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Novel Human Umbilical Vein Endothelial Cells (HUVEC)-Apoptosis Inhibitory Phytosterol Analogues: Insight into Their Structure-Activity Relationships

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Design, synthesis and insight into the structure-activity relationships (SAR) of phytosterol analogues as novel antiapoptotic agents are described. In particular, the non-branched alkyl chain at C24 and the pseudosugar moiety at C3 hydroxyl group turned out crucial for the inhibition of human umbilical vein endothelial cells (HUVEC) apoptosis.

Key words: Antiapoptotic agent, Phytosterol, HUVEC

INTRODUCTION

Blood vessel is a monolayer of endothelial cell, which is located at the interface between vascular and perivascular compartments. The endothelial cells have two different roles in communicating blood with underlying tissues and acting as a barrier between intravascular and extravascular compartment. Such a local feature of the endothelial cell assures the simultaneous and constant exposure of the cells to a wide variety of stimuli, which have the potential to induce or prevent apoptosis of the cells (Bazzoni, 2006). It has been reported that apoptosis of endothelial cells has been increased in experimental and human cardiovascular diseases models (Stefanec, 2000). Also, apoptosis occurs in the retina degenerative diseases such as retinitis pigmentosa (Wong, 1994), anterior ischemic optic neuropathy (Levin and Louhab, 1996), glaucoma (Kerrigan et al., 1997) and human diabetic retinopathy (Barber et al., 1998). Considering these preliminary reports, inhibition of VEC apoptosis has been considered as a novel approach for treatment of various physiological disorders such as cardiovascular and retinal vascular diseases. There were a few reports for development of antiapoptotic agents from the small molecules (Liu et al., 2009; Tan et al., 2009) to natural products (Alvarez et al., 1997; Chen et al., 2010). Recently, we reported that ginsenoside Rg3 and Rk1 have antiapoptotic activity on human endothelial cell (Min et al., 2006). Based on these results, we have also developed a series of synthetic analogues, derived from easily accessible cholesterol as potent antiapoptotic agents, which substantially replaced the protopanaxdiol moiety of Rk1. In particular, the novel ginsenoside equivalents, such as 4,6-di-*O*-acetyl-2,3-dideoxyhex-2-enopyran and tetrahydropyran cholesterol analogue exhibited excellent cell survival activities, which are equipotent to that of ginsenoside Rk1 (Lee et al., 2010). With the results in hands, we evaluated the potent ginsenoside equivalents for their bioactivity to maintain tight junction integrity and prevent retinal vascular leakage. Interestingly, cholesterol analogue SAC-0601 (**1**) significantly reduced retinal vascular leakage in diabetic retinopathy mice model via stabilizing tight junctions (Maharjan et al., 2011). On the basis of these preliminary studies, we have designed and synthesized the diverse phytosterol analogues to confirm the function of sterol as a substitute for the protopanaxdiol moiety. There were three major considerations; 1) effect of carbohydrate moiety at C3 hydroxyl group, 2) steric influence of alkyl chain at C24, and 3) influence of the steroidal skeleton, as shown at Fig. 1.

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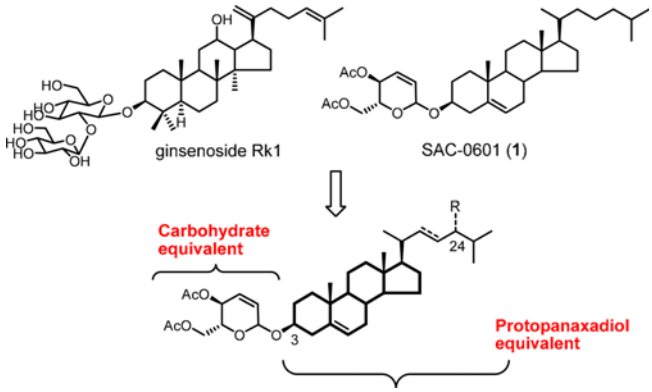


Fig. 1. Strategy for development of novel antiapoptotic agents

MATERIALS AND METHODS

General procedure

Unless noted otherwise, all starting materials were obtained from commercial suppliers and were used without further purification. The reactions were performed under an argon atmosphere. Flash column chromatography was performed using silica gel 60 (230-400 mesh, Merck) with indicated solvents. Thin-layer chromatography was performed using 0.25 mm silica gel F254 plates (Merck). Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl. Dichloromethane was distilled from calcium hydride. All solvents used for routine isolation of products and chromatography were reagent grade and distilled. Reaction flasks were oven dried at 120°C. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL LNM-LA 300 (300 MHz), or Brucker FT-NMR AVANCE 400 (400 MHz) spectrometer as solutions in deuteriochloroform ($CDCl_3$). Chemical shifts were expressed in parts per million (ppm, δ) and tetramethylsilane (TMS) was used as an internal standard. ^{1}H-NMR data were reported in order of chemical shift, multiplicity (s, singlet; br, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet and/or multiple resonance), number of protons and coupling constant in herz (Hz). Infrared spectra were recorded on Jasco FT/IR-4200 or Perkin-Elmer 1710 FT-IR spectrometer. Low resolution mass spectra were obtained on VG Trio-2 GC-MS.

General procedure for dihydropyran derivatives

To a solution of 3,4,6-triacetyl glucal (3 eq) in THF (6 mL) was added phytosterol (1 eq) and $BF_3 \cdot OEt_2$ (3 eq). After stirring for 2 h, the reaction mixture was quenched with saturated $NaHCO_3$ and then diluted with EtOAc. The organic phase was washed with H_2O and brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification of the residue via flash column chromatography on silica gel (EtOAc-hexanes = 1:7) afforded the corresponding dihydropyran derivatives.

General procedure for hydrogenation of physterols

To a solution of phytosterol in EtOAc was added 10% palladium on activated carbon. The resulting mixture was hydrogenated for 24 h and then filtered through a pad of Celite. Purification of the residue via flash column chromatography on silica gel (EtOAc-hexanes = 1:30) afforded the corresponding phytostanol.

(3*S*,10*S*,13*R*,17*R*)-17-((2*R*,5*R*)-5-Ethyl-6-methylheptan-2-yl)-10,13-dimethylhexadecahydro-1*H*-cyclopenta[α]phenanthren-3-ol (Sitostanol, 3)

92%; white solid: FT-IR (neat) ν_{max} 3536, 1465, 1384, 764 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 3.55 (m, 1H), 1.93 (d, 1H, *J* = 12.1 Hz), 1.82-0.72 (m, 47H), 0.58 (s, 3H); LR-MS (FAB+) *m/z* 417 (M+H).

(3*S*,5*S*,10*S*,13*R*,17*R*)-10,13-dimethyl-17-((*R*)-6-methylheptan-2-yl)hexadecahydro-1*H*-cyclopenta[α]phenanthren-3-ol (Cholestanol, 5)

98%; white solid: FT-IR (neat) ν_{max} 3589, 2932, 1467, 1376, 758 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 3.55 (m, 1H), 1.93 (d, 1H, *J* = 12.1 Hz), 1.82-0.72 (m, 43H), 0.58 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 71.3, 56.4, 56.2, 54.3, 44.8, 42.5, 40.0, 39.4, 38.1, 36.9, 36.1, 35.7, 35.4, 32.0, 31.4, 28.7, 27.9, 24.1, 23.8, 22.7, 24.1, 22.5, 21.2, 18.6, 12.2, 12.0; LR-MS (FAB+) *m/z* 417 (M+H).

((2R,3S)-3-Acetoxy-6-((3S,10R,13R,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[α]phenanthren-3-yloxy)-3,6-dihydro-2*H*-pyran-2-yl)methyl acetate (1)

79%; white solid: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 5.83 (m, 2H), 5.33 (br, 1H), 5.27 (dd, 1H, *J* = 9.2 Hz, 1.5 Hz), 5.14 (br, 1H), 4.17 (m, 3H), 3.53 (m, 1H), 2.37 (m, 2H), 2.07 (s, 3H), 2.05 (s, 3H), 1.99-1.01 (m, 26H), 0.97 (s, 3H), 0.84 (dd, 9H, *J* = 6.6, 14.3 Hz), 0.65 (s, 3H); FT-IR (neat) ν_{max} 2938, 1464, 1377, 1261, 1199, 1024 cm^{-1}; LR-MS (FAB+) *m/z* 621 (M+Na).

((2*R*,3*S*)-3-Acetoxy-6-((3*S*,10*R*,13*R*,17*R*)-17-((2*R*,5*S*,*E*)-5-ethyl-6-methylhept-3-en-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[α]phenanthren-3-yloxy)-3,6-dihydro-2*H*-pyran-2-yl)methyl acetate (7)

79%; white solid: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 5.81 (m, 2H), 5.30 (m, 2H), 5.16 (br, 1H), 5.10 (d, 1H, *J* = 8.4 Hz), 4.98 (dd, 1H, *J* = 8.4, 15.0 Hz), 4.17 (m, 3H), 3.53

(m, 1H), 2.35 (m, 2H), 2.07 (s, 3H), 2.05 (s, 3H), 2.02-1.81 (m, 6H), 1.68 (m, 1H), 1.53 (m, 9H), 1.25-0.75 (m, 22H), 0.67 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 170.7, 170.2, 140.6, 138.2, 129.2, 128.8, 128.0, 121.7, 92.7, 76.5, 66.7, 65.3, 63.1, 56.7, 55.8, 51.1, 50.1, 42.1, 40.4, 40.3, 39.5, 37.0, 36.6, 31.8, 28.8, 28.1, 25.3, 24.2, 21.1, 21.0, 20.9, 20.9, 20.7, 19.2, 18.9, 12.2, 11.9; LR-MS (FAB+) *m/z* 647 (M+Na).

((2*R*,3*S*)-3-Acetoxy-6-((3*S*,10*R*,13*R*,17*R*)-17-((2*R*,5*R*)-5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[α]phenanthren-3-yloxy)-3,6-dihydro-2*H*-pyran-2-yl)methyl acetate (8)

82%; white solid: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 5.81 (m, 2H), 5.32 (d, 1H, *J* = 4.8 Hz), 5.25 (d, 1H, *J* = 9.3 Hz), 5.14 (s, 1H), 4.16 (m, 3H), 3.51 (m, 1H), 2.34 (m, 2H), 2.06 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 2.00-1.81 (m, 5H), 1.74-1.00 (m, 22H), 0.97 (s, 3H), 0.88 (d, 3H, *J* = 6.2 Hz), 0.80 (q, 6H, *J* = 6.9 Hz), 0.64 (s, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 170.4, 169.9, 140.5, 128.6, 128.2, 121.6, 92.5, 77.9, 66.6, 65.1, 62.9, 56.2, 55.8, 49.9, 45.5, 42.1, 40.2, 39.5, 36.9, 36.4, 35.9, 33.7, 31.7, 31.6, 28.9, 28.0, 25.8, 24.1, 22.8, 20.8, 20.7, 20.6, 19.6, 19.1, 18.8, 18.6, 18.5, 12.0, 11.8; LR-MS (FAB+) *m/z* 649 (M+Na).

((2*R*,3*S*)-3-Acetoxy-6-((3*S*,10*S*,13*R*,17*R*)-17-((2*R*,5*R*)-5-ethyl-6-methylheptan-2-yl)-10,13-dimethylhexadecahydro-1*H*-cyclopenta[α]phenanthren-3-yloxy)-3,6-dihydro-2*H*-pyran-2-yl)methyl acetate (9)

78%; white solid: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 5.80 (m, 2H), 5.26 (d, 1H, *J* = 9.2 Hz), 5.14 (s, 1H), 4.16 (m, 3H), 3.59 (m, 1H), 2.06 (s, 3H), 2.04 (s, 3H), 1.93 (m, 1H), 1.82-0.77 (m, 46H), 0.61 (s, 3H); LR-MS (FAB+) *m/z* 651 (M+Na).

((2*R*,3*S*)-3-Acetoxy-6-((3*S*,5*S*,10*S*,13*R*,17*R*)-10,13-dimethyl-17-((*R*)-6-methylheptan-2-yl)hexadecahydro-1*H*-cyclopenta[α]phenanthren-3-yloxy)-3,6-dihydro-2*H*-pyran-2-yl)methyl acetate (10)

82%; white solid: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 5.82 (m, 2H), 5.26 (d, 1H, *J* = 9.2 Hz), 5.14, (s, 1H), 4.18 (m, 3H), 3.60 (m, 1H), 2.07 (s, 3H), 2.05 (s, 3H), 1.93 (d, 1H, *J* = 12.1 Hz), 1.78-0.93 (m, 30H), 0.85 (dd, 9H, *J* = 6.5, 10.08 Hz), 0.77 (s, 3H), 0.61 (s, 3H); LR-MS (FAB+) *m/z* 623 (M+Na).

2-((3*S*,10*R*,13*R*,17*R*)-17-[(1*R*)-1,5-Dimethylhexyl]-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[α]phenanthren-3-yloxy)tetrahydro-2*H*-pyran (11)

To a solution of cholesterol (100 mg, 0.26 mmol) in CH_2Cl_2 (1.5 mL) was added dihydropyran (0.18 mL, 1.97 mmol) and *p*-toluene sulfonic acid (12 mg, 0.07 mmol). After stirring for 4 h, the reaction mixture was quenched with H_2O, and then diluted with EtOAc. The organic phase was washed with H_2O and brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification of the residue via flash column chromatography on silica gel (EtOAc-hexanes = 1:20) afforded 82 mg (68%) of **7** as a white solid: FT-IR (neat) ν_{max} 2921, 1460, 1112 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 5.32 (br, 1H), 4.69 (br, 1H), 3.88 (m, 1H), 3.54-3.43 (m, 2H), 2.34-2.28 (m, 2H), 2.00-1.66 (m, 7H), 1.59-1.00 (m, 24H), 0.98 (s, 3H), 0.86 (ddd, 9H, *J* = 15.3, 6.4, 1.2 Hz), 0.65 (s, 3H); LR-MS (FAB+) *m/z* 471 (M+H).

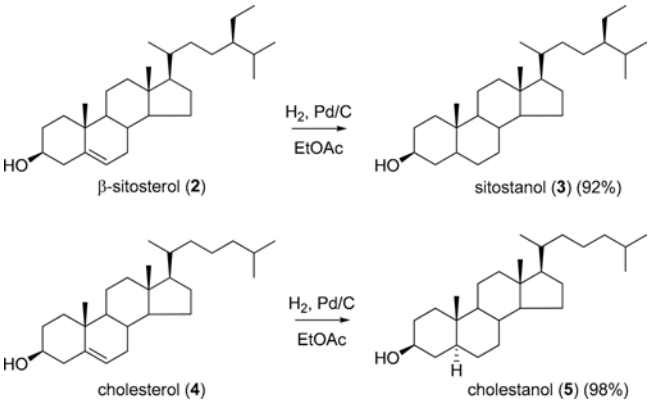
MTT and morphological assay of viable endothelial cells

Human umbilical vein endothelial cells (HUVECs) (3×10^5 cells/well) were plated onto 24-well plates in M199 media containing 20% fetal bovine serum. Next day, the cells were switched to serum free media and treated with or without various synthetic compounds at concentration of 10 μg/mL. After 48 h, cells were washed and photographed using optical microscope (Olympus 1X50-58F2) at 10X magnification. Cells were then incubated with serum free media containing 100 μg/mL MTT for 4 h. Upon incubation, the soluble yellow MTT is converted to insoluble dark blue formazan crystals by dehyrdrogenase enzymes of the viable cells. The dark blue crystals were then solubilized by adding DMSO-Ethanol (1:1) and the absorbance was measured at 540 nm using ELISA plate reader (FLUO star Omega).

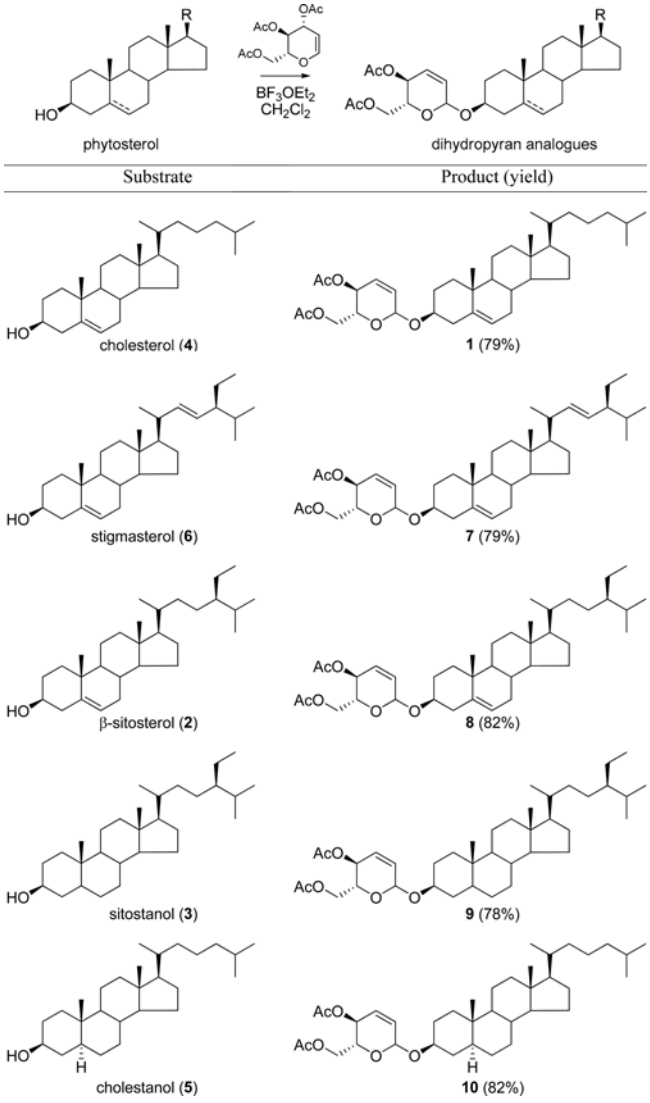
RESULTS AND DISCUSSION

Herein, we report design, synthesis and biological evaluation of a series of phytosterol analogues as novel antiapoptotic agents. Considering the previous results that ginsenoside and cholesterol analogues consisting of steroidal backbone exhibited potent antiapoptotic effects, we focused on the synthesis of the phytosterol analogues, which have the structurally similar skeleton to the steroidal backbone of ginsenoside and cholesterol analogues. Our work commenced with comparison of activities of the phytosterols and their analogues connected to the dihydropyran moieties to elucidate the influence of the carbohydrate moieties at C3 hydroxyl group as well as their steroidal backbones of the analogues on antiapoptotic activities. Thus, we synthesized the 4,6-di-*O*-acetyl-2,3-dideoxyhex-2-enopyran derivatives, which provided potent antiapoptotic activity on HUVEC in preliminary studies. The struc-

tural variation was also anticipated to reveal the affect of the side chain at C24 of the phytosterols on the biological activities. As illustrated in Scheme 1, our initial studies involved preparation of the phytostanols from phytosterols by hydrogenation. Sitostanol (**2**) and cholestanol (**4**) were synthesized by hydrogenation of β-sitosterol and cholesterol in the presence of Pd/C. The stereochemistry at C5 of cholestanol was confirmed by comparison of its spectral data with those reported in literature (Jursic et al., 2010).



Scheme 1. Synthesis of dihydrophytosterols

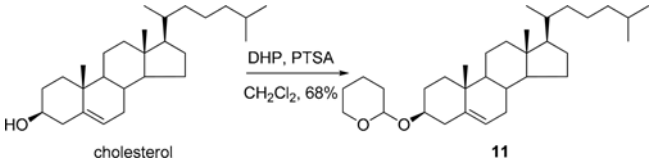


Scheme 2. Glycosylation of phytosterols

Next, dihydropyran analogues **1**, **7**, **8**, **9** and **10** were synthesized by an addition of 3,4,6-*tri*-*O*-acetyl-*D*-glucal to the corresponding phytosterol (**2**, **3**, **4**, **5** and **6**) solutions in the presence of boron trifluoride as described in Scheme 2.

The tetrahydropyran analogues of cholesterol were also prepared, as described in Scheme 3 (Lee et al., 2010). With phytosterols and the dihydropyran phytosterol analogues in our hands, the MTT assay was carried out to evaluate their antiapoptotic activities on HUVEC. The results are summarized in Fig. 1.

In order to evaluate the effects of the analogues on HUVEC apoptosis, the standard procedure in which HUVEC apoptosis was induced by deprivation of serum was used (Hogg et al., 1999). As previously reported (Lee et al., 2010), the cholesterol analogues (**1** and **11**) showed the desired antiapoptotic activities on HUVECs at 10 μg/mL treatment. In particular, the dihydropyran cholestanol analogue (**10**) also exhibited antiapoptotic activity on endothelial cells. However, the dihydropyran analogues of other phytosterols such as analogues **7**, **8**, and **9** showed no significant antiapoptotic activities on HUVEC. This result supported that the



Scheme 3. Synthesis of tetrahydropyran analogues

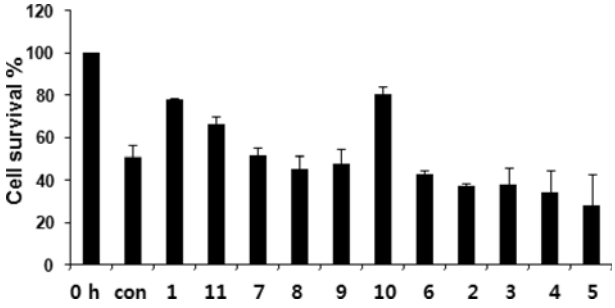


Fig. 2. MTT assay on HUVECs. Antiapoptotic activities of the synthesized analogues on HUVECs at 10 μg/mL for 48 h treatment of analogue. 0 h, the viability of the cells cultured in the medium without any derivatives. con, the viability of the cells cultured in the medium containing DMSO used as a vehicle control.

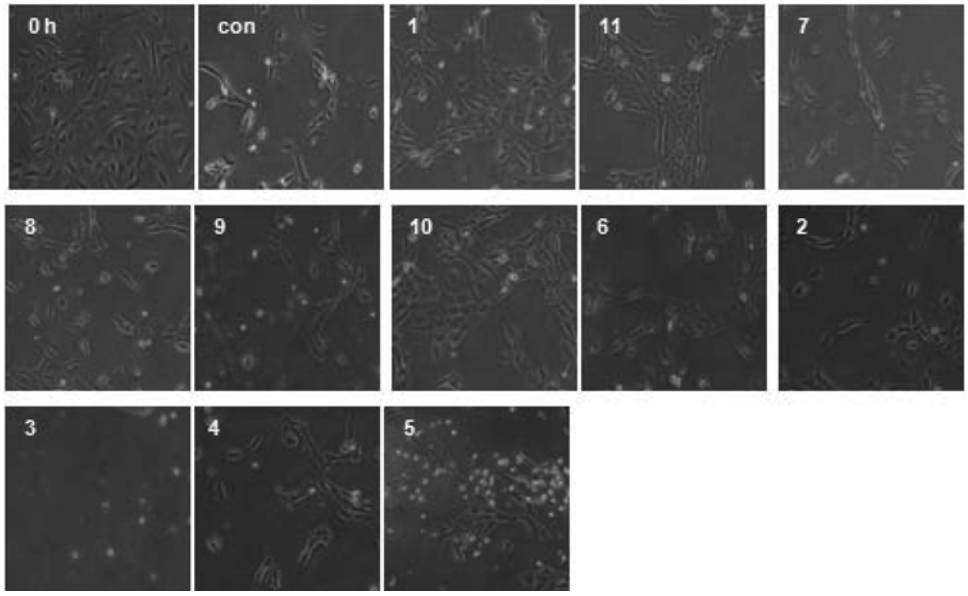


Fig. 3. Morphology of HUVEC. Morphological changes of HUVECs at 10 μg/mL for 48 h treatment of the analogues. 0 h, the viability of the cells cultured in the medium without any derivatives. con, the viability of the cells cultured in the medium containing DMSO used as a vehicle control.

non-branched side chain at C24 is essential for the inhibition of HUVEC apoptosis. In contrast, the bulkier upper side chain in steroidal backbone seems not beneficial for enhancement of the antiapoptotic activity. Moreover, it has been suggested that the glucal moiety at C3 hydroxyl group is crucial for antiapoptotic activity on the basis of analysis of all phytosterols (**2**, **3**, **4**, **5** and **6**) and the dihydropyran analogues (**1**, and **7-10**). However, presence of the olefin in steroidal skeleton seems not crucial for antiapoptotic activities, considering insignificant difference between the activities of the dihydropyran analogues of cholesterol **1** and cholestanol **10**, as well as sitosterols **8** and **9**. In addition, olefin in the upper side chain seems not beneficial for the antiapoptotic effect (**7** and **8**). Finally, we analyzed the morphological changes associated with physiological and pathological processes in HUVECs. As the cells detach from the culture dish bottom and become round, they might undergo the apoptosis processes. On the other hand, HUVEC elongation and bending into rings indicate microvessel formations (Oishi et al., 2004). In our experiments, the HUVEC elongations were clearly observed on treatments with the analogues **1**, **11** and **10**. This result supported that the tested analogues are capable of inhibiting HUVEC-apoptosis.

In conclusion, we have designed and synthesized a series of novel phytosterol analogues as novel anti-apoptotic agents. Evaluation of the synthesized analogues for antiapoptotic activities on HUVECs provided an insight into the structure-activity relationship of phytosterol analogues, which allowed us to identify the readily available phytosterols as an excellent equivalent to the protopanaxadiol backbone of ginsenoside. Our report would also provide an important basis for development of the highly potent antiapoptotic agents with appropriate pharmacological properties. The mechanistic studies as well as developments of potent antiapoptotic agents are in good progress.

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