Stereoisomers ginsenosides-20(S)-Rg3 and -20(R)-Rg3 differentially induce angiogenesis through peroxisome proliferator-activated receptor-gamma

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Stereoisomers of ginsenosides-20(S)-Rg3 and -20(R)-Rg3 differentially induce angiogenesis through peroxisome proliferator-activated receptor-gamma

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Abstract

Background—Ginsenosides are the major constituents that are responsible for most of the pharmacological actions of ginseng. However, some ginsenosides exist as stereoisomer pairs, detail and molecular exposition based on the structural difference of ginsenoside stereoisomers has not been emphasized in many studies. Here we explore the differential functional differences of ginsenoside Rg3 stereoisomers on angiogenesis.

Methods and Results—In this study, we demonstrated the differential angiogenic activities of 20(S)-Rg3 and 20(R)-Rg3 stereoisomers. 20(S)-Rg3 at micromolar concentration promotes human endothelial cells proliferation, migration and tube formation in vitro, as well as ex vivo endothelial sprout. The effects induced by 20(S)-Rg3 are significantly more potent than 20(R)-Rg3. These effects are partially mediated through the activation of AKT/ERK-eNOS signaling pathways. Moreover, we identified peroxisome proliferator-activated receptor-gamma (PPARγ) is the molecular target of ginsenoside Rg3, by using PPARγ competitive ligand-binding assay and reporter gene assay. The PPARγ agonist activity of 20(S)-Rg3 is 10-fold higher than 20(R)-Rg3 possibly due to the critical hydrogen bonding between 20(S)-Rg3 and tyrosine-473 in the ligand binding domain of PPARγ. Knockdown of PPARγ by specific small interference RNA abolished the 20(S)-Rg3-induced angiogenesis, indicating that PPARγ is responsible for the angiogenic activity of Rg3.

Conclusions—The present study elucidated the differential angiogenic effects of Rg3 stereoisomers by acting as partial agonist of PPARγ. The results shed light on the structural difference between two ginsenoside stereoisomers that can lead to significant differential physiological outcomes which should be carefully considered in the future development of ginsenoside-based therapeutics.

Key Words: ginsenoside ■ Rg3 ■ stereoisomer ■ angiogenesis ■ PPARγ
Introduction

Panax ginseng C. A. Meyer is the most popular herbal medicine widely used as general tonic to improve health and vitality. Ginsenosides are the pharmacological active phytochemicals of ginseng which belong to a family of triterpenoid saponins. Three types of ginsenosides have been classified, they are protopanaxadiols (e.g., Rb1, Rb2, Rg3 and Rh2), protopanaxatriols (e.g., Re, Rg1 and Rg2), and oleanolic acid derivatives. Depend on the processing methods, composition of different ginsenosides in a ginseng may be alternated; steaming of fresh ginseng for 2 to 3 h can produce red ginseng and enrich the amount several ginsenosides species such as Rg3, the amount of Rg3 may increase from 0.37 % to 1.32 % (w/w). Structurally, ginsenosides contain a hydrophobic triterpenoid skeleton attached with hydrophilic sugar moieties or hydroxyl groups at carbon-3, -6 and -20. Protopanaxadiol ginsenosides 20(S)-Rg3 and 20(R)-Rg3 are epimeric pair that differ in the position of hydroxyl group on the chiral center at carbon-20 (C20) (Figure 1A and B). During chemical synthesis of ginsenosides, the major ginsenosides, such as Rb1, Rb2, and Rd can be readily converted into mixture of 20(S)-Rg3 and 20(R)-Rg3 stereoisomers by either acid treatment or heating, but the isolation of each isomer from the mixture is rather difficult. Indeed, ginsenosides Rg2, Rg3, Rh1, Rh2, and Rs3 all exist as stereoisomer pairs. Although stereoselectivity of ginsenosides on ion channel, effects on coronary artery contractions, anti-tumor effects, osteoclastgenesis inhibition, and endothelial cell apoptosis have been described previously; detail and molecular exposition based on the structural difference of ginsenoside stereoisomers has not been emphasized.

The mechanistic action of ginsenoside has been firstly suggested that Rg1 can interact with glucocorticoid receptor. After that, other ginsenosides such as Rb1 and Rg3 were demonstrated to activate estrogen receptor, and peroxisome proliferator-activated receptor (PPAR). Nuclear receptors belong to a class of ligand dependent transcriptional factors which regulate diverse physiological functions. The natural ligands of PPAR are polyunsaturated fatty acids. PPARs control many important metabolic functions, and PPAR is the best-studied member of the family. PPAR is predominantly expressed in adipocytes and some other metabolic active tissues. It is also initially found to be critical in lipid homeostasis and adipocyte differentiation. Recent studies have implicated the role of PPAR on regulating angiogenesis. The commercially available anti-diabetic drug rosiglitazone, which is a PPAR agonist, can increase endothelial progenitor cells functions via upregulating the Akt-eNOS signaling pathway. Rosiglitazone also promotes angiogenesis and increases eNOS expression in endothelial cell after focal cerebral ischemia. Similarly, prolonged treatment with troglitazone, another PPAR agonist, increases endothelial nitric oxide (eNOS) production though PPAR-dependent signaling pathway. These studies highlighted the potential role of PPAR on angiogenesis. Moreover, it also pointed the interesting therapeutic potential for PPAR agonist.

Angiogenesis is defined as the formation of new blood capillaries from pre-existing vascular networks. It is an essential physiological process during embryogenesis, pregnancy and wound healing. Besides, angiogenesis is also contributed to the pathogenesis of diabetic retinopathy, rheumatoid arthritis and tumor development. Distinct but tightly regulated events are involved during angiogenesis, including degradation of extracellular matrix, proliferation, migration of endothelial
cells, and formation of capillary lumen. In the present study, we presented evidences to support the differential angiogenic properties of Rg3 stereoisomers at micromolar level on human endothelial cells. In addition, we identified PPARγ as the molecular target which mediated the differential actions of Rg3 stereoisomers.
Methods

Analyses of Ginsenosides by UPLC-MS
Analyses of ginsenoside Rg3 stereoisomers were carried out on a Waters ACQUITY UPLC™ system (Waters Corp., MA, USA) which was equipped with a binary solvent delivery system and a sample manager coupled to Bruker MicroTOF mass spectrometer with an ESI source. The chromatography was performed on an Acquity BEH C\textsubscript{18} column (2.1 × 100 mm, 1.7μm). The mobile phase consisted of water containing 0.1 % formic acid (A) and acetonitrile containing 0.1 % formic acid (B). Optimized UPLC elution condition as following was employed: linear gradient from 28 % to 55 % B at 0-15 min, then increase to 70 % B at 15-19 min, and finally from 70 to 100 % B at 19 – 19.5 min. The ESI-MS data were acquired in negative mode and conditions for MS analysis were as follows: end plate offset, -500 V; capillary voltage, 4500 V; nebulizing gas (N\textsubscript{2}) pressure, 1.5 bar; drying gas (N\textsubscript{2}) flow rate, 8.0 L/min; drying gas temperature, 200 °C; mass range, m/z 300 - 1500.

Cell Culture and Treatment
Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Walkersville, MD, USA) and were maintained in passages 2 – 8 to ensure genetic stability. Cells were cultured in M199 medium supplemented with heparin (90 mg/L), heat-inactivated FBS (20 %, v/v), ECGS (20 μg/ml), and PS (1 %, v/v), and kept at 37 °C in a humidified 5 % CO\textsubscript{2} incubator. HUVECs were seeded and treated with ginsenoside Rg3 in M199 containing 1 % FBS. Ginsenosides were obtained from Fleton (Chengdu, China) with purity over 98 %.

Cell Proliferation Assay
HUVECs (1 × 10\textsuperscript{4} cells/well) were seeded onto 96-well plates overnight. After drug treatment for the indicated time, cell proliferation was determined by the (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and bromodeoxyuridine (BrdU) incorporation ELISA. The MTT assay was based on the cleavage of the yellow tetrazolium salt to purple formazan crystal by living cells. Briefly, cells were incubated with MTT solution (0.5 mg/ml) in assay medium for 4 h. The formazan product was solubilized by DMSO after complete removal of medium; absorbance at wavelength of 540 nm and 690 nm (reference) was measured by the microplate reader (Infinite F200, Tecan; Männedorf, Switzerland). Cell proliferation was also determined by using BrdU incorporation kit (Roche Biochemicals, Milan, Italy). The cells were labeled with BrdU (10 μM) for 2 h according to the manufacturer’s protocol. Absorbance at 450 nm was measured by microplate reader.

Cell Migration Assay
HUVECs (3 × 10\textsuperscript{4} cells/well) were seeded on 96-well plates overnight. An artificial wound was created by mechanical scratching of the cell monolayer using home-made mechanical wounder.\textsuperscript{18} The sharp wound in each well was captured. Culture medium was replaced with fresh medium containing ginsenoside Rg3 (0.01 – 30 μM), cells were then incubated for further 24 h and the wounded area of each well was captured again. Images at 0 and 24 h were analyzed using Java’s Image J software (http://rsb.info.nih.gov). The migration of cells towards the denuded area was expressed as percentage of recovery. Percentage of recovery = A\textsubscript{0} – A\textsubscript{24} / A\textsubscript{0} × 100 % where A\textsubscript{0} is the wounded area measured immediately after scratching, A\textsubscript{24} is the wounded area measured 24 h after wounding.
Tube Formation Assay
The 96-well plate first precoated with growth factor reduced-Matrigel (BD Bioscience, San Jose, CA, USA) was allowed to solidify at 37 °C for 1 h. HUVECs were then plated with or without drug in 1 % FBS supplemented medium. Images of each well were captured after 24 h. The angiogenic activities were determined by counting the branch points of tubes formed in each well.

Aortic Ring Sprouting Assay
The rat aortic rings were prepared and treated as described previously. Briefly, aortic fragments were first isolated from rat, and the divided fragments were sandwiched in two layer of growth factor reduced Matrigel matrix in 96-well. The cultures were incubated in 200 µl endothelial growth medium containing ECGS (200 mg/ml) or Rg3 stereoisomers. Culture medium was refreshed on day 4 and the microvascular outgrowths were determined according to their morphology. Images were captured using digital camera attached on a stereomicroscope.

Western Blot Analysis
Treated or untreated cells were washed with ice-cold PBS twice, and were lysed by ice-cold cell lysis buffer (Novagen, USA) supplemented with protease inhibitor cocktail (1 %, v/v) and phosphatase inhibitor cocktail (0.5 %, v/v) (Calbiochem, San Diego, CA, USA). The cells were scraped off by rubber policeman, and the protein in the supernatant was collected after centrifugation. The concentration of protein was determined by Dc protein assay (Bio-Rad, Hercules, CA, USA). Equal amount of protein was separated by 10 % SDS-PAGE followed by electroblotting onto nitrocellulose transfer membrane. The membrane blocked with blocking buffer was then incubated with primary antibodies. The washed membrane was then further incubated with horseradish peroxidase-conjugated goat-anti-rabbit IgG or horseradish peroxidase-conjugated goat-anti-mouse IgG (Invitrogen). The protein band was visualized by Chemiluminescent Western Detection Kit (Bio-Rad).

PPARγ time-resolved fluorescence resonance energy transfer (TM-FRET) competitive binding assay
PPARγ LanthaScreen™ TR-FRET competitive binding assay (Invitrogen) was used to study the binding between ginsenosides Rg3 and PPARγ. This assay allowed high-throughput robust screening of ligands for PPARγ. The method is based on the principle that when two suitable fluorophores are brought within close proximity of each other, excitation of the first fluorophore (donor) can result in energy transfer to the second fluorophore (acceptor). This energy transfer resulted in an increase in the fluorescence emission of the acceptor and a decrease in fluorescence emission of the donor. In this assay, the GST-tagged PPARγ-LBD was combined with terbium-labeled anti-GST antibody. When a green fluorescent pan-PPAR ligand (tracer) is bound to the receptor, energy transfer from the antibody to the tracer occurs, and a high TR-FRET ratio is observed. The ability of GW1929 (1 pM – 100 µM), a PPARγ specific agonist, and ginsenoside Rg3 (10 nM – 100 µM) to displace the tracer from LBD was tested, which results in a loss of FRET ratio. The binding curves were fitted with a two-state one-site competition model by the Prism software (GraphPad software Inc., San Diego, CA, USA).

PPARγ Reporter Gene Assay
PPARγ reporter gene assay were performed in human dermal fibroblast (HDFs). Cells were seeded in 24-well plate overnight, then cells were starved in serum-free Opti-MEM for another 24 h and transiently transfected with reporter plasmid peroxisome proliferator-activated receptor response element (PPRE)X3-TK-luc (contains three copies of DR-1 response elements upstream of a luciferase reporter) (250 ng/well) (Addgene plasmid 1015), expression plasmid pSG5-PPARγ (125 ng/well), and pSG5-RXRα (125 ng/well) using Lipofectamine LTX transfection reagent (1.5 µl/well) (Invitrogen). After 6 h of transfection, cells were washed once with Opti-MEM and further incubated with troglitazone (PPARγ agonist) or ginsenosides for 24 h. Cells were lysed by passive reporter lysis buffer; luciferase activity was measured using Luciferase Assay System (Promega, Madison, WI, USA) with microplate luminometer (Tecan). Luciferase activity was normalized by amount of protein of cell lysate.

Small Interference RNA Transfection
Cells were transiently transfected with PPARγ-siRNA (50 nM) complexed with RNAiMAX transfection reagent (1.5 µl/well) (Invitrogen) in Opti-MEM for 24 h. Cells were rinsed with Opti-MEM followed by drug treatment. Non-sequence-specific negative control siRNA (Dharmacon, Lafayette, CO, USA) was used in parallel with PPARγ-siRNA.

Statistical Analysis
All results were expressed as mean ± standard derivation (S.D.) of at least 3 independent experiments. Statistical differences between two groups were analyzed by Student’s t test.
Results

Characterization of Rg3 Stereoisomers by UPLC-MS

To demonstrate the purity of each ginsenoside Rg3 stereoisomers, UPLC-MS was used. Under the optimized conditions mentioned in materials and methods, 20(S)-Rg3 and 20(R)-Rg3 can be separated with good resolution (Figure 2B and C). 20(S)-Rg3 and 20(R)-Rg3 were characterized by comparing the values of UPLC retention time with those of the standards.

Differential Angiogenic Effects of Rg3 Stereoisomers

Differential HUVECs proliferation was observed after treatment with ginsenosides-Rg3 stereoisomers for 24 h. 20(S)-Rg3 (15 µM) increased 50 % of cell proliferation, while only 10 % of cell proliferation was stimulated by the same concentration of 20(R)-Rg3 (Figure 3A). Furthermore, by using the BrdU incorporation assay, which is a direct indicator for DNA synthesis during cell proliferation, 20(S)-Rg3 (15 µM) stimulated 20 % of HUVECs DNA synthesis; while 20(R)-Rg3 showed a lower rate of DNA synthesis when compared with solvent control (Figure 3B). Then the migration of Rg3 stereoisomers on cell motility was further examined in cell migration and tube formation assay. As shown in Figure 3C, only 20(S)-Rg3 (15 – 30 µM) can increase endothelial cells migration, while 20(R)-Rg3 exerted no significant effect on cell migration. In the tube formation assay, result showed that micromolar level of 20(R)-Rg3 could also induce tubular network formation. However, the extent of tube formation was not as much as in the case of 20(S)-Rg3, in which the average number of tube induced by 20(S)-Rg3 was about 90, while in the case of 20(R)-Rg3, it could only induce 60 tubes in each well (Figure 3D, lower panel). Furthermore, the differential angiogenic properties between 20(R)-Rg3 and 20(S)-Rg3 were further evaluated by ex vivo culturing of rat aortic fragments; the endothelial cell sprouting was examined. ECGS, as a positive control, induced microvascular sprouting when compared with untreated control; while 20(S)-Rg3 (10 µM) can also increase microvascular sprouting but not 20(R)-Rg3 (Figure 3E). These findings demonstrated that 20(S)-Rg3 at micromolar level is a lot more potent than 20(R)-Rg3 in promoting angiogenesis.

20(S)-Rg3 Activates AKT, ERK and eNOS to Induce HUVECs Proliferation

The signaling pathways activated by Rg3 to induce angiogenesis were then studied. Rapid and sustained phosphorylation of ERK in HUVECs were found after 20(S)-Rg3 (15 µM) treatment within 2 h; whereas no effect was observed in 20(R)-Rg3 treated cells (Figure 4A). In addition, sequential increase of phosphorylation of AKT at Ser473 and eNOS at Ser1177 was found after 60 min of 20(S)-Rg3 treatment. Again, 20(R)-Rg3 did not shown similar effect. Moreover, the pretreatment of L-NAME, PD98059 and LY294002, which are the eNOS, ERK and PI3K pharmacological inhibitors, respectively, could partially but significantly abolish 20(S)-Rg3-induced HUVECs proliferation (Figure 4B), indicated that activation of eNOS, AKT and ERK were involved in 20(S)-Rg3-induced HUVECs proliferation.

Rg3 Stereoisomers Differentially Activate PPARγ
To examine the direct binding of Rg3 stereoisomers on PPARγ, time-resolved fluorescence resonance energy transfer (TR-FRET) high-throughput screening assay was performed (Figure 5A). GW1929, a potent synthetic PPARγ agonist, is served as a positive control. Binding of ligand on PPARγ leads to displacement of fluoromone from the ligand binding domain, which will result in decrease of fluorescent polarization. The binding assay demonstrated that only 20(S)-Rg3 but not 20(R)-Rg3 can bind on PPARγ. Although the binding affinity of 20(S)-Rg3 is much less specific when compared with GW1929, direct binding was observed when the concentration of 20(S)-Rg3 is increased to about 10 μM, which is the concentration capable to induce angiogenesis. To demonstrate 20(S)-Rg3 is a functional ligand of PPARγ, PPARγ luciferase reporter gene assay was performed. After treating with Rg3 in HDFs which were transiently transfected with reporter plasmid containing PPRE, 20(S)-Rg3 significantly increased luciferase activity in a dose-dependent manner (0.1 μM to 10 μM) which was comparable with that of troglitazone (5 μM). 20(R)-Rg3 only induced 1.5-fold luciferase activity at 10 μM (Figure 5B). Therefore, the PPARγ transcriptional activity activated by 20(S)-Rg3 is about 10-times more potent than 20(R)-Rg3. This suggested that the structural difference between 20(S)-Rg3 and 20(R)-Rg3 is critical for effective activation of PPARγ. Using computational modeling, the docking results suggest that both 20(R)-Rg3 and 20(S)-Rg3 occupy similar spatial position as that of the anti-diabetic drug rosiglitazone in the ligand-binding domain (LBD) of PPARγ at the C20 chiral center of the molecule locating close to the helix-12, and their disaccharide groups are proximal to the β-sheet of the protein (Figure 6). Furthermore, it can be seen that the hydroxyl group at the C20 chiral center of the 20(S)-Rg3 interacts with Tyr473 through hydrogen bond. Similar interaction is also observed in the crystal structure of PPARγ in complex with rosiglitazone in which the thiazole group of the drug hydrogen bonds to the side chain of Tyr473. In contrast, the sterically strained binding pocket does not allow the 20(R)-Rg3 to interact optimally with Tyr473. Interaction with Tyr473 in helix-12 of PPARγ is required for the functional activity of full agonists. Mutation of Tyr473 to alanine abolishing the full agonistic activity of PPARγ has been reported. The distinctive ligand-binding modes may contribute to the different binding affinity and the observed biological activity of the stereoisomers.

**Angiogenic Effects of Rg3 is Abolished by PPARγ-siRNA**

To verify the role of PPARγ in Rg3-induced angiogenesis, PPARγ-siRNA was used to deplete PPARγ expression (Figure 7A). PPARγ-specific siRNA can significantly suppress the 20(S)-Rg3-induced HUVECs proliferation (Figure 7B), migration (Figure 7C), tube formation (Figure 7D) and ERK/AKT/eNOS phosphorylation (Figure 7E). In fact, no significant effect was found on 20(R)-Rg3-treated cells. These results clearly demonstrated that PPARγ is essential for 20(S)-Rg3-induced angiogenesis. In addition, the 20(S)-Rg3-induced HUVECs proliferation, migration and eNOS, ERK, and AKT phosphorylation can also be inhibited by pretreatment with PPARγ-specific antagonist, GW9662 (online-only Data Supplement Figure 1). Furthermore, pretreatment with PPARα or PPARβ/δ specific antagonists, GW6471 and GSK0660 (online-only Data Supplement Figure 2A and 2B) exerted no inhibitory effect on Rg3-induced HUVECs proliferation, which further confirmed that the specific role of PPARγ in Rg3-induced angiogenesis.
Discussion

Ginsenosides account for the multiple pharmacological effects of ginseng. Single ginsenoside, such as, Rg3 and Rh2 are suggested to possess anti-tumor and angiomodulatory activity.24-26 The anti-inflammatory effects of Rh1,27 and neuroprotective effects of Rg2 were also reported.28 However, these ginsenosides exist as stereoisomers. The importance of drug stereoselectivity is gaining greater attention in recent years,29 but little is known about the pharmacology of ginsenosides’ stereoisomers. Many studies did not report the stereospecificity of the ginsenosides, whether they are in the form of racemate mixture, or the particular ratio of ginsenosides’ stereoisomers. The disregard of stereochemistry of ginsenosides may lead to disparate results in vitro when compared with in vivo assessments of potency.30-32 It is because even both stereoisomers of a ginsenoside possess the same functional group at C20, but the difference in three-dimensional structure may affect the binding affinity of corresponding binding site. It has been shown that 20(S)-Rh2, the immediate metabolite of 20(S)-Rg3, exhibits greater cellular uptake than 20(R)-Rh2 properly due to greater affinity with stereospecific ABC transporter.33 In the present study, we have identified PPARγ as the molecular target which mediates the angiogenic effects of ginsenoside Rg3 stereoisomer. Because PPAR ligand displays a higher degree of stereoselectivity,34 here we have showed that Tyr473 seems to be critical to distinguish the differential pharmacological activity of two Rg3 stereoisomers based on the result of computational modeling. It has been recently demonstrated that 20(S)-Rg3 but not 20(R)-Rg3 showed measurable binding to PPARγ using a novel polarization anisotropy complemented with total internal reflection fluorescence microscopy technique.35 From the results of reporter gene assay, both Rg3 stereoisomers activate PPARγ transcription; however, 20(S)-Rg3 showed 10-fold more potent than 20(R)-Rg3. Hence, this indicated that the C20 of Rg3 may be important for stable docking of Rg3 onto the receptor, so as to elicit downstream activities.

PPARγ has diverse role on regulation of glucose and lipid metabolism, inflammation, and immune response. Ligands of PPARγ are reported to have both angiogenesis-promoting and anti-angiogenic effects depends on interactions with co-activator and co-repressor.36 Ginseng extract and ginsenosides are suggested to interact with PPARγ in recent years. Among the 30 types of ginsenosides, RF is first described to regulate lipoprotein metabolism through PPARα;37 subsequently, several ginsenosides including Rg3, Rh2 and PPT are demonstrated to regulate adipogenesis by activating PPARγ and related signalling.38-40 In fact, from the result of PPARγ reporter gene assay, 20(S)-Rg3 can activate PPARγ at micromolar level. Although the binding affinity of 20(S)-Rg3 is much lower than those synthetic agonists such as rosiglitazone (EC50 = 0.043 µM) and troglitazone (EC50 = 0.55 µM), 20(S)-Rg3 may act as a partial agonist of PPARγ. As several reports have shown that, rosiglitazone, which previously used for treating type II diabetes, may increase the risk of myocardial infraction and death from cardiovascular diseases.41 Along the line, a dietary supplement containing 20(S)-Rg3 may act as an alternative or compensation of PPARγ agonist, which on one hand acts as PPARγ agonist, on the other hand, avoiding the adverse effects induced by those full agonists.

Rg3 were suggested to increase eNOS phosphorylation via estrogen receptor-dependent PI3K signaling.42 In this report, we showed that ginsenoside Rg3
differentially induces ERK/AKT-eNOS signaling pathways via activation of PPARγ, and induces HUVECs proliferation, migration, and tube formation. The ERK and AKT are upstream kinases which can phosphorylate eNOS at Ser1177,\(^\text{1,43}\) the activation of ERK/AKT and eNOS by 20(S)-Rg3 may enhance NO production and contribute to the rapid vasodilating effects of \textit{Panax ginseng}.\(^\text{44}\) The phosphorylation of ERK/AKT-eNOS by Rg3 is PPARγ dependent, as demonstrated by PPARγ specific inhibitor. However, linkage between PPARγ and ERK/AKT phosphorylation is still not clear in this model. Indeed, increasing evidences suggested that natural and pharmacological PPARγ ligands induce rapid non-transcriptional effects in different cell type due to the extranuclear trafficking of PPARγ, and direct interaction of PPARγ with ERK and its upstream kinases was demonstrated.\(^\text{45}\) In addition, ERK and AKT can be phosphorylated by several receptor tyrosine kinases (RTK) such as c-Met, EGFR, VEGFR or FGFR, and PPARγ has been demonstrated to interact with RTKs to phosphorylate ERK.\(^\text{46}\) So, Rg3 may activate RTKs through binding with PPARγ to activate the downstream ERK/AKT-eNOS signaling pathways.

Although previous study from our laboratory demonstrated that ginsenoside 20(R)-Rg3 can reduce angiogenic activity of HUVECs, by suppressing cell proliferation, chemoinvasion and anti-MMP expression.\(^\text{19}\) The concentration of 20(R)-Rg3 used in that study is in nanomolar level, which is different from the micromolar concentration used in the present study. It is not surprise that a single compound can act on different targets at different concentrations. It was showed that genistein, the soy phytoestrogen, can activate estrogen receptor at low concentration (< µM) and inhibit adipogenesis; however, at high concentration (> µM), it promotes adipogenesis through acting as a ligand of PPARγ.\(^\text{47}\) The authors further suggested that may explain why genistein functions only at certain level, as genistein plasma level of humans consuming diets without soy is only 40 nM, but can reach 4 µM in Japanese who consume high amount of soy products. This may also imply the contradictory activities of Rg3 on modulating angiogenesis. If low concentration is applied, it may act on an unidentified target and exert its angiosuppressive effects; but may promote angiogenesis at high concentration through binding with PPARγ. In addition to the concentration differences, the conditions of drug treatment may also lead to the different observation. The endothelial cells are treated in high serum and are stimulated with VEGF, which mimic the tumor angiogenesis condition in the previous study. But in the present study, endothelial cells are treated with low serum concentration, which may resemble the normal and non-activated status (quiescence) of endothelial cells. So, ginsenoside Rg3 may suppressive angiogenesis when endothelial cells are activated, but may promote angiogenesis when the cells are unstimulated. This phenomenon is also consistent with the Yin and Yang actions of Chinese medicine which emphasize the importance of balance.

In summary, ginsenoside stereoisomers 20(S)-Rg3 and 20(R)-Rg3 differentially induce angiogenesis, in which 20(S)-Rg3 significantly induces HUVECs proliferation, cell migration and tube formation, while 20(R)-Rg3 showed no effect. The underlying mechanisms may due to the differential activation of PPARγ and the downstream ERK/AKT-eNOS signaling. This study can enhance our understanding on the importance of ginsenoside stereochemistry and the angiogenic activity of Rg3. More importantly, the results stress the slight structural difference between two ginsenoside stereoisomers can lead to significant differential
physiological outcomes which should be careful considered in the future development of ginsenoside-based therapeutics.
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Figure legends

Figure 1. Chemical structures of ginsenoside (A) 20(S)-Rg3 and (B) 20(R)-Rg3.

Figure 2. UPLC-TOF-MS chromatograms of (A) racemic Rg3, (B) 20(S)-Rg3, and (C) 20(R)-Rg3.

Figure 3. Differential effects of Rg3 stereoisomers on angiogenesis. HUVECs were treated with either form of Rg3 stereoisomers for 24 h. Cell proliferation was determined by (A) MTT proliferation assay and (B) BrdU incorporation assay. Values are expressed as the percentage of cell proliferation relative to the solvent control. (C) Rate of cell migration was determined as described in materials and methods (lower panel), representative photomicrographs of wounded monolayers of HUVECs (upper panel). (D) Effect of tube formation after Rg3 treatment, average number of tubes formed in each well (lower panel), representative photomicrographs of tube formation. (E) Differential effect of Rg3 stereoisomers on ex vivo endothelial sprout, representative photomicrographs of microvascular sprouting in indicated conditions. Values are presented as mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs DMSO control.

Figure 4. Rg3-induced proliferation of HUVECs is dependent on eNOS, AKT, and ERK1/2 activation. (A) Differential phosphorylation of eNOS, Akt, and ERK. HUVECs were treated with either form of Rg3 stereoisomers for the indicated time. Phosphorylated and total form of eNOS, Akt and ERK was detected by Western blot analysis. A representative Western blot of three independent experiments was shown. (B) Effects of various inhibitors on the proliferation of HUVECs. HUVECs were pretreated with indicated concentration of L-NAME (eNOS inhibitor), LY294002 (PI3K inhibitor), or PD98059 (ERK inhibitor) for 1 h, then co-incubated with either form of Rg3 stereoisomers for another 24 h. Cell proliferation was determined by MTT proliferation assay. Values are presented as mean ± SD of three independent experiments. ***p<0.001 vs DMSO control. ###p<0.001 vs 20(S)-Rg3 treatment alone.

Figure 5. Differential binding and activation of PPARγ by stereoisomers of Rg3. (A) The competitive binding of Rg3 stereoisomers were determined by a time-resolved fluorescence resonance energy transfer (TR-FRET) high-throughput screening assay. GW1929 served as a positive control. Values are presented as mean ± SD of two independent experiments. (B) HDFs were transiently transfected with luciferase reporter plasmid containing three copies of PPRE together with PPARγ and RXRα expression plasmids. Luciferase reporter activity was measured 24 h after incubation of transfected cells with troglitazone and different concentrations of 20(R)-Rg3 and 20(S)-Rg3. Troglitazone was used as positive controls. Values are presented as mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs DMSO control.

Figure 6. Docking structures of (A) 20(S)-Rg3 and (B) 20(R)-Rg3 in the LBD of PPARγ. The ginsenosides and side chain of Tyr473 in helix-12 (H-12) are shown in sticks. Crystal structure of rosiglitazone in PPARγ (PDB: 1ZGY) is aligned and shown in grey for comparison. The docked 20(S)-Rg3 adopts a position similar of rosiglitazone in the protein and the hydroxyl group at the C20 position interacts with
the side chain of Tyr473 (dotted line). The docked 20(R)-Rg3 occupies similar position as the 20(S)-Rg3 in the protein but only the methyl group at the C20 position points towards Tyr473 and does not form any effective hydrogen bond. The protein backbones are rendered as faded out ribbons. Hydrogen atoms are not shown for clarity. Both Rg3 stereoisomers were docked into the protein by using Autodock 4.0.48 while the receptor protein structure is directly obtained from the protein databank (PDB: 2I4J).49

Figure 7. Rg3 induced angiogenic effects are dependent on PPARγ activation. HUVECs were transiently transfected with PPARγ-siRNA (50 nM) for 24 h before treatment of ginsenosides. (A) PPARγ-siRNA inhibited PPARγ protein expression. PPARγ-siRNA abolished 20(S)-Rg3-induced (B) HUVECs proliferation, (C) HUVECs migration, (D) tube formation and (E) eNOS, AKT and ERK phosphorylation. Values are presented as mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs DMSO control. #p<0.05, ##p<0.01, ###p<0.001 vs 20(S)-Rg3 treatment alone.
Figure 1.

(A) 20(S)-Rg3

(B) 20(R)-Rg3
Figure 2.
Figure 3.

(A) Cell proliferation (%)

(B) BrdU incorporation (%)

Variations in cell proliferation and BrdU incorporation with different concentrations of Rg3.
Figure 4.

(A) 

(B) 

Cell proliferation (%)
Figure 5.

(A)

(B)

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<tr>
<td>Troglitzone (5 μM)</td>
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<tr>
<td>20(S)-Rg3 (μM)</td>
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</tr>
<tr>
<td>20(R)-Rg3 (μM)</td>
<td>- - - 0.1 1 10</td>
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Statistical significance:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001
Figure 6.
Figure 7.

(A)

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</tr>
<tr>
<td>siPPARγ (50 nM)</td>
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(B)

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<tr>
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<td>NS-siRNA (50 nM)</td>
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<td>PPARγ siRNA (50 nM)</td>
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**Significance:**

- *** p < 0.001
- ** p < 0.01