based therapy, interferons, interleukins, and a number of new promising antitumor agents (1–4). With respect to antitumor agents, some marine secondary metabolites are known to be among the most promising for chemotherapy of cancer (5–7). Polyprenylated 1,4-benzoquinones and hydroquinones are commonly found in a variety of organisms and play an important role in photosynthesis, electron transport, and as antioxidants (8,9). The previously described marine polyprenylbenzoquinones and hydroquinones contain a terpenoid portion ranging from one to nine prenyl units. These quinones have been isolated from brown algae of the order Fucales (10–13), sponges (14–17), alcyonaceans (18), gorgonaceans (19), and ascidians belonging to the genus *Aplidium* (20–25). Brown algae contain diprenyl-, triprenyl-, and tetraprenylquinones and hydroquinones (10–13). Sponges contain prenylated 1,4-benzoquinones and hydroquinones with linear and longer (up to nine prenyl units) terpenoid side chains (14–17). Ascidians of the genus *Aplidium* have previously yielded about a dozen prenylated quinones and related compounds (20–25).

The purpose of the present work was to study the cancer-preventive activity and to establish the corresponding structure–activity relationships (SARs) of a group of prenylated quinones resembling ubiquinones in their structures, but differing in that they have shorter polyprenyl side chains and modified quinone moieties. Two of these compounds, 3-demethylubiquinone Q_2 (**1**) and its 2′,3′-*cis*-isomer, 2,3-dimethoxy-5-(3′,7′-dimethyl-octa-2′(*Z*),6′-dienyl)-[1,4]benzoquinone (**2**), were isolated from the Far Eastern ascidian *Aplidium glabrum* (26), whereas compounds **3**–**14** are synthetic analogs of **1**. Distinct from previous investigations on related compounds, we studied the proapoptotic properties and the cancer-preventive activities of these compounds by using mouse cell lines and methods of flow cytometry or DNA laddering for assessing apoptosis and the well-accepted

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ABBREVIATIONS: 3-demethylubiquinone Q2, 2,3-dimethoxy-5-(3′,7′-dimethyl-octa-2′(E),6′-dienyl)-[1,4]benzoquinone (1); EGF, epidermal growth factor; FBS, fetal bovine serum; MEM, minimum essential medium; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

anchorage-independent assay using JB6 P$^+$ Cl41 cells in soft agar for investigation of the cancer-preventive activity.

MATERIALS AND METHODS

General Procedures

^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WM-250 spectrometer at 250 and 62.9 MHz and on a Bruker DPX 300 at 300 and 75 MHz, respectively. High-resolution electron impact mass spectrometry (HREIMS) was obtained on an AMD-604S mass spectrometer. High-performance liquid chromatography (HPLC) separations were conducted on a DuPont 8800 chromatograph equipped with differential refractometer using an Ultrasphere Si column. The infrared (IR) spectra were measured on a Bruker FT-IR "Vector 22" spectrophotometer. Ultraviolet (UV) spectra were determined in CCl_4 on a Cecil CE 7200 spectrophotometer. The onset of apoptosis was analyzed by flow cytometry using the Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA, USA). The MTS reduction assay to determine cell viability was measured using the Multiskan MS microplate reader (Labsystems, Helsinki, Finland). Cell colonies in the anchorage-independent transformation assay were scored using the LEICA DM IRB inverted research microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) and Image-Pro Plus software, version 3.0 for Windows (Media Cybernetics, Silver Spring, MD, USA). The luminescence assay for p53, AP-1, and NF-κB nuclear factor-dependent transcriptional activity was measured using the Luminoscan Ascent Type 392 microplate reader (Labsystems).

Reagents

Minimum essential medium (MEM) and Dulbecco's modified Eagle's medium were from Gibco Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Gemini Bio-Products (Calabasas, CA, USA). Penicillin/streptomycin and gentamicin were from Bio-Whittaker (Walkersville, MD, USA); L-glutamine was from Mediatech, Inc. (Herndon, VA, USA). Epidermal growth factor (EGF) was from Collaborative Research (Bedford, MA, USA). Luciferase assay substrate and Cell Titer 96 Aqueous One Solution Reagent (MTS) for the cell proliferation assay were from Promega (Madison, WI, USA). The Annexin V-FITC Apoptosis Detection Kit was from Medical & Biological Laboratories (Watertown, MA, USA). Silica gel L (40/100 μm) for low-pressure column liquid chromatography was from Chemapol (Prague, Czech Republic). Silica gel plates for thin-layer chromatography (4.5 × 6.0 cm, 5–17 μm) were from Sorbfil (Sorbpolymer, Krasnodar, Russia).

Cell Culture

The JB6 P$^+$ Cl41 mouse epidermal cell line and its stable transfectants Cl41–NF-κB, Cl41–AP-1, and Cl41–p53 (PG-13) were cultured in monolayers at 37°C and 5% CO_2 in MEM containing 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Syntheses of Quinones 1–14

Step A

Boric trifluoride etherate (0.5 ml) was added to the stirred mixture containing 1 mmol of the corresponding phenol (**15**–**17**) and 4 mmol of *trans*-geraniol (**18**) or *trans*-farnesol (**19**) in 10 ml of absolute ether. The mixture was kept for 12 h at room temperature, and then 30 ml of water was added to the mixture and products were extracted with ether (3 × 15 ml). The extract was washed with 10% NaCl and dried over Na_2SO_4. The solvent was removed, and the residue was separated using column chromatography on silica gel. Prenylated phenols were eluted with the solvent system gradient hexane/acetone, 50:1 → 20:1. The yield of mixtures of the purified prenylphenols was about 60%. These mixtures were used for step 2 of the syntheses. The prenylated phenols **20**, **21**, **23**, and **28** were isolated as individual compounds by HPLC on an Ultrasphere Si (Altex, Berkeley, CA, USA), 4.6 mm × 25 cm column in the system hexane/ethyl acetate, 7:1.

Step B

A solution of cerium-ammonium nitrate (CAN; 0.9 mmol) in 3 ml of the mixture CH_3CN/H_2O, 1:2, was added to the cooled (0°C) stirred solution of the corresponding prenylated phenol in 7 ml of CH_3CN. After being stirred at 0°C for 1–2 h, the mixture was poured into 25 ml of 10% NaCl and extracted with ether (3 × 15 ml).

The extract was dried over Na_2SO_4 and evaporated. The corresponding prenylated [1,4]-benzoquinones **1**–**10** and **12**–**14** were separated by preparative thin-layer chromatography on silica gel in the system hexane/acetone, 8:1. Yields of target products were about 70% at this stage. Total yield calculated for two stages was about 45%.

*Step C (Synthesis of Prenylated Hydroquinone **11**)*

A solution of $Na_2S_2O_4$ (3 mmol) in 3 ml of water was added to 1 mmol of the prenylquinone **7** in 7 ml of acetone. The mixture was stirred for 1 h, diluted with water, and extracted with ether (3 × 15 ml). The extract was dried over Na_2SO_4 and evaporated. As a result, hydroquinone **11** was obtained.

3-Demethylubiquinone Q_2 or 2,3-dimethoxy-5-(3′,7′-dimethyl-octa-2′(*E*),6′-dienyl)-[1,4]benzoquinone (**1**): Yellow oil, HREIMS *m*/*z* 304.1655 $[M]^+$, calcd. for $C_{18}H_{24}O_4$ 304.1675, IR ($CHCl_3$): 1675, 1657, 1603. ^{1}H-NMR ($CDCl_3$, 250 MHz) δ: 6.34 (t, $J = 1.7$, 1H, H-6); 5.13 (m, 1H, H-2′); 5.08 (m, 1H, H-6′); 4.02 (s, 3H, OMe); 4.00 (s, 3H, OMe); 3.10 (dd, $J = 7.3, 1.7$, 2H, H-1′); 2.09 (m, 2H, H-5′); 2.08 (m, 2H, H-4′); 1.70 (d, $J = 1.2$, 3H, H-8′); 1.62 (d, $J = 1.2$, 3H, H-10′); 1.60 (br. s, 3H, H-9′). ^{13}C-NMR ($CDCl_3$, 62.9 MHz) δ: 16.20 (q, C-10′), 17.79 (q, C-9′), 25.77 (q, C-8′), 26.52 (t, C-5′), 27.17 (t, C-1′), 39.72 (t, C-4′), 61.20 (q, OMe), 61.30 (q, OMe), 117.78 (d, C-2′), 123.98 (d, C-6′), 130.45 (d, C-6), 131.95 (s, C-7′), 140.17 (s, C-3′), 144.91 (s, C-2 or C-3), 145.16 (s, C-3 or C-2), 146.92 (s, C-5), 184.38 (s, C-4 or C-1), 184.54 (s, C-1 or C-4).

2,3-Dimethoxy-5-(3′,7′-dimethyl-octa-2′(*Z*),6′-dienyl)-[1,4]benzoquinone (**2**): Yellow oil, HREIMS *m*/*z* 304.1662 $[M]^+$, calcd. for $C_{18}H_{24}O_4$ 304.1675, ^{1}H-NMR ($CDCl_3$, 250

MHz) δ: 6.37 (t, *J* = 1.7, 1H, H-6); 5.13 (m, 1H, H-2′); 5.07 (m, 1H, H-6′); 4.02 (s, 3H, OMe); 4.00 (s, 3H, OMe); 3.11 (br. d, *J* = 7.1, 1.2, 2H, H-1′); 2.04 (m, 2H, H-5′); 2.04 (m, 2H, H-4′); 1.75 (q, *J* = 1.2, 3H, H-10′); 1.66 (br. d, *J* = 1.2, 3H, H-8′); 1.59 (d, *J* = 1.2, 3H, H-9′). ^{13}C-NMR ($CDCl_3$, 62.9 MHz) δ: 17.79 (q, C-9′), 22.76 (q, C-10′), 25.77 (q, C-8′), 26.52 (t, C-5′), 27.27 (t, C-1′), 32.01 (t, C-4′), 61.20 (q, OMe), 61.30 (q, OMe), 117.78 (d, C-2′), 123.98 (d, C-6′), 130.45 (d, C-6), 131.95 (s, C-7′), 140.17 (s, C-3′), 144.91 (s, C-2 or C-3), 145.16 (s, C-3 or C-2), 146.92 (s, C-5), 184.38 (s, C-4 or C-1), 184.54 (s, C-1 or C-4).

2-Methoxy-3-(3′,7′-dimethyl-octa-2′,6′-dienyl)-[1,4]benzoquinone (**3**): Yellow oil, HREIMS *m*/*z* 274.1558 [M]$^+$, calcd. for $C_{17}H_{22}O_3$ 274.1569, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 6.68 (d, *J* = 10.0, 1H, H-5); 6.59 (d, *J* = 10.0, 1H, H-6); 5.05 (m, 2H, H-2′, H-6′); 4.02 (s, 3H, OMe); 3.15 (br. d, *J* = 7.3, 2H, H-1′); 2.01 (m, 4H, H-4′, H-5′); 1.73 (br. s, 3H, Me); 1.65 (br. s, 3H, Me); 1.58 (br. s, 3H, Me).

2-Methoxy-6-(3′,7′-dimethyl-octa-2′,6′-dienyl)-[1,4]benzoquinone (**4**): Yellow oil, HREIMS *m*/*z* 274.1576 [M]$^+$, calcd. for $C_{17}H_{22}O_3$ 274.1569, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 6.45 (q, *J* = 2.1, 1H, H-5); 5.87 (d, *J* = 2.4, 1H, H-6); 5.15 (m, 1H, H-2′); 5.08 (m, 1H, H-6′); 3.82 (s, 3H, OMe); 3.14 (br. d, *J* = 7.3, 2H, H-1′); 2.07 (m, 4H, H-4′, H-5′); 1.70 (br. s, 3H, Me); 1.63 (br. s, 3H, Me); 1.60 (br. s, 3H, Me).

2-Methoxy-5-(3′,7′-dimethyl-octa-2′,6′-dienyl)-[1,4]benzoquinone (**5**): Yellow crystals, HREIMS *m*/*z* 274.1582 [M]$^+$, calcd. for $C_{17}H_{22}O_3$ 274.1569, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 6.46 (t, *J* = 1.7, 1H, H-6), 5.92 (s, 1H, H-3), 5.15 (m, 1H, H-2′), 5.08 (m, 1H, H-6′), 3.82 (s, 3H, OMe), 3.14 (br. d, *J* = 7.3, 2H, H-1′), 2.08 (m, 4H, H-4′, H-5′), 1.70 (br. s, 3H, Me), 1.62 (br. s, 3H, Me), 1.60 (br. s, 3H, Me).

2-(3′,7′-Dimethyl-octa-2′,6′-dienyl)-[1,4]benzoquinone (**6**): Yellow oil, HREIMS *m*/*z* 244.1454 [M]$^+$, calcd. for $C_{16}H_{20}O_2$ 244.1463, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 6.77 (d, *J* = 10.2, 1H, H-6); 6.69 (dd, *J* = 10.2, 2.3, 1H, H-5); 6.53 (q, *J* = 1.9, 1H, H-3); 5.15 (m, 1H, H-2′); 5.07 (m, 1H, H-6′); 3.13 (br. d, *J* = 7.5, 2H, H-1′); 2.08 (m, 4H, H-4′, H-5′); 1.69 (br. s, 3H, Me); 1.62 (br. s, 3H, Me); 1.60 (br. s, 3H, Me).

2,3-Dimethoxy-5-(3′,7′,11′-trimethyl-dodeca-2′,6,′10′-trienyl)-[1,4]benzoquinone (**7**): Yellow oil, HREIMS *m*/*z* 372.2316 [M]$^+$, calcd. for $C_{23}H_{32}O_4$ 372.2300, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 6.34 (t, *J* = 1.8, 1H, H-6); 5.10 (m, 3H, H-2′, H-6′, H-10′); 4.02 (s, 3H, OMe); 4.00 (s, 3H, OMe); 3.11 (br. d, *J* = 7.3, 2H, H-1′); 2.05 (m, 8H, H-4′, H-5′, H-8′, H-9′); 1.68 (br. s, 3H, Me); 1.62 (br. s, 3H, Me); 1.60 (br. s, 6H, 2Me).

2-Methoxy-6-(3′,7′,11′-trimethyl-dodeca-2′,6′,10′-trienyl)-[1,4]benzoquinone (**8**): Yellow oil, HREIMS *m*/*z* 342.2182 [M]$^+$, calcd. for $C_{22}H_{30}O_3$ 342.2195, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 6.45 (q, *J* = 2.0, 1H, H-5); 5.87 (d, *J* = 2.4, 1H, H-3); 5.15 (m, 1H, H-2′); 5.10 (m, 2H, H-6′, H-10′); 3.81 (s, 3H, OMe); 3.14 (br. d, *J* = 7.3, 2H, H-1′); 2.06 (m, 8H, H-4′, H-5′, H-8′, H-9′); 1.67 (br. s, 3H, Me); 1.63 (br. s, 3H, Me); 1.60 (br. s, 6H, 2Me).

2-Methoxy-5-(3′,7′,11′-trimethyl-dodeca-2′,6′,10′-trienyl)-[1,4]benzoquinone (**9**): Yellow crystals, HREIMS *m*/*z* 342.2172 [M]$^+$, calcd. for $C_{22}H_{30}O_3$ 342.2195, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 6.47 (t, *J* = 1.7, 1H, H-6); 5.93 (s, 1H, H-3); 5.16 (m, 1H, H-2′); 5.10 (m, 2H, H-6′, H-10′); 3.82 (s, 3H, OMe); 3.14(br. d, *J* = 7.3, 2H, H-1′); 2.05 (m, 8H, H-4′, H-5′, H-8′, H-9′); 1.68 (br. s, 3H, Me); 1.62 (br. s, 3H, Me); 1.60 (br. s, 6H, 2Me).

2-Methoxy-3-(3′,7′,11′-trimethyl-dodeca-2′,6′,10′-trienyl)-[1,4]benzoquinone (**10**): Yellow oil, HREIMS *m*/*z* 342.2212 [M]$^+$, calcd. for $C_{22}H_{30}O_3$ 342.2195, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 6.68 (d, *J* = 10.0, 1H, H-6); 6.57 (d, *J* = 10.0, 1H, H-5); 5.07 (m, 3H, H-2′, H-6′, H-10′); 4.02 (s, 3H, OMe); 3.16(br. d, *J* = 7.3, 2H, H-1′); 2.01 (m, 8H, H-4′, H-5′, H-8′, H-9′); 1.73 (br. s, 3H, Me); 1.67 (br. s, 3H, Me); 1.60 (br. s, 3H, Me); 1.57 (br. s, 3H, Me).

2,3-Dimethoxy-5-(3′,7′,11′-trimethyl-dodeca-2′,6′,10′-trienyl)-benzene-1,4-diol (**11**): Yellow oil, HREIMS *m*/*z* 374.2472 [M]$^+$, calcd. for $C_{23}H_{34}O_4$ 374.2457, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 6.49 (s, 1H, H-6); 5.31 (s, 1H, OH); 5.30 (m, 1H, H-2′); 5.17 (s, 1H, OH); 5.12 (m, 2H, H-6′, H-10′); 3.91 (s, 3H, OMe); 3.88 (s, 3H, OMe); 3.28 (br. d, *J* = 7.6, 2H, H-1′); 2.05 (m, 8H, H-4′, H-5′, H-8′, H-9′); 1.70 (br. s, 3H, Me); 1.68 (br. s, 3H, Me); 1.60 (br. s, 6H, 2Me).

2,3-Dimethoxy-5,6-bis-(3′,7′-dimethyl-octa-2′,6′-dienyl)-[1,4]benzoquinone (**12**): Yellow oil, HREIMS *m*/*z* 440.2944 [M]$^+$, calcd. for $C_{28}H_{40}O_4$, 440.2927, 1H-NMR (250 MHz, $CDCl_3$) δ: 5.04 (m, 2H, H-2′, H-2″); 4.94 (m, 2H, H-6′, H-6″); 3.99 (s, 6H, 2OMe); 3.19 (br. d, *J* = 6.8, 2H, H-1′, H-1″); 2.00 (m, 8H, H-4′, H-5′, H-4″, H-5″); 1.73 (br. s, 6H, 2Me); 1.66 (br. s, 6H, 2Me); 1.58 (br. s, 6H, 2Me).

2-Methoxy-5,6-bis-(3′,7′,11′-trimethyl-dodeca-2′,6′,10′-trienyl)-[1,4]benzoquinone (**13**): Yellow oil, HREIMS *m*/*z* 546.4048 [M]$^+$, calcd. for $C_{37}H_{54}O_3$, 546.4073, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 5.87 (s, 1H, H-3); 5.00 (m, 6H, H-2′, H-6′, H-10′, H-2″, H-6″, H-10″); 3.79 (s, 3H, OMe); 3.22 (br. d, *J* = 6.8, 4H, H-1′, H-1″); 2.01 (m, 16H, H-4′, H-5′, H-8′, H-9′, H-4″, H-5″, H-8″, H-9″); 1.73 (m, 3H, Me); 1.67 (m, 9H, 3Me); 1.60 (m, 12H, 4Me).

2-Methoxy-3,5-bis-(3′,7′,11′-trimethyl-dodeca-2′,6′,10′-trienyl)-[1,4]benzoquinone (**14**): Yellow oil, HREIMS *m*/*z* 546.4052 [M]$^+$, calcd. for $C_{37}H_{54}O_3$, 546.4073, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 6.33 (t, *J* = 1.2, 1H, H-6); 5.09 (m, 6H, H-2′, H-6′, H-10′, H-2″, H-6″, H-10″); 4.00 (s, 3H, OMe); 3.14 (m, 4H, H-1′, H-1″); 2.04 (m, 16H, H-4′, H-5′, H-8′, H-9′, H-4″, H-5″, H-8″, H-9″); 1.74 (m, 3H, Me); 1.68 (m, 9H, 3Me); 1.60 (m, 12H, 4Me).

2,3,4-Trimethoxy-6-(3′,7′-dimethyl-octa-2′,6′-dienyl)-phenol (**20**): Pale yellow oil, HREIMS *m*/*z* 320.1974 [M]$^+$, calcd. for $C_{19}H_{28}O_4$ 320.1987, IR (CCl_4): 3541, 2935, 1498, 1464 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 250 MHz) δ: 6.44 (s, 1H, H-5), 5.45 (s, 1H, OH), 5.31 (m, 1H, H-2′), 5.11 (m, 1H, H-6′), 3.95 (s, 3H, OMe), 3.86 (s, 6H, OMe), 3.79 (s, 3H, OMe), 3.31 (br. d, *J* = 7.1, 2H, H-1′), 2.07 (m, 4H, H-4′, H-5′), 1.72 (d, *J* = 1.2, 3H, Me), 1.67 (d, *J* = 1.2, 3H, Me), 1.60 (d, *J* = 0.7, 3H, Me). ^{13}C-NMR ($CDCl_3$, 62.9 MHz) δ: 16.12 (q, C-10′), 17.66 (q, C-9′), 25.66 (q, C-8′), 26.73 (t, C-5′), 27.90 (t, C-1′), 39.75 (t, C-4′), 56.62 (q, OMe), 60.89 (q, OMe), 61.16 (q, OMe), 108.30 (d, C-5), 121.61 (s, C-6), 121.98 (d, C-2′ or C-6′), 124.20 (d C-6′ or C-2′), 128.89 (s, C-1), 131.41 (s, C-7′), 136.59 (s, C-4), 140.04 (s, C-3′), 140.81 (s, C-3 or C-2), 146.14 (s, C-2 or C-3).

2,3,4-Trimethoxy-6-(3′,7′,11′-trimethyl-dodeca-2′,6′,10′-trienyl)-phenol (**21**): Pale yellow oil, HREIMS *m*/*z* 388.2648 [M]$^+$, calcd. for $C_{24}H_{36}O_4$ 388.2635, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 6.44 (s, 1H, H-5); 5.45 (s, 1H, OH); 5.32 (br. t, *J* = 7.3, 1H, H-2′); 5.12 (m, 2H, H-6′, H-10′); 3.95 (s, 3H, OMe); 3.87 (s, 3H, OMe); 3.79 (s, 3H, OMe); 3.31(br. d, *J* = 7.3, 2H, H-1′); 2.06 (m, 8H, H-4′, H-5′, H-8′, H-9′); 1.72 (br. s, 3H, Me); 1.67 (br. s, 3H, Me); 1.60 (br. s, 6H, 2Me).

3,4-Dimethoxy-6-(3′,7′-dimethyl-octa-2′,6′-dienyl)-phenol (**23**): Pale yellow oil, HREIMS *m/z* 290.1876 $[M]^+$, calcd. for $C_{18}H_{26}O_3$ 290.1882, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 6.73 (s, 1H, H-2); 6.49 (s, 1H, H-5); 5.27 (br t, *J* = 7.3, 1H, H-2′); 5.15 (s, 1H, OH); 5.10 (m, 1H, H-6′); 3.87 (s, 3H, OMe); 3.79 (s, 3H, OMe); 3.24 (br. d, *J* = 7.3, 2H, H-1′); 2.05 (m, 4H, H-4′, H-5′); 1.68 (br. s, 6H, 2Me); 1.60 (br. s, 3H, Me).

2,4-Dimethoxy-6-(3′,7′-dimethyl-octa-2′,6′-dienyl)-phenol (**28**): Pale yellow oil, HREIMS *m/z* 290.1898 $[M]^+$, calcd. for $C_{18}H_{26}O_3$ 290.1882, ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 6.36 (d, *J* = 2.7, 1H, H-3 or H-5); 6.31 (d, *J* = 2.7, 1H, H-3 or H-5); 5.33 (br. t, *J* = 7.1, 1H, H-2′); 5.26 (s, 1H, OH); 5.11 (br. t, *J* = 6.6, 1H, H-6′); 3.86 (s, 3H, OMe); 3.75 (s, 3H, OMe); 3.35 (br. d, *J* = 7.3, 2H, H-1′); 2.08 (m, 4H, H-4′, H-5′); 1.72 (br. s, 3H, Me); 1.67 (br. s, 3H, Me); 1.60 (br. s, 3H, Me).

Cell Viability Assay

JB6 P^+ Cl41 cells were cultured overnight in 96-well plates (6000 cells/well) using 5% FBS-MEM. Then the medium was replaced with 0.1% FBS-MEM containing the quinones at different concentrations in a volume of 0.1 ml, and the cells were incubated with the quinone solutions for 22 h. Then 20 μl of the MTS reagent was added into each well. The MTS reduction was measured 2 h later spectrophotometrically at 492 and at 690 nm as a background using the Multiskan MS microplate reader (Labsystems). For each compound, two independent experiments with five samples for each concentration were performed. (see Table S1, available online at www.springerlink.com; search for DOI: 10.1007/s11095-005-8813-4; Electronic Supplementary Materials can be found at the end of the article).

Anchorage-Independent Assay

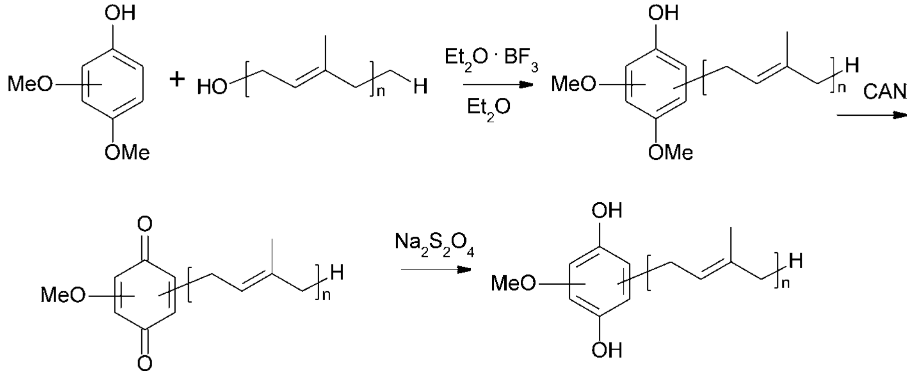
The cancer-preventive effects of the quinones were evaluated in 6-well plates using JB6 P^+ Cl41 cells, activated with EGF (10 ng/ml) or 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA; 20 ng/ml). JB6 P^+ Cl41 cells (8×10^3/ml) were treated with the indicated concentrations of the quinones in 1 ml of 0.33% basal medium Eagle (BME) agar containing 10% FBS over 3.5 ml of 0.5% BME agar containing 10% FBS and indicated concentrations of the quinones. The cultures were maintained in a 37°C, 5% CO_2 incubator for 1 week (JB6 P^+ Cl41 cells, activated with EGF) or 2 weeks (JB6 P^+ Cl41 cells, activated with TPA). Cell colonies were then scored using the LEICA DM IRB inverted research microscope (Leica Mikroskopie und Systeme GmbH) and Image-Pro Plus software, version 3.0 for Windows (Media Cybernetics). For each compound, two independent experiments in triplicate for each concentration were performed. (see Table S2, available online at www.springerlink.com; search for DOI: 10.1007/s11095-005-8813-4; Electronic Supplementary Materials can be found at the end of the article).

Apoptosis Assay Using Flow Cytometry

JB6 P^+ Cl41 cells (3×10^5 cells/dish) were grown in 6-cm dishes for 24 h in 5% FBS-MEM. Then cells were treated with different concentrations of the compounds dissolved in 0.1% FBS medium for 3 h. Then the medium was removed, and attached cells were harvested with 0.025% trypsin in 0.1% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS). Trypsinization was stopped by adding 2 ml of 5% FBS in PBS. Cells were then washed by centrifugation at 1000 rpm (170 rcf) for 5 min and processed for detection of apoptosis using Annexin V-FITC and propidium iodide staining according to the manufacturer's protocol. In brief, 1–5 × 10^5 cells were collected after centrifugation and resuspended in 500 μl of 1× binding buffer (Annexin V-FITC Apoptosis Detection Kit, Medical & Biological Laboratories). Then, 5 μl of Annexin V-FITC and 5 μl of propidium iodide were added, and the cells were incubated at room temperature for 5 min in the dark and were analyzed by flow cytometry. For each compound, two independent experiments in duplicate for each concentration were performed. (see Table S3, available online at www.springerlink.com; search for DOI: 10.1007/s11095-005-8813-4; Electronic Supplementary Materials can be found at the end of the article).

Apoptosis Assay Using DNA Ladder Method

JB6 Cl 41 cells were grown in 10-cm dishes and treated with 3-demethylubiquinone Q_2 (**1**) when cells were 80% confluent. The cells were incubated with quinone **1** for 24 h. Then, both detached and attached cells were harvested by scraping followed by centrifugation. The obtained cells



Scheme 1. The general scheme of synthesis of quinones **1**–**14**.

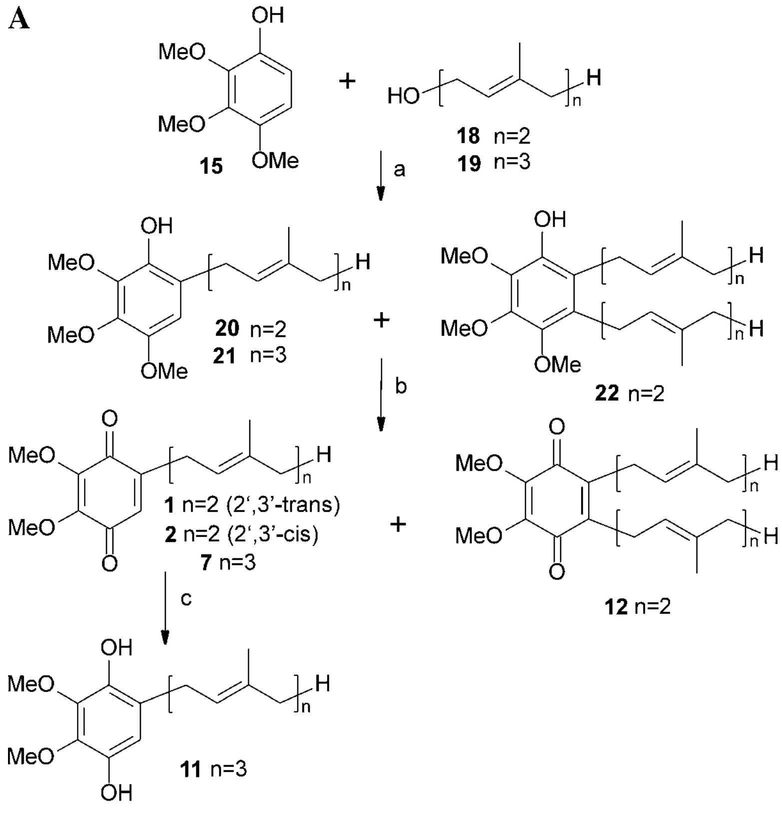
were disrupted with lysis buffer (5 mM Tris–HCl, pH 8.0, 20 mM EDTA, and 0.5% Triton X-100) and left on ice for 45 min. After centrifugation at 14,000 rpm (45 min, 4°C), the supernatant fraction containing fragmented DNA was extracted twice with phenol/chloroform/isopropyl alcohol (25:24:1, v/v/v) and once with chloroform. Then the fragmented DNA was precipitated overnight at −20°C after addition of two volumes of 100% ethanol and 1/10 volume of 5 M NaCl. The DNA pellet was saved by centrifugation at 14,000 rpm for 45 min, washed once with 70% ethanol, dried, and resuspended in Tris–EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). After addition of 100 μg/ml RNAse A (Sigma), the mixture was incubated at 37°C for 2 h. The DNA fragments were separated by 1.8% agarose gel electrophoresis. DNA laddering in the gel was stained with ethidium bromide and photographed under UV light. Two independent experiments were performed.

The Effect of Quinones 1 and 3–14 on p53-, AP-1-, and NF-κB-Dependent Transcriptional Activities

The ability of quinones **1** and **3–14** to influence AP-1-, NF-κB-, and p53-dependent transcriptional activities in the mouse JB6 Cl41 cell line was evaluated using the luciferase method. Viable JB6-LucPG-13, JB6-LucAP-1, or JB6-LucNF-κB cells (6×10^3) suspended in 100 μl 5% FBS-MEM were added into each well of a 96-well plate. Plates were incubated for 24 h and then treated with various concentrations of quinones in 100 μl of 0.1% FBS-MEM. After incubation with quinones for 24 h, the cells were extracted for 1 h at room temperature with 100 μl/well of lysis buffer [0.1 M potassium phosphate buffer at pH 7.8, 1% Triton X-100, 1 mM dithiothreitol (DTT), 2 mM EDTA]. Then 30 μl of lysate from each well was transferred in a plate used for luminescent analysis, and luciferase activity was measured using 100 μl/well of the luciferase assay buffer [1 mM D-luciferase, pH 6.1–6.5; 40 mM Tricin, 2.14 mM magnesium carbonate $(MgCO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$, 5.34 mM $MgSO_4 \cdot 7H_2O$, O, 66.6 mM DTT, 1.06 mM adenosine triphosphate, 0.54 mM coenzyme A, 0.2 mM EDTA, pH 7.8] and the Luminoscan Ascent Type 392 microplate reader (Labsystems). For each compound, two independent experiments with five samples for each concentration were performed. (see Table S4, available online at www.springerlink.com; search for DOI: 10.1007/s11095-005-8813-4; Electronic Supplementary Materials can be found at the end of the article).

Statistics

The statistical computer program Statistica 6.0 for Windows (StatSoft, Inc., Tulsa, OK, USA, 2001) was used for analysis of the obtained data. Nonparametric Mann–



a: Et_2O BF_3, Et_2O, r.t., 12 h; b: CAN, $MeCN$-H_2O, 0oC,1.5 h;
c: $Na_2S_2O_4$, acetone-H_2O, r.t., 1h

Scheme 2. Syntheses of quinones **1**, **2**, **7**, **11**, **12** (A); **3**, **5**, **9**, **10**, **14** (B); and **4**, **8**, **13** (C).

Whitney *U* test was used to compare two independent groups of data. Nonparametric Spearman rank-order correlations method was used to compute single nonparametric correlations. Method of regressions was used to compute IC_{50} or inhibition of the number of the colonies C_{50} ($INCC_{50}$) in corresponding experiments.

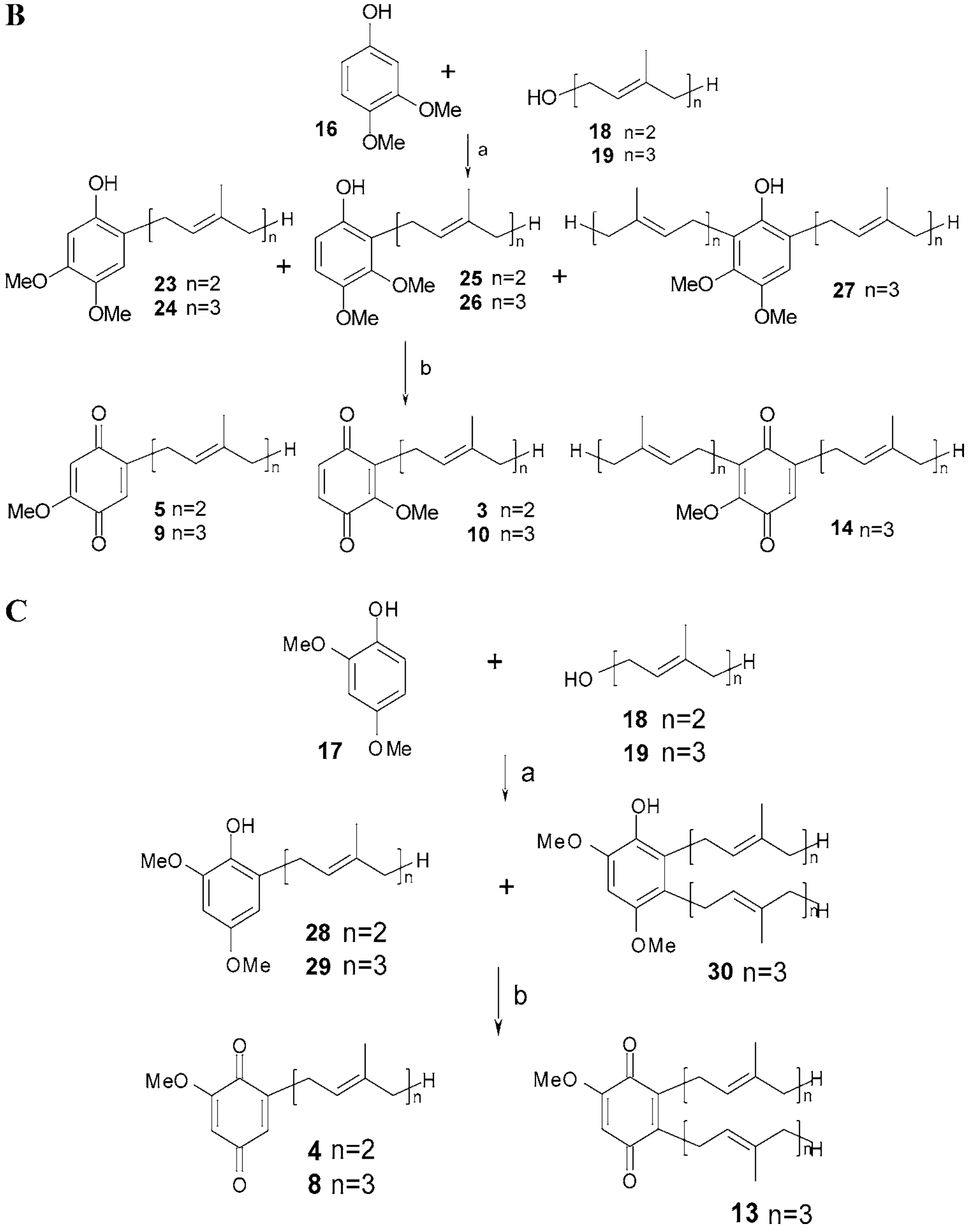
RESULTS

Syntheses of Polyprenylquinones 1 Through 14

Syntheses of polyprenylquinones **1**–**14** were carried out in accordance with Scheme 1. The first stage yielded prenylated phenols through the alkylation of corresponding available methoxyphenols by *trans*-geraniol (**18**) or *trans*-farnesol (**19**) in the presence of boric trifluoride etherate as an acidic catalyst as was described earlier (27). Under these conditions, total yields of the alkylation products were about 60%. The second stage, the oxidative demethylation of the fractions obtained with CAN, yielded mixtures of the corresponding quinones, which were further separated to obtain individual compounds. The third stage, reduction of the obtained quinones with $Na_2S_2O_4$, was used only in one case and yielded the corresponding prenylated hydroquinone (**11**).

In particular, prenylquinones **1**, **2**, **7**, **11**, and **12** were synthesized from 2,3,4-trimethoxyphenol (**15**, Scheme 2A), prenylquinones **3**, **5**, **9**, **10**, and **14** from 3,4-dimethoxyphenol (**16**, Scheme 2B), and prenylquinones **4**, **8**, and **13** from 2,4-dimethoxyphenol (**17**, Scheme 2C). The previously known 2-(3′,7′-dimethyl-octa-2′,6′-dienyl)-1,4-benzoquinone (**6**) (not shown on scheme) was synthesized as earlier described (27).

Target 2′-3′-*trans*-prenylquinones **1** and **3**–**14** contained less than 6% of the corresponding 2′-3′-*cis*-isomers as



Scheme 2. Continued.

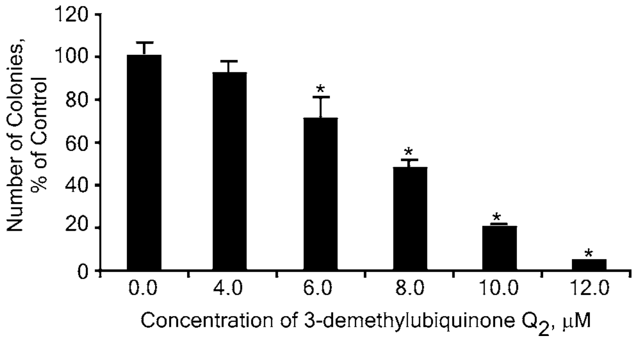


Fig. 1. The inhibition of epidermal growth factor (EGF)-induced JB6 P^+ Cl41 cell transformation by demethylubiquinone Q_2 (**1**) in soft agar (anchorage-independent assay). JB6 P^+ Cl41 cells (8×10^3/ml in 6-well plates) were activated with EGF (10 ng/ml), treated with the indicated concentrations of quinone (**1**), maintained for 1 week, and cell colonies were then scored as described in "Materials and Methods". Data represent the percentage of EGF-activated, quinone (**1**)-treated cell colonies compared to percentage of EGF-activated, untreated cells. Each bar represents the mean ± SD from six samples of two independent experiments. * indicates a significant inhibition by quinone (**1**) ($p < 0.05$) compared to EGF-activated untreated control.

impurities. The formation of these isomers may be explained by the isomerization of geraniol and farnesol or corresponding products of alkylation at the action of boric trifluoride etherate at the stage of prenylation of phenols. As a rule, impurities of *cis*-isomers were not separated from the major products (*trans*-isomers) except for the mixture of **1** and **2**, which was separated by HPLC. To the best of our knowledge, among all the above-mentioned polyprenylquinones, only compounds **1**, **6**, **7**, and **8** were described previously (22,27–29). Intermediate prenylated phenols **20**, **21**, **23**, and **28** were obtained as individual substances. The structures of the target prenylquinones **3**–**14** as well as the intermediate prenylphenols **20**, **21**, **23**, and **28** were established using NMR spectroscopy in comparison with spectra of **1** and **2** (26).

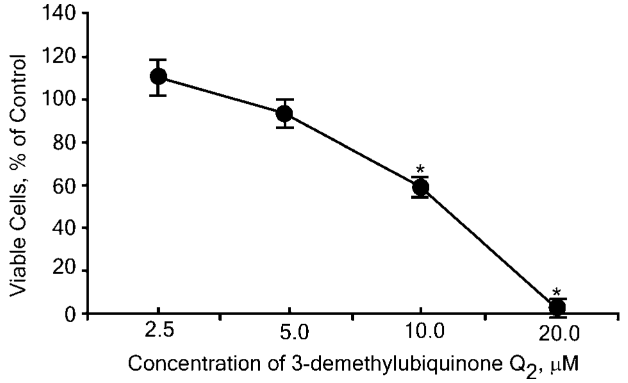


Fig. 2. The effect of demethylubiquinone Q_2 (**1**) on JB6 P^+ Cl41 cell viability. The cells were cultured in 96-well plates, as described in "Materials and Methods". Then, the medium was replaced with 0.1% FBS-MEM containing the indicated concentrations of quinone (**1**). The cells were incubated with the quinone (**1**) for 22 h. The methanethiosulfonate reagent was then added, and its reduction was measured spectrophotometrically 2 h later. Data represent the percentage of quinone (**1**)-treated viable cells compared to percentage of untreated control cells. Each data point represents the mean ± SD from ten samples of two independent experiments. * indicates a significant decrease in viability induced by quinone (**1**) ($p < 0.05$) compared to untreated control cells.

3-Demethylubiquinone Q_2 and Synthetic Polyprenylquinones 3–14 Inhibit Malignant JB6 P^+ Cl41 Cell Transformation

Both natural and synthetic compounds **1** and **3**–**14** were assayed for cancer-preventive activity using the anchorage-independent JB6 P^+ Cl41 cell transformation assay in a soft agar. Based on our previous experience (30–39), inhibition of cell transformation is a good indication that a compound will have an effective cancer-preventive activity. Toxicity of each compound for JB6 Cl41 cells was determined by the MTS cell viability assay. For one of the quinones, natural 3-demethylubiquinone Q_2 A (**1**), the corresponding data are shown in Figs. 1 and 2. Using the obtained data and statistical computer program Statistica 6.0, the corresponding regressions were built, and the IC_{50} for decreased cell viability and the $INCC_{50}$ for inhibition of cell transformation were determined for each quinone studied. These data are summarized in Table I. The obtained results indicated that all quinones studied inhibited cell transformation induced by EGF or TPA in dose-dependent manner in JB6 Cl41 cells. For some of the quinones, the dose that inhibited malignant transformation by 50% was below that which was toxic (Table I). For example, for 3-demethylubiquinone Q_2, the corresponding doses were 7.3 and 11.4 μM. To understand the possible inhibitory signaling pathway activated by the quinones studied, we then investigated whether the JB6 cells were undergoing apoptosis induced by quinones.

The Quinones Induce Apoptosis in JB6 Cl41 Cells

The ability of the quinone compounds to induce apoptosis was determined by flow cytometry. The results indicated that quinones **1** and **3**–**14** induced apoptosis in JB6

Table I. IC_{50} and $INCC_{50}$ of the Quinones **1** and **3**–**14** for JB6 Cl41 P^+ Cells

Compounds	IC_{50} (μM)	$INCC_{50}$ (μM)
Structural group 1		
Quinone **1**	11.4	7.3
Quinone **3**	45.1	15.1
Quinone **4**	8.3	6.6
Quinone **5**	11.8	3.1
Quinone **6**	23.4	14.7
Structural group 2		
Quinone **7**	5.1	19.4
Quinone **8**	4.7	16.7
Quinone **9**	8.6	7.4
Quinone **10**	25.5	24.6
Quinone **11**	4.6	51.7
Structural group 3		
Quinone **12**	>140[a]	29.2
Quinone **13**	>15[a]	54.7
Quinone **14**	99.6	95.3

$INCC_{50}$: inhibition of the number of the colonies C_{50}.
[a] In all calculations the designated numbers were used.

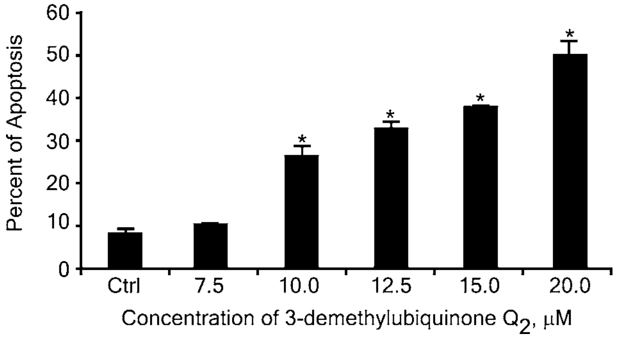


Fig. 3. The induction of apoptosis by demethylubiquinone Q_2 (**1**) in JB6 P^+ Cl41 cells measured by flow cytometry. The cells (3 × 10^5/dish) were grown in 6-cm dishes and treated with the indicated concentrations of quinone (**1**) as described in "Materials and Methods". Cells were harvested and processed for detection of apoptosis using Annexin V-FITC and propidium iodide staining according to the manufacturer's protocol. Each bar represents the mean ± SD from four samples of two independent experiments. * indicates a significant increase in apoptosis by quinone (**1**) ($p < 0.05$) com-pared to untreated control cells.

Cl41 cells in a dose-dependent manner (Fig. 3). For the natural 3-demethylubiquinone Q_2 A (**1**), apoptosis was also demonstrated by DNA laddering in JB6 Cl41 cells (Fig. 4).

Polyprenylquinones 1 and 3–14 Inhibit p53 and Induce AP-1 or NF-κB Transcriptional Activity

Several key transcription factors, including the p53 tumor suppressor protein, AP-1, or NF-κB, are often implicated in the induction or inhibition of apoptosis by various stimuli, including chemopreventive compounds or drugs. Therefore, we then studied the effect of quinones **1** and **3**–**14** on these three transcription factors. JB6 Cl41 cell lines stably expressing a luciferase reporter gene controlled by an AP-1, NF-κB, or p53 DNA binding sequence were used. To study the effect of the substances on the nuclear factors-dependent transcriptional activity, we used broad range of the concentrations. In Table II, we showed only maximal significances of the corresponding induction or inhibition. These significances were achieved at the concentrations of the substances that correlated well with their IC_{50} for the corresponding cells. Quinones **1** and **3**–**14** showed a significant (up to an 8-fold) induction of AP-1- or NF-κB-dependent transcriptional activation and a substantial (up to 4-fold) inhibition of p53-dependent transcriptional activity (see Table II).

Structure–Activity Relationships for Polyprenylquinones 1 and 3–14

One of the major goals of the present investigation was to establish the SARs for the quinones studied. The SARs of quinones **1** and **3**–**14** were studied using statistical analysis (Statistica 6.0). The quinone compounds **1** and **3**–**14** were divided into three groups according to the number of isoprene units included in their terpenoid parts (see Table I). Group 1 consists of quinones **1** and **3**–**6**, which have two isoprene units (10 carbon atoms) in their side chains. Group 2 (quinones **7**–**11**) contains three isoprene units (15 carbon atoms) in their terpenoid parts, and group 3 (quinones **12**–**14**) have four to six isoprene units (20–30 carbon atoms) in their side chains. Significant differences and correlations between the data regarding the biological activities obtained for different structural groups of the quinones were determined using the nonparametric Spearman correlation method and the Mann–Whitney *U* test and the data in Table I.

Our results indicated that the biological activity of these quinones depends on the length of the terpenoid side chains in the molecule. Statistical analysis of data from Table I indicates that quinones in group 1 averaged an IC_{50} of 20.0 ± 15.2 μM for toxicity in JB6 Cl41 cells. Quinones in group 2 had an average IC_{50} of 9.7 ± 9.0 μM. Finally, the quinones in group 3 showed an average IC_{50} of 84.9 ± 63.8 μM. These results suggest that quinones **7**–**11** (group 2), with three isoprene units in their terpenoid portion, are on the whole more toxic against JB6 cells than the quinones in group 1 (quinones **1** and **3**–**6**), which have only two isoprene units in their side chains. The opposite conclusion can be drawn when comparing the IC_{50} of quinones in groups 1 and 3 or for those in groups 2 and 3. The quinones of groups 1 or 2 are significantly more toxic for JB6 Cl41 cells than those of group 3. Therefore, we conclude that when the length of the terpenoid portion increases as from group 1 to group 2, the toxicity of the quinones of this series also increases; but it decreases dramatically by further increasing the length of the terpenoid portion as from group 2 to group 3.

In the statistical analysis of the effect of these quinones on EGF-induced cell transformation in JB6 Cl41 cells, we found that the quinones in group 1 displayed an average

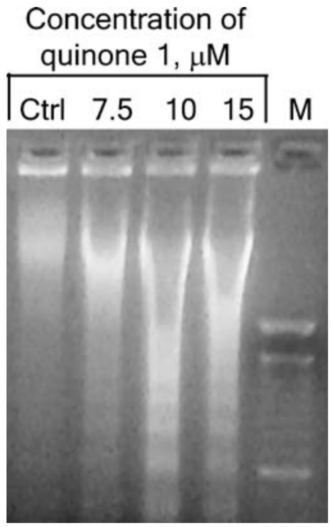


Fig. 4. The induction of apoptosis by demethylubiquinone Q_2 (**1**) in JB6 Cl41 cells determined by the method of DNA laddering. JB6 Cl41 cells were grown in 10-cm dishes, treated with the indicated concentrations of quinone (**1**) for 24 h, and harvested as described in "Materials and Methods." The isolated DNA fragments were separated by 1.8% agarose gel electrophoresis. DNA laddering in the gel was stained with ethidium bromide and photographed under ultraviolet light. A representative experiment is shown.

Table II. The Effect of the Quinones **1** and **3**–**14** on AP-1-, NF-κB-, and p53-Dependent Transcriptional Activity in JB6 Cl41 Cells

Compounds	AP-1-dependent transcriptional activity (% of untreated control)	NF-κB-dependent transcriptional activity (% of untreated control)	p53-dependent transcriptional activity (% of untreated control)
Structural group 1			
Quinone **1**	190.3	216.9	71.7
Quinone **3**	159.8	194.8	55.8
Quinone **4**	293.3	197.9	36.3
Quinone **5**	721.7	201.2	31.8
Quinone **6**	880.7	421.7	47.0
Structural group 2			
Quinone **7**	125.0	99.2	24.6
Quinone **8**	97.2	138.8	57.9
Quinone **9**	252.2	223.0	26.9
Quinone **10**	107.7	179.3	31.5
Quinone **11**	84.0	403.5	22.8
Structural group 3			
Quinone **12**	139.5	225.7	[a]
Quinone **13**	[a]	[a]	[a]
Quinone **14**	198.5	549.7	36.5

[a] Significant differences were not determined.

INCC$_{50}$ of 9.4 ± 5.3 μM, and the quinones in group 2 averaged an INCC$_{50}$ of 24.0 ± 16.7 μM. Group 3 again possessed minimal activity among all three groups of compounds with an average INCC$_{50}$ of 59.7 ± 33.3 μM (Table I). Therefore, based on this analysis and the results of the Mann–Whitney U test, group 1 quinones had the most potent effect on inhibition of cell transformation ($p = 0.0283$ *vs.* group 2; $p = 0.0253$ *vs.* group 3; $p = 0.0084$ *vs.* groups 2 and 3), and group 3 was the least effective. A significant correlation was observed between the length of the terpenoid portion and INCC$_{50}$ ($p = 0.0002$, $R = 0.8556$). These results indicate that when the length of the terpenoid portion increases, the INCC$_{50}$ values for cell transformation also increase.

We then compared toxicity with cell transformation in JB6 P$^+$ Cl41 cells and found that the quinones in group 1 displayed an average INCC$_{50}$ (inhibition of cell transformation) 1.4–4 times less than the IC$_{50}$ (toxicity) for the corresponding cells (Table I). On the other hand, the majority of quinones in group 2 showed an INCC$_{50}$ (inhibition of cell transformation) at doses 4–10 times higher than the IC$_{50}$ values (toxicity; Table I). Based on these data, we can conclude that quinones of group 1 are distinctly more toxic to transformed JB6 cells than to normal JB6 cells. In contrast, the quinones in group 2 are more toxic to normal JB6 cells than to those that have been transformed. Therefore, the quinones in group 1 have more potential with respect to cancer-preventive activity than the quinones from group 2.

We further showed that the activity of these quinones depends on the position of the methoxy group relative to the terpenoid part. We selected several pairs of structurally similar quinones, which have the methoxy groups in the same position. These pairs are as follows: (1) *ortho*-analogs, quinones **3** and **10**; (2) *meta*-analogs, quinones **4** and **8**; and (3) *para*-analogs, quinones **5** and **9**. Based on the data from Tables I and II, we conclude that the cancer-preventive activity and the effect of quinones on AP-1 transcriptional activity increased in the line of *ortho* → *meta* → *para*. The INCC$_{50}$ had the following values: for quinones **3** and **10**, 15.1 and 24.6 μM, respectively; for quinones **4** and **8**, 6.6 and 16.7 μM, respectively; and for quinones **5** and **9**, 3.1 and 7.4 μM, respectively.

Induction of AP-1 transcriptional activity by the *ortho* compounds **3** and **10** averaged 133.8% of control, and for the induction by the *meta* derivatives **4** and **8**, the average was 187.7% of control. The *para* derivatives **5** and **9** had the highest induction of AP-1 activation at 486.9% of control. The *ortho*-disubstituted quinones **3** and **10** are the least active compounds not only in the induction of AP-1 transcriptional activity but also in the inhibition of cell transformation compared with the *meta*- and *para*-analogs. Among the *para*-disubstituted derivatives, quinone **5** having two isoprene units in the side chain showed better activities compared with quinone **9** having three isoprene units in the side chain. Indeed, the *para*-disubstituted quinones **5** and **9** showed an INCC$_{50}$ of 3.1 and 7.4 μM, respectively, against EGF-induced JB6 P$^+$ Cl41 cell transformation. Quinone **5** also demonstrated a higher induction of AP-1 transcriptional activity (721.7%) compared with quinone **9** (252.2%).

DISCUSSION

In spite of the fact that polyprenylated 1,4-benzoquinones and hydroquinones are very common in nature, to the best of our knowledge, only one related marine metabolite, 2-(3-methylbuten-2-yl)-[1,4] hydroquinone (20), was earlier studied for its cancer-protective properties. To establish the cancer-preventive activity of this metabolite, the modified Ames assay for mutagenicity of benzo(*a*)-pyrene, aflatoxin B1, or UV against *Salmonella typhimurium* was used (40). However, biological tests examining transformation of animal cells after treatment with tumor promoters were not used in this study. For the first time in the present work, the cancer-preventive properties for a large group of the newly synthesized and earlier known polyprenylated benzoquinones were studied using mouse epithelial JB6 P^+ Cl41 cells and mouse embryonic fibroblasts (MEFs).

In our study the quinones having two isoprene units in the side chain showed specific effects against the malignantly transformed JB6 Cl41 cells compared with normal cells. The active doses differed up to 4-fold.

We established structure–activity relationships for the quinones studied with respect to cytotoxic or cancer-preventive properties. Our present study indicated that cytotoxicity of quinones increased with the number of carbon atoms from quinones having two prenyl units in their side chain to their analogs having three prenyl units and then decreased for compounds with four to six isoprene units. The observed SARs do not fully correspond to that earlier established for related nonmethoxylated quinones. A series of nonmethoxylated prenylated quinones with side chains containing from one to eight prenyl units was synthesized by an Italian research group and studied along with related marine natural compounds (27). Resembling our study, the toxicity of these compounds in brine shrimp and fish lethality assays was shown to first increase and then again to decrease with the length of the terpenoid part. But distinct from our study, quinones containing two isoprene units in the terpenoid portion were reported to be the most toxic among all studied compounds (27). No one has previously established the SARs for any series of prenylquinone compounds with respect to cancer-preventive activity. Our study showed that cancer-preventive activity decreased when the polyprenyl side chain became longer. The most active cancer-preventive polyprenylquinones, among those studied herein, have a side chain containing two isoprene units.

No previous evidence exists in the literature that shows that any polyprenyl quinone induces apoptosis in any cell line. Using flow cytometry and the DNA laddering method, we showed that quinones **1** and **3**–**14** induced apoptosis in JB6 Cl41 cells and MEFs. The tumor suppressor protein p53, which is a part of the cell's emergency team and functions to negatively regulate cell growth following DNA damage, is often involved in apoptosis induced by various stimuli including chemopreventive agents and drugs (41–45). However, in our study, quinones **1** and **3**–**14** did not activate p53, but instead, most of the quinones studied demonstrated significant inhibition of p53-dependent transcriptional activity. In addition, these compounds induced a substantial activation of AP-1- or NF-κB-dependent transcriptional activities (Table II). The AP-1 transcription factor regulates a variety of cellular processes, including proliferation, differentiation, and apoptosis, and has been considered primarily to be an oncogene (33,36–38,46,47). Recently, some of the AP-1 proteins, such as Jun-B and c-Fos, were shown to have tumor-suppressor activity both *in vitro* and *in vivo* (48,49). Activation of another AP-1 protein, c-Jun, is required for the induction of Fas L-mediated apoptosis in PC12 and human leukemia HL-60 cells (50,51). Activation of both AP-1 and NF-κB nuclear factors is necessary for DNA damaging agents- and ceramide-induced apoptosis in T lymphocytes and Jurkat T cells (52,53). The balance between AP-1 family members, c-Jun, and ATF-2 governs the choice between differentiation and apoptosis in PC12 cells (54). Anticancer drugs, such as vinblastine, which inhibit microtubules, activate AP-1 in human KB-3 carcinoma cells. This activation is required for efficient apoptosis induced by these drugs (55,56). NF-κB, a member of a family of highly regulated dimeric transcription factors, is involved in the activation of a large number of genes that respond to infections, inflammation, and other stressful situations. NF-κB is reported to be involved in both induction and inhibition of apoptosis (52,53,57–59). Our study therefore suggests that apoptosis induced by quinones **1** and **3**–**14** occurs independently of p53 activation, but instead may be related to the induction of AP-1 and NF-κB transcriptional activity.

CONCLUSION

Our results show that methoxylated polyprenylquinones and their synthetic analogs represent a new prospective group of marine secondary metabolites as cancer-preventive compounds. They show also cytotoxic properties and induce apoptosis of JB6 P^+ Cl41 cells and MEFs. The most active of these compounds are potent inducers of AP-1 and NF-κB activation and, at the same time, inhibitors of p53 transcriptional activities.

In our study, therefore, the cancer-preventive effects of quinones **1** and **3**–**14** (Table I) may be explained by the induction of p53-independent apoptosis.

We also found that quinones having a side chain of 10-carbon atom length showed specificity in the inhibitory effect for transformed JB6 P^+ Cl41 cells in contrast to quinones with 15 or 20–30 carbon atoms in the side chain. We conclude therefore that a further search for cancer-preventive agents may be promising among diprenylated analogs of the compounds studied. Taking into consideration that these compounds were active against transformation of the epithelial JB6 cells, we suggest that some quinones studied may be used as themselves or as models for the development of promising skin cancer-preventive agents.

Taking into account the data from Table I, and SAR based on the position of the methoxy-groups, we also conclude that quinone **5**, which has a diprenylated side chain in the *para*-position relative to the methoxy-group, is the most potent among all the quinones studied with respect to cancer-preventive effect.

ACKNOWLEDGMENTS

This work was supported in part by The Hormel Foundation and National Institutes of Health grants CA81064, CA77646, and CA88961. The Russian co-authors are grateful for financial support by the RFFI Grant no. 05-04-48246, Russian Federation President Grant no. 725.2003.4, Program of Presidium of RAS "Molecular and Cell Biology" Grant no. 05-I-05-005, and FEB Grant no. 05-III-A-05-129.

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