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Growth Inhibitory Effect of (E)-2,4-bis(*p*-hydroxyphenyl)-2-Butenal Diacetate through Induction of Apoptotic Cell Death by Increasing DR3 Expression in Human Lung Cancer Cells

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Abstract

The Maillard Reaction Products (MRPs) are chemical compounds which have been known to be effective in chemoprevention. Death receptors (DR) play a central role in directing apoptosis in several cancer cells. In our previous study, we demonstrated that (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal, a MRP product, inhibited human colon cancer cell growth by inducing apoptosis via nuclear factor-κB (NF-κB) inactivation and G_2/M phase cell cycle arrest. In this study, (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate, a new (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal derivative, was synthesized to improve their solubility and stability in water and then evaluated against NCI-H460 and A549 human lung cancer cells. (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate reduced the viability in both cell lines in a time and dose-dependent manner. We also found that (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate increased apoptotic cell death through the upregulation of the expression of death receptor (DR)-3 and DR6 in both lung cancer cell lines. In addition to this, the transfection of DR3 siRNA diminished the growth inhibitory and apoptosis inducing effect of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate on lung cancer cells, however these effects of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate was not changed by DR6 siRNA. These results indicated that (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate inhibits human lung cancer cell growth via increasing apoptotic cell death by upregulation of the expression of DR3.

Key Words: (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate, Human lung cancer cells, Apoptosis, DR6

INTRODUCTION

Lung cancer is one of the most common cancers worldwide. It is the leading cause of cancer deaths in men and women world. Many antitumor compounds have been developed, but treatment options in patients with advanced stage of lung cancer have limited efficacy and are associated with significant side effects and reduced quality of life. Therefore, developing novel alternatives for this malignancy is of great importance.

Apoptosis pathways by activated death receptors (DRs) of tumour necrosis factor (TNF) family such as Fas, tumour necrosis factor receptor (TNFR) 1, TNFR2 or the other receptors death receptor (DR) 3, DR4, DR5 and DR6 are implicated in diverse diseases including cancers. Alpha-tocopherol ether-linked acetic acid (α-TEA) induced apoptotic cell death via up-regulation of Fas, DR5, and Fas-associated protein with death domain (FADD) in human breast cancer cells (Li *et al.*, 2010). Fucoidan increased apoptosis of HT-29 and HCT116 human colon cancer cells via increased expression of DRs (Kim *et al.*, 2010). Celastrol also enhanced anti-cancer effect of TNF-related apoptosis-inducing ligand (TRAIL)/Apo-2L via up-regulation of DR4 and DR5 expression in human ovarian and colon cancer cells (Zhu *et al.*, 2010). In addition, Flavokawain B induced apoptosis via increasing DR5 expression in prostate cancer cell line (Tang *et al.*, 2010). Therefore, targeting DR-mediated apoptosis has emerged as an effective strategy for cancer therapy. However, certain types of cancer cells are intrinsically resistant to DR-mediated apoptosis. In an effort to identify agents that can sensitize cancer cells to DR-induced apoptosis, we have identified (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate with anticancer activity, as an effective sensitizer of DR-mediated apoptosis.

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Recently, we synthesized 4-bis(*p*-hydroxyphenyl)-2-butenal by maillard reaction (MR) with tyrosine and fructose by high temperature and pressure treatment (Hwang *et al.*, 2011). Moreover, in our previous study, the ability of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal as potential antitumor agents have been demonstrated. (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal inhibited the growth of human colon cancer cells through induction of apoptotic cell death by nuclear factor-κB (NF-κB) inactivation and G_2/M phase cell cycle arrest. Our another study also showed that (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal has anti-inflammatory effects through inhibition of IκB kinase β (IKKβ)/NF-κB and signal transducer and activator of transcription 3 (STAT3) activity in neuronal cells (Lee *et al.*, 2011). Althourgh (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal showed remarkable anti-cancer and anti-inflammatory activity *in vitro* and *in vivo* test, it has low solubility and stability in water. Because of these disadvantages, we have developed a new derivative of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal to increase the benefits of the chemical, with good results, and evaluated their effect as potential anti-tumor agents on human lung cancer cells.

MATERIALS AND METHODS

Synthesis of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate

$Pd(OAc)_2$ (208 mg, 0.93 mmol), tributyl phosphine (685 μl, 2.78 mmol) in t-Butanol was degassed under argon gas for 10 min, until the solution becomes clear pale yellow, then added 4-(oxiran-2-yl) phenyl acetate (5.5 g, 30.87 mmol) and stirred the reaction mixture at 85°C for 12 h. After completion of the reaction, the solvent was removed in *vacuo*. Followed by column purification (3:1 hexanes: ethyl acetate) yielded (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate as pale yellow liquid (2 g, 45%). ^{1}H NMR (500 MHz, $CDCl_3$) δ. 9.66 (s, 1H), 7.29 (d, 2H, *J*=14.5 Hz), 7.2 (dd, 4H, *J*=3.5, 14.5 Hz), 7.07 (d, 2H, *J*=14.5 Hz), 6.88 (t, 1H, *J*=12.5 Hz), 3.72 (d, 1H, *J*=12.5 Hz), 2.34 (s, 3H), 2.32 (s, 3H). HRMS (ESI) *m/z* $[M+H]^+$ cacld 338.1154, found 339.1183. The structure is shown in Fig. 1. The molecular weight of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate is 338.35. (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate was dissolved in 0.05% DMSO and used at the treatment dose of 10-40 μg/ml.

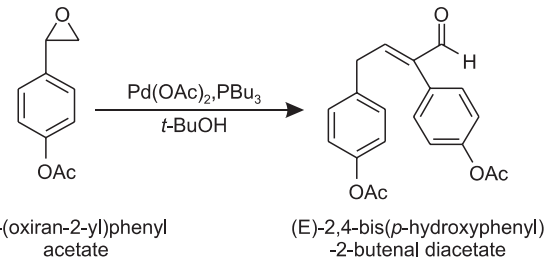

Fig. 1. Scheme of synthesis of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate.

Cell culture

NCI-H460 and A549 human lung cancer cells were obtained from the American Type Culture Collection (Cryosite, Lane Cove NSW, Australia). All cell lines were grown in RPMI1640 with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2 humidified air.

Cell viability assay

Cell viability was assessed using hematocytometer by trypan blue exclusion method. Trypsinized cells were incubated with 0.4% trypan blue solution. Subsequently, a drop of suspension was placed into a Neubauer chamber and the living cancer cells were counted. Cells that showed signs of staining were considered to be dead, whereas those that excluded trypan blue were considered viable. More than 200 cells per group were scored and each assay was carried out in triplicate.

Detection of apoptosis

Cells were cultured on 8-chamber slides. After treatment with (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate (10-40 μg/ml) for 48 h, the cells were washed twice with PBS and fixed by incubation in 4% paraformaldehyde in PBS for 1 h at room temperature. Apoptotic cells were determined by the morphological changes after DAPI staining under a fluorescence microscopic (DAS microscope, Leica Microsystems, Inc., Deefield, IL, USA). Apoptosis was also evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining assay. TUNEL assays were performed by using the in situ Cell Death Detection Kit (Roche Diagonostics GmbH, Mannheim, Germany) according to manufacturer's instructions. Total number of cells in a given area was determined by using 4',6-diamidino-2-phenylindole (DAPI) and apoptotic cells by TUNEL staining. The apoptotic index was determined as the number of DAPI-stained TUNEL-positive cells divided by the total cell number counted ×100.

Western blot analysis

Cultured cells were washed twice with 1× phosphate buffered saline (PBS), followed by the addition of 1 ml of PBS, and the cells were scraped into a cold Eppendorf tube. Cells were homogenized with lysis buffer [50 mM Tris pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 1 mM PMFS, 10 μl/ml aprotinin, 1% igapel 630 (Sigma-Aldrich, St. Louis, MO, USA), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA and 0.5% sodium deoxycholate], and centrifuged at 13,000 rpm for 10 min. The protein concentration was measured by the Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories Inc, Hercules, CA, USA), and equal amount of proteins (50 μg) were separated on a SDS/12%-polyacrylamide gel, and then transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Blots were blocked for 2 h at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] solution containing 0.05% tween-20. The membrane was incubated with specific antibodies against TNFR1, TNFR2, DR3, DR4, DR5, DR6 and β-actin (1:500 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The blot was then incubated with the corresponding conjugated anti-rabbit and anti-mouse immunoglobulin G-horseradish peroxidase (1:2,000 dilution, Santa Cruz Biotechnology Inc.). Immunoreactive proteins were detected with the enhanced chemiluminescence (ECL) western blotting detection system.

Transfection

Oligonucleotides of scrambled, DR3 and DR6 siRNA were purchased from Santa Cruz Biotechnology Inc.. Cells were transfected with 100 nM of scrambled, DR3 or DR6 siRNA (Santa Cruz Biotechnology Inc.) using WelFect-EX™ plus transfection reagent (WelGENE, Inc.) prepared in serum-free culture medium at 37°C for 4 h. After 4 h, complete medium was added and cells were further cultured for 24 h.

Data analysis

Statistical analysis Data were analyzed using one-way analysis of variance followed by Tukey test as a post hoc test. Differences were considered significant at $p<0.05$.

RESULTS

(E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate inhibited NCI-H460 and A549 human lung cancer cell growth

To confirm the inhibitory effect of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate on human lung cancer cell growth, we analyzed cell viability by cell counting assay. (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate inhibited NCI-H460 and A549 human lung cancer cells in a concentration- and time-dependent manners (Fig. 2). IC_{50} values of cell growth inhibition at 24, 48 and 72 h were >40, 29 and 13 μg/ml in NCI-H460 human lung cancer cells. IC_{50} values of cell growth inhibition at 24, 48 and 72 h were >40, 24 and 17 μg/ml in A549 human lung cancer cells.

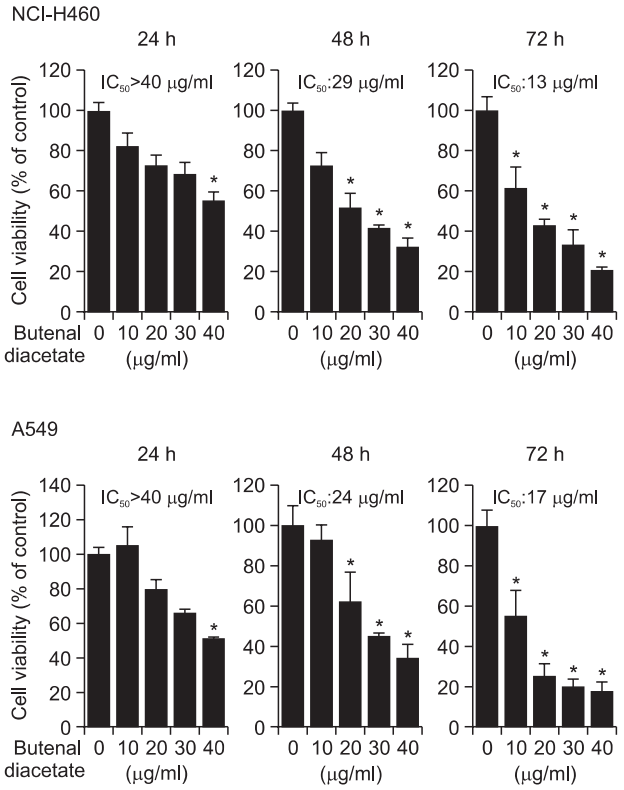

Fig. 2. Cell viability of human lung cancer cells by (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate. NCI-H460 and A549 human lung cancer cells were incubated with (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate at concentrations of 10 to 40 μg/ml for 24, 48 and 72 h. Cells were harvested by trypsinization and stained with 0.2% trypan blue. Relative cell survival rate was determined by counting live and dead cells. The data are expressed as the mean ± SD of three experiments with replicates. *$p<0.05$, versus the control group.

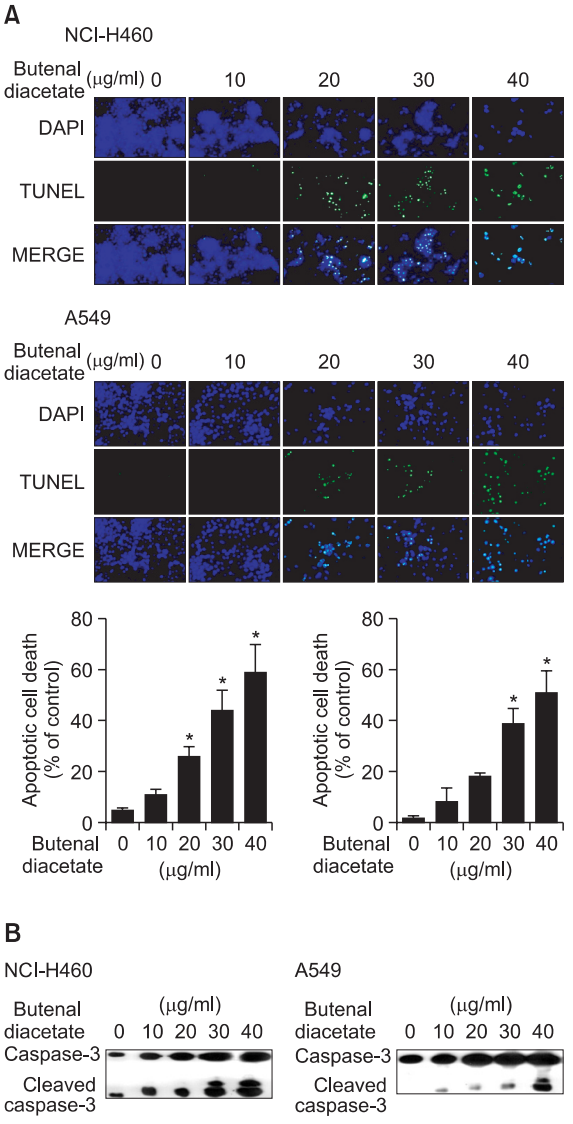

Fig. 3. Apoptotic cell death and expression of apoptosis-related proteins of lung cancer cells by (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate. (A) Human lung cancer cells were treated with several concentration of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal for 48 h; and apoptotic cells were examined with a fluorescence microscope after TUNEL staining. Total number of cells in a given area was determined by DAPI nuclear staining. The apoptotic index was determined as the number of DAPI-stained TUNEL-positive cells counted. The data are expressed as the mean ± SD of three experiments with replicates. *$p<0.05$, versus the untreated group. Magnification, ×100. (B) Equal amounts of total proteins (50 μg/lane) were subjected to 12% SDS-PAGE. Expression of cleaved caspase-3 was detected by Western blotting using specific antibodies. The blot is representative of three independent experimental results.

(E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate induced apoptotic cell death and the expression of apoptosis regulatory proteins

To delineate whether the inhibition of cell growth by (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate was due to the induction of apoptotic cell death, we evaluated changes in the chromatin morphology of cells using DAPI staining. To further characterize the apoptotic cell death by (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate, we performed TUNEL staining assays, and then the labeled cells were analyzed by fluorescence microscopy. (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate treated cells were labeled by TUNEL assay and were increased fluorescence intensity in both NCI-H460 and A549 human lung cancer cells (Fig. 3A). Apoptotic cells number (DAPI-positive TUNEL stained cells) on NCI-H460 lung cancer cell was increased to 5, 8, 22, 41 and 59% by 0, 10, 20, 30 and 40 μg/ml (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate, respectively (Fig. 3A). Apoptotic cells number on A549 lung cancer cell was increased to 2, 6, 19, 40 and 56% by 0, 10, 20, 30 and 40 μg/ml (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate, respectively (Fig. 3A). To figure out the relationship between the induction of apoptosis by (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate and the expression of apoptotic gene, the expression of apoptosis related protein was investigated. Expression of apoptotic proteins such as cleaved caspase-3 was increased in both NCI-H460 and A549 human lung cancer cells (Fig. 3B).

(E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate regulated the expression of DRs

The extrinsic pathway of apoptosis requires induction of death receptors (Kang *et al.*, 2011; Zhu *et al.*, 2011). Therefore, we investigated the effect of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate on the expression of death receptors. The expression of DR3 and DR6 was highly induced by (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate in a concentration-dependent manner in both NCI-H460 and A549 lung cancer cells, however the expression of DR5 was elevated in NCI-H460, but not in A549 lung cancer cells. In addition, (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate did not increased the expression of other DRs in both lung cancer cells (Fig. 4).

Inhibition of DR3 diminished the growth inhibitory and apoptosis inducing effects of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate

In order to investigate involvement of DR3 or DR6 in the (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate-induced cancer cell growth, the effects of DR3 or DR6 siRNA on (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate-induced cell growth inhibition were analyzed by direct counting of the cell number using trypan blue dye exclusion assay. Transfection of DR3 siRNA diminished the growth inhibitory effect of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate on lung cancer cells, however these effects of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate was not changed by DR6 siRNA (Fig. 5A). The increased apoptotic cell death by (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate were also decreased in NCI-H460 lung cancer cells treated with DR3 siRNA compared to the (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate alone treated cancer cells (Fig. 5B). In agreement with these findings, the increased expression of cleaved caspase-3 protein by (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate were also inhibited in NCI-H460 lung cancer cells treated with DR3 siRNA compared to the (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate alone treated cancer cells (Fig. 5C).

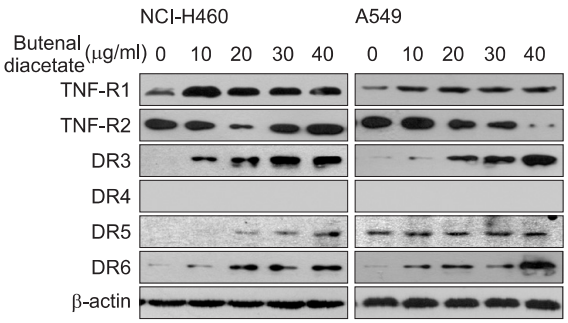

Fig. 4. Expression of DR-related proteins of lung cancer cells by (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate. The cells were treated with different concentrations (10-40 μg/ml) of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate at 37°C for 48 h, and then harvested. Expression of DRs was detected by western blotting using specific antibodies. β-Actin protein was used as an internal control. Each blot is representative of three independent experimental results.

DISCUSSION

In the present study, we found that (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate inhibited lung cancer cell growth and increased apoptotic cell death through induction of DR3 expression in NCI-H460 and A549 human lung cancer cells. Knockdown of DR3 by siRNA diminished the growth inhibitory and apoptosis inducing effect of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate on lung cancer cells. Taken together, our data show that induction of apoptosis could be involved in (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate-induced cell growth inhibition in lung cancer cells, and induction of DR3 expression could provide a specific and causative mechanism for the inhibition of lung cancer cell growth by (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate, suggesting that (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate can be a useful agent for treatment of lung cancer cell growth.

It is well-known that apoptosis can be induced by stimulation of DRs including TNFR1/2, DR3, DR4, DR5, DR6 and Fas by their respective ligands (Kang *et al.*, 2011; Zhu *et al.*, 2011). Therefore, these receptors emerged as attractive targets for anti-cancer therapeutics. Several compounds such as α-TEA, celastrol, fucoidan, flavokawain B induced apoptotic cell death of cancer cells through increasing DR expression (Kim *et al.*, 2010; Li *et al.*, 2010; Tang *et al.*, 2010; Zhu *et al.*, 2010). Moreover, recent studies have demonstrated that anti-cancer drug-induced apoptotic cell death could be related with increase of DR3 expression. Lupeol (Lup-20(29)-en-3H-ol), a novel dietary triterpene, resulted in significant inhibition of cell viability in a dose-dependent manner and caused apoptotic death of hepatocellular carcinoma SMMC7721 cell line with activation of caspase-3 expression through increasing DR3 expression (Zhang *et al.*, 2009). IL-32 enhanced the cytotoxic effect of natural killer (NK)-92 cells on the cancer cells through activation caspase-3 by upregulation of DR3 (Park *et al.*, 2012). Similar to these results, our results sug-

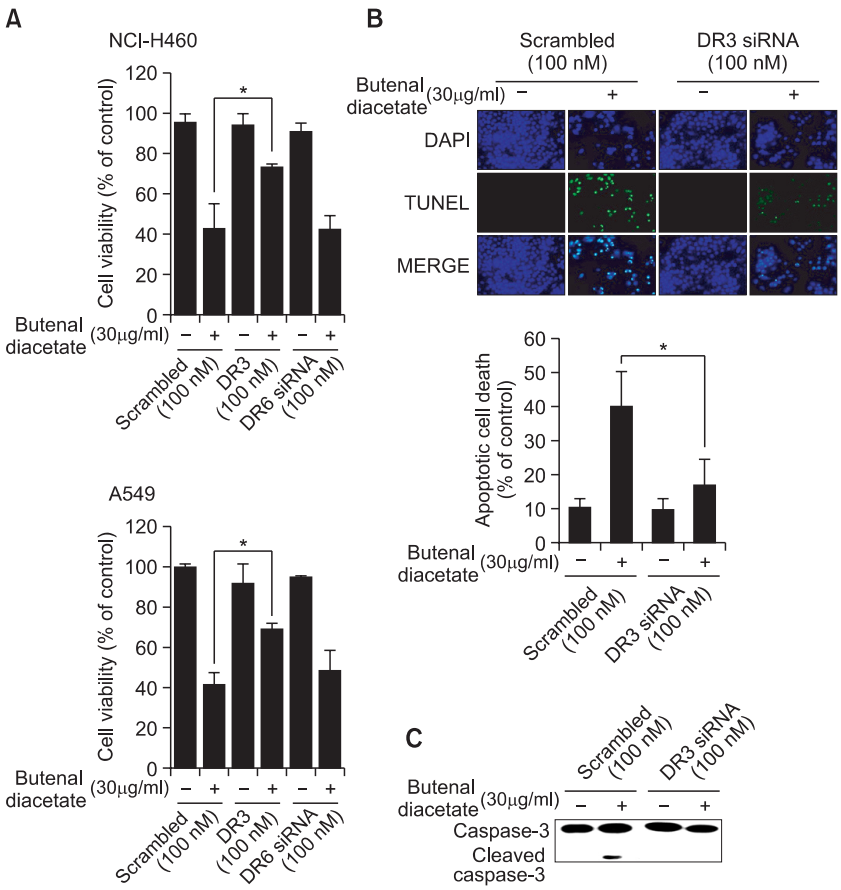

Fig. 5. Effect of DR3 or DR6 siRNA on cell viability and apoptotic cell death in (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate-treated lung cancer cells. The cells were transfected with scrambled, DR3 or DR6 siRNA (100 nM), treated 30 μg/ml of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate at 37°C for 48 h, and then harvested. (A) Cell viability was determined by direct cell counting using trypan blue exclusion. Values are the mean ± SD of three experiments, each performed in triplicate. **p*<0.05 indicates statistically significant differences from control group. (B) Apoptotic cells were examined by fluorescence microscopy after DAPI and TUNEL staining. **p*<0.05 indicates statistically significant differences from control group. (C) The expression of apoptotic proteins was detected by western blotting.

gested that (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate-induced apoptosis is related with DR3. As shown in Fig. 4A, the expression of DR3 and DR6 was highly induced by (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate in both NCI-H460 and A549 lung cancer cells, however the expression of DR5 was elevated in NCI-H460 in a concentration-dependent manner, but not in A549 lung cancer cells. In addition, (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate did not increased the expression of other DRs in both lung cancer cells. To study the role of DR3 and DR6 expression in (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate-induced lung cancer cell growth inhibition and apoptosis induction, we examined the effect of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate with and without knockdown of DR3 or DR6 by transfection with siRNA. Knockdown of DR3 diminished the growth inhibitory and apoptosis inducing effect of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate on lung cancer cells. In contrast, these effects of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate on cancer cell growth was not changed by knockdown of DR6 in both lung cancer cells. These data indicate DR3 may be a critical death receptor discriminating the responses between both lung cancer cells against (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate.

Caspases play a critical role in apoptosis by DRs (Kaufmann and Earnshaw, 2000; Elrod and Sun, 2008; Sun, 2011). Hesperetin, a flavonoid from citrus fruits, exhibited a potential anticancer activity against human cervical cancer cell lines *in vitro* through the reduction in cell viability and the induction of apoptosis via caspase-3 activation by increasing Fas expression (Alshatwi *et al.*, 2012). Genistein, one of several known isoflavones, enhanced apoptosis in A549 lung cancer cells induced by trichostatin A by increasing the expression of cleaved caspase-3 via up-regulation of TNFR-1 DR signaling (Wu *et al.*, 2012). Similarly, we showed that the caspase-3 was activated by treatment of (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate. To further investigate the involvement of DRs in (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate induced apoptotic cell death, we employed siRNA of DR3 or DR6, and found that (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate-induced apoptotic cell death of human lung cancer cells was abolished by DR3, but not DR6 siRNA. For these reasons, (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate may be effective for inducing lung cancer cell death through activation of DR3 mediated cell death signals.

In conclusion, our results demonstrated that (E)-2,4-bis(*p*-hydroxyphenyl)-2-butenal diacetate can inhibit the growth of human lung cancer cells through apoptotic cell death via increasing DR3 expression, suggesting its potential role in human lung cancer treatment.

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