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Esculetin exerts anti-proliferative effects against non-small-cell lung carcinoma by suppressing specificity protein 1 *in vitro*

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Abstract. Esculetin, a coumarin derivative, is a phenolic compound isolated from *Artemisia capillaris*, *Citrus limonia*, and *Euphorbia lathyris*. Although it has been reported to have anti-inflammatory, anti-oxidant, and anti-proliferative activities in several human cancers, its anti-proliferative activity against non-small-cell lung carcinoma (NSCLC) and the molecular mechanisms involved have not been adequately elucidated. In this study, we used two NSCLC cell lines (NCI-H358 and NCI-H1299) to investigate the anti-proliferative activity and apoptotic effect of esculetin. Our data showed that esculetin-treated cells exhibited reduced proliferation and apoptotic cell morphologies. Intriguingly, the transcription factor specificity protein 1 (Sp1) was significantly suppressed by esculetin in a dose- and time-dependent manner. Furthermore, the levels of p27 and p21, two key regulators of the cell cycle, were up-regulated by the esculetin-mediated down-regulation of Sp1; the level of a third cell-cycle regulator, survivin, was decreased, resulting in caspase-dependent apoptosis. Therefore, we conclude that esculetin could be a potent anti-proliferative agent in patients with NSCLC.

Key words: Apoptosis — Esculetin — Non-small-cell lung carcinoma — Specificity protein 1

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Introduction

Lung cancer is a major cause of cancer death worldwide, accounting for about one million deaths each year. Its incidence is strongly associated with cigarette smoking, with approximately 90% of lung cancers being generated by tobacco use (Jemal et al. 2011). Lung cancer is categorized into two morphologic groups: small-cell lung carcinoma and non–small-cell lung carcinoma (NSCLC). About 85% of the patients with lung cancer have NSCLC, and about half this group present with advanced metastatic disease (stage IV; Molina et al. 2008). Furthermore, NSCLC is mostly an insidious disease, with no detectable symptoms until it has spread too far to be cured. When symptoms do develop, they may be due to the lung tumor itself or its effects on tissues outside the lung or to the spread of cancerous cells to other sites, including the lymph nodes, adrenal gland, bone, ovary, pancreas, liver, and brain (DeVita et al. 2005). The major treatment for NSCLC is surgery combined with radiation and chemotherapy, but many patients are not eligible for surgical treatment (Saintigny and Burger 2012). Chemotherapy by itself has effectively cured NSCLC. Recent attempts to develop effective strategies to prevent NSCLC have focused on dietary agents, including coumarin derivatives (Lopez-Gonzalez et al. 2004).

Strategies to minimize the side effects of cancer have recently involved the use of natural products (Mateen et al. (2013). Esculetin (6,7-dihydroxycoumarin) is a coumarin derivative produced by various plant materials (e.g., Artemisia capillaris, Citrus limonia, and Euphorbia lathyris) that are traditionally used as folk medicines (Chang et al. 1996; Masamoto et al. 2003). Interestingly, esculetin has been found to have a variety of biological activities, including anti-oxidant, anti-inflammatory, anti-proliferation, and anti-edematous effects (Fylaktakidou et al. 2004; Yun et al. 2011; Lee et al. 2013). However, the molecular mechanisms in esculetin's potential anti-proliferative activity in NSCLC have not been clarified.

The transcription factor specificity protein 1 (Sp1) expressed in mammalian cells is a member of the specificity protein/Krüppel-like factor transcription factor family, which interacts with the GC-rich regions and binds to DNA through three Cys^2/His^2-type zinc fingers in the C-terminal domain (Suske 1999; Li and Davie 2010). Sp1 target genes are mainly associated with cell proliferation, cell cycle and arrest, apoptosis, and tumorigenesis (Zhang et al. 2005; Wang et al. 2009; Kong et al. 2010; Nam et al. 2012). Furthermore, Sp1 is often over-expressed in several types of cancer cells, such as in gastric, thyroid, lung, and colorectal cancers, but not in normal cells (Chiefari et al. 2002; Wang et al. 2003; Chuang et al. 2009; Colon et al. 2011; Pathi et al. 2012). For these reasons, ways to inhibit Sp1 have been explored for the prevention or treatment of human cancers. Thus, Sp1 suppression may be a logical approach to treating cancer.

In this regard, to verify the therapeutic potential Sp1, this study examines whether esculetin regulates Sp1 target proteins can induce apoptosis by suppressing the level of Sp1 in NCI-H358 and NCI-H1299 cells.

Materials and Methods

Cell culture

Human NCI-H358 and NCI-H1299 cell lines acquired from the American Type Culture Collection (Manassas, VA, USA) were grown in RPMI-1640 (Welgene, Deagu, Korea), supplemented with 10% fetal bovine serum (FBS) and 100 U/ml each of penicillin and streptomycin (Gibco, Grand Island, NY, USA) in appropriate concentrations at 37°C with 5% CO_2 in a fully humidified atmosphere.

Cell viability assay

The effect of esculetin on cell viability was estimated using a MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay kit (Promega, Madison, WI, USA). NCI-H358 (3.5×10^3) and NCI-H1299 (2.5×10^3) cells were seeded overnight on a 96-well microtiter plate and were then treated with esculetin for 24 or 48 h. The absorbance was measured using an Absorbance Microplate Reader (BioTek, Winooski, VT, USA) at 490 nm, and each of the measurements was carried out in triplicate. The viability of esculetin-treated cells was represented as a percentage after being normalized to the viability of untreated control cells.

DAPI staining

The nuclear condensation and fragmentation levels were observed *via* nucleic acid staining with DAPI (4'-6-diamidino-2-phenylindole). The NCI-H358 and NCI-H1299 cells were treated with various concentrations of esculetin and were harvested *via* trypsinization, washed with ice-cold phosphate buffered saline (PBS), and then fixed in 100% methanol at room temperature for 20 min. The cells were spread on a slide and stained with a DAPI solution (Sigma-Aldrich, St. Louis, MO, USA). Fluorescence images were then taken using a confocal laser microscope (FluoView FV10i, Olympus Corp., Tokyo, Japan).

Annexin V/propodium iodide (PI) staining

Cells were seeded onto a 100-mm^2 dish containing NCI-H358 (3×10^6) and NCI-H1299 (2×10^6) cells and then

treated for 48 h with various concentrations of esculetin (0, 50, 100 and 150 μg/ml). Both adherent and floating cells were harvested and washed once with PBS. To detect apoptosis, we incubated the cells with Annexin V/PI for 15 min at room temperature in the dark. Apoptotic and necrotic cells were analyzed by flow cytometry (BD FACSVerse, BD Biosciences, Oxford, UK) using the FITC Annexin V Apoptosis Detection Kit 1 (BD Biosciences). The experiment was conducted independently in triplicate.

Immunocytochemistry analysis

The cells were seeded on sterilized glass coverslips in four-well tissue culture plates for 24 h and incubated with various concentrations of esculetin (0, 50, 100 and 150 μg/ml) for 48 h. The cells were fixed/permeabilized with cytotoxic solution (BD Biosciences) for 20 min at 4°C. After washing twice with PBS, the cells were incubated with anti-Sp1 and in 1% BSA at 4°C overnight. The cells were again washed with PBS and were subsequently incubated with Alexa Fluor 488 conjugated anti-rabbit IgG secondary antibodies (1:400 dilution) (Life Technologies, Carlsbad, CA, USA) in 1% bovine serum albumin (BSA) for 1 h at room temperature. Cell nuclei were stained with DAPI. Finally, the stained cells were mounted using a solution that contained 10% glycerol and were then observed under a laser microscope (Olympus).

Western blot analysis

Protein expression levels were analyzed by means of Western blotting. After the NCI-H358 and NCI-H1299 cells were treated with esculetin, they were washed with ice-cold PBS and then lyzed in ice-cold Mammalian Protein Extraction Reagent (M-PER, Thermo Scientific, Rockford, IL, USA) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). The soluble fraction of the cell lysates was isolated by centrifugation at 13,000 × *g* for 15 min in a microfuge. The BCA Protein Assay Kit (Thermo Scientific) was used to measure the protein concentration in the supernatants, and equal amounts of protein samples were separated by 10%

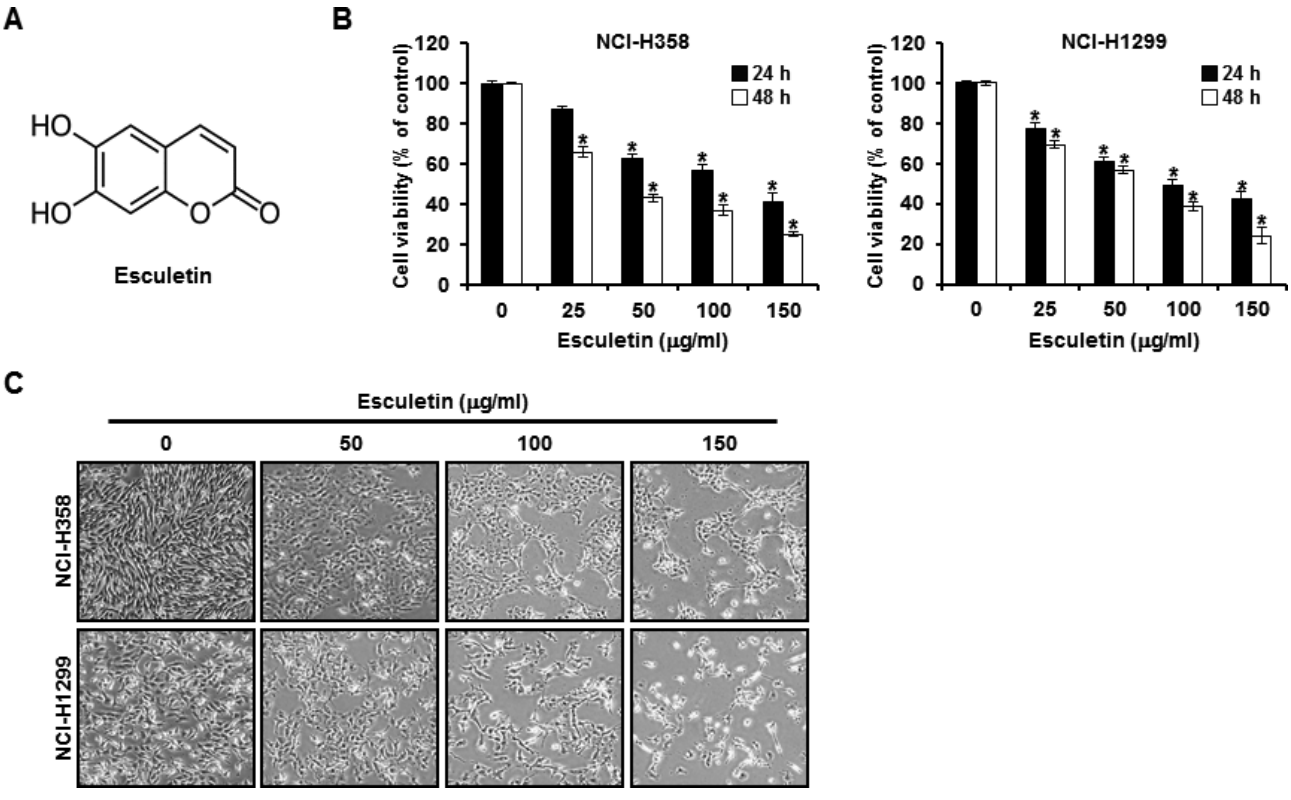

Figure 1. The effect of esculetin on cell viability in NSCLC cells. **A.** Chemical structure of esculetin. **B.** The NCI-H358 and NCI-H1299 cells were treated with different concentrations of esculetin (0, 50, 100 and 150 μg/ml) for 24 and 48 h, and cell viability was assessed using the MTS assay. The asterisk indicates a significant difference relative to negative control (untreated) cells (* $p < 0.05$). **C.** Cellular morphological changes in NCI-H358 and NCI-H1299 cells with or without esculetin treatment (in concentrations of 0, 50, 100 and 150 μg/ml) for 48 h (magnification ×40).

or 12% SDS-polyacrylamide gel electrophoresis and were then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), which were blocked for 30 min at room temperature with 5% (v/v) skim milk in TBS buffer containing 0.1% Tween-20. The samples were then incubated with primary antibodies overnight at 4°C. The membranes were washed five times for 10 min with TBS-T and were incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG or anti-goat IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then washed five times for 10 min with TBS-T, and the chemiluminescence signals were enhanced using a Pierce ECL Western Blotting Substrate (Thermo Scientific) and detected using ImageQuant Las4000 Mini (GE Healthcare Life Sciences, Buckinghamshire, UK).

Statistical analysis

Results were presented as means ± standard deviation (SD) of triplicate independent experiments. Statistical significance was assessed using a Student's *t*-test. A value $p < 0.05$, as compared with the non-treated control, was considered statistically significant.

Results

Esculetin inhibits cell viability of NSCLC cells

The structure of esculetin is shown in Fig. 1A. The effects of esculetin on cell proliferation in the NCI-H358 and NCI-H1299 cells lines were verified by MTS assay. As shown in

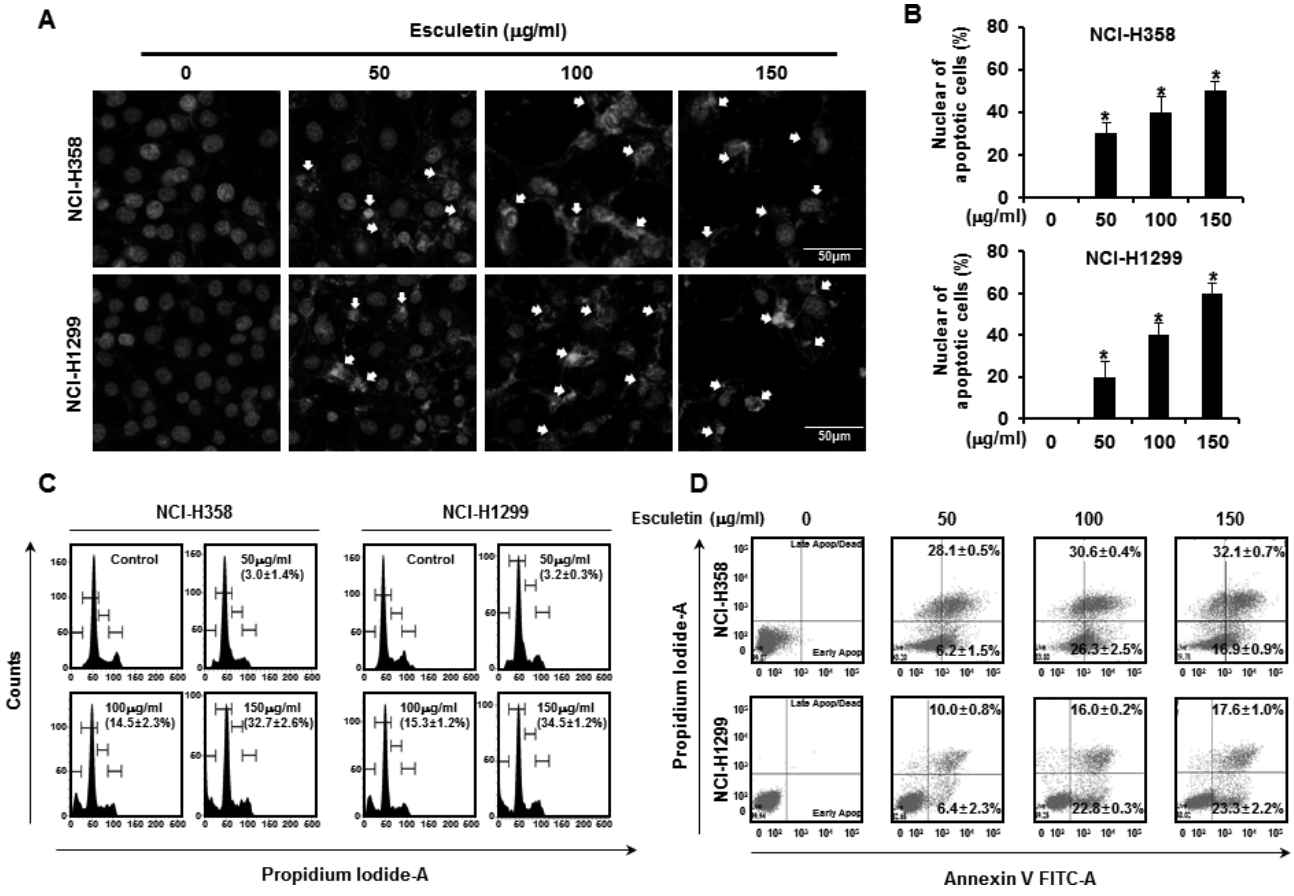

Figure 2. The effect of esculetin on induction of apoptosis in NSCLC cells. The NCI-H358 and NCI-H1299 cells were incubated with different concentrations of esculetin (0, 50, 100 and 150 μg/ml) for 48 h. **A.** Fluorescence microscopic images of DAPI staining. Scale bar = 50 μm. **B.** DNA fragmentation and nuclear condensation were quantified, and data represent mean percentages ± SD (* $p < 0.05$). **C.** NCI-H358 and NCI-H1299 cells were treated with 50, 100 and 150 μg/ml esculetin or DMSO (vehicle), and the cells were washed, fixed, stained with propidium iodide (PI) and analyzed for DNA content by flow cytometry 48 h after treatment. The ratio of apoptotic cells was measured by flow cytometry after PI staining. **D.** Quantitative detection of Annexin V/7-AAD positive cells was performed with the Muse Cell Analyzer. Cells stained with Annexin V only were defined as early apoptotic (lower right); Annexin V and 7-AAD double-stained cells were defined as late apoptotic (upper right).

Fig. 1B, the efficiency of esculetin in altering the viability of NCI-H358 and NCI-H1299 cells was assayed after 24 and 48 h of incubation in media containing esculetin at different concentrations (25, 50, 100 and 150 μg/ml). The IC_{50} value of esculetin at 48 h was estimated to be 58.1 μg/ml in the NCI-H358 and 43.9 μg/ml in the NCI-H1299 cells. Thus, esculetin decreased cell proliferation of NCI-H358 and NCI-H1299 cells in a dose- and time-dependent manner for 24 and 48 h. To investigate morphological changes, NCI-H358 and NCI-H1299 cells were treated with three different concentrations of esculetin (50, 100 and 150 μg/ml) for 48 h. The apoptotic features included cell rounding, cytoplasmic blebbing, and irregularities in a dose-dependent manner (Fig. 1C). These results indicate that esculetin inhibited the growth of human NSCLC cells.

Esculetin induces apoptosis of NSCLC cells

To determine whether esculetin induced apoptosis in the two NSCLC cell lines, we carried out confocal laser microscopic analysis of the esculetin-treated NCI-H358 and NCI-H1299 cells to visualize the apoptotic morphological changes using DAPI staining. The results indicated the presence of nuclear condensation and perinuclear apoptotic bodies in the NCI-H358 and NCI-H1299 cells, after esculetin treatment at concentrations of 0, 50, 100 and 150 μg/ml for 48 h (Fig. 2A, B). The cell cycle distribution was analyzed through the FACS analysis. As shown in the graphs of Fig. 2C, there was significant increase in the number of sub-G1 cells in NCI-H358 cells: 3.0 ± 1.4, 14.5 ± 2.3 and 32.7 ± 2.6% in the presence of 50, 100 and 150 μg/ml of esculetin,

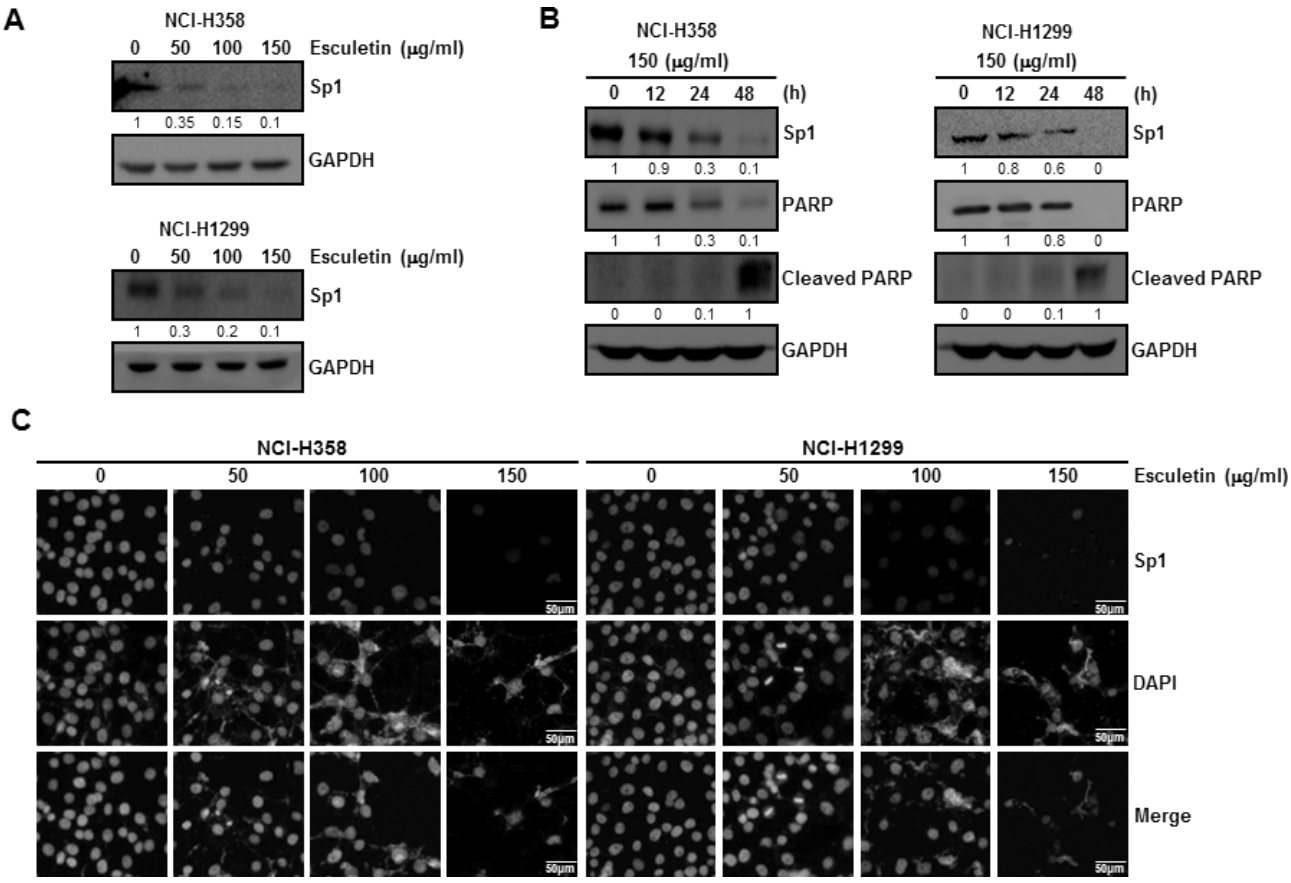

Figure 3. Effect of esculetin on the level of Sp1 expression in NSCLC cells. **A.** NCI-H358 and NCI-H1299 cells were treated with different concentrations of esculetin (0, 50, 100 and 150 μg/ml) for 48 h. The cell lysates were separated by SDS-PAGE, and the membranes were then transferred from SDS-PAGE gels subjected to Western blot analysis to detect Sp1. An equal loading protein was confirmed using GAPDH. **B.** The NCI-H358 and NCI-H1299 cells were treated with 150 μg/ml of esculetin for 0, 12, 24 and 48 h and were then evaluated to determine the expression levels of Sp1 and PARP. Equal loading protein was confirmed using GAPDH. **C.** Immunofluorescence microscopic analysis of NCI-H358 and NCI-H1299 cells treated with different concentrations of esculetin (0, 50, 100 and 150 μg/ml) for 48 h, after which the cells were immunostained with anti-Sp1. Signals were detected with Alexa Fluor 488-conjugated anti-rabbit secondary antibody. DAPI was used for nuclear staining. Scale bar = 50 μm.

respectively, in comparison to untreated control cells. An increase in the number of sub-G1 cells was also observed in NCI-H1299 cells: 3.2 ± 0.3, 15.3 ± 1.2 and 34.5 ± 1.2% in the presence of 50, 100 and 150 μg/ml of esculetin, respectively, in comparison to untreated control cells. Cells stained with Annexin V only were defined as early apoptotic and Annexin V (lower right) and 7-AAD double-stained cells were defined as late apoptotic (upper right). Esculetin displayed marked effects to induce apoptosis of NCI-H358 and NCI-H1299 cells in a dose-dependent manner (Fig. 2D). Treatment of the NCI-H358 cells with 50, 100 and 150 μg/ml of esculetin for 48 h resulted in 6.2 ± 1.5, 26.3 ± 2.5 and 16.9 ± 0.9% of early apoptotic cells (lower right) and 28.1 ± 0.5, 30.6 ± 0.4 and 32.1 ± 0.7% of late apoptotic cells (upper right), respectively. Similarly, treatment of NCI-H1299 cells with esculetin also led to 6.4 ± 2.3, 22.8 ± 0.3 and 23.3 ± 2.2% of early apoptotic cells (lower right) and 10.0 ± 0.8, 16.0 ± 0.2 and 17.6 ± 1.0% of late apoptotic cells (upper right) at the same three concentrations as above, respectively. Apparently, esculetin-mediated apoptosis of NCI-H358 and NCI-H1299 cells, at least in part, contributed to its anti-proliferative effects.

Esculetin suppresses expression of Sp1 in NSCLC cells

Previous studies have shown that higher levels of transcription factor Sp1 expression were associated with human cancer development (Sankpal et al. 2011). Therefore, to examine Sp1 expression, NCI-H358 and NCI-H1299 cells were treated with different concentrations of esculetin (0, 50, 100 and 150 μg/ml) for 48 h. Esculetin suppressed Sp1 expression levels in a dose-dependent manner (Fig. 3A). To further investigate the time-dependent expression levels of Sp1, we treated the cells with 150 μg/ml esculetin at four different times (0, 12, 24 and 48 h). Esculetin also suppressed Sp1 expression levels in a time-dependent manner (Fig. 3B). Immunocytochemical analysis revealed decreased levels of Sp1 expression in the NCI-H358 and NCI-H1299 cells in a dose-dependent manner (Fig. 3C). Our results indicated that suppression of Sp1 by esculetin treatment leads to apoptotic cell death.

Esculetin regulated expression of cell-cycle and apoptosis--related proteins in NSCLC cells

Cyclin/cyclin-dependent kinase (CDK) complexes play an important role in cell-cycle regulation (Boxem et al. 1999; Boxem and van den Heuvel 2001). When we examined the expression levels of the cell-cycle regulatory proteins p21, p27, and survivin, we found that expression of p21 and p27 increased distinctively and that of survivin decreased after treatment with 150 μg/ml of esculetin for 48 h (Fig. 4). Furthermore, to determine whether treatment with esculetin regulated the expression of apoptosis-related proteins, we performed Western blot analyses. As shown in Fig. 5, activated caspase-3, poly (ADP-ribose) polymerase (PARP), and Bcl-xL decreased in esculetin-treated NSCLC cells, whereas expression of the anti-apoptotic proteins Bax, cleaved PARP, and cleaved caspase-3 increased. These data suggest that esculetin treatment of NSCLC decreases Sp1, resulting in growth arrest and apoptotic cell death.

Discussion

Since ancient times natural products have been widely used in clinical trials as a source of chemotherapeutic drugs (Cragg et al. (1997). Esculetin, a natural compound isolated from coumarin, is an attractive new therapeutic candidate for cancer prevention (Chang et al. 1996; Masamoto et al. 2003). Several potentially useful properties of esculetin have been reported such as its anti-oxidant, anti-inflammatory, and neuroprotective activities and its anti-proliferative, cell cycle arrest effects in oral cancer, human malignant melanoma, colon cancer, breast cancer, leukemia, cervical cancer, and human myeloma (Kloosterboer et al. 2004; Kim et al. 2008; Yang et al. 2010; Park et al. 2011; Subramaniam and Ellis 2013; Cho et al. 2015;

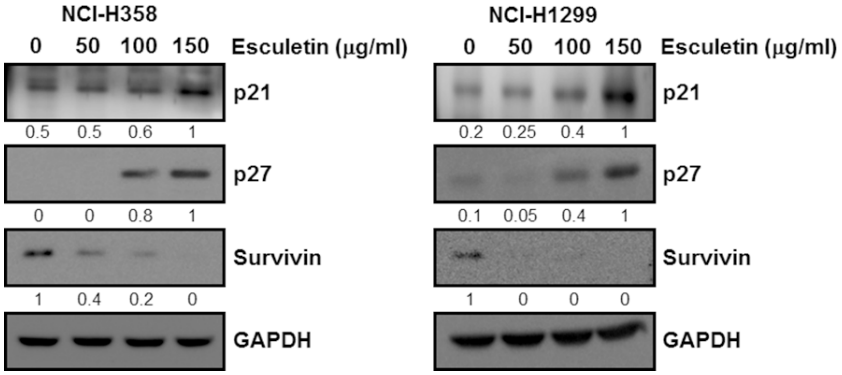

Figure 4. The effect of esculetin on the expression of cell cycle–related proteins in NSCLC cells. The NCI-H358 and NCI-H1299 cells were incubated without or with different concentrations of esculetin (0, 50, 100 and 150 μg/ml) for 48 h. The cell lysates were determined using Western blot analysis with antibodies against p21, p27, and survivin. Equal loading protein was confirmed using GAPDH. Values were obtained by means of ImageJ densitometry.

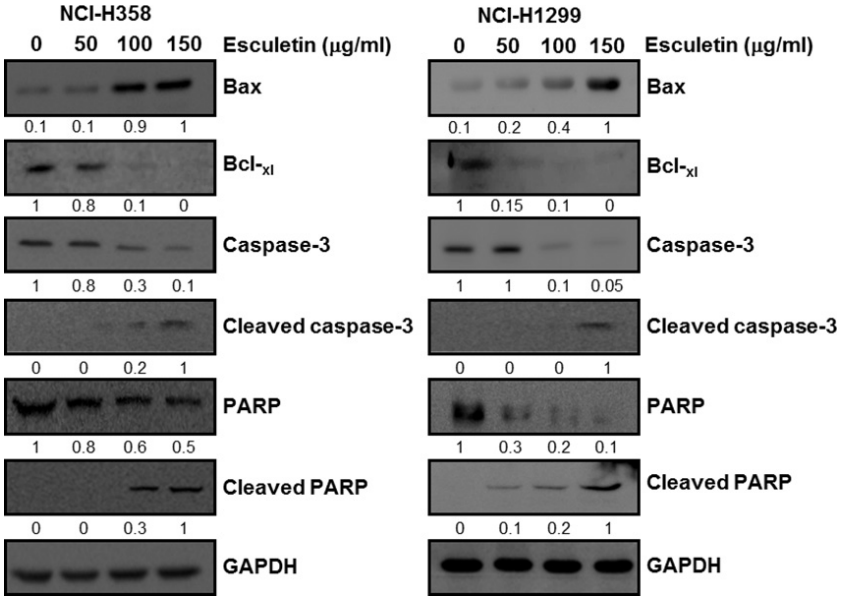

Figure 5. The effect of esculetin on the expression of apoptosis-related proteins in NSCLC cells. The NCI-H358 and NCI-H1299 cells were treated without or with different concentrations of esculetin (0, 50, 100 and 150 μg/ml) for 48 h. The cell lysates were evaluated by Western blot analysis using Bax, Bcl-xL, caspase-3, cleaved caspase-3, PARP, and cleaved PARP antibodies. Equal loading protein was confirmed using GAPDH. The values were measured using ImageJ densitometry.

Jeon et al. 2015). Despite numerous studies on a variety of cancer cells, the anti-proliferative effects of esculetin on NSCLC cells are not well understood.

In this study, we extensively explored the apoptotic effects of esculetin, at different times and concentrations, in two NSCLC cell lines, NCI-H358 and NCI-H1299. Most cases of NSCLC, a type of lung cancer, are diagnosed at an advanced stage when the disease is non-resectable. Our results showed that esculetin reduced the viability of NCI-H358 and NCI-H1299 cells, with a decrease in the size of the cells (morphologically seen as rounding) in a dose-dependent manner. DAPI staining and Annexin V cytometry revealed that esculetin induced significant apoptosis.

The transcription factor Sp1 is involved in a variety of cellular functions, including cell-cycle regulation, apoptosis, and differentiation. Many studies have reported that Sp1 is over-expressed in rapidly proliferating cancer cells, such as in human breast cancer, pancreatic cancer, colorectal cancer, gastric carcinoma, thyroid carcinoma, and hepatocellular carcinoma (Lietard et al. 1997; Chiefari et al. 2002; Wang et al. 2003; Abdelrahim et al. 2004; Hosoi et al. 2004; Mertens-Talcott et al. 2007). Sp1 appears to play a key role in tumor survival for numerous cancer cells and thus represents an attractive target for cancer treatment. Our data showed that esculetin induced apoptosis in NCI-H358 and NCI-H1299 cells by suppressing Sp1 expression levels in a dose- and time-dependent manner, confirming p21, p27, and survivin as Sp1 target proteins. Several human cancers frequently show down-regulation of p21 and p27, which are characterized as negative regulators of cell-cycle progression and G1-phase arrest that result from the interaction of cyclins/CDK complexes (Sherr and Roberts 1999; Murray 2004). Survivin is a member of the IAP (inhibitor of apoptosis) family and is a key regulator of mitosis and both G1 and G2 check points and apoptosis (Ambrosini et al. 1997; Altieri 2008). In this regard, our results indicate that esculetin increased the expression levels of p21 and p27 but decreased those of survivin in a dose-dependent manner. In agreement with these findings, esculetin reduced the expression levels of Bcl-xL but increased those of Bax, cleaved caspase-3, and cleaved PARP.

Conclusions

Esculetin inhibited NSCLC cell proliferation by suppressing Sp1, leading to the up-regulation of p21 and p27 and the down-regulation of survivin. Furthermore, esculetin regulated apoptosis-related proteins including Bax, Bcl-xL, caspase-3, cleaved caspase-3, PARP, and cleaved PARP. Esculetin thus exerted a potent anti-proliferative effect in NSCLC by triggering Sp1-mediated apoptotic signaling. Therefore, esculetin may be considered a drug or natural supplement candidate for the prevention of NSCLC.

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