

## Biphenylalkylacetylhydroquinone Ethers Suppress the Proliferation of Murine B16 Melanoma Cells

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**Abstract.** Hydroquinone, an activator of caspase-9 activity via reactive oxygen species, and farnesol, a post-translational down-regulator of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity suppress the growth of murine B16 melanoma cells. Our previous studies have shown that farnesyl-O-acetylhydroquinone has a markedly greater growth-suppressive activity than that predicted by the responses to the parent compounds. Perillyl alcohol, a modulator of small G-protein activity, and biphenyl compounds, activators of Fas-mediated death pathways, suppress B16 growth. A similar synergistic increase in the potency of each compound when ether-linked to acetylhydroquinone is reported. Perillyl-O-acetylhydroquinone, biphenylethyl-O-acetylhydroquinone and biphenylpropyl-O-acetylhydroquinone had dose-dependent impacts on the proliferation of B16 cells with 50% inhibitory concentration (IC<sub>50</sub>) values of 8.0, 4.2 and 1.4 μmol/L, respectively. The growth-suppression effected by biphenylpropyl-O-acetylhydroquinone was accompanied by a dose-dependent arrest at the G1/S interface of the cell cycle, an impact greater than that previously reported for farnesyl-O-acetylhydroquinone (IC<sub>50</sub>=2.5 μmol/L). These new hydroquinone derivatives may have potential in cancer chemoprevention and/or therapy.

Hydroquinone (1-5) and *trans, trans*-farnesol (farnesol) (6-11) suppress the growth of neoplastically-derived cell lines (1-11) and with less potency, the growth of cell lines derived from normal tissues (1, 10, 11). Preclinical trials have found no impact of the sesquiterpenoid on the growth of normal tissues (12).

**Abbreviations:** IC<sub>50</sub>, the concentration required to suppress the increase in the population of cells by 50%; FBS, fetal bovine serum; HBSS, Hanks'-balanced salt solution; MEM, Eagle's minimum essential medium.

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Caspase-dependent and caspase-independent processes initiate apoptosis in hydroquinone-treated cells, the former tracing to the activation of caspase-9, the latter to the release of apoptotic proteins from mitochondrial membranes perturbed by reactive oxygen species (5). Cells incubated in the presence of farnesol are arrested at the G1/S-phase of the cell cycle (13); cells escaping this arrest undergo apoptosis (8, 14, 15). The post-transcriptional down-regulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase activity signaled by farnesol starves cells of prenyl-pyrophosphate intermediates of the mevalonate pathway that are essential for cells to move through the cell cycle (16).

We recently evaluated the impacts of hydroquinone and farnesol on the growth of murine B16 melanoma cells and found respective 50% inhibitory concentration (IC<sub>50</sub>) values of 40 and 33 μmol/L (17). Synthesized farnesyl-O-acetylhydroquinone (Figure 1A) proved to have a much greater growth-suppressive activity (IC<sub>50</sub>=2.5 μmol/L) than that of either parent compound. We suggest that this increase in potency reflects the impacts of the distinct actions of the two components.

Perillyl alcohol also suppressed the growth of neoplastically-derived cell lines (6, 18-22), while a preclinical trial found no impact of this cyclic monoterpene on the growth of normal tissues (12). The tumor-suppressive action of perillyl alcohol has been traced to the inhibited expression (23, 24) and processing (25) of small G-proteins.

A natural biphenyl compound, magnolol, suppressed the growth of B16 cells *in vitro* (26) and *in vivo* (27). The sites of the biphenyl action, the Fas-mediated death pathway, the activation of caspases, and anti-angiogenesis (26), appear to be distinct from the Ras farnesylation that was proposed for perillyl alcohol (25).

In view of the dramatic increase in the efficacy achieved with the synthesis of an agent comprised of two constituents, each with a distinct mode of chemopreventive action, perillyl-O-acetylhydroquinone (Figure 1B) and two biphenylalkyl acetylhydroquinones, biphenylethyl-O-acetylhydroquinone

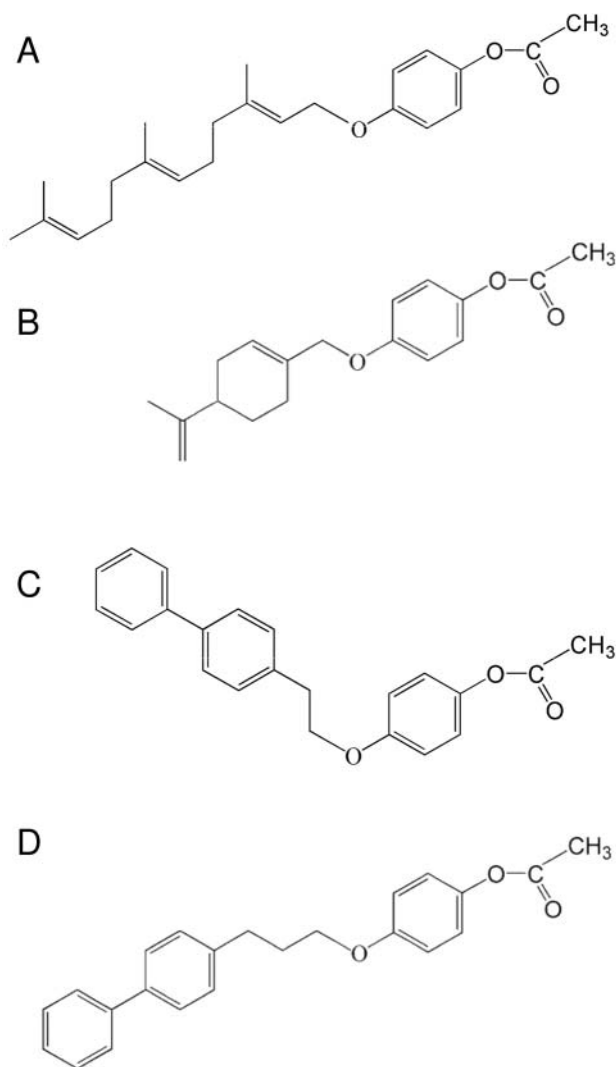


Figure 1. Schematic representation of the structures of acetylhydroquinone derivatives farnesyl-O-acetylhydroquinone (A), perillyl-O-acetylhydroquinone (B), biphenylethyl-O-acetylhydroquinone (C) and biphenylpropyl-O-acetylhydroquinone (D).

(Figure 1C) and biphenylpropyl-O-acetylhydroquinone (Figure 1D) were synthesized and the growth-suppressive activity was evaluated.

## Materials and Methods

**Chemical synthesis.** Lovastatin was a gift from Merck Research Laboratories (Rahway, NJ, USA). Perillyl alcohol was purchased from Aldrich (Germany). Biphenylpropanol and biphenylethanol were synthesized by  $\text{LiAlH}_4$  reduction of the corresponding carboxylic acids as reported for biphenylethanol (28). The acetic acid 4-hydroxyphenyl ester was synthesized *via* the Bayer-Villiger oxidation procedure (17). The ethers were synthesized by a

Mitsunobu type coupling (29). All compounds were characterized for identity and purity using nuclear magnetic resonance, infrared spectroscopy and mass spectrometry (data not shown).

**Cell proliferation assay.** The proliferation of murine B16 melanoma cells was measured by using CellTiter 96<sup>®</sup> Aqueous One Solution (Promega, Madison, WI, USA) as previously described (17). Briefly, the B16 melanoma cells, purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma) and 80 mg gentamicin/L (Sigma) at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$ , were seeded at 1000 cells/0.1 mL medium/well in 96-well tissue culture plates (Fisher Scientific Company LLC, Houston, TX, USA). At 24 h the medium was decanted from each well and replaced with 0.1 mL fresh medium containing the test agents (perillyl-O-acetylhydroquinone, biphenylethyl-O-acetylhydroquinone and biphenylpropyl-O-acetylhydroquinone) that were pre-dissolved in dimethyl sulfoxide (DMSO). All cultures contained 1 mL/L of DMSO. Cells were further incubated for an additional 48 h. The 72-h cell populations were determined by adding 20  $\mu\text{L}$  of CellTiter 96<sup>®</sup> Aqueous One Solution to each well; plates were held in the dark at 37°C for 2 h and then read at 490 nm with a SPECTRAmax<sup>®</sup> 190 multi-plate reader with SOFTmax<sup>®</sup> PRO version 3.0 (Molecular Devices, Sunnyvale, CA, USA). Absorbances from wells containing cell-free medium were used as baselines and were deducted from absorbances of other cell-containing wells.

Biphenylpropyl-O-acetylhydroquinone was subsequently re-evaluated for its dose-dependent impact on B16 cell proliferation using the Guava<sup>®</sup> ViaCount<sup>®</sup> assay (Guava Technologies Inc., Hayward, CA, USA). B16 cells, inoculated in 6-well plates (Fisher Scientific) at  $1 \times 10^5$  cells/well in 3 mL medium, were allowed to attach for 24 h. The culture medium was then replaced with fresh medium containing various concentrations of biphenylpropyl-O-acetylhydroquinone pre-dissolved in DMSO. Following a 48-h incubation the B16 cells were harvested by trypsinization, centrifuged and resuspended in 0.2 mL phosphate-buffered saline (PBS) to which 1  $\mu\text{L}$  Guava<sup>®</sup> ViaCount<sup>®</sup> Flex dye was added and mixed. Following a 5-min incubation the cell suspension was subjected to a ViaCount<sup>®</sup> assay using a Guava<sup>®</sup> EasyCyte flow cytometer to determine the numbers of viable cells. Guava<sup>®</sup> ViaCount<sup>®</sup> Flex contains a dual-dye to differentiate viable and non-viable cells.

**Microscopy.** Photomicrographs of representative fields of monolayers of B16 melanoma cells were obtained with a Nikon Eclipse TS 100 microscope (Nikon Corporation, Tokyo, Japan) equipped with a Nikon Coolpix 995 digital camera (Nikon Corporation).

**Cell cycle analysis.** The B16 cells were seeded in 6-well plates (Fisher Scientific) at  $1 \times 10^5$  cells/well with 3 mL medium/well and incubated for 24 h. The medium was then decanted and cultures replenished with fresh medium containing biphenylpropyl-O-acetylhydroquinone that had been dissolved in DMSO. Following an additional 24-h incubation adherent cells were harvested by trypsinization and pelleted by low speed centrifugation. The cell pellets ( $> 3 \times 10^4$  cells) were fixed in 1 mL 70% ethanol at 4°C for 60 min, washed in 1 mL PBS and re-suspended in 400  $\mu\text{L}$  PBS containing 0.5 mg RNase A (Sigma). Following gentle mixing a 10  $\mu\text{L}$  aliquot of propidium iodide (Sigma, 1 g/L in PBS) was added. The cells were incubated in the

Table I.  $IC_{50}$  values of acetylhydroquinone derivatives in B16 melanoma cells.

	$IC_{50}$ ( $\mu\text{mol/L}$ )	n
Farnesyl-O-acetylhydroquinone	$2.5 \pm 0.6^a$	8
Perillyl-O-acetylhydroquinone	8.0	2
Biphenylethyl-O-acetylhydroquinone	4.2	2
Biphenylpropyl-O-acetylhydroquinone	$1.4 \pm 0.3$	6

Values are mean  $\pm$  SD. <sup>a</sup>McAnally *et al.* (17)

dark at room temperature for 15 min and then held at 4 °C in the dark for flow cytometric analysis (30). Aliquots of  $5 \times 10^3$  cells were analyzed for DNA content using a Guava EasyCyte flow cytometer (Guava Technologies, Inc.). The distribution of cells in the G1, S, and G2/M-phases of the cell cycle was determined using MultiCycle AV software (Phoenix Flow Systems, San Diego, CA, USA).

**Guava Nexin™ Assay for apoptosis.** The B16 cells were seeded in 6-well plates (Fisher Scientific) at 200,000 cells/well with 3 mL medium/flask and incubated for 24 h. The medium was then decanted and cultures replenished with fresh medium containing biphenylpropyl-O-acetylhydroquinone or lovastatin pre-dissolved in DMSO for the experimental groups and solvent only for the control cells. Following an additional 6, 12, or 24-h incubation, the medium was decanted and adherent cells harvested by trypsinization and pelleted by refrigerated centrifugation at 300 g for 10 min. The cell pellets were washed and resuspended in Nexin™ buffer (Guava Technologies, Inc.) at  $1 \times 10^6$  cells/mL. In each well of a 96-well plate 20  $\mu\text{L}$  of cell suspension was mixed with 2.5  $\mu\text{L}$  of Annexin V-PE and 2.5  $\mu\text{L}$  of Nexin 7-amino-actinomycin D (7-AAD) solutions according to the manufacturer's instructions (Guava Technologies, Inc.) and kept in the dark on ice for 20 min. The plate was then loaded into a Guava EasyCyte flow cytometer (Guava Technologies, Inc.) and  $5 \times 10^3$  cells per sample were analyzed by using the Guava ExpressPlus program. Annexin V is a phospholipid-binding protein that has high affinity for phosphatidylserine (31) translocated from the internal face to the outer surface of cell membranes at the early stage of apoptosis (32). 7-AAD selectively permeates late stage apoptotic and dead cells. Therefore cells that are viable (Annexin V- and 7-AAD-), early apoptotic (Annexin V+ and 7-AAD-), and late apoptotic or dead (Annexin V+ and 7-AAD+) can be separated and percentages of these cell populations quantified.

**Statistics.** One-way analysis of variance (ANOVA) was performed to assess the differences between groups. Differences in means were analyzed by Dunnett's test. Levels of significance were designated as  $p < 0.05$ .

## Results

Adding the acetylhydroquinone moiety to perillyl alcohol gave an  $IC_{50}$  value for perillyl-O-acetylhydroquinone of 8.0  $\mu\text{mol/L}$  (Table I). The  $IC_{50}$  values estimated for the biphenylalkyl ethers, biphenylethyl-O-acetylhydroquinone and biphenylpropyl-O-acetylhydroquinone, were 4.2  $\mu\text{mol/L}$  and  $1.4 \pm 0.3$   $\mu\text{mol/L}$ , respectively.

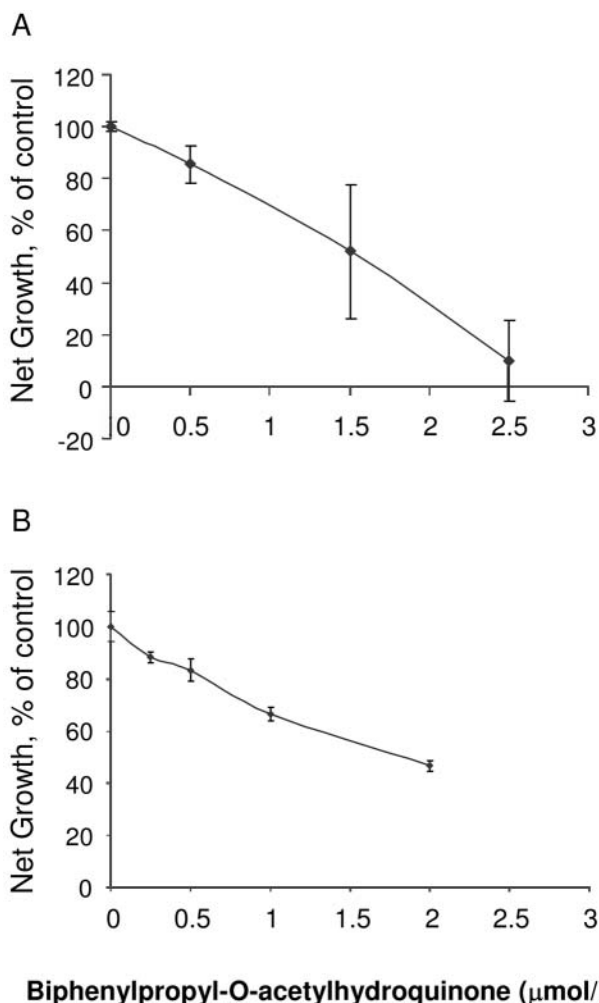


Figure 2. Representative growth curves of B16 melanoma cells showing the concentration-dependent suppression of B16 cell proliferation by biphenylpropyl-O-acetylhydroquinone. Cell proliferation was measured by CellTiter 96® Aqueous One Solution (A) and Guava® ViaCount® assay (B) after 48-h incubation. Values are mean  $\pm$  SD,  $n=3$ .

Figure 2A shows the concentration-dependent suppression of the proliferation of B16 cells by biphenylpropyl-O-acetylhydroquinone, the most potent growth suppressor among the three derivatives. There was a near linear decrease in cell growth following a 48-h incubation as the concentration of biphenylpropyl-O-acetylhydroquinone increased from 0 to 2.5  $\mu\text{mol/L}$ . Biphenylpropyl-O-acetylhydroquinone induced 15%, 47% and 91% growth suppression at 0.5, 1.5 and 2.5  $\mu\text{mol/L}$ , respectively. The growth suppression measured by CellTiter 96® Aqueous One Solution (Figure 2A) was also confirmed by using Guava® ViaCount® assay. At 0.25, 0.5, 1 and 2  $\mu\text{mol/L}$  biphenylpropyl-O-acetylhydroquinone induced growth suppression of 13%, 18%, 34%, and 54%, respectively (Figure 2B).

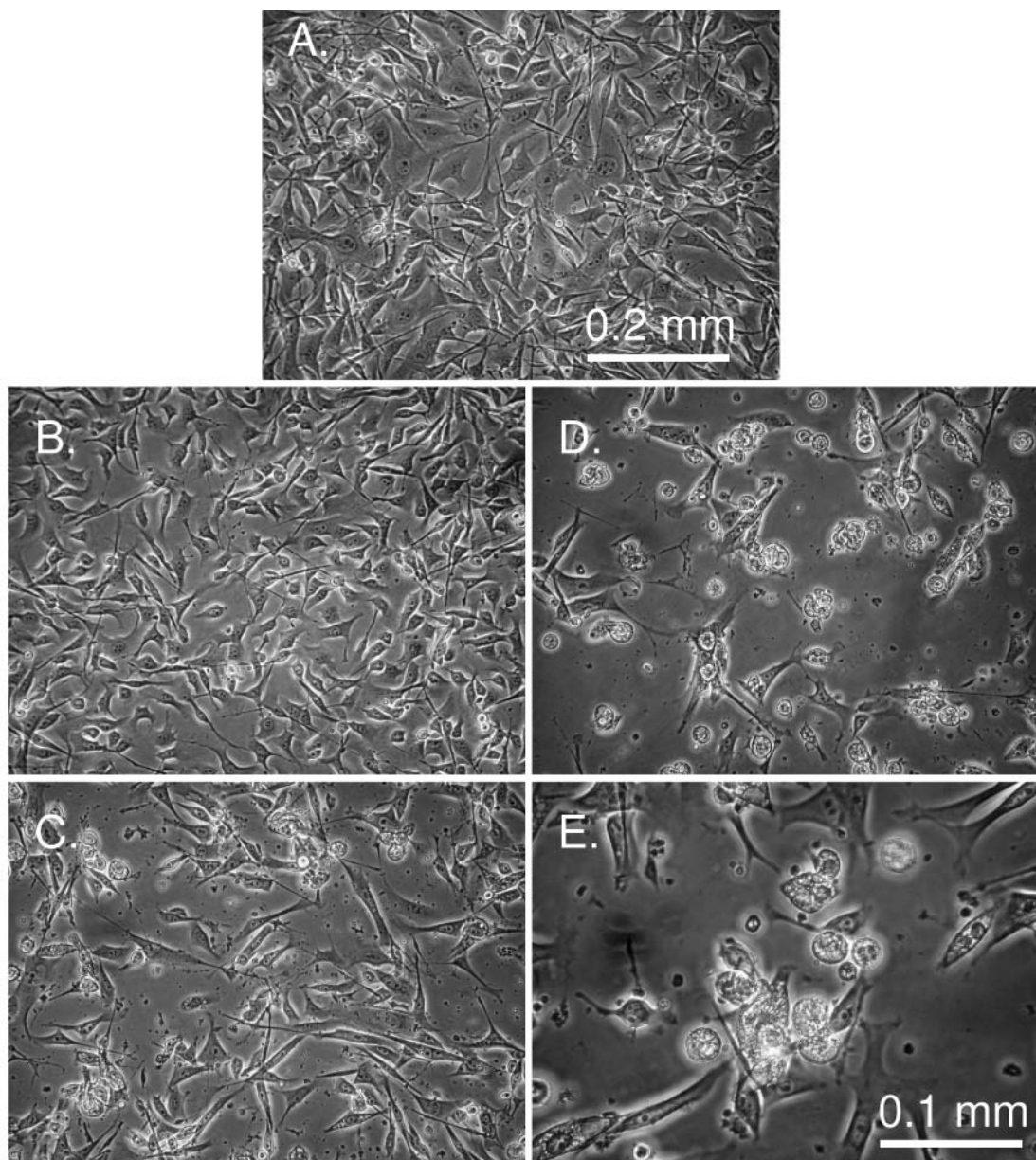
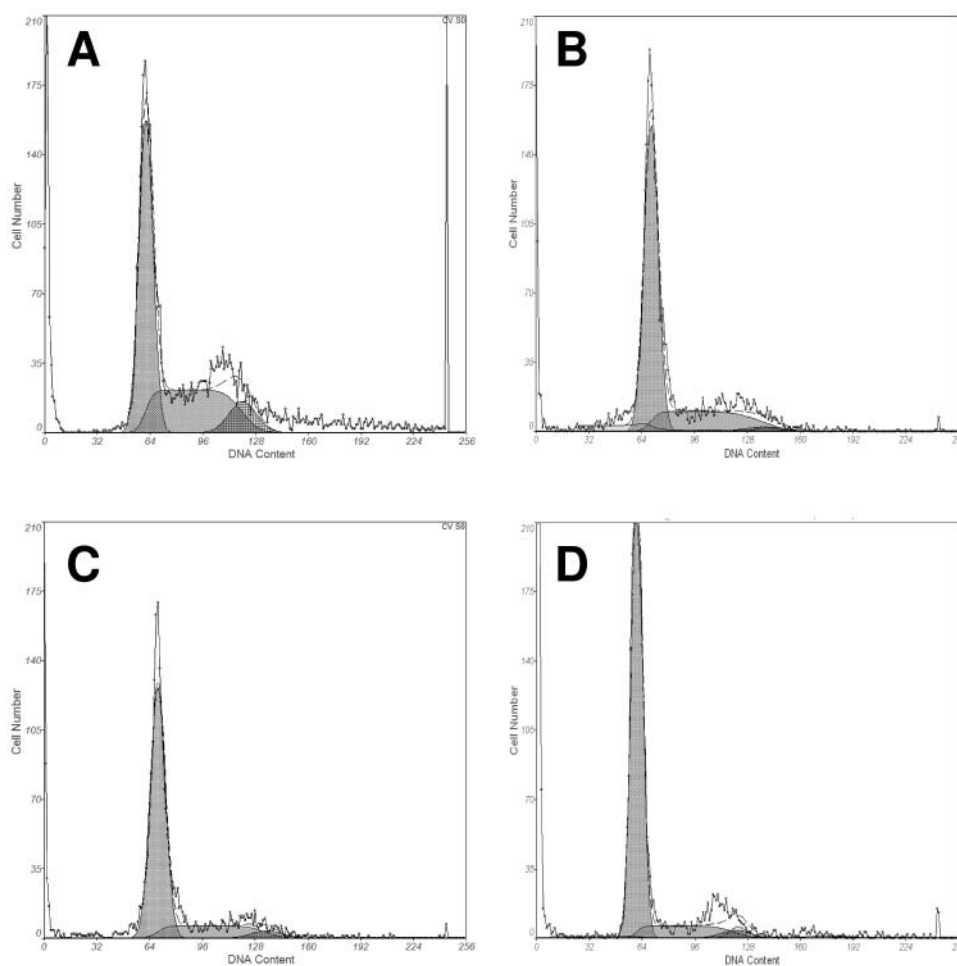


Figure 3. Concentration-dependent inhibition of B16 melanoma cell proliferation shown in photomicrographs of B16 cells after a 24-h incubation with 0 (A), 0.5 (B), 1 (C), and 2 (D and E)  $\mu\text{mol/L}$  biphenylpropyl-O-acetylhydroquinone. Morphological changes including cell elongation and rounding are most evident in the photomicrographs with 2  $\mu\text{mol/L}$  biphenylpropyl-O-acetylhydroquinone (D and higher magnification, E).

The photomicrographs shown in Figure 3 reflect the impact of biphenylpropyl-O-acetylhydroquinone on B16 cells following a 24-h incubation. The untreated cells (Figure 3A) exhibited the characteristic contact inhibition-disabled growth of B16 melanoma cells. Increasing the concentration of biphenylpropyl-O-acetylhydroquinone from 0 to 2  $\mu\text{mol/L}$  yielded a decrease in cell density and an increase in cell rounding, morphological changes more evident in Figure 3E under a higher magnification.

The impact of biphenylpropyl-O-acetylhydroquinone on the distribution of B16 cells in the cell cycle is shown in Figure 4. Increasing the biphenylpropyl-O-acetylhydroquinone concentration resulted in major shifts in the distribution of cells in the phases of cell cycle. The proportion of cells in the G1-phase increased from 50 to 70.9, 74.8 and 76.7%, while concomitantly the proportion of cells in the S-phase decreased from 42.1 to 23.4, 21.1 and 18.0%, respectively with 0, 0.5, 1, and 2  $\mu\text{mol/L}$



$\mu\text{mol/L}$	G1%	S%	G2%	G1/S
0	50.0 $\pm$ 2.3	42.1 $\pm$ 5.8	7.9 $\pm$ 4.2	1.2
0.5	70.9 $\pm$ 9.9	23.4 $\pm$ 7.1	5.8 $\pm$ 2.9	3.0
1.0	74.8 $\pm$ 9.9	21.1 $\pm$ 8.5	4.0 $\pm$ 3.3	3.5
2.0	76.7 $\pm$ 3.4	18.0 $\pm$ 3.7	5.3 $\pm$ 1.9	4.3

Figure 4. A representative analysis of the concentration-dependent impact of biphenylpropyl-O-acetylhydroquinone on the 24-h cell cycle distribution of B16 melanoma cells. Cells incubated with 0 (A), 0.5 (B), 1 (C) or 2 (D)  $\mu\text{mol/L}$  of biphenylpropyl-O-acetylhydroquinone for 24 h were analyzed for DNA content by flow cytometry. Values are mean  $\pm$  SD,  $n=6$ .

biphenylpropyl-O-acetylhydroquinone. The G1/S ratio, an indicator of G1 arrest, increased from 1.2 for untreated cells to 3.0, 3.5 and 4.3 for cells treated with 0.5, 1 and 2  $\mu\text{mol/L}$  biphenylpropyl-O-acetylhydroquinone, respectively.

The impact of biphenylpropyl-O-acetylhydroquinone was compared with that of lovastatin, an inhibitor of HMG CoA reductase (16), on the initiation of apoptosis in B16 cells. The cells were incubated for 24 h in the presence of 1.4  $\mu\text{mol/L}$  biphenylpropyl-O-acetylhydroquinone or 1.5  $\mu\text{mol/L}$

lovastatin. The proportions of viable, that is, adherent, cells in the washed control, biphenylpropyl-O-acetylhydroquinone and lovastatin cultures were 88.0, 85.8 and 82.3%, respectively. Within the population of cells harvested after the dead cells were washed off the plates the proportion of early apoptotic cells did not differ between treatments (Figure 5). The proportions of late apoptotic cells in the control and biphenylpropyl-O-acetylhydroquinone-treated cells were similar. The proportion of late apoptotic cells in

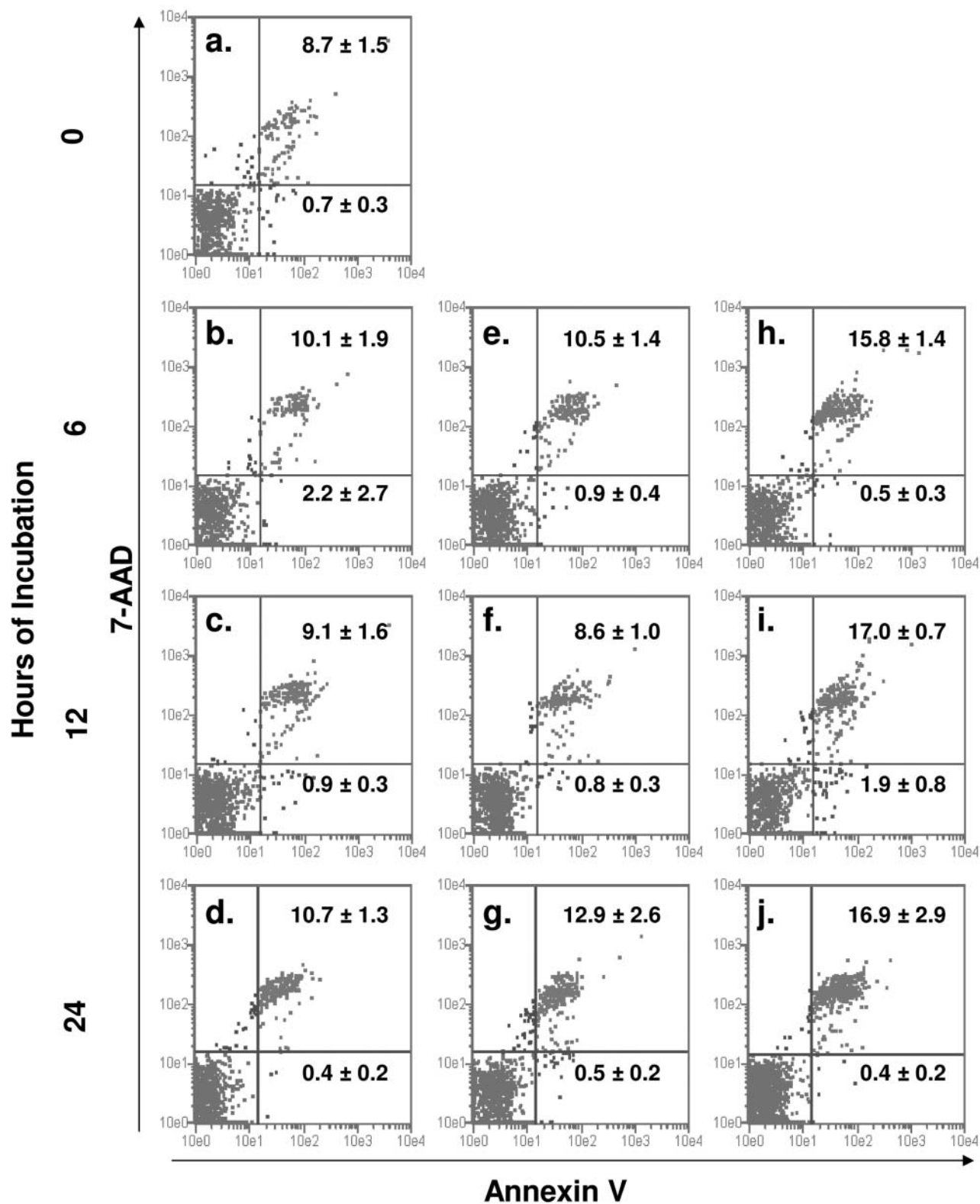


Figure 5. Representative plots showing the differential impacts of biphenylpropyl-O-acetylhydroquinone and lovastatin on the initiation of apoptosis in murine B16 melanoma cells. B16 cells were incubated with solvent only (a-d), biphenylpropyl-O-acetylhydroquinone (1.4 μmol/L, e-g) or lovastatin (1.5 μmol/L, h-j) for 6, 12 or 24 h. The percentages of early apoptotic cells (Annexin V+/7-AAD-) and late apoptotic and necrotic cells (Annexin V+/7-AAD+) in each dot plot are indicated in the corresponding quadrants. Values are mean ± SD, n=3.

the lovastatin culture ( $16.9 \pm 2.9\%$ ) was significantly greater than that in the control ( $10.7 \pm 1.3\%$ ,  $p < 0.05$ ). Similar findings were observed at 6 and 12 h.

## Discussion

The  $IC_{50}$  values reported herein for biphenylpropyl-O-acetylhydroquinone ( $1.4 \mu\text{mol/L}$ ), biphenylethyl-O-acetylhydroquinone ( $4.2 \mu\text{mol/L}$ ), and perillyl-O-acetylhydroquinone ( $8.0 \mu\text{mol/L}$ ), fall in the same range reported by others for terpenylquinones and terpenylhydroquinones (33, 34). All these values are lower than those of hydroquinone, reportedly 40 (17) to  $250 \mu\text{mol/L}$  (3).

In the present and previous (17) studies, the relative potencies of perillyl alcohol, geraniol and farnesol ( $IC_{50}$  values, 250, 160 and  $33 \mu\text{mol/L}$ , respectively) and those of their acetylhydroquinone derivatives ( $IC_{50}$  values, 8, 5.1 and  $2.5 \mu\text{mol/L}$ , respectively) have now been evaluated. The  $IC_{50}$  of perillyl-O-acetylhydroquinone was 31- and 5-fold lower than those of perillyl alcohol ( $250 \mu\text{mol/L}$ ) (35) and hydroquinone ( $40 \mu\text{mol/L}$ ) (17), respectively. Acetylation of hydroquinone ring to prevent autoxidation is reported to have no impact on biological potency of the hydroquinone (33).

The  $IC_{50}$  values of biphenylethyl-O-acetylhydroquinone ( $4.2 \mu\text{mol/L}$ ) and biphenylpropyl-O-acetylhydroquinone ( $1.4 \mu\text{mol/L}$ ) were several fold lower than those reported for the natural biphenyl, magnolol ( $30\text{-}100 \mu\text{mol/L}$ ) (26) and hydroquinone and fell on either side of that of farnesyl-O-acetylhydroquinone ( $2.5 \mu\text{mol/L}$ ) (17). The morphological changes in the B16 cells produced by biphenylpropyl-O-acetylhydroquinone were reminiscent of those induced by farnesyl-O-acetylhydroquinone, which induced cell cycle arrest at G1-phase in B16 cells (17). Biphenylpropyl-O-acetylhydroquinone induced B16 cell cycle arrest at G1-phase at concentrations as low as  $0.5 \mu\text{mol/L}$ , an impact absent in hydroquinone- or magnolol-treated cells, but reminiscent of, though to a greater extent than, that induced by farnesol, farnesyl derivatives (13) and lovastatin (17). The G1/S ratio of 4.3 induced by  $2 \mu\text{mol/L}$  biphenylpropyl-O-acetylhydroquinone was much greater than the 3.1 which resulted from a longer incubation with an equal molar concentration of farnesyl-O-acetylhydroquinone (17). The essential role of mevalonate in cell cycle progression through G1-phase was manifested by the lovastatin-induced cell cycle arrest at G1-phase shown by others (36) and by our previous study (17). The basis for the biphenylpropyl-O-acetylhydroquinone-induced G1-phase arrest remains to be determined.

Farnesol induces apoptosis in tumor cells by limiting the pool of mevalonate-derived intermediates required for viability (37, 38); cells respond in a similar fashion to lovastatin. Consistent with earlier reports that hydroquinone at  $2\text{-}3 \mu\text{mol/L}$  suppresses apoptosis (39) and caspase-3 activity (40), apoptosis was not

observed when biphenylpropyl-O-acetylhydroquinone was applied at its  $IC_{50}$  value,  $1.4 \mu\text{mol/L}$ . At levels as high as  $50\text{-}75 \mu\text{mol/L}$  hydroquinone is capable of inducing massive apoptosis that is dependent on (1, 3) or independent of caspase activation (4), which is likely to be a cell type-specific event. The biphenylpropyl-O-acetylhydroquinone-mediated growth suppression shown here is therefore likely to be attributable to cell cycle arrest.

In the present study biphenylpropyl-O-acetylhydroquinone demonstrated an  $IC_{50}$  of  $1.4 \mu\text{mol/L}$  in B16 melanoma cells. The toxicity of biphenylpropyl-O-acetylhydroquinone to normal cells and efficacy of biphenylpropyl-O-acetylhydroquinone *in vivo* remain to be investigated. Nonetheless, farnesyl-O-acetylhydroquinone with a higher  $IC_{50}$  of  $2.5 \mu\text{mol/L}$  *in vitro* showed tumor-suppressive potential *in vivo* (17). Biphenylalkyl-O-acetylhydroquinones may hold potential in cancer chemoprevention and/or therapy.

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