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In vitro study of 2,3-dehydrosilybin and its galloyl esters as potential inhibitors of angiogenesis

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2,3-Dehydrosilybin exhibits substantial anticancer and antiangiogenic effects, which can be potentially improved by semi-synthetic modification such as esterification with gallic acid. The aim of this study was to examine the potential antiangiogenic effect of 2,3-dehydrosilybin and its galloyl esters (3-*O*-galloyl-2,3-dehydrosilybin; 7-*O*-galloyl-2,3-dehydrosilybin; 20-*O*-galloyl-2,3-dehydrosilybin and 23-*O*-galloyl-2,3-dehydrosilybin) and to determine which molecular mechanism could be responsible for their activity. The effect on cell proliferation, tube formation, signal transduction pathways (PI3K/Akt and ERK) and the cell cycle was studied in human microvascular endothelial cells (HMEC). The results showed that all compounds decreased the growth of HMEC, but the strongest effect was observed for 20-*O*-galloyl-2,3-dehydrosilybin at 5 µmol/l. In addition, at 5 and 10 µmol/l, this was the only compound that significantly inhibited HMEC tube formation. Based on an assessment of Akt and ERK1/2 expression, we suggest that 20-*O*-galloyl-2,3-dehydrosilybin influences the angiogenic process through the Akt pathway.

1. Introduction

The use of chemotherapeutic agents is limited by side effects and drug resistance. Natural compounds can potentially help to avoid these problems. Flavonolignans are one of the most promising groups of natural compounds that exhibit chemopreventive and chemotherapeutic effects (Cho et al. 2013b).

Silymarin extracted from *Silybum marianum* (milk thistle) fruits has been traditionally used to protect the liver from toxic substances and to treat liver diseases such as hepatitis or cirrhosis (Gabrielova et al. 2010). The main component of the silymarin complex is silybin (SB). It has been reported that SB exhibits anti-inflammatory, anti-angiogenic, antiproliferative and antioxidant activities (Cho et al. 2013a). The oxidized form of SB, 2,3-dehydrosilybin (DHS), was shown to possess a stronger antioxidant activity than SB (Huber et al. 2008) due to significant ROS scavenging properties (Gabrielova et al. 2010; Gazak et al. 2004; Svobodova et al. 2007). In addition, a notable anticancer efficacy of DHS has been demonstrated in cell culture models of epithelial cancers including skin, bladder, colon, prostate and lung (Deep and Agarwal 2013; Singh et al. 2008a), but also in a mouse ectopic allograft model of basal cell carcinoma (Tilley et al. 2016). DHS anticancer activity is potentially related to the inhibition of cellular glucose uptake (Zhan et al. 2011) or to antiangiogenic effects of DHS (Gazak et al. 2011).

Angiogenesis is essential for the growth of both primary and metastatic tumors. Tumor angiogenesis requires endothelial cell proliferation, migration and the formation of a capillary sprout and tubes with a new basement membrane (Hanahan and Weinberg 2011; Shimo et al. 1999). All these processes are controlled by specific angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiopoietin, which are produced by tumor and stromal cells and are important regulators of angiogenesis (Alon et al. 1995; Hanahan and Folkman 1996; Javerzat et al. 2002; Rarova et al. 2012). The process of angiogenesis is controlled by two major signaling pathways, *i.e.*, PI3K/Akt and ERK (Dai et al. 2009). Akt is an important signaling molecule associated with the survival and migration of endothelial cells through activating the nuclear factor (NF)-κB (Beraud et al. 1999; Kane et al. 1999). It is induced by VEGF and bFGF (Shiojima and Walsh 2002). In addition, Akt can promote the upregulation of Bcl-2 expression, through which growth factors induce cell survival (Pugazhenthi et al. 2000). The ERK pathway is activated by VEGF and FGF and is involved in the regulation of cell motility and survival (Huber et al. 2008; L'Hote and Knowles 2005; Wu et al. 2000). Understanding the mechanism involved in angiogenic processes could lead to potential cancer therapies (Folkman et al. 1989; Weitensteiner et al. 2013).

Agarwal et al. (2013) suggested that the biological activity of DHS could be enhanced through suitable chemical modifications. For this reason, we performed a structure-activity relationship study with methylated and galloylated DHS on human umbilical vein endothelial cells (HUVECs) and demonstrated that galloylated DHS derivatives

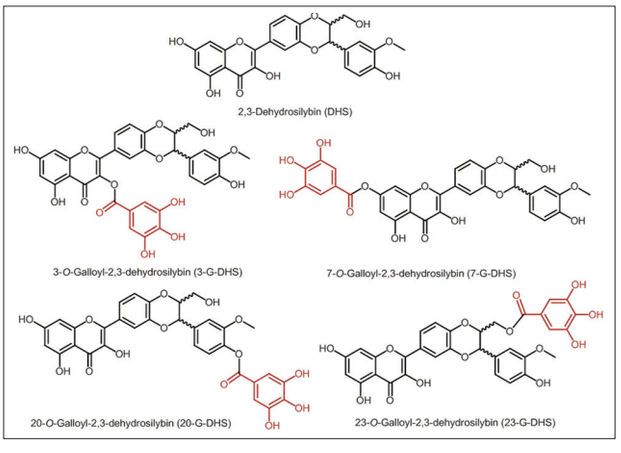

Fig. 1: Chemical structures of 2,3-dehydrosilybin and its galloyl esters.

show promise and should be further evaluated as anti-angiogenic agents (Karas 2015). We therefore focused on studying the influence of 2,3-dehydrosilybin (DHS) and its galloyl esters 3-*O*-galloyl-2,3-dehydrosilybin (3-G-DHS), 7-*O*-galloyl-2,3-dehydrosilybin (7-G-DHS), 20-*O*-galloyl-2,3-dehydrosilybin (20-G-DHS) and 23-*O*-galloyl-2,3-dehydrosilybin (23-G-DHS) on angiogenesis *in vitro*. Normal and cancerous tissues are dependent on their blood supply. Endothelial cells are involved in the formation of new blood vessels and are essential for blood supply (Yi et al. 2014). Therefore human microvascular endothelial cells (HMEC) were chosen as an appropriate cell model for the study of angiogenesis. We studied the processes necessary for the inhibition of angiogenesis; cell growth inhibition, cell cycle arrest, induction of apoptosis, inhibition of capillary tube organization and inhibition of the signal transduction pathways PI3K/Akt and ERK. This work had two main aims; i) to investigate whether the galloylation of DHS improves its biological activity and ii) to elucidate possible mechanisms by which these compounds might influence the angiogenic process.

2. Investigations and results

2.1. Effect of 2,3-dehydrosilybin and its galloyl esters on cell proliferation

Based on cell viability MTT assays performed by us previously on HUVEC (Karas et al. 2016), concentrations of compounds displaying less than 50 % toxicity were used for further testing on HMECs.

Cell proliferation was studied after 72 h according to the National Cancer Institute (NCI) protocols for angiogenesis. All compounds decreased the growth of HMEC, but only DHS and 20-G-DHS inhibited the growth of HMEC in a dose-dependent manner (0, 1, 3, 5, and 10 µmol/l, Fig. 2). The greatest reduction in HMEC growth was observed after treatment with 20-G-DHS at 5 and 10 µmol/l (Fig. 2). However, the most significant effect of 20-G-DHS was observed at 5 µmol/l. Based on the proliferation assay, concentrations of 5 and 10 µmol/l were chosen for further testing of the antiangiogenic activity of compounds on HMEC.

2.2. Effect of 2, 3-dehydrosilybin and its galloyl esters on tube formation

The tube formation assay showed that of all the tested compounds, only 20-G-DHS at 5 and 10 µmol/l inhibited the tube formation. This compound at 10 µmol/l significantly reduced total tube length. In addition, 10 µmol/l 20-G-DHS significantly suppressed the total number of loops compared to the control cells (Fig. 3). The remaining compounds had no significant effect on tube formation at the tested concentrations (data not shown). Therefore only 20-G-DHS was subjected to further investigation.

2.3. Effect of 20-O-galloyl-dehydrosilybin on cell cycle

The influence of 20-G-DHS on the cell cycle was studied by flow cytometric analysis after 6, 24 and 48 h. After 6 h, 20-G-DHS at 5 and 10 µmol/l significantly decreased the cell number in the S phase compared to control cells (Fig. 4). After 24 h, 5 and 10

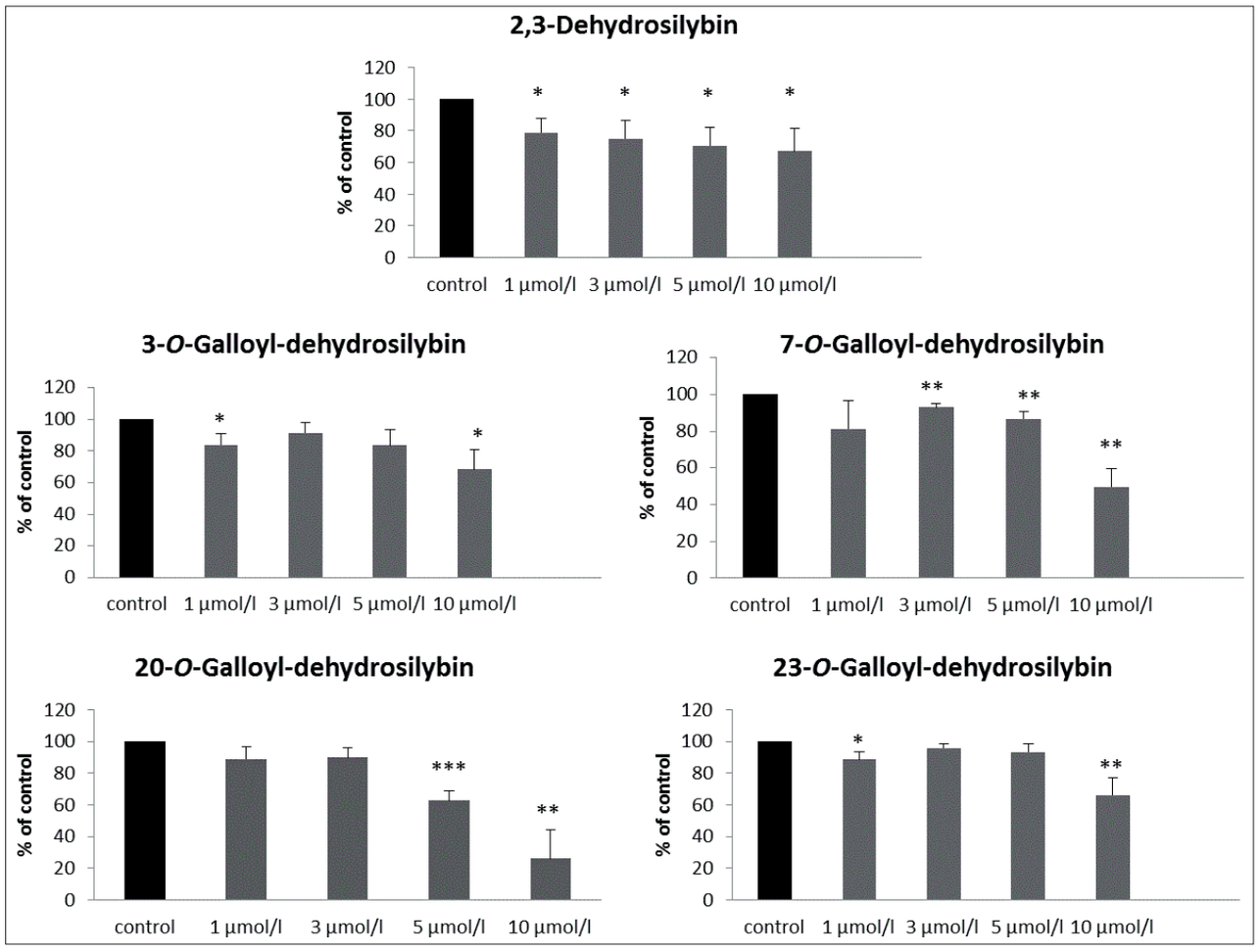

Fig. 2: Effect of 2,3-dehydrosilybin and its galloyl esters on HMEC proliferation. Cells were treated with 2,3-dehydrosilybin and its galloyl derivatives (1, 3, 5, and 10 µmol/l) for 72 h. Untreated cells were used as the control. Data are means ± SD of three independent experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly decreased versus control cells.

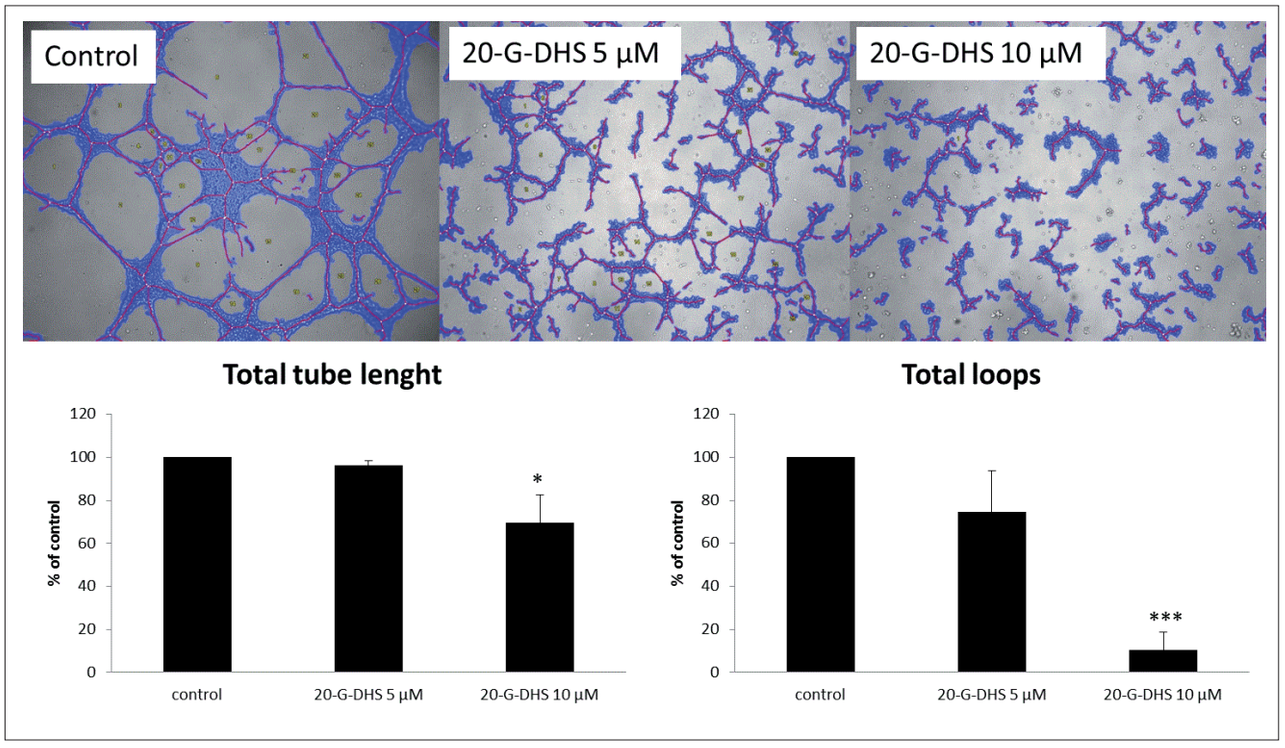

Fig. 3: The effect of 20-O-galloyl-2,3-dehydrosilybin (20-G-DHS) on the formation of tubes by HMEC-1 cells after 16 -20 h. Untreated cells were used as the control. Cells treated with 20-G-DHS (5 µmol/l and 10 µmol/l) are depicted in photos from the tube formation assay. Data are means ± SD of three independent experiments performed in triplicate. * $p < 0.05$, *** $p < 0.001$, significantly decreased versus control cells.

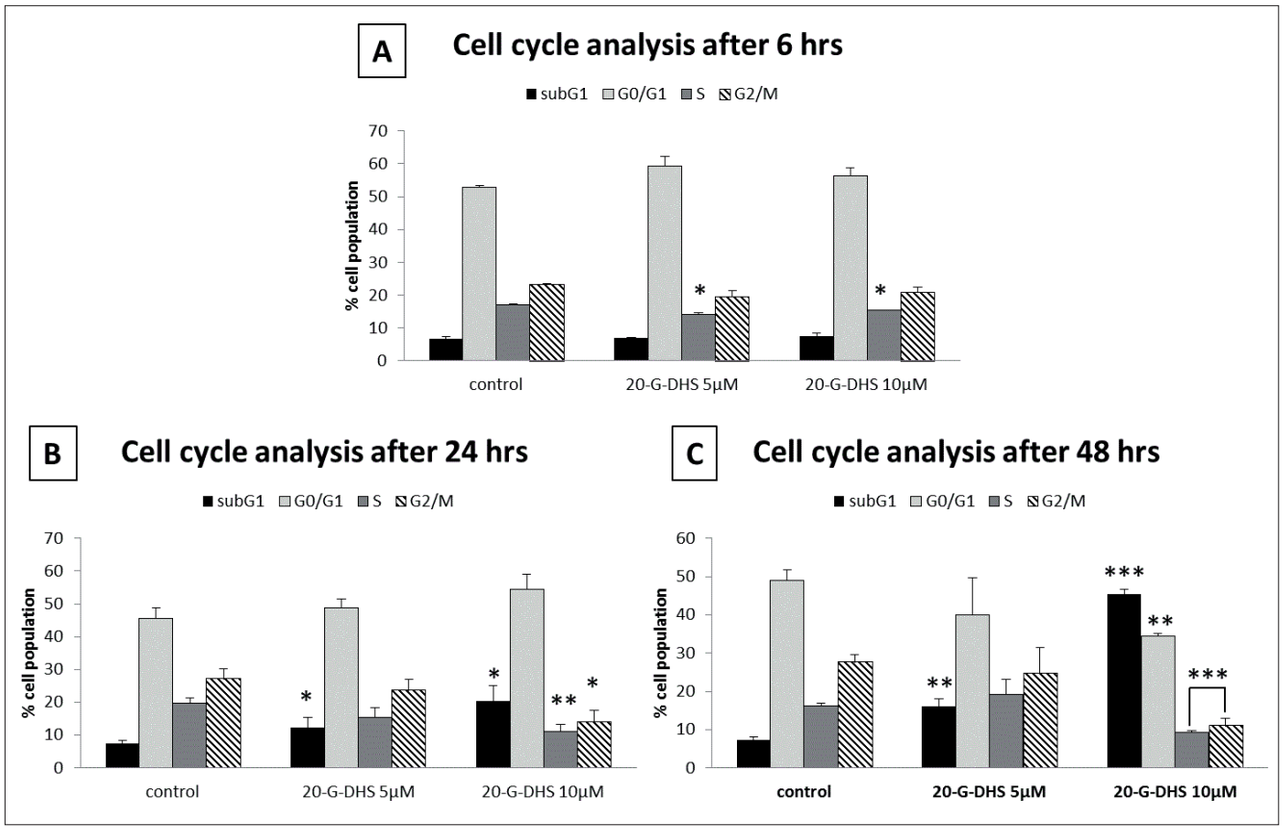

Fig. 4: Flow cytometric analysis of cell cycle in HMEC-1 cells treated with 20-O-galloyl-2,3-dehydrosilybin (20-G-DHS, 5 µmol/l and 10 µmol/l) for 6 (A), 24 h (B) and 48 h (C). Untreated cells were used as the control. The graphs present the proportion of cells in the sub G1, G0/G1, S and G2/M fractions. The data shown are the means of four independent experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly decreased or increased versus control cells.

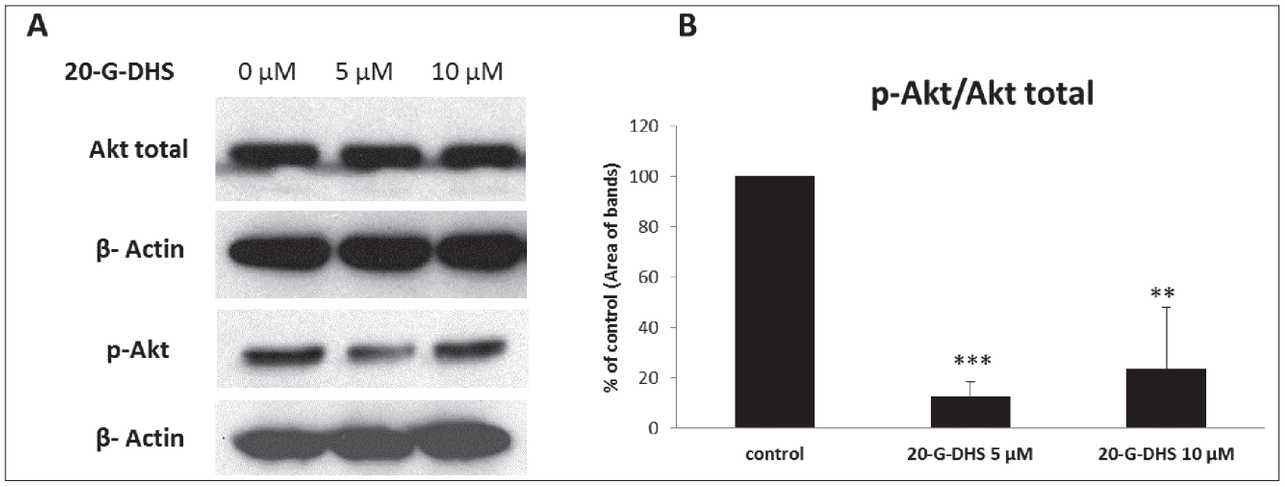

Fig. 5: Effect of 20-O-galloyl-2,3-dehydrosilybin (20-G-DHS) on the phosphorylation of Akt in HMEC-1 cell monolayer. Cells were treated with 20-O-galloyl-2,3-dehydrosilybin (5 µmol/l and 10 µmol/l) for 6 h. Untreated cells were used as the control. (A) After treatment, proteins in whole cell lysates (25 µg/lane) were analyzed by western blotting, and Akt and actin were visualized by chemiluminescent detection. Representative results are shown. (B) After treatment, the expression of Akt was determined by densitometric analysis. Data are means ± SD of four independent experiments. ** $p < 0.01$, *** $p < 0.001$ significantly decreased versus control cells.

µmol/l 20-G-DHS caused a significant increase in the number of apoptotic cells in the subG1 phase compared to the control cells (Fig. 4). Moreover 10 µmol/l 20-G-DHS caused a significant reduction in the cell number in the S and G2/M-phases compared to the control cells after 24 h (Fig. 4). After 48 h, both concentrations of 20-G-DHS increased the apoptotic cell number in subG1 compared to the control cells (Fig. 4). In addition, 10 µmol/l 20-G-DHS significantly decreased the cell number of the G0/G1, S and G2/M phases compared to the control cells (Fig. 4).

2.4. Effect of 20-O-galloyl-dehydrosilybin on phosphorylation of Akt and ERK1/2

The phosphorylation of Akt and ERK1/2 was determined using western blotting after 6 h. 20-G-DHS had no effect on the phosphorylation of ERK1/2 at any of the tested concentrations (data not shown). On the other hand, 5 and 10 µmol/l 20-G-DHS significantly decreased Akt phosphorylation in HMEC after 6 h compared to the control cells (Fig. 5). The strongest effect on Akt phosphorylation was observed for 5 µmol/l 20-G-DHS (Fig. 5).

3. Discussion

In many cases, an increase in angiogenesis is connected with a poor prognosis of solid tumors (Singh et al. 2008b). The antiangiogenic effects of SB and DHS have been observed in a number of models, such as skin and bladder cancer (Singh et al. 2008a; Tilley et al. 2016). Current studies have demonstrated that the presence of a galloyl moiety in the structure of flavonoids (e.g., catechins) could be an important prerequisite for their significant antiangiogenic properties (Agarwal et al. 2013; Gazak et al. 2011). Therefore the aim of this study was to examine the potential antiangiogenic effect of DHS and its galloyl esters (3-G-DHS; 7-G-DHS; 20-G-DHS and 23-G-DHS) as well as the potential molecular mechanisms responsible.

Inhibition of endothelial cell proliferation is an important anticancer effect of natural compounds or drugs, because it can modulate angiogenesis (Nor and Polverini 1999). Several studies presented the effect of different compounds on the inhibition of endothelial cell proliferation. For example, the inhibition of HUVEC and HMEC growth was reported for SB (25, 50 and 100 µmol/l, Singh et al. 2005) as well as for natural brassinolides (Rarova et al. 2012). Furthermore, HMEC proliferation was reduced by red raspberry extract (Sousa et al. 2015). Other studies showed that the growth of HUVEC significantly decreased after treatment with silymarin (50 and 100 µg/ml), cysteine-rich buccal gland protein, chebulagic acid or natural compound codonolactone (Jiang et al. 2000, 2016; Lu and Basu 2015; Wang et al. 2015). In addition, many studies investigated the effect of SB and DHS on cancer cell line proliferation. SB inhibited the growth of several prostate carcinoma cells (Davis-Searles et al. 2005; Tyagi et al. 2002) and the growth of colorectal cancer SW480 cells (Deep and Agarwal 2013). While comparing the effect of DHS and SB, DHS was more effective at inhibiting human cancer cell lines from the bladder, colon and prostate, namely HTB9, HCT116 and PC3, than SB (Agarwal et al. 2013). A similar trend was observed in murine basal cell carcinoma cells, but in this case both compounds decreased the production of proliferation biomarkers in an ectopic allograft model *in vivo* (Tilley et al. 2016). Our results confirmed the antiproliferative activity of DHS and its galloyl esters, because all the tested compounds suppressed the growth of HMEC. The same effect was observed in the study by Gažák et al. (2011), where SB and its galloyl derivatives decreased the proliferation of HUVEC. In addition, 7-*O*-galloylsilybin (7-G-SB) exerted the most potent inhibitory effect on HUVEC (Gazak et al. 2011).

The formation of the vascular network plays a crucial role in the evaluation of angiogenesis (Shimo et al. 1999). Therefore several works focused on testing the potential inhibition of tube formation in endothelial cells, where capillary-like structure formation was suppressed after treatment with various compounds (Jiang et al. 2016; Lu and Basu 2015; Rarova et al. 2012; Sousa et al. 2015; Wang et al. 2015). Also silymarin (50 and 100 µg/ml) inhibited the tube formation of HUVEC and SB (25-100 µmol/l) inhibited tube formation in HUVEC and in HMEC (Jiang et al. 2000; Singh et al. 2005). Moreover, SB was able to inhibit the tube formation in a co-culture of HMEC with glioblastoma cells (Chakrabarti and Ray 2015). The compounds tested in this study inhibited tube formation. However, at the tested concentrations, only 20-G-DHS had a significant effect on the inhibition of tube formation in HMEC. In contrast, when the effect of SB and its galloyl esters on the tube formation of HUVECs was studied, the strongest inhibitor was the 7-galloyl ester, followed by 20-galloylsilybin (Gazak et al. 2011).

Deregulation of the cycle progression exhibited by transformed cells is another target for chemotherapeutics (Singh et al. 2008a). Moreover, the induction of apoptosis can be used to inhibit new blood vessel formation in tumors, and it was suggested that cell cycle arrest could have a role in the inhibition of cell growth (Singh et al. 2005). Silymarin induced apoptosis in HUVEC in a dose-dependent manner (50 and 100 µg/ml, Jiang et al. 2000) and treatment with SB (25-100 µmol/l) for 24, 48 and 72 h caused apoptotic cell death in HUVEC and in HMEC (Singh et al. 2005). The same effect on apoptosis in HUVEC was shown for other natural compounds, such as brassino-

lides or codon lactone (Jiang et al. 2016; Rarova et al. 2012). A similar effect was observed for DHS and SB when tested on human cancer cells. DHS induced significant cell death in HepG2 and FIB cells, and SB (100 µmol/l) initiated apoptosis in HT29 cells (Agarwal et al. 2003; Thongphasuk et al. 2008). Furthermore, silymarin flavonolignans also exhibited effects on the cell cycle. For example, silybin A, silybin B and isosilybin A (5-30 µmol/l) caused G1 arrest and G2/M arrest in a dose-manner in HUVECs after 24 h (Deep et al. 2012). SB also (25, 50 and 100 µmol/l) caused a strong time- and dose-dependent increase in cell number in the G1 phase in HUVEC and HMEC, and inducted a time- and dose-dependent decrease in cell number in the S and G2/M phases in both cell lines after 24, 48 and 72 h (Singh et al. 2005). Our results gave a similar trend for 20-G-DHS, which increased the number of apoptotic cells (subG1 phase) in a dose- and time-dependent manner in HMEC. Moreover, 20-G-DHS induced a slight accumulation of cells in the G0/G1 phase after 6 h and 24 h. This could be the reason for the significant reduction in cell number in the subsequent phases (S and G2/M phase). On the other hand, after 48 h 20-G-DHS decreased the cell number in the G0/G1, S and G2/M phases in a dose-dependent manner.

Subsequently, we wanted to elucidate the mechanism by which 20-G-DHS induces the inhibition of angiogenesis in endothelial cells. Hence its effect on PI3K/Akt and ERK pathways was studied. It was reported that SB at 10 and 25 µmol/l slightly suppressed the phosphorylation of Akt, but at 50 µmol/l strongly inhibited it (Singh et al. 2005). Our results suggest that 20-G-DHS inhibits angiogenesis by influencing the PI3K/Akt pathway, because the phosphorylated form of Akt was suppressed in HMEC, whereas the phosphorylated form of ERK1/2 was unchanged. Accordingly, the level of phosphorylated Akt and ERK1/2 was shown to be influenced by the concentration of red raspberry extract. ERK1/2 was decreased at lower concentrations (10 and 25 µg/ml), while Akt was suppressed at the higher concentration (50 µg/ml) in HMEC (Sousa et al. 2015). In our study, we did not observe the influence of concentration on the phosphorylation of Akt and ERK1/2 reported by others.

Taken together, the results of this study demonstrate that the galloylation of DHS enhanced its antiproliferative and antiangiogenic effects in endothelial cells. Substitution at the C-20 OH leads to the greatest antiangiogenic activity at the tested concentrations (5 and 10 µmol/l). In addition, our results suggest that 20-G-DHS inhibited angiogenesis through inhibiting the PI3K/Akt pathway.

4. Experimental

4.1. Materials

Endothelial Cell Growth Medium was purchased from Promocell, Germany. Fetal calf serum (FCS) and basic fibroblast growth factor (bFGF), heparin, epidermal growth factor (EGF), propidium iodide, Tris–HCl, NaCl, NaF, Nonidet P-40, PMSF, SDS, deoxycholic acid, Tween-20, methanol and ethanol were purchased from Sigma-Aldrich, USA. M199 medium was purchased from PAN Biotech, Germany. Ibidi µ-slides (15-well) were purchased from Ibidi GmbH, Germany. Matrigel® was purchased from Corning GmbH, Germany. Nitrocellulose membranes were purchased from Bio-Rad Laboratories, USA. The specific primary antibodies (β-tubulin, p42/44 MAPK, phospho-p44/42 MAPK, Akt antibody and phospho-Akt antibody) were purchased from Cell Signaling Technology, USA. Anti-β-actin monoclonal antibody was purchased from Millipore, USA. Secondary antibody goat anti- mouse IgG1-HRP was purchased from Biozol, Germany and secondary antibody goat anti- rabbit IgG1 from Jackson ImmunoResearch, USA.

4.2. Compounds

All semi-synthetic DHS galloyl derivatives (Fig. 1) were kindly provided by prof. V. Křen (Institute of Microbiology, Academy of Sciences of the Czech Republic). Their synthesis is described elsewhere (Karas et al. submitted). Stock solutions of the tested compounds (10 mM) were prepared in DMSO and stored at -20 °C.

4.3. Cell culture

Human microvascular endothelial cells (HMEC, cell line CDC/EU.HMEC-1), kindly provided by the Centers for Disease Control and Prevention (Atlanta, USA), were cultured in Endothelial Growth Medium, containing 10 % inactivated fetal calf serum (FCS) and growth factors (basic fibroblast growth factor 1.0 ng/ml, heparin 0.004 mg/ml, and epidermal growth factor 0.1 ng/ml). M199 medium was used as a starvation medium. Untreated cells were used as controls.

4.4. Proliferation assay

The proliferation assay was performed according to the National Cancer Institute (NCI) protocols for angiogenesis. Briefly, HMEC were seeded into 96-well plates (15×10^3 cells/well). The next day, control cells were stained with crystal violet solution (0.5% crystal violet in 20% methanol) for 10 min to determine the initial cell number. Then HMEC were treated with increasing concentrations of the tested compounds (0, 1, 3, 5 and 10 µmol/l) for 72 h. After incubation, cells were also stained with crystal violet solution. Unbound crystal violet was removed by rinsing with distilled water, and cells were subsequently air dried. Next, crystal violet, which mainly binds to DNA, was eluted from cells with 0.1 mol/l sodium citrate in 50% ethanol. The absorbance of crystal violet is proportional to the cell number and was determined spectrophotometrically with a Sunrise ELISA reader (Tecan Trading AG) at 540 nm.

4.5. Tube formation

HMEC were seeded into 6-well dishes (25×10^4 cells/well), and immediately treated with the tested compounds at 5 and 10 µmol/l. After 24 h of incubation, cells treated with the compounds were trypsinized and added into the wells of Ibidi µ-slides (15-well) coated with Matrigel®. After 16-20 h, HMEC were stained with propidium iodide (10 µg/ml) and images were taken using a TILLvisiON-system (Lochham, Germany) connected to an Axiovert 200 microscope (Zeiss, Germany). The formation of tubes was expressed as the number of tubes and number of nodes using specific software (S.CO LifeScience, Garching, Germany).

4.6. Flow cytometry

HMEC were seeded into 6-well dishes (25×10^4 cells/well), and immediately incubated with 20-G-DHS at 5 and 10 µmol/l. After 6, 24 and 48 h, the cells were detached with trypsin, washed and stained overnight at 4 °C in 0.1% [m/v] sodium citrate, 0.1% [v/v] Triton X-100, and 50 µg/ml propidium iodide in phosphate buffered saline (PBS). DNA content was assessed with a flow cytometer (FACSCalibur, Becton Dickinson, Germany). The distribution of cells in the subG1 (apoptotic cells), the G0/G1, S and the G2/M peak was quantified using histogram analysis in FlowJo software (Tree Star, USA).

4.7. SDS-polyacrylamide gel electrophoresis and immunoblotting

HMEC were seeded into 6-well plates (25×10^4 cells/well) and grown to confluence. Then 20-G-DHS (5 and 10 µmol/l) was added and the HMEC were incubated with the compound for 6 h. For immunoblotting, harvested cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.4, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 30 mM PMSF, 1 mM SDS, 6.4 mM deoxycholic acid). The concentrations of protein in lysates were quantified by the Bradford method. Lysates were diluted with sample electrophoresis buffer. Proteins were then separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were blocked with 5% (w/v) BSA in Tris-buffered saline (TBS) with 0.1% Tween-20 for 2 h, probed with the specific primary antibody (p42/44 MAPK, phospho-p44/42 MAPK, Akt antibody and phospho-Akt antibody) and incubated overnight. The membranes were then probed with horseradish peroxidase conjugated secondary antibodies (goat anti mouse IgG1 and goat anti rabbit IgG1) and visualized with chemiluminescent detection reagent. To confirm equal protein loading, immunodetection was performed with anti-β-actin and β-tubulin polyclonal antibodies. The protein expressions in treated cells were compared to untreated cells. Semi-quantitative analyses of the immunoblots were evaluated using the software ImageJ 1.49n.

4.8. Statistical analysis

Results were expressed as mean±standard deviation (SD) of three or four independent experiments performed in duplicate or triplicate. Statistical analysis was carried out using the software Statistica version 12.0 by one-way ANOVA and Student *t*-test. The differences between means were considered to be statistically significant at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

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Conflict of interest: The authors declare that there are no conflicts of interest.

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