Verticinone Induces Cell Cycle Arrest and Apoptosis in Immortalized and Malignant Human Oral Keratinocytes

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Although verticinone, a major alkaloid isolated from the bulb of Fritillaria ussuriensis, has been shown to induce differentiation in human leukemia cells, the exact mechanism of this action is not completely understood in cancer cells. Verticinone was used to conduct growth and apoptosis-related experiments for two stages of oral cancer on immortalized human oral keratinocytes (IHOKs) and primary oral cancer cells (HN4). The procedures included MTT assay, three-dimensional (3-D) raft cultures, Western blotting, cell cycle analysis, nuclear staining and cytochrome c expression related to the apoptosis signaling pathway.

Verticinone inhibited the proliferation of immortalized and malignant oral keratinocytes in a dose- and time-dependent manner. In 3-D organotypic culture, verticinone-treated cells were less mature than the control cells, displaying low surface keratinization and decreased epithelial thickness. The major mechanism by which verticinone inhibits growth appears to be induced apoptosis and G0/G1 cell cycle arrest. This finding is supported by the results of the cell cycle analysis, FITC-Annexin V staining, DNA fragmentation assay and Hoechst 33258 staining. Furthermore, the cytosolic level of cytochrome c was increased, while the expression of Bcl-2 protein was gradually down-regulated and Bax was up-regulated, accompanied by caspase-3 activation. The data suggests that verticinone may induce apoptosis through a caspase pathway mediated by mitochondrial damage in immortalized keratinocytes and oral cancer cells.

Keywords: verticinone; anticancer effects; oral cancer; immortalized keratinocyte; apoptosis; cell cycle.

INTRODUCTION

Carcinomas of the oral cavity account for 3–5% of all malignancies worldwide and are increasing in incidence. Squamous cell carcinomas (SCC) are the most frequent head and neck malignancies. Oral cancer is treated by surgery, radiation, chemotheraphy, or a combination of these methods. However, these treatments result in adverse systemic and cytotoxic effects, as well as the development of therapeutic resistance (Schliefhake, 2003; Marcus et al., 2004). Therefore, alternative chemotherapies, with minimal or no side effects, should be considered for oral cancer (Yamachika et al., 2004).

Plants are well-established sources of clinically relevant antitumor compounds, and efforts are under way worldwide to discover new plant-based anticancer agents. The use of certain herbs as alternative cancer therapies has attracted attention because of their reduced toxicity and cost benefit. Several clinical trials using herbs or natural products are under way in the USA (McCann, 1997; Cassileth and Chapman, 1996). Some herbal medicines, or their constituents, have been reported to directly inhibit cancer cell proliferation (Kim et al., 2005; Lee et al., 2006). For example, Dipaola et al. (1998) demonstrated the clinical efficacy of an estrogenic herbal combination on prostate cancer. Thus, the discovery of new herbal medicines that may induce apoptosis is highly desirable for cancer treatments, offering fewer side effects, nontoxic agents, or both.

Bulbus Fritillariae (‘beimu’ in Chinese), which is derived from the bulbs of various Fritillaria species (Liliaceae), has been used for thousands of years as one of the most important antitussive and expectorant drugs in traditional Chinese medicine (Shang and Liu, 1995; Chen et al., 1996). The major constituents in beimu are isosteroidal alkaloids (Xu et al., 1990; Ding et al., 1996). Verticinone and verticinone are the majoralkaloids in the commonly used Fritillaria herbs (Xu et al., 1990; Ding et al., 1996). Other alkaloids, including isoverticin, ebeiedine and ebeiedinone, have also been isolated from various Fritillaria species (Xu et al., 1990; Ding et al., 1996; Li et al., 1999; Kaneko et al., 1988).
Although Pae et al. (2002) reported that verticinone exhibits growth-inhibition and differentiation-inducing effects on human leukemia HL-60 cells, there has been no investigation of the effects of verticinone on solid tumor cells such as immortalized human oral keratinocytes (IHOK) and oral cancer cells.

The aim of this study was to elucidate verticinone-induced changes in the growth, cell cycle and expression of apoptosis-related protein in IHOK and oral cancer cells (HN4). In addition, organotypic 3-dimensional cultures of stratified epithelium were used as an in vitro tissue model to study the effect of verticinone.

**MATERIALS AND METHODS**

**Reagents.** Dulbecco's modified Eagle's medium (DMEM), KGM medium (Cambrex), fetal bovine serum (FBS) and other tissue culture reagents were purchased from Gibco BRL Co (Grand Island, NY), Anti-caspase-3, Bel-2, Bax, cytochrome-c, actin and the other antibodies were purchased from Santa Cruz (Santa Cruz, CA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33258 and the other chemicals were obtained from Sigma Chemical Co (St Louis, MO).

**Plant material.** The bulbs of *Fritillaria ussuriensis* Maxim were purchased from the Herbal Medicine Cooperative Association of Jeonbuk Province, Republic of Korea, in September 2000. A voucher specimen (No. DH 21) was authenticated by Professor Tae-Oh Kwon, College of Life Science and Natural Resources, Wonkwang University, and deposited in the Herbarium of the Professional Graduate School of Oriental Medicine, Wonkwang University (Republic of Korea).

**Extraction and isolation.** Dried bulbs of *F. ussuriensis* (500 g) were extracted with MeOH for 24 h. The MeOH extract was concentrated, suspended in H₂O, and sequentially partitioned with n-hexane, EtOAc and BuOH. The BuOH fraction (fr.) (3.9 g) was subjected to octadecyl functionalized silica gel flash column (2.5 × 40 cm) chromatography (Park et al., 1995). The column was eluted with a stepwise gradient with 250 mL aliquots of MeOH in H₂O (from 20% to 100% in 10% increments), followed by 300 mL of 50% MeOH in CH₂Cl₂, affording fractions 1–10. The fractions eluted with 50% to 80% MeOH in H₂O (fr. 5 to fr. 8) were combined, and the dried residue (595.2 mg) was further purified by Sephadex LH-20 column chromatography [3 × 50 cm; eluted with 11 of n-hexane–toluene–MeOH (3:1:1); collecting 7 mL fractions]. Fractions of similar composition as determined by TLC were pooled. The fraction eluted between 385 mL and 532 mL (87.2 mg) was then further purified by chromatography on a silica gel column (3.0 × 5.0 cm; 63–200 μm particle size) chromatography eluted with CHCl₃–MeOH–NH₄Cl (13:1:0.2) to afford verticinone (1, 8.0 mg, Rₜ = 0.4). The fraction eluted between 533 mL and 630 mL (103.5 mg) was then further purified by chromatography on a silica gel column (3.0 × 5 cm; 63–200 μm particle size) chromatography eluted with EtOAc–CHCl₃–NH₄Cl (8:2:0.5) to afford isoverticinone (2, 10.5 mg, Rₜ = 0.3).

The structure of the verticinone compound was identified by comparison of spectral properties (MS, ¹H- and ¹³C-NMR) with those reported in the literature (Xu et al., 1990; Ding et al., 1996).

**Cell culture.** Normal human oral keratinocytes (NHOK) and gingival fibroblasts (GF) were isolated from an explant culture technique from patients undergoing orthodontic treatment by previously described methods (Somerman et al., 1988). Informed consent from donors was obtained for use of the tissues. Patients signed the corresponding informed consent approved by the Institutional Review Board at Wonkwang University for the use of the tissues. Primary NHOK cultures were prepared from separated keratinizing oral epithelial tissue. The NHOK cells were cultured in keratinocyte growth medium (KGM) containing a low level (0.15 mM) of Ca²⁺ and a supplementary growth factor bullet kit (Cambrex, East Rutherford, NJ). Epithelial cells were isolated from the basal surface of the epithelial tissue of approximately 25 mm² × 0.5 mm in size by trypsin digestion. The duration of the enzymatic digestion was limited to 3 min to avoid harvesting cells from the suprabasal layers, as suggested previously (Kang et al., 2000).

For the primary culture of GF, gingival tissues were cut into 1 mm² explants and placed on 100 mm culture dishes (Nunc, Naperville, IL, USA) containing 10 000 U/mL of penicillin G sodium, 10 000 μg/mL of streptomycin sulphate, 25 μg/mL of amphotericin B and 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Grand island, NY, USA) at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. After 2 or 3 days, the cells started to outgrow from the explants. When the primary cell culture reached confluence, the cells were detached with 0.025% trypsin and 0.05% EDTA, diluted with culture medium, and then subcultured in a ratio of 1:4. Primary NHOK and GF cell cultures between the 5th and 7th passages were used in this study.

HPV-immortalized human oral keratinoicyte (IHOK) cells (passage 60–80), HNSCC4 (=HN4) and HNSCC12 (=HN12) cells were examined. IHOK cells were derived by transfecting normal human gingival epithelial cells with PLXSN vector containing the E6/E7 open reading frames of HPV type 16, following methods described previously (Park et al., 1995; Lee et al., 2005). The immortalized oral keratinocytes were cultured in the keratinocyte growth medium (Cambrex, MD), and HaCaT, HN4 and HN12 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Biofluid, Rockville, MD) containing 10% fetal bovine serum (FBS, Gibco, USA) with 100 U/mL penicillin and 100 U/mL streptomycin (Life Technologies, Gaithersburg, MD). Although HaCaT cells are immortalized and are genetically abnormal (Boukamp et al., 1998), they retain many features of keratinocyte differentiation and represent a skin keratinocyte model. Cell line HN4 from a primary TₙNₘM₀ carcinoma of the mouth floor and HN12 from metastatic carcinoma of the oral cavity (Cardini et al., 1985) were derived in the laboratory of Dr John F. Ensley (Wayne State University). All the cell lines were grown at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The cells were dissociated with 0.25% trypsin just before transfer for experiments and were counted using a hemocytometer.

**MTT cell viability assay.** Viable cells were detected using MTT dye, which forms blue formazan crystals that are
reduced by mitochondrial dehydrogenase that is present in living cells. Briefly, 2 × 10^6 cells were seeded in a 96-well plate and cultured overnight. Serial dilutions of verticinone were added, and the cells were treated for 1, 2 and 3 days. After treatment, 50 μL of MTT solution (2 mg/mL in PBS) was added to each well and incubated for 4 h. The plates were then centrifuged at 200 × g for 10 min and the supernatant was discarded. To each well, 50 μL of DMSO was added. The plates were shaken until the crystals dissolved. Reduced MTT was measured spectrophotometrically at 570 nm in a dual beam microtiter plate reader.

Raft culture of epithelial cells with verticinone. Dermal equivalent collagen gels were prepared as described previously (Chung et al., 1997; Lee et al., 2005) using type I collagen (Nitta Gelatin, Osaka, Japan). Confluent monolayer cells were trypsinized using 0.5% trypsin/EDTA (Gibco, Grand Island, NY) and seeded on a freshly prepared gel containing type I collagen matrix and primary human gingival fibroblasts. The gel was kept submerged in a Millicell (Becton Dickinson, USA) for 5 days, and fed with fresh medium every 24 h. Then, the gel was lifted to the liquid/air interface in order to have the nutrients supplied vertically from the bottom to top and to induce keratinocyte differentiation mimicking a stratified epithelium. The raft culture was maintained with or without verticinone treatment for 14 days, and the organotypic tissue was fixed entirely in 10% buffered formalin, sectioned in 4 μm thicknesses, and stained for microscopic analysis and photography.

Propidium iodide (PI) staining. Cells (5 × 10^5) were cultured at 37 °C for 3 days with or without verticinone in medium that contained 10% FBS. The cells were harvested, washed with PBS, fixed with 75% ethanol at 4 °C for 2 h, and treated with 0.25 mg/mL RNase A (Sigma) at 37 °C for 1 h. After washing, the cells were stained with 500 g/mL PI at room temperature for 10 min. Analysis was performed on a flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). The percentages of cells in each stage of the cell cycle were determined by analysing the staining profiles of viable cells using a cell fit analysis program.

FITC-Annexin V and propidium iodine double staining. After dispensing with 1 × 10^5 cells on the 6-well plate and treating each trial group with the reagents, the cell pellet was prepared in a FACStar tube containing annexin V-FITC solution and incubated at 37 °C in a CO2 incubator. The PI solution (without NP-40) was then added, and the ratio of PI-positive and annexin V-positive cells was measured in the flow cytometer.

Morphological analysis of apoptosis by staining with Hoechst 33258. To confirm morphological changes of the nuclei, the cells were cultured in the 60 mm dishes overnight, and then washed twice in the DMEM. The cells were incubated with verticinone for 3 days. Hoechst 33258 solution was added to 20 μg/mL and the cells were incubated for 30 min. The cells were observed by fluorescence microscopy (Olympus, Tokyo, Japan).

DNA fragmentation assay. Cell pellets (3 × 10^5 cells) were resuspended in 500 μL of lysis buffer (0.5% Triton X-100, 10 mM EDTA, 10 mM Tris-HCl, pH 8.0) at room temperature for 15 min and centrifuged at 16 000 × g for 10 min. DNA was then extracted using a Wizard genomic DNA purification kit (Promega), precipitated with ethanol and resuspended in Tris/EDTA buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). DNA was analysed after separation by gel electrophoresis (1.5% agarose).

Western blot analysis. Protein samples (50 μg) were mixed with an equal volume of 2 × SDS sample buffer, boiled for 5 min, and then separated using 8–15% SDS-gels. After electrophoresis, the proteins were transferred to nylon membranes. The membranes were blocked in 5% dry milk (1 h), rinsed and incubated with the primary antibodies (diluted 1:500–1:1000) followed by the secondary antibodies in TBS for 1 h at room temperature. Finally, each protein was detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, USA).

Evaluation of cytochrome c release from mitochondria. For the analysis of cytochrome c release from mitochondria, stimulated cells (1 × 10^5) were trypsinized and then washed with ice-cold buffer A (250 mM sucrose, 20 mM HEPES-KOH, 1 mM EDTA, 1 mM EGTA, 2 μg/mL leupeptin, 1 μg/mL pepstatin pH 7.4). Cells were resuspended in 200 μL of buffer A and carefully homogenized using a Dounce homogenizer. The homogenates were separated into cytosol (supernatant) and mitochondrial fractions (pellet) by differential centrifugation as described previously (Thornberry et al., 1998). Cytosolic proteins were then subjected to immunoblot analysis using the anti-cytochrome c monoclonal antibody as described above.

RESULTS

Effect of verticinone on cell viability

As verticinone is known to inhibit growth in leukemia cells, the effects of this verticinone were examined on the growth of normal, immortalized and malignant cells by MTT assay. Interestingly, the viability of GF was not significantly affected by verticinone (Fig. 1A). The growth of skin (HaCaT) and oral immortalized keratinocytes (IHOK) was inhibited in a dose- and time-dependent fashion at verticinone concentrations ranging from 1 to 50 μg/mL on days 1, 2 and 3 of culture. At days 2 and 3, IHOK cells were more sensitive to growth inhibition by verticinone than were HaCaT cells (Fig 1C, 1D).

The study next investigated the effect of verticinone on the growth of HN4 (primary oral cancer) and HN12 (metastatic oral cancer) cells, and compared the results with IHOKs. The growth of both HN4 and HN12 cells was also inhibited in the presence of verticinone. Of the two cancer cell lines, the metastatic HN12 line was more resistant than the primary HN4 cell line (Fig 1E, F).

The verticinone concentration resulting in a 50% inhibition of cell growth (IC50) was calculated after 1, 2 and 3 days of exposure (Table 1). The IC50 of IHOK and HN4 cells at 3 days was lower than that of HN12, suggesting that IHOK and HN4 cells are more susceptible to verticinone-induced toxicity than are HN12 cells.
Morphological appearance of organotypic cultures treated with verticinone

In organotypic culture, control IHOK and HN4 cells showed stratification, with well-preserved morphological differentiation. At six or more cell layers, the cells showed good polarity of differentiation and a distinct keratin layer (Fig. 2A, C). In contrast, verticinone-treated IHOK and HN4 cells showed only one or two cell layers, less keratinization, and less stratification (Fig. 2B, D). Light microscopic evaluation confirmed that verticinone at 50 μg/mL had an inhibitory effect on keratinocyte growth, as was evident from diminished keratinization and a reduction in the cell layer number from six or more for the control to one or two, regardless of the keratinocyte type.

Apoptosis induction by verticinone treatment

To clarify whether the verticinone-induced decreases in viability and growth rate were attributable to apoptosis, checks were made by cell cycle analysis for patterns characteristic of apoptosis (Fig. 3A), FITC-Annexin V and PI staining (Fig. 3B), nuclear staining with Hoechst 33258 (Fig. 3C) and DNA fragmentation assay (Fig. 3D).

First, cell cycle analysis of keratinocytes treated with 50 μg/mL verticinone for 3 days demonstrated that a distinct, quantifiable population of G0G1 fraction cells increased in the verticinone-treated IHOK and HN4 cells (Fig. 3A). Thus, verticinone delayed the progression of cells from G0G1 to the S-phase and induced cells toward apoptosis.

Second, flow cytometric analysis using Annexin V/PI double-staining revealed that the percentage of FITC-Annexin V-positive/PI-negative IHOKs and HN4 cells that were apoptotic, rather than necrotic, increased over the control level after 3 days of treatment with 50 μg/mL verticinone (Fig. 3B). Thus, verticinone induced apoptosis in both IHOKs and HN4 cells. The induction of apoptosis by verticinone was greater in oral cancer cells (29%) than in immortalized keratinocytes (12%).

Table 1. Verticinone concentration required for 50% growth inhibition (IC50)

<table>
<thead>
<tr>
<th>Days (μg/mL)</th>
<th>HaCaT</th>
<th>IHOK</th>
<th>HN4</th>
<th>HN12</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26.22 ± 0.15</td>
<td>34.10 ± 0.34</td>
<td>20.42 ± 0.4</td>
<td>52.05 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>22.07 ± 0.14</td>
<td>20.03 ± 0.43</td>
<td>17.29 ± 0.18</td>
<td>35.22 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>19.58 ± 0.19</td>
<td>12.04 ± 0.42</td>
<td>16.19 ± 0.55</td>
<td>28.06 ± 0.24</td>
</tr>
</tbody>
</table>

Figure 1. Effects of verticinone on cell viability of normal human gingival fibroblasts (GF, A), normal human oral keratinocytes (NHOK, B), immortalized skin keratinocytes (HaCaT, C), immortalized human oral keratinocytes (IHOK, D), primary oral cancer (HN4, E) and metastatic oral cancer (HN12, F) cells. Cells as measured by MTT assay. Results are expressed as mean ± SD of three independent experiments. * Statistically significant difference compared with the control group, p < 0.05.
Lastly, the DNA fragmentation assay of cells treated with 50 μg/mL verticinone for 3 days showed DNA laddering, indicative of apoptosis, in both IHOKs and HN4 cells (Fig. 3D).

Collectively, these results indicate verticinone-induced apoptosis in both IHOKs and HN4 cells. HN4 cells were more sensitive to apoptotic induction by verticinone than were IHOKs.

Western blot analysis for cell cycle progression and apoptosis-related proteins

The expression of proteins involved in cell cycle progression and apoptosis was assessed by western blot analysis. In this study, the levels of p53 and p21 proteins increased in the verticinone-treated cells, whereas pRb protein decreased (Fig. 4A). Interestingly, the p16 protein level did not change in the verticinone treated IHOK and HN4 cells.

The balance between anti-apoptotic and pro-apoptotic Bcl-2 family members is critical in determining the susceptibility of cells to apoptotic signals (Oh et al., 1992). Therefore, the influence of verticinone on Bcl-2 family proteins was examined and it was found that verticinone down-regulated Bcl-2 but up-regulated Bax protein in both cell lines (Fig. 4B).
In order to understand the death-signaling pathway underlying the verticinone-induced apoptotic DNA fragmentation, mitochondrial cytochrome c release into the cytoplasm was investigated, being known frequently to be involved in a chemical-induced apoptotic signaling pathway, and the resultant activation of the caspase cascade, including caspase-3, leading to poly (ADP-ribose) polymerase (PARP) degradation (Ashkenazi and Dixit, 1999; Nagata, 1997; Sun et al., 1999). As shown in Fig. 4B, cytochrome c was weakly detectable in the cytosolic fraction of control cells, but the level of cytochrome c released from the mitochondria increased in cells treated with 50 μg/mL verticinone for 3 days. Coincident with the release of mitochondrial cytochrome c into the cytoplasm, caspase-3 activation was detected in verticinone-treated IHOKs and HN4 cells, but caspase-9 was not activated (data not shown). As a downstream target of active caspase-3 during apoptosis induction, PARP is reportedly cleaved into two fragments (Lazebnik et al., 1994). After treatment with 50 μg/mL verticinone, PARP cleavage was detected in IHOKs and HN4 cells, along with the activation of caspase-3 (Fig. 4B). These results suggest that verticinone-induced apoptosis involves the mitochondrial cytochrome c-dependent activation of caspase-3.

DISCUSSION

Verticinone has been reported to induce differentiation in human leukemia cells (Pae et al., 2002), but the effects of verticinone on solid tumor cells, especially immortalized keratinocytes and oral cancer cells, have not been examined. The study investigated the influence of verticinone on cell proliferation, the cell cycle and apoptosis in HPV16 E6/E7-immortalized human oral keratinocytes (IHOKs) and primary oral cancer cells (HN4).

The current study revealed that verticinone treatment inhibited the proliferation of NHOK, IHOKs, HaCaT, HN4 and HN12 cells in a dose- and time-dependent manner (Fig. 1). However, in this study, selective cell death was found in IHOK and HN4 cells treated with relatively high concentrations of verticinone, and these events appeared to be more pronounced in IHOK and HN4 cells than in NHOK cells. These results suggest that oral cancer cells are more sensitive to verticinone than are immortalized (IHOK, HaCaT) and normal cells (GF, NHOK).

The 3-D culture method is a useful model for investigating the pharmacological influence of drugs, such as verticinone, on the morphology, function and differentiation of keratinocytes in vitro (Lee et al., 2006; Lee et al., 2005; Chung et al., 1997). With verticinone treatment, the 3-D organotypic raft culture of the immortalized and malignant keratinocytes showed less epithelial maturation, less surface keratinization and decreased epithelial thickness, compared with the control group (Fig. 2). Additionally, the 3-D culture study showed that relatively long-term treatment (2 weeks) with verticinone completely disrupted the growth and differentiation of the keratinocytes used in this study. To determine the mechanism of the antiproliferative activity of verticinone, the study investigated whether verticinone possessed the potential to induce cellular apoptosis in immortalized and malignant oral keratinocytes. The investigations demonstrated that verticinone at micromolar concentrations can trigger apoptosis in IHOKs and oral cancer cells. Cell cycle analysis showed that immortalized and malignant oral keratinocytes display an increased fraction of G0/G1 cells after verticinone treatment (Fig. 3A). FITC-Annexin V and PI double-staining revealed that the apoptotic Annexin V+/PI population of immortalized and malignant oral keratinocytes increases after exposure to verticinone (Fig. 3B). IHOKs and oral cancer cells underwent morphological and nuclear changes characteristic of apoptosis (Fig. 3C), and a DNA fragmentation assay produced DNA ladders typical of apoptotic cells (Fig. 3D). However, the induction of apoptosis by verticinone was more prominent in malignant oral keratinocytes (HN4) than in IHOKs.
To elucidate the mechanism of the growth inhibitory effect of verticinone, its effect on other cell cycle regulatory proteins was investigated. The ability of cells to arrest in G1 response to DNA damage depends on the accumulation of the tumor suppressor p53 (Lowe et al., 1993). This p53-dependent G1 arrest is largely mediated through the induction of the CDK inhibitor p21 (Pavlletich, 1999), which appears to be induced in a p53-dependent manner. The data showing an induction of p21<sup>WAF1</sup> by verticinone appears to be p53 dependent in IHOK and HN4 cells (with wild-type p53). The levels of other cell cycle inhibitor, p16, was not significantly changed by verticinone, which result is consistent with the effects of trichostatin A in lung cancer cells (Mukhopadhyay et al., 2006) and inhibitors of DNA polymerase in gastric cancer cells (Murakami et al., 2003). The non significant effect on p16 expression, suggests that p21 is an important mediator by which verticinone may arrest cell proliferation. p21<sup>WAF1</sup> is a universal inhibitor of CDK, which phosphorylates pRb (Hartwell and Kaston, 1994). In the present study, verticinone promoted the dephosphorylation of pRb, being consistent with evidence that demonstrates a requirement for pRb in G1 arrest induced by a variety of DNA-damaging agents (Harrington et al., 1998). Therefore, it is plausible that verticinone induces p53-dependent p21 expression and leads to a loss in pRb activity, resulting in the G<sub>G1</sub> arrest.

Bcl-2 may act to control apoptosis by influencing the permeability of intracellular membranes and the cytochrome c release from mitochondria (Yang et al., 1997). Furthermore, during the apoptotic process, the permeability of intracellular membranes and cytochrome c release from mitochondria (Yang et al., 1997). Therefore, it is plausible that verticinone induces p53-dependent p21 expression and leads to a loss in pRb activity, resulting in the G<sub>G1</sub> arrest.

References

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