Anti-proliferative Effects of β-ionone on Human Lung Cancer A-549 Cells

Sun Min Lee¹, Young Sook Km³, Wook Jin Jang¹, Abdur Md. Rakib², Tae Woo Oh¹, Bon Hyun Km¹, So Young Km³, Jeong Ok Km³ and Yeong Lae Ha¹

¹Division of Applied Life Science (BK21 Plus), Graduate School, and Institute of Agriculture & Life Science, Gyeongsang National University, Jinju 660-701, Korea  
²Department of Biochemistry and Molecular Biology, Faculty of Science, University of Rajshahi, Rajshahi-6205, Bangladesh  
³School of Food Science, International University of Korea, Jinju 660-759, Korea  
⁴HK Biotech Co Ltd, Jinju 660-544, Korea

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The anti-proliferative activity of β-ionone was investigated on human non-small lung cancer A-549 cells (designated A-549 cells). A-549 cells were treated with various concentrations of β-ionone (1, 5, 10, and 15 μM) for two, four, and six days. Biochemical markers related to the growth inhibition of A-549 cells by β-ionone were measured at the second day of incubation. β-Ionone inhibited the growth of A-549 cells by dose-and time-dependent manners, resulting in an IC₅₀ of 5.0 μg/ml at the second day of incubation. β-Ionone induced apoptosis by a dose-dependent manner. β-Ionone increased levels of p53, p21, and Bax proteins, but suppressed expression of the Bel-2 protein. Similarly, β-ionone enhanced cytochrome c release from the mitochondria to the cytosol, and induced activation of caspase-9 and -3. Additionally, β-ion-one reduced dPLA₂ and COX-2 protein levels. These results suggest that the β-ionone inhibits the proliferation of A-549 cells through reciprocal regulation of Bax and Bel-2 gene expression and suppression of dPLA₂ and COX-2 protein expressions.

Key words: β-Ionone, human non small lung cancer A-549 cells, apoptosis, dPLA₂, COX-2

Introduction

The consumption of fruits and vegetables is associated with a protective action for many human cancers [4]. This might be attributed to the phytochemicals widely distributed in fruits and vegetables. One such chemical is β-ionone, (E)-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one (Fig 1), which imparts a desirable flavor note to foods and physiological activities [30]. β-Ionone is a common intermediate of more than 22,000 isoprenoids in many fruits, vegetables and grains, and also a precursor for carotenoids, where two molecules of β-ionone combine to form β-carotene [9]. Dietary studies reveal that β-ionone exhibits chemopreventive and antitumor activities [13, 32].

β-ionone is the major aroma molecule formed in a Port wine supplemented with β-carotene [10]. Such a β-ionone is produced from enzymatic degradation of carotenoids and photo-oxidation or thermal oxidation of carotenoids in food systems to provide characteristic flavors [24, 29]. In addition, β-ionone is a fragrance ingredient used in many fragrance compounds. It may be found in fragrances used in decorative cosmetics, fine fragrances, shampoos, toilet soaps and other soaps, as well as in non-cosmetic products such as household cleaners and detergents [16].

β-Ionone exhibits various biological activities, including anticarcinogenic, antimutagenic and antifungal activities [6, 11]. Antitumor activities of β-ionone have been demonstrated in melanoma, breast cancer and chemical-induced rat carcinogenesis [13, 22, 32]. β-Ionone also inhibits the growth of various human cancer cells including breast and gastric

*Corresponding author  
Tel: +82-55-772-1964, Fax: +82-55-772-1969  
E-mail: ylha@gnu.ac.kr  
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Fig. 1. Chemical structure of β-ionone, (E)-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one.
tumor cells [8, 21] and murine tumor cells [26]. Its exact mechanisms are not clear, but apoptosis is one of mechanistic actions of β-ionone on SGC-7901 gastric cancer cells, B16 murine tumor cells, and other cancer cells [8, 21, 26]. It is interesting to note that, in spite of the volatility of β-ionone, the compound's anticarcinogenic activity has not been investigated on lung cancer cells.

Lung cancer is one of the most frequent neoplasms caused by smoking in Korea and most parts of the world [28]. Chemoprevention is always used as the main treatment for advanced lung cancer, and has been under intense investigation [18]. The anti-proliferative activity of β-ionone on various types of cancer cells could be a useful way to prevent lung cancers in the general population. Since β-ionone is a desirable volatile flavor compound, it is very easy to handle and to contact with lung adenocarcinoma following inhalation to suppress cell proliferation. Presently, we determined whether β-ionone exhibits anti-proliferative activity and can induce apoptosis of non-small cell lung cancer (NSCLC) A-549 cell (designate A-549 cell), which is the principal carcinoma cell attributed to smoking related lung cancer.

Materials and Methods

Materials

A549 cell (ATCC CCL-185) was purchased from the Korea Cell Line Bank (Seoul, Republic of Korea). Dulbecco's Modified Eagle Medium/Ham' F-12 nutrient mixture (DMEM/F12), fetal bovine serum (FBS), and a mixture of penicillin (10,000 U/ml) and streptomycin (10,000 µg/ml), and trypsin were obtained from Gibco BRL (Grand Island, NY). Phosphate buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and Nonidet P-40 were purchased from Amresco (Solon, OH). β-Ionone, Hoechst 33258, ribonuclease A (RNase A), propidium iodide (PI), leupeptin, phenyl methane sulfonyl fluoride (PMSF) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies to β-actin, caspase-3, cytochrome c and COX-2, and goat anti-rabbit IgG-horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Bax, Bcl-2, p53, and p21 were obtained from Delta Biolabs (Gilroy, CA). Antibody to cPLA2 was obtained from Cell Signaling Technology (Danvers, MA). The enhanced chemiluminescence (ECL) Western blotting kit was purchased from Amersham Biosciences (Buckingham, UK). The protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). All other reagents used were obtained from Sigma-Aldrich.

Cell culture and treatment

A549 cells were cultured in DMEM/F12 medium supplemented with 10% FBS and penicillin/streptomycin (10,000 U/ml and 10,000 µg/ml, respectively) at 37°C in a humidified atmosphere with 5% CO2 [18]. Cells were grown to 80% confluence prior to treatment with 0.1% trypsin-0.06 mM EDTA to disperse the cells. The dispersed cells were collected by centrifugation (1,000× g 10 min, 4°C). For cytotoxicity test, the collected cells were seeded into a 24-well culture plate (Nunc, Rochester, NY) at a density of 2×10^5 cells per well and incubated for 24 h. Cells, replaced by FBS-free culture medium, were treated with β-ionone (0, 1, 5, 10, and 15 µM) in DMSO for 0, 2, 4, and 6 days, repeating every 2 days. For the other experiments, 10 ml of cell solution (3×10^5 cells/ml), replaced by FBS-free culture medium, were incubated with β-ionone (0, 1, 5, 10, and 15 µM) in 100 mm-diameter culture dishes (Nunc) for 2 days. The cells were trypsinized with 0.1% trypsin-0.06 mM EDTA to disperse them. The dispersed cells were collected by centrifugation (1,000× g 10 min, 4°C) and resuspended in PBS for further use. Exponentially growing cells were used throughout the experiments.

Cytotoxicity tests

The MTT-based assay was used to estimate viable cell number [14]. The absorbance was measured at 570 nm using an Anthos model 2020 microplate reader (Anthos Labtech Instruments, Wals., Austria). Cytotoxicity was represented as a growth rate, which is the percentage ratio of cell numbers at given incubation days to cell numbers at 0 day.

Morphological examination

The morphological changes of the cells were examined using an inverted phase-contrast microscope (Olympus, Tokyo, Japan).

Flow cytometry analysis

The cells were fixed by adding ice-cold 70% ethanol (200-500 µl) in a drop-wise manner while gently vortexing, after which they were stored at -20°C until analysis. The fixed cells were rinsed with PBS and then incubated in 500
μl PI solution (10 mM Tris, pH 8, containing 1 mM NaCl, 0.1% Nonidet P-40, 0.7 μg/ml RNase A, and 0.05 μg/ml PI) for 30 min at 37°C [8]. The analyses of cell apoptosis and cell cycle in 20,000 cells were subsequently performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with CellQuestPro software (BD Biosciences).

**Hoechst 333258 staining**

Apoptotic nuclear morphology was assessed using Hoechst 333258 staining as described previously [15]. Briefly, cells were fixed with 4% paraformaldehyde in PBS for 30 min at 4°C. After washing three times with PBS, the nuclei of cells were stained with Hoechst dye (1 mg/ml) for 15 min. The cells were observed using a fluorescence microscope using an Olympus DP70 Digital Camera System.

**Western blot analysis**

Cells were lysed in a buffer (50 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 1 mM EDTA, 10 mM sodium fluoride, and 1% Triton X-100) that contained the protease inhibitors leupeptin (10 μg/ml) and PMSF (2 mM). The protein concentration was determined according to the method described by the Bio-Rad protein assay manual. Western blotting was performed as described previously [4, 15]. Bound antibodies were visualized with the aid of ECL Western blotting detection kit. The relative protein levels were determined using the software of Kodak ID Scientific Imaging System (Rochester, NY).

**Mitochondrial cytochrome c release assay**

Mitochondrial and cytosolic fractions from cells were prepared by a method reported earlier [15]. Briefly, the cells were harvested and washed with PBS, and then resuspended at 5×10^6 cells/ml in an extraction buffer (20 mM HEPES, pH 7.5 containing 10 mM KCl, 15 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 PMSF, and 250 mM sucrose) and homogenized using a Kontes Pellet Pestle Motor homogenizer (Vineland, NJ). The homogenate was centrifuged at 1,000×g for 10 min at 4°C and the supernatants were centrifuged at 10,000×g for 15 min (4°C) using a Beckman ultracentrifuge (Gagny, France), and the pellets were resuspended in 0.5 ml of extraction buffer (designated the mitochondrial fraction). The supernatants separated at 10,000×g were further centrifuged at 100,000×g for 1 h (4°C), and the resulting supernatants were collected (designated the cytosol fraction). Cytochrome c of both fractions was analyzed by probing with an anti-cytochrome antibody using the standard protocol of Western blot analysis [15].

**Statistical analysis**

Data are expressed as means ± SD. Statistical analysis of the results was made using an one-way analysis of variance (ANOVA), and the significance at p<0.05 was analyzed by Duncan's multiple range tests.

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**Fig. 2. Effects of β-ionone on the growth (A) and morphological changes (B) of A-832 cells. Line identification: control (●); 1 μM β-ionone (○); 5 μM β-ionone (▲); 10 μM β-ionone (▼); and 15 μM β-ionone (■). Values are expressed as mean ± SD (n=6).** Morphological changes of A-549 cells treated various concentrations of β-ionone for 48 h were observed using a phase-contrast microscope. Means with different small letters at same incubation times represent significances at p<0.05 by Duncan's multiple range tests.
Results

Anti-proliferative activity of β-ionone on A-549 cells

Fig. 2 shows the anti-proliferative effects of β-ionone on A-549 cells treated with various concentrations (1, 5, 10, and 15 μM) for up to 6 days, as assessed in the MTT-based assay. β-Ionone reduced A-549 cell growth in dose- and time-dependent manners, and resulted in an IC₅₀ of 5.0 μg/ml by 2 days of incubation (Fig. 2A). To examine the morphological changes of A-549 cells resulting from β-ionone treatment, cells were incubated in medium containing various concentrations of β-ionone (1, 5, 10, and 15 μM) for 48 h, followed by phase contrast microscopy examination (Fig. 2B). A-549 cells treated with β-ionone displayed a condensed cell cytoplasm and were smaller due to membrane shrinkage and rounding up of cells. This β-ionone-mediated morphological change was dose-dependent. In high concentrations of β-ionone, cells lost the ability to adhere to the plate surface. These observations are consistent with an anti-proliferative action of β-ionone on A-549 lung cancer cells (Fig. 2A).

Anti-apoptotic effects of β-ionone on A-549 cells

To elucidate the anti-proliferative actions of β-ionone on A-549 cells, apoptosis induction was investigated in cells incubated in medium containing various concentrations of β-ionone (1, 5, 10, and 15 μM) for 48 h (Fig. 3). Increased apoptosis of A-549 cells treated with β-ionone was evident by flow cytometry, with decreased portions of G0/G1, S, and G2/M phase DNA in the β-ionone-treated cells, as compared to control cells, which resulted in a concomitant increase in the proportion of apoptotic DNA, which is the sub-G1 phase DNA (Fig. 3A). The proportion of apoptotic DNA in β-ionone-treated cells was increased in a dose-dependent manner as compared to control cells. The apoptotic

<table>
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<th>Treatment (μM)</th>
<th>% of sub-G1 (apoptotic) DNA</th>
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<tr>
<td>Control</td>
<td>6.54</td>
</tr>
<tr>
<td>1</td>
<td>9.02</td>
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<tr>
<td>5</td>
<td>32.35</td>
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<td>10</td>
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Fig. 3. Flow cytometric analysis (A) and Hoechst staining (B) of A-549 cells treated with β-ionone. Cells, treated with various concentrations of β-ionone for 48 h, were analyzed apoptotic DNA contents by flow cytometry, and nuclei morphological changes by Hoechst 33258 staining.
DNA contents in sub-G1 cycle of cells were 65, 9.0, 32.4, 38.0, and 40.3% of cells treated with control, 1, 5, 10, and 15 μM β-ionone, respectively (Fig. 3A). These results support the suggestion that β-ionone actively arrests progression of the cell cycle and induces apoptosis in A-549 cells. These results were further supported by Hoechst 33258 staining (Fig. 3B), which revealed the biochemical hallmarks of apoptosis [2]. The morphological features of apoptosis including condensation of chromatin and fragmentation of the nucleus into discrete masses were evident in β-ionone-treated cells. The efficacy of β-ionone was dose-dependent. These results supported the suggestion that the anti-proliferative action of β-ionone might be, in part, due to the induction of apoptosis, with respect to arresting cell cycle in A-549 cells.

Expression or activation of anti-apoptotic proteins on A-549 cells by β-ionone

The expression of p53, p21, Bax, and Bcl-2 genes at the level of protein production and the activation of caspase-9 and -3 enzymes were measured to establish the role of apoptosis in A-549 cells treated for 48 h with various concentrations of β-ionone (1, 5, 10, and 15 μM) by Western blotting (Fig. 4, 5). The p53 tumor suppressor protein was significantly increased (p<0.05) in cells treated with β-ionone, relative to control cells (Fig. 4A). The p53 level in the cells treated with 15 μM β-ionone was increased 2.2-fold in comparison with the control treatment. Likewise, β-ionone treatment significantly elevated the level of expression of p21 (p<0.05), which is a cell cycle regulator Cdk inhibitor, as compared to control cells (Fig. 4B). Such an upregulation

Fig. 4. Expression of p53 (A), p21 (B), Bax/Bcl-2 (C) and Cytochrome c (D) proteins in β-ionone-treated A-549 cells. Cells, treated with various concentrations of β-ionone for 48 h, were analyzed by Western blotting. Values are expressed as mean ± SD (n=3). Means with different small letters on same lines represent significances at p<0.05 by Duncan’s multiple range tests.
of both p53 and p21 protein expression was closely related to the anti-proliferative effects (Fig. 2) and cell apoptosis (Fig. 3) of β-ionone.

Bax can promote the release of cytochrome c from the internal spaces of the mitochondria into the cytoplasm, which is one of the key executioners of apoptosis [12], followed by activation of caspase-3, which is one of the key executioners of apoptosis and cell cycle arrest [31]. Thus, the quantity of Bax, Bcl-2, cytochrome c, caspase-9 and caspase-3 proteins was determined by Western blotting from A-549 cells treated with various concentrations of β-ionone (1, 5, 10, and 15 μM) for 48 h. As shown in Fig. 4C, Bax protein expression in A-549 cells was proportionally increased (p<0.05) with β-ionone concentration, relative to that in control cells. Meanwhile, Bcl-2 protein expression in cells treated with β-ionone was reduced (p<0.05) by the concentrations of β-ionone. These results support the suggestion that the anti-proliferate effect and apoptosis observed in A-549 cells were directly related to the abundance of these two proteins. Much of the evidence to date suggests that an increased Bax/Bcl-2 ratio makes cells more susceptible to apoptosis [15]. The ratio of Bax to Bcl-2 in β-ionone treated cells was found to be 1.4 and 1.8 at 10 and 15 μM β-ionone, respectively.

The release of cytochrome c from the internal space of the mitochondria into the cytoplasm of A-549 cells treated with β-ionone is depicted in Fig. 4D. The cytochrome c levels in mitochondria were significantly decreased and concomitantly increased in the cytosol in a β-ionone dose-dependent manner. The relative ratio of cytochrome c in the internal space of mitochondria to cytosol was 2.0 at 15 μM β-ionone. These results support the suggestion that β-ionone disrupts the mitochondrial membrane, releasing cytochrome c into the cytosol of cells. Caspases play key roles in apopto-

![Fig. 5. Activation of Caspase-9 (A), caspase-3 (B), cPLA2 (C) and COX-2 (D) proteins in A-549 cells treated with β-ionone. Cells, treated with various concentrations of β-ionone for 48 h, were analyzed by Western blotting. Values are expressed as mean ± SD (n=3). Means with different small letters on same lines represent significances at p<0.05 by Duncan's multiple range tests.](image-url)
is, with the initiation of caspase-9 in the caspase-dependent pathway induce cell apoptosis [20]. To determine whether increases in caspase activities coincided with apoptotic cell death, the protein levels of caspase-9 and caspase-3 were measured in cells treated with β-ionone (1, 5, 10, and 15 μM) (Fig. 5A, SB). Both protein levels were significantly elevated (p<0.05) in a β-ionone dose-dependent manner. These results support the suggestion that the cytochrome c release is followed by the activation of caspase-9 and caspase-3.

Reduction of cPLA2 and COX-2 proteins

Arachidonic acid metabolites are closely related to carcinogenesis in animal models and to cancer cell proliferation [7]. Hence, we measured cPLA2 and COX-2, which are related to arachidonic acid metabolism in cancer cells. The expression of cPLA2 and COX-2 proteins in A-549 cells treated with 1, 5, 10, and 15 μM β-ionone was significantly (p<0.05) lowered, relative to that of control cells (Fig. 5C, SD). The efficacy of β-ionone on the suppression of cPLA2 protein expression was greater than that of COX-2 protein expression. At lower concentrations (1-10 μM) of β-ionone, cPLA2 expression was less than 73%, relative to control treatment. At highest concentration of 15 μM β-ionone, cPLA2 expression was approximately 30%, but COX-2 expression was about 50%, relative to control treatment.

Discussion

β-Ionone exhibits anti-proliferative activity on various cancer cells by induction of apoptotic pathways, but there have been no reports on the anti-proliferative effects of β-ionone in lung cancer cells. Presently, in β-ionone-treated A-549 cancer cells, the inhibitory efficacy of β-ionone was dose dependent and could be, in part, associated with the induction of apoptosis. These effects could be attributed to β-ionone itself and/or its metabolites, since β-ionone is metabolized to 3-oxo-β-ionone, 3-oxo-β-ionol, dihydro-3-oxo-β-ionol, and 3-hydroxy-β-ionol, and glucuronides of 3-oxo-β-ionol and dihydro-3-oxo-β-ionol in vivo systems [16].

The molecular mechanism by which β-ionone inhibits the proliferation of A-549 cells is not yet well-understood. However, β-ionone induced apoptosis through the mitochondrial dysfunction pathway, related to the reciprocal expressions of the Bax and Bcl-2 protein genes (Fig. 4). β-Ionone elevated the expressions of the p53, p21, and Bax proteins, and considerably reduced the expression of Bcl-2 protein, followed by releasing mitochondrial cytochrome c to cytosol. β-Ionone also elevated caspase-9 and caspase-3 proteins (Fig. 5A, SB), which executed apoptosis (Fig. 2). The up-regulated production of p53 and p21 genes, which play critical roles in the induction of apoptosis [23, 25], might reciprocally regulate the expressions of the Bax and Bcl-2 proteins in response to β-ionone. The elevated expression of the Bax protein leads to the activation of caspase-3 from procaspase-3 by caspase-9, which is activated by cytochrome c bound with deoxy ATP and Apaf-1 [17]. The activation of caspase-3 leads to the cleavage of the poly ADP ribose polymerase (116 kDa) protein, thus yielding the characteristic 85 kDa fragment for the induction of apoptosis [5]. Given these results, the β-ionone-mediated anti-proliferation can be explained, in part, by the alteration in the caspase-dependent pathway caused by Bax, Bcl-2 and caspase-3. Our findings are in agreement with the results derived from gastric cancer cells, murine tumor cells, and A-549 tumor cells [20, 26, 31].

Although the up-regulation of p53 and p21, reciprocal regulation of Bax and Bcl-2, and activation of caspase-3 might be sufficient to shift the balance toward apoptosis in the anti-proliferative activity, the expression and/or activation of cPLA2 and COX-2, which are closely related to arachidonic acid metabolisms, are also associated with apoptosis of A-549 cells in response to β-ionone. β-Ionone treatment suppressed the expression of cPLA2 and COX-2 proteins (Fig. 5C, SD). These suppressions may reduce the biosynthesis of PGE2 from arachidonic acid via the cyclooxygenase and prostaglandin H synthase pathways [19]. It is evident that arachidonic acid and PGE2 are anti-apoptotic effects in various cancer cells and chemical-induced carcinogenesis in animal models [27], confirming our data obtained in the present study. In addition, cPLA2 is cleaved by caspase-3 and/or a related caspase in cells undergoing apoptosis [1, 33]. As a consequence, the activated caspase-3 might be involved in the cleavage of cPLA2, leading to lower arachidonic acid content and PGE2 contents with response to the lowered COX-2 protein level in A-549 cells treated with β-ionone. Hence, in addition to the mitochondrial damage-dependent pathway involved in β-ionone-induced apoptosis in A-549 cells, an additional potential mechanism is a reduction in the synthesis of arachidonic acid and arachidonate-derived eicosanoids, which is associated with the stimulation of cancer cell growth.

In the present study, β-ionone exhibits anticancer activity...
in human lung cancer cell, A-549. Additionally, \(\beta\)-ionone has been shown antitumor activity in melanoma, breast cancer and chemical-induced rat carcinoma [13, 20, 32] and human breast and gastric tumor cells [8, 21]. Given these antitumor activities of \(\beta\)-ionone on various types of cancer cells could be a useful way to prevent lung cancers in the general population. Hence, it is noteworthy that the consumption of fruits and vegetables, and foods containing \(\beta\)-ionone might reduce human lung cancer risk through the prevention of cancer developments.

In conclusion, \(\beta\)-ionone inhibits the proliferation of A-549 cells by inducing apoptosis through the reciprocal expressions of the Bcl-2 and Bax pathways, and by reducing the expression of cPLA2 and COX-2 proteins. The precise mechanistic action and signaling event involved in \(\beta\)-ionone-induced apoptosis in A-549 cells remains to be determined. \(\beta\)-Ionone holds promise as a useful way to prevent lung cancers due its ease of handling and ready contact with lung adenocarcinoma through inhalation of a desirable flavored compound of \(\beta\)-ionone.

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References

**초록**: β-ionone의 인체 비스페임세포 A-549에 대한 anti-proliferative 효과

이선임, 김영숙, 정연주, 박두호, 리키브, 오태우, 김보현, 김성일, 김정옥, 허영래

1) 경상대학교 대학원 응용생명과학부 (BK21 Plus), 2) 방글라데시 라사히대학교 생명과학 및 분자생물학과, 3) 한국국제대학교 식물과학과, 4) 바이오텍 HK