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Identification of a structure specific Bcl-2 phosphorylating homoisoflavone molecule from Vietnamese coriander (*Polygonatum odoratum*) that induces apoptosis and G2/M cell cycle arrest in breast cancer cell lines

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This paper is dedicated to our beloved friend and mentor the Late Prof. Robert T. Rosen.

Abstract

Bcl-2 is an anti-apoptotic protein which is over-expressed in many cancers. Modulating Bcl-2 expression is one of the most important strategies in the combat of cancer. We have isolated and identified a structure specific homoisoflavone from Vietnamese coriander (*Polygonatum odoratum* or Solomon seal) root which induces Bcl-2 phosphorylation, thereby causing mitotic arrest in breast cancer cells. Bioassay-directed fractionations resulted in a biologically active fraction for Bcl-2 phosphorylation. HPLC separation followed by mass spectrometry and NMR studies identified two compounds. We currently combine the chemistry and biological activity of Vietnamese coriander extracts by using bioassay directed fractionation to identify the structure specific Bcl-2 phosphorylating molecules, as well as their molecular mechanism of action. Only one molecule was responsible for Bcl-2 phosphorylation; it was identified as 2,3-dihydro3-[(15-hydroxyphenyl) methyl]-5,7-dihydroxy-6(8-dimethyl-4H-1-benzopyran-4-one) or 8-methyl-dihydrobenzopyranone (8-methyl-DBP, MW 314). The effect on Bcl-2 was structure specific, because 2,3 dihydro3-[(15-hydroxyphenyl) methyl]-5,7-dihydroxy-6-methyl-8-methoxy-4H-1-benzopyran-4-one or 8-methoxy-dihydrobenzopyranone (8-methoxy-DBP, MW 330), in contrast to 8-methyl-DBP, was not capable of Bcl-2 phosphorylation. Pure 8-methyl-DBP induced Bcl-2 phosphorylation in breast tumor cells, causes G2/M cell cycle arrest, up regulates the expression of p21 and p53 proteins and decreases cell viability demonstrated by a clonogenic assay. Therefore, these data demonstrate that Vietnamese coriander root contains 8-methyl-dihydrobenzopyrone (8-methyl-DBP, MW 314), which induces Bcl-2 phosphorylation, apoptosis, and G2/M cell cycle arrest in breast tumor cells.

Keywords: Bcl-2; Vietnamese coriander; p21; p53; Apoptosis; Homoisoflavone; *Polygonatum odoratum*

1. Introduction

Herbs, spices, condiments, phytochemicals and medicinal plants are often used for the treatment and prevention of many human maladies (Heber, 2004; Lai & Roy, 2004; Lampe, 2003; Risberg, Lund, Wist, Daasa, & Wilsgaard, 1998). Many natural products have been used as chemopreventive and chemotherapeutic agents to combat cancer by inducing apoptosis in cancer cell lines (Kelloff et al., 2000; Sporn & Suh, 2000). The roots of Vietnamese coriander (*Polygonatum odoratum* or commonly called Solomon seal or Vietnamese mint) have been used for a variety of therapeutic purposes in traditional Chinese medicine (Lin, Han, & Liao, 1994). It has been used as a crude medicinal agent in the treatment

Abbreviations: 8-methyl-DBP, 8-methyl-dihydrobenzopyranone; 8-methoxy-DBP, 8-methoxy-dihydrobenzopyranone; PBS, phosphate buffered saline; TEA, triethylamine.

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of analeptic (Tomoda, Yoshiko, Tanaka, & Uno, 1971) and as a nutritious tonic in Asia. In addition to China, *P. odoratum* grows throughout Asia including Thailand and Vietnam where it is known as Pak pai or Vietnamese mint. *P. odoratum* is a weed that grows throughout the southern United States and is commonly used as a condiment. *P. odoratum* alcohol extracts have been used as an immunopotentiator of mice injured by burns (Xiao, Cui, Ning, & Zhao, 1990). Compounds that have been previously identified in *P. odoratum* include quercitol (Lazer, Gheta, & Grigorescu, 1971), flavonoids (Yang, Chen, Chen, Yang, & Liu, 2005), azetidine 2-carboxylic acid (Fowden, 1956), mucous polysaccharides (Tomoda et al., 1971), and steroidal compounds (Lin et al., 1994; Sugiyama, Nakano, Tomimatsu, & Nohara, 1984) including furostanol glycoside (Qin, Li, Wang, & Si, 2004) and diosgenin (Okanishi, Akahori, Yasuda, Takeuchi, & Iwao, 1975).

Although the above compounds were isolated and no mechanistic studies have been reported, the whole root ethanol extract was tested for anti-cancer properties with a specific mechanism of targeting the anti-apoptotic protein Bcl-2. Bcl-2 is a 26 kDa protein that blocks cell death by inhibiting cytochrome c release from mitochondria, a critical event in the cell apoptotic pathway (Reed, 1997). Over expression of Bcl-2 protects cells from death and lowering Bcl-2 increases cell death and sensitivity to chemotherapy (Reed, 1997). Recent studies suggest that drugs which decrease Bcl-2 expression, or inactivate the molecule through phosphorylation, induce apoptosis (Adam, Crepin, & Israel, 1997; DiPaola et al., 1999; Hadlar, Chintapalli, & Croce, 1996; Haldar, Jena, & Croce, 1995; Rafi et al., 2005; Rafi et al., 2002). Current active chemotherapy agents, such as taxol, vincristine, and vinblastine can induce Bcl-2 phosphorylation, alter microtubule structure in cancer cells and cause programmed cell death (Adam et al., 1997; DiPaola et al., 1999; Haldar, Basu, & Croce, 1997; Haldar et al., 1996, 1995). Since Bcl-2 expression in tumor cells may be responsible for chemotherapy resistance, novel agents that abrogate Bcl-2 function through phosphorylation are attractive candidates for study. This research involves bioassay-directed fractionation of *P. odoratum*, whose crude extracts were found to phosphorylate Bcl-2 protein in breast cancer cell lines. This work is an extension of a previously published chemistry paper in an ACS symposium book series (Vastano et al., 2002). We currently combine the chemistry and biological activity of Vietnamese coriander extracts by using bioassay directed fractionation to identify the structure specific Bcl-2 phosphorylating molecules, as well as their molecular mechanism of action. Therefore, studying condiments such as Vietnamese coriander for effects on Bcl-2 may lead to the identification of novel chemical structures on which newer anti-cancer agents can be derived.

2. Experimental

2.1. Materials

The roots of *P. odoratum* were imported from Guangdong, China and purchased from Jasmine Enterprises located in Flagstaff, AZ (Rutgers Chrysler Herbarium voucher number CHR 30683). T47D (breast cancer), and MCF-7 (breast cancer) cells were obtained from the American Type Culture Collection (ATCC). Cells were maintained at 37 °C in an atmosphere of 5% CO_2 and grown in RPMI 1640 supplemented with fetal bovine serum (FBS), 50 units penicillin, and 50 μg/mL streptomycin. Cells were routinely checked and found to be free of contamination by mycoplasma.

2.2. General procedures

^{1}H NMR and ^{13}C NMR spectra were obtained on a VXR-200 instrument and mass spectra were obtained using atmospheric pressure chemical ionization (APCI) in the negative-ion mode and by electron ionization (EI). APCI MS analysis was performed on a Micromass Platform II system (Micromass Co.) equipped with a Digital DECPc XL560 computer for analysis of data. The ion source temperature was set at 150 °C and the probe temperature was set at 450 °C. The sample cone voltage was 10 V and the corona discharge was 3.2 kV. Direct probe EI-MS was performed on a Finnigan MAT 8230 high resolution mass spectrometer. HPLC analysis was performed on a Varian Vista 5500 Liquid Chromatograph pump coupled to a Varian 9065 Polychrom diode array detector. Fractionation of purified compounds was obtained on a Waters 600E HPLC pump coupled to a Milton Roy Spectro Monitor 3100 variable wavelength detector. Selecto Scientific silica gel (100–200 mesh particle size) was used for column chromatography. All column fractions were screened on Whatman silica gel thin-layer chromatography (TLC) plates (250 μm thickness, 60 A silica gel medium) with compounds revealed under fluorescent light. The column packing and TLC plates were purchased from Fisher Scientific (Springfield, NJ). All solvents used for extraction and isolation were of HPLC grade and purchased from Fisher Scientific.

2.3. Extraction and isolation procedures

The roots of *P. odoratum* were dried in a vacuum oven prior to being ground into a powder. The powdered roots of *P. odoratum* were extracted with methanol and concentrated under vacuum using rotary evaporation. The remaining concentrate was re-extracted and partitioned with acidified ethyl acetate (3% HCl). After biological evaluation, the ethyl acetate extract of *P. odoratum* was determined to have strong bio-activity.

The ethyl acetate extract was dried and then chromatographed on a silica gel column such that bio-assay directed

fractionation could be performed. Elution was performed using a solvent mixture of chloroform/methanol with an increasing amount of methanol (30:1, 20:1, 10:1, 8:1, 7:1, 5:1, 3:1, 1:1, 1:5, 1:15, 1:25, 0:1; each 500 mL) (Chen, Wang, Rosen, & Ho, 1999). Successive fractions were collected and tested for biological activity. The fraction eluted with 30:1 (v/v) chloroform/methanol was determined to be most active. This fraction was then rechromatographed on a second silica gel column. Elution of the 30:1 (v/v) chloroform/methanol fraction was performed using a solvent mixture of hexane/chloroform/methanol with increasing amounts of chloroform and methanol (50:50:0, 40:60:0, 30:70:0, 20:80:0, 0:100:0, 0:90:10, 0:0:100). Successive fractions were collected tested for biological activity.

The fraction eluted with 20:80:0 (v/v/v) hexane/chloroform/methanol was determined to be the most active fraction. Silica gel TLC plates and analytical HPLC were used to screen this fraction for purity. Separation was performed on a Discovery C18 reversed phase column (250 mm × 4.6 mm, 5 μm) with a column guard purchased from Supelco (Bellefonte, PA). The solvent program was a gradient system: A, water with 0.15% triethylamine (TEA) purchased from Sigma Chemical Co. and 0.18% formic acid (FA) purchased from Fisher Scientific; B, acetonitrile. The elution program at 1 mL min^{-1} was as follows: 100% A to 100% B (0–35 min); 100% B (35–55 min). The wavelengths monitored were 220–320 nm with a Varian 9065 diode array detector. HPLC analysis indicated the presence of two major compounds in the 20:80:0 (v/v/v) hexane/chloroform/methanol fraction, peaks 1 and 2. Final separation of pure compounds was obtained using semi-preparative HPLC on a Zorbax Rx-C18 reversed phase column (9.4 mm × 240 mm, 5 μm) purchased from MacMod Analytical. Compounds were eluted by an isocratic solvent system containing 60% water with 0.15% TEA and 0.18% FA; 40% acetonitrile. The solvent program was at 4 mL min^{-1} and the wavelength monitored was 254 nm. Analytical HPLC concluded that peaks 1 and 2 were isolated as pure compounds and needed no further purification.

2.4. Bcl-2 expression and phosphorylation

Analysis of Bcl-2 protein by Western blots was determined as previously described (Rafi et al., 2000). Protein identification was made using a monoclonal Bcl-2 primary antibody (DAKO Corp) and secondary goat anti-mouse horseradish peroxidase conjugated antibody (Bio-Rad Laboratories Richmond, CA). The phosphorylation of Bcl-2 was determined by mobility shifts in Western blot as described by Haldar et al. (1996, 1995).

2.5. P21 and p53 expression

Analysis of p21 and p53 proteins were determined by Western blots. Protein identification was made using a monoclonal p21 and p53 primary antibody (DAKO Corp) and secondary goat anti-mouse horseradish peroxidase conjugated antibody (DAKO Corp).

2.6. Cell cycle analysis

Cells were treated with 8-methyl-DBP and 8-methoxy-DBP for 24 h, and incubated with 10 μM BrdU for 45 min at 37 °C. Cells were then washed with ice-cold phosphate buffered saline (PBS), resuspended in 200 μL of PBS, and fixed with cold 70% ethanol. The cells were resuspended, incubated for 30 min in 2 N hydrochloric acid/0.5% Triton X-100 in PBS, and neutralized by rinsing once in 0.1 M sodium tetraborate (pH 8.5). Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Becton-Dickinson) was added (10 μg per sample) in 50 μL of 0.5% Tween 20/1% BSA in PBS and incubated for 30 min. The cells were washed and resuspended in 1 mL of PBS containing 5 μg/mL propidium iodide. Fluorescence intensity was determined by quantitative flow cytometry, and profiles were generated on Becton Dickinson FACS. A minimum of 10,000 cells were analyzed using Modifit LT (Verily Software House, Inc., Topsham, ME).

2.7. Clonogenic assay

Monolayer cultures of MCF-7 were harvested by treatment with Trypsin–EDTA and washed in medium. Five hundred cells were seeded in 60 × 15 mm tissue culture petri dishes with various concentrations of 8-methyl-DBP and 8-methoxy-DBP and incubated for 14 days at 37 °C. Thereafter, cells were incubated for 3 min in methylene blue (2.5 g methylene blue trihydrate in 50% ethanol). Colonies were counted by hand and confirmed using Bio-Rad Quantity one software (Bio-Rad Laboratories Inc.). The IC_{50} values were obtained by extrapolation by linear regression analysis.

3. Results

3.1. Vietnamese coriander root extract induced Bcl-2 phosphorylation

To determine the effect of Vietnamese coriander root on mitosis we measured the effect of Vietnamese coriander root on Bcl-2 phosphorylation. Various extracts from whole Vietnamese coriander root were assessed by immunoblotting. The ethyl acetate extract of Vietnamese coriander root induced Bcl-2 phosphorylation as shown in Fig. 1. The ethyl acetate extract was subjected to bioassay directed fractionation using silica gel column chromatography and yielded an active fraction upon dilution with 30:1 (v/v) chloroform/methanol. This fraction was then rechromatographed on a second silica gel column and elution with 20:80:0 (v/v/v) hexane/chloroform/methanol yielded the active fraction. As shown in Fig. 1, the ethyl acetate extract, 30:1 (v/v) fraction and 20:80:0 (v/v/v) fraction

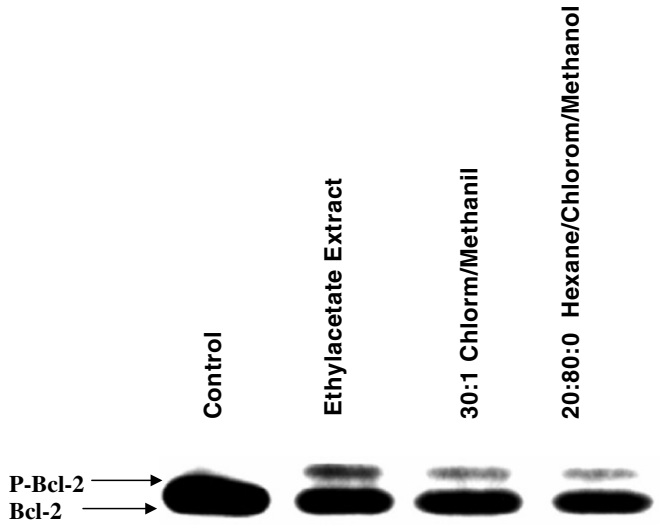

Fig. 1. The effect of Vietnamese coriander root extracts on Bcl-2. Immunoblot of MCF-7 cells treated with ethylacetate extract, 30:1 (v/v) chloroform/methanol extract, 20:80:0 (v/v/v) hexane/chloroform/methanol fractions, and control (EtOH) for 12 h. Cells were lysed and analyzed with a monoclonal Bcl-2 antibody.

induced Bcl-2 phosphorylation, as demonstrated by a slow migrating band, in contrast to the vehicle control.

3.2. Extraction and identification of active compound capable of Bcl-2 phosphorylation

The 20:80:0 (v/v/v) hexane/chloroform/methanol fraction was evaporated to dryness under nitrogen at room temperature. The sample was then reconstituted in methanol and analyzed by reversed phase HPLC. The HPLC conditions are shown in Section 2. The HPLC chromatogram of the 20:80:0 (v/v/v) hexane/chloroform/methanol fraction at 302 nm is presented in Fig. 2. The 20:80:0 (v/v/v) hexane/chloroform/methanol fraction was also analyzed by APCI LC-MS in the negative ion mode. It was determined that peak 1 had a molecular weight of 330 as evident by the pseudomolecular ion at m/z 329

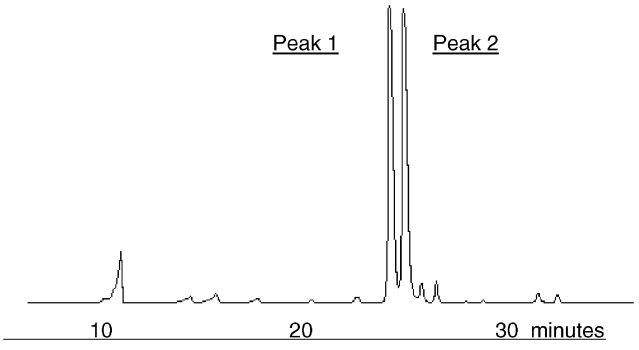

Fig. 2. Reversed-phase HPLC chromatogram of *Polygonatum odoratum* 20:80:0 (v/v/v) hexane/chloroform/methanol fraction at 302 nm. Analytical C18 reversed-phase HPLC column (9.4 mm × 4.6 mm, 5 μm) eluted by a gradient solvent system results in peak 1 and peak 2.

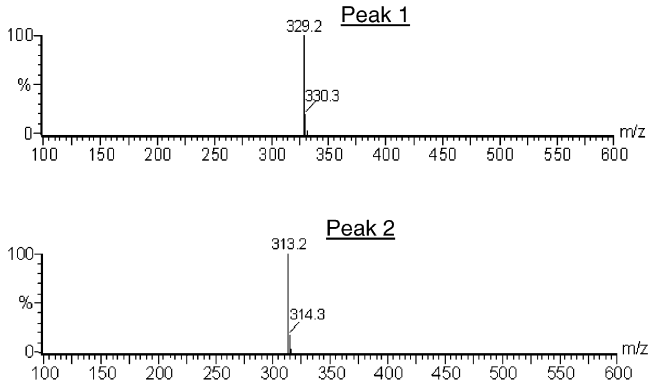

Fig. 3. APCI negative ion mass spectrum of Peak 1, m/z 329 = $[M-H]^-$ and Peak 2, m/z 313 = [M−H].

($[M-H]^-$), as shown in Fig. 3. Peak 2 had a molecular weight of 314 and gave a pseudomolecular ion at m/z 313 ($[M-H]^-$), as shown in Fig. 3.

For ultimate proof of structure, isolation and purification of the 20:80:0 (v/v/v) hexane/chloroform/methanol fraction was necessary for NMR studies. The final purification of the dihydrobenzopyranones was performed using reversed phase, semi-preparative HPLC. The HPLC parameters for purification are shown in Section 2. Peak 1 was determined to be 2,3-dihydro-3-[(15-hydroxyphenyl)-methyl]-5,7-dihydroxy-6-methyl-8-methoxy-4H-1-benzopyran-4-one (8-methoxy-DBP, MW 330) and Peak 2 was determined to be 2,3-dihydro-3-[(15-hydroxyphenyl)-methyl]-5,7-dihydroxy-6,8-dimethyl-4H-1-benzopyran-4-one (8-methyl-DBP, MW 314). The structures of both dihydrobenzopyranones, as shown in Fig. 4 were determined by NMR studies. Electron ionization mass spectrometry was used to help confirm the identification of peaks 1 and 2. The fragmentation patterns of both peaks 1 and 2 were determined by EI-MS, shown in Fig. 5. It was determined that peak 1 had a molecular weight of

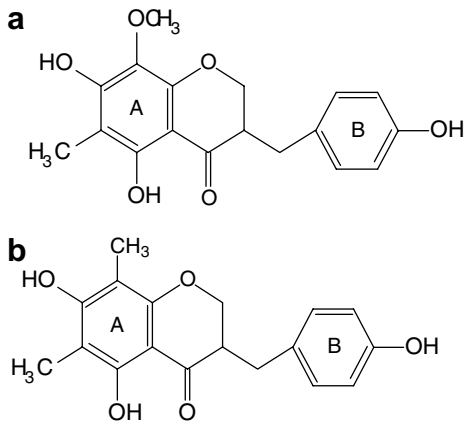

Fig. 4. Structures of dihydrobenzopyranones isolated from *Polygonatum odoratum* identified by mass spectrometry and NMR; (a) peak 1; 2,3-dihydro-3-[(15-hydroxyphenyl)methyl]-5,7-dihydroxy-6-methyl-8- methoxy-4H-1-benzopyran-4-one; (b) peak 2; 2,3-dihydro-3-[(15-hydroxyphenyl)methyl]-5,7-dihydroxy-6,8-dimethyl-4H-1- benzopyran-4-one.

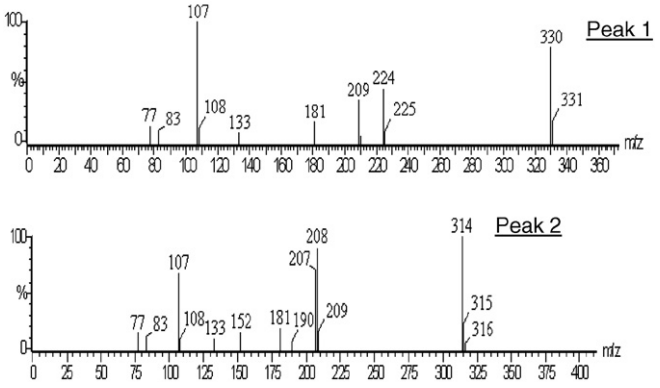

Fig. 5. EI mass spectrum of Peak 1, $m/z = 330$ [M^+], 224 ([$M^{\cdot+}$-substituted tropylium ion + H]), 209 (m/z 224 – CH_3), and 107 (base peak) (substituted tropylium ion) and peak 2, $m/z = 314$ [M^+] (base peak), 208 ([$M^{\cdot+}$-substituted tropylium ion + H]), and 107 (substituted tropylium ion).

330, as evident by the m/z 330 [M^+], (shown in Fig. 5). Other major fragments from peak 1 include m/z 224 [$M^{\cdot+}$-substituted tropylium ion + H], m/z 209 (m/z 224 – CH_3), and m/z 107 (base peak) (substituted tropylium ion). Peak 2 had a molecular weight of 314 as indicated by the m/z 314 [M^+] (base peak) with major fragments of m/z 208 [$M^{\cdot+}$-substituted tropylium ion + H], and m/z 107 (substituted tropylium ion). The concentration of both dihydrobenzopyranones in the powdered roots of *P. odoratum* was approximately 30 μg/g.

3.3. NMR data for isolated compounds

The structures of Peak 1 (8-methoxy-DBP, MW 330) and Peak 2 (8-methyl-DBP, MW 314) were determined using the following ^{1}H NMR and ^{13}C NMR data. Peak 1: 2,3-Dihydro-3-[(15-hydroxyphenyl)methyl]-5,7-dihydroxy-6-methyl-8-methoxy-4H-1-benzopyran-4-one (8-methoxy-DBP): High Res. MS Experimental = 330.110648 and Theoretical = 330.110339, $C_{18}H_{18}O_6$; EI-MS: m/z = 330 [M^+], 224 ([$M^{\cdot+}$-substituted tropylium ion + H]), 209 (m/z 224 – CH_3), and 107 (base peak) (substituted tropylium ion); APCI, m/z 329 ([M−H]$^-$); ^{1}H NMR δ 1.97 (3H, s, 18-H), δ 2.68 (1H, dd, $J = 13.5$, 10.3 Hz, 11a-H), δ 2.81 (1H, m, 3-H), δ 3.10 (1H, dd, $J = 13.6$, 4.2 Hz, 11b-H), δ 3.73 (3H, s, 19-H), δ 4.15 (1H, dd, $J = 11.1$, 6.7 Hz, 2a-H), δ 4.30 (1H, dd, $J = 11.1$, 3.9 Hz, 2b-H), δ 6.74 (2H, d, $J = 8.2$ Hz, 14, 15-H), δ 7.07 (2H, d, $J = 8.2$ Hz, 13, 17-H), ^{13}C NMR δ 7.52 (C-18), δ 33.23 (C-11), δ 47.80 (C-3), δ 61.84 (C-19), δ 70.39 (C-2), δ 102.34 (C-10), δ 105.28 (C-6), δ 116.40 (C-14, 16), δ 129.16 (C-8), δ 130.15 (C-12), δ 131.16 (C-13, 17), δ 152.95 (C-9), δ 157.21 (C-15), δ 158.63 (C-7)*, δ 159.20 (C-5)*, δ 199.63 (C-4). (*Assignments may be interchanged with one another.)

8-Methoxy-DBP was determined to have an elemental formula of $C_{18}H_{18}O_6$ by the ^{13}C NMR spectrum, the APCI LC-MS, which exhibited a pseudomolecular ion at m/z 329 ([M−H]$^-$), and the high resolution EI-MS. The ^{1}H NMR spectrum showed two doublets at δ 6.74 (2H, d, $J = 8.2$ Hz) and δ 7.07 (2H, d, $J = 8.2$ Hz), due to the protons of a *p*-disubstituted B ring. The ^{13}C NMR spectrum also showed the corresponding carbon signals (δ_C 157.21, 131.6, 130.15, and 116.40). The ^{13}C NMR spectra also gave six carbon signals assigned for a hexasubstituted benzoyl group (A ring) (δ_C 159.20, 158.63, 152.95, 129.16, 105.28, and 102.34). This data is in accordance with the absence of any other proton signals but those for the B ring. In addition to the signals above, the ^{1}H and ^{13}C NMR spectra exhibited the presence of one methyl group substituted on the A ring (δ_H 1.97, s; δ_C 7.52), one methoxy group (δ_H 3.73, S; δ_C 61.84), one carbonyl group (δ_C 199.63), one oxygenated methylene group (d_H 4.15, 4.30, 1H each, dd; δ_C 70.39), one methylene group (d_H 2.68, 3.10, 1H each, dd; δ_C 33.23), and one methine group (δ_H 2.81, m, δ_C 47.80).

The NMR information above indicates this compound has an unusual skeleton. The ^{1}H-^{1}H COSY and HMBC NMR spectra showed the presence of two moieties: (A ring)–O–CH_2(2)–CH(3)–CH_2(11)-(B ring) and (A ring)-CO(4)-CH(3)-. These moieties were connected by the following long range ^{1}H-^{13}C correlation ions: C-3/H-11, C-4/H-2, C-11/H-2, and C-12/H-11. Furthermore, since the chemical shift of C-4 was 199.63, one hydroxyl group should be substituted at C-5. The substitution sites of the methoxyl group and the methyl group were determined by the information from the ^{13}C NMR and HMBC spectra.

Peak 2: 2,3-Dihydro-3-[(15-hydroxyphenyl)methyl]-5,7-dihydroxy-6,8-dimethyl-4H-1- benzopyran-4-one (8-methyl-DBP): High Res. MS Experimental = 314.115729 and Theoretical = 314.115424, $C_{18}H_{18}O_5$; EI-MS: $m/z = 314$ [M^+] (base peak), 208 ([$M^{\cdot+}$-substituted tropylium ion]), and 107 (substituted tropylium ion); APCI, m/z 313 ([M−H]$^-$); ^{1}H NMR δ 1.98 (3H, S, 18-H)*, δ 2.00 (3H, s, 19-H)*, δ 2.69 (1H, dd, $J = 13.6$, 10.2 Hz, 11a-H), δ 2.83 (1H, m, 3-H), δ 3.09 (1H, dd, $J = 13.6$, 4.0 Hz, 11b-H), δ 4.12 (1H, dd, $J = 11.5$, 6.8 Hz, 2a-H), δ 4.26 (1H, dd, $J = 11.5$, 4.0 Hz, 2b-H), δ 6.74 (2H, d, $J = 8.4$ Hz, 14, 15-H), δ 7.07 (2H, d, $J = 8.4$ Hz, 13, 17-H), ^{13}C NMR δ 7.71 (C-18)$^{\#}$, δ 8.17 (C-19)$^{\#}$, δ 33.45 (C-11), δ 48.00 (C-3), δ 70.33 (C-2), δ 102.04 (C-10), δ 104.24 (C-6)$^{\$}$, δ 106.36 (C-8)$^{\$}$, δ 116.66 (C-14, 16), δ 130.61 (C-12), δ 131.43 (C-13, 17), δ 157.64 (C-15), δ 164.25 (C-9), δ 168.20 (C-5)$^{\Delta}$, δ 168.27 (C-7)$^{\Delta}$, δ 199.60 (C-4). ($^{*\Delta\$\#}$Correlating assignments may be interchanged with one another.)

The ^{13}C NMR and by the APCI LC-MS spectrum, which gave a pseudomolecular ion at m/z 313 ([M−H]$^-$), confirms the molecular weight of this compound as 314 and corresponds to the molecular formula $C_{18}H_{18}O_5$. A comparison of the ^{1}H and ^{13}C NMR spectra of this compound to those of Peak 1 differ only by the absence of the methoxyl group and the presence of an additional methyl group (δ_H 2.00 or 1.98; δ_C 7.71 or 8.17) on the A ring. The corresponding changes also occurred: the chemi-

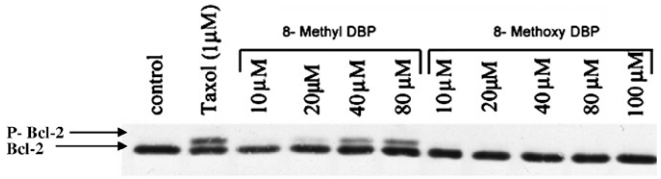

Fig. 6. Effect of 8-methyl-DBP compared to 8-methoxy-DBP on Bcl-2 proteins. Immunoblot of MCF-7 cells treated with taxol, 8-methyl-DBP (10–80 μM) or 8-methoxy-DBP (10-100 μM) for 12 h. Cells were lysed and analyzed with a monoclonal Bcl-2 antibody.

cal shifts of C-5, C-7, and C-9 shifted from 159.20, 158.63, and 152.95 in Peak 1 to 168.20, 168.27, and 164.25 in Peak 2. The data presented for peak 2 is in agreement with previous reports of this compound (Huang, Gan, Wu, & Lin, 1997). No effort was made to determine the stereochemistry of these compounds.

3.4. 8-Methyl-dihydrobenzopyranone (8-methyl-DBP) was identified as the active component of Vietnamese coriander root

To determine active compound(s) from Vietnamese coriander root extract capable of Bcl-2 phosphorylation, fractions were initially collected and assessed by HPLC. As shown in Fig. 1, Vietnamese coriander root extract was subjected to column chromatography using 30:1 (v/v) chloroform/methanol and 20:80:0 (v/v/v) hexane/chloroform/methanol which yielded the active fraction, as described above. Analysis by NMR and mass spectroscopy identified the active compound as 8-methyl-DBP (MW 314) in peak 2. The effect of 8-methyl-DBP (Fig. 6) on Bcl-2 phosphorylation was structure specific, because 8-methoxy-DBP (Fig. 6), in contrast to 8-methyl DBP, was not capable of Bcl-2 phosphorylation, as shown in Fig. 6. As shown in Fig. 6, 8-methyl-DBP induced Bcl-2 phosphorylation similar to paclitaxel (taxol) in contrast to 8-methoxyDBP. Given these data, 8-methyl-DBP (Fig. 4b) was the subject of further biological assessment.

3.5. 8-Methyl-DBP induced Bcl-2 phosphorylation and G2/M cell cycle arrest

To confirm the activity of the pure compound, 8-methyl-DBP was studied further. As shown in Fig. 6, 8-methyl-DBP induced phosphorylation of Bcl-2 in breast cancer cell lines. Prior studies demonstrated that anti-microtubule agents such as paclitaxel and vinca alkaloids induce Bcl-2 phosphorylation, in association with G2/M cell cycle arrest (Blagosklonny, Schulte, Nguyen, Trepel, & Neckers, 1996; Haldar et al., 1996, 1995; Lin et al., 1994). To confirm that pure 8-methyl-DBP induced G2/M cell cycle arrest, T47D tumor cells were treated with 8-methyl-DBP and assessed by flow cytometry. As shown in Fig. 7, 8-methyl-DBP (MW 314) (Fig. 7c) induced a G2/M cell cycle arrest similar to that of the known antimicrotubule agent paclitaxel (Fig. 7b). As compared with 8-methyl-DBP, 8-methoxy-DBP was not capable of causing G2/M cell cycle arrest (Fig. 7d).

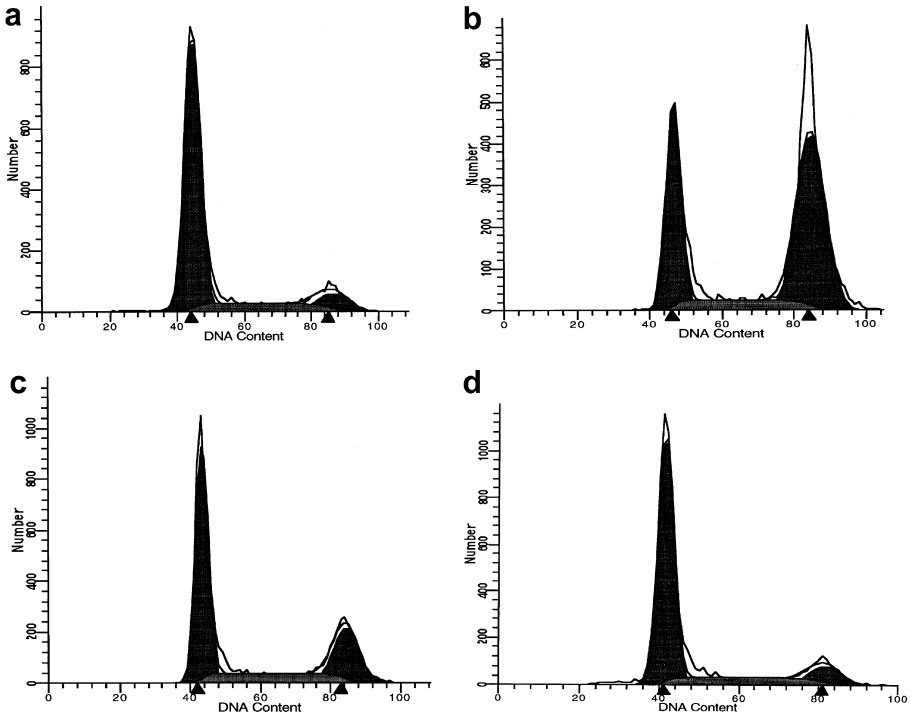

Fig. 7. Effect of 8-methyl-DBP on tumor cell cycle. Cell cycle analysis of T47D cells were treated using vehicle control (a), 10 μM Taxol (b), 50 μM 8-methyl-DBP (c), and 50 μM of 8-methoxy-DBP (d) for 12 h.

3.6. Effect of 8-methyl-DBP on p53 and p21

To assess the effect on cell cycle regulatory proteins such as p53 and p21, the cells were treated with 8-methyl-DBP and analyzed using western blot. 8-methyl-DBP slightly increased the expression of p53 and p21 proteins at higher concentrations (40–80 μM), whereas the expression of p53 and p21 proteins was not altered by 8-methoxy-DBP (Fig. 8). Earlier studies have shown that Genestein, an isoflavonid present in soy proteins did not induce p53 and p21 proteins in breast cancer cell lines (Liao, Pan, Guh, & Teng, 2004). Expression of p53 and its downstream protein, p21 were upregulated with this homoisoflavone (MW 314) along with G2/M cell cycle arrest and Bcl-2 phosphorylation.

3.7. Effect of 8-methyl DBP on cell growth

To assess the cytotoxicity of 8-methyl-DBP, tumor cells were treated with pure 8-methyl-DBP and assessed for cell viability by clonogenic assays. 8-methyl-DBP was purified and assessed for cytotoxicity by clonogenic assay. Although similar in structure, as shown in Fig. 4, 8-methyl-DBP was more cytotoxic than 8-methoxy-DBP (Table 1) and therefore 8-methyl-DBP was used for further mechanistic studies. Treatment with 8-methyl-DBP and 8-methoxy-DBP for 14 days inhibited clonogenic growth of MCF-7 cells with an IC_{50} of approximately 30–100 (μM) (Table 1). 8-methyl-DBP was more cytotoxic (30 μM) than 8-methoxy-DBP (90 μM).

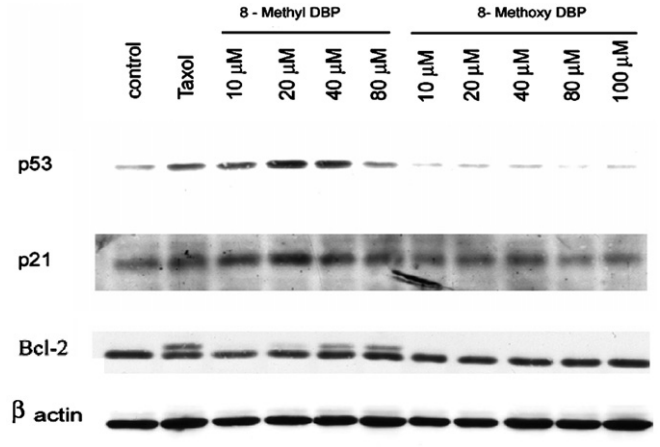

Fig. 8. The effect of 8-methyl-DBP on p53 and p21 protein. Immunoblot of MCF-7 cells treated using 10–80 μM 8-methyl-DBP, 10–100 μM 8-methoxy-DBP or vehicle control for 12 h. Cells were lysed and assayed with p53, p21, Bcl-2 and Beta-actin monoclonal antibodies.

Table 1
Cell viability assay of MCF-7 cells assessed by clonogenic assay after treatment with various concentrations of 8-methyl-DBP and 8-methoxy-DBP for 14 days

Cell line	IC50 8-methyl-DBP (μM)	IC50 8-methoxy-DBP (μM)
MCF-7	30	90

4. Discussion

Extensive bioassay directed fractionation has resulted in the isolation and identification of two dihydrobenzopyranones. Dihydrobenzopyranones are a small class of phenolic compounds also referred to as homoisoflavones or 3-benzyl-4-chromanones. These compounds have been isolated from various genera including those of the Lilaceae family (Heller & Tamm, 1981). Several dihydrobenzopyranone analogues have been identified in the bulbs of *Muscari comosum* (Adinolfi et al., 1984; Adinolfi et al., 1985) *Eucomis bicolor* (Bohler & Tamm, 1967), *Eucomis autumnalis* (Sidwell & Tamm, 1970), and *Eucomis punctata* (Finckh & Tamm, 1970), as well as in the heartwood of *Pterocarpus marsupium* (Jain, Sharma, Kumar, Rajwanshi, & Babu, 1997). Dihydrobenzopyranones are known to be antimutagenic (Wall, Wami, Manikumar, Taylor, & McGivney, 1989), anti-inflammatory (Della, Del, Tubaro, Barone, & Parrilli, 1989) and of chemotaxonomic interest (Heller & Tamm, 1981). Anti-microtubule agents such as Taxol from willow bark are widely used for the treatment of malignancy (DiPaola et al., 1999; Haldar et al., 1997, 1996, 1995). Most of the known drugs such as paclitaxel, docetaxel, vincristine, and vinblastine are large complex structures with molecular weights of over 800. In our study, we demonstrated for the first time that Vietnamese coriander root has biological activity similar to that of known anti-microtubule drugs, and we identified the active compound as 8-methyl-DBP (peak 2, molecular weight 314), which induces G2/M cell cycle arrest, Bcl-2 phosphorylation, and causes apoptosis similar to known anti-microtubule agents.

Previous studies have demonstrated that Vietnamese coriander root extract has other potent biological effects (Lin et al., 1994; Sugiyama et al., 1984; Tomoda et al., 1971; Xiao et al., 1990). For example, an alcohol extract of Vietnamese coriander root was found to act as an immunopotentiator in mice injured by burns (Xiao et al., 1990). In our study, isolation of active compounds in Vietnamese coriander root with biological activity on Bcl-2 protein and the cell cycle required more hydrophobic solvents. Haldar et al. demonstrated that paclitaxel, vincristine, docetaxel, and vinblastine induced Bcl-2 phosphorylation, which inactivated Bcl-2 function (Haldar et al., 1997, 1996, 1995). Although phosphorylation of Bcl-2 is associated with anti-microtubule activity, the relationship of Bcl-2 phosphorylation and apoptosis remains unclear (Ling, Tomos, & Perez-Soler, 1998; Srivastava, Mi, Hardwick, & Longo, 1999). For example, some investigators have demonstrated that paclitaxel induced apoptosis requires the phosphorylation of Bcl-2 (Srivastava et al., 1999). In contrast, other investigators have reported that phosphorylation of Bcl-2 is a marker of G2/M Phase events and not a determinant of apoptosis (Ling et al., 1998). In agreement with this latter study, 8-methyl-DBP induced G2/M cell cycle arrest (Fig. 7). 8-methyl-DBP also induced apoptosis as demonstrated in Table 1. Further studies

would also be warranted to determine whether 8-methyl-DBP enhances the effect of known anti-microtubule agents capable of Bcl-2 phosphorylation.

8-Methyl-DBP slightly increased the expression of p53 and p21 proteins, whereas the expression of p53 and p21 proteins was not altered by 8-methoxy-DBP. Earlier studies have shown that Genistein, an isoflavonoid present in soy proteins did not induce p53 and p21 proteins in breast cancer cell lines (Liao et al., 2004). Expression of p53 and its downstream protein, p21 were upregulated with this homoisoflavone along with G2/M cell cycle arrest. Our study is first to show that a homoisoflavone induces p53 and p21 proteins and causes G2/M cycle arrest. Given the structural complexity of known agents capable of Bcl-2 phosphorylation, such as paclitaxel and vinblastine, the study of structure and activity of this simple homoisoflavone compound may improve our ability to develop novel clinically active agents.

The observation of methylene group between the A and B rings in dihydrobenzopyranones (Fig. 4a and b) is of interest as this structure is different than an isoflavone, where the A and B rings are directly connected. It is likely that the addition of the methylene group changes the conformation of the molecule and positively affects bioactivity. Bioassays of the purified compounds determined that 8-methyl-DBP had increased cytotoxicity and induced Bcl-2 phosphorylation, where as 8-methoxy-DBP had less cytotoxicity and did not induce Bcl-2 phosphorylation. These analogs can serve as lead compounds in the synthesis of more potent analogs in the development of improved therapeutic agents for cancer treatment, which have specific molecular targets.

In conclusion, these data demonstrate for the first time that Vietnamese coriander root has biological activity capable of Bcl-2 phosphorylation and G2/M cell cycle arrest. The molecule causing this activity was identified as 8-methyl-DBP (MW 314), which was purified, and induced Bcl-2 phosphorylation, apoptosis, and G2/M cell cycle arrest in a manner similar to that of the more complex known anti-microtubule agents. These effects were structurally specific because 8-methoxy-DBP (MW 330) in contrast to 8-methyl-DBP, was incapable of Bcl-2 phosphorylation. Further studies of the structure–activity relationship of 8-methyl DBP as a parent compound are warranted.

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