## 20(S)-ginsenoside Rh2 inhibits hepatocellular carcinoma by suppressing angiogenesis and the GPC3-mediated Wnt/β-catenin signaling pathway

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<th>Journal:</th>
<th><em>Acta Biochimica et Biophysica Sinica</em></th>
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<td>Manuscript ID</td>
<td>ABBS-2023-512.R3</td>
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<tr>
<td>Manuscript Type:</td>
<td>Original Article</td>
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<tr>
<td>Date Submitted by the Author:</td>
<td>24-Dec-2023</td>
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<td>Complete List of Authors:</td>
<td>Xu, Lin fei; Affiliated Hospital of Weifang Medical University Li, Jing; Affiliated Hospital of Weifang Medical University Hou, Ningning; Affiliated Hospital of Weifang Medical University Han, Fang; Affiliated Hospital of Weifang Medical University Sun, Xiangdong; Affiliated Hospital of Weifang Medical University Li, Qin ying; Affiliated Hospital of Weifang Medical University,</td>
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<td>Keywords:</td>
<td>20(S)-Rh2, Angiogenesis, Hepatocellular carcinoma, GPC3/Wnt/β-catenin</td>
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Original Article

20(S)-Ginsenoside Rh2 inhibits hepatocellular carcinoma by suppressing angiogenesis and the GPC3-mediated Wnt/β-catenin signaling pathway

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Running title: Anti-liver cancer effect of the ginsenoside Rh2

Received: 29 October 2023
Accepted: 14 December 2023

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Abstract

20(S)-Ginsenoside Rh2 has significant anti-tumor effects in various types of cancers, including human hepatocellular carcinoma (HCC). However, its molecular targets and mechanisms of action remain largely unknown. Here, we aim to elucidate the potential mechanisms by which Rh2 suppresses HCC growth. We first demonstrate the role of Rh2 in inhibiting angiogenesis. We observe that Rh2 effectively suppresses cell proliferation and induces apoptosis in HUVECs. Furthermore, Rh2 significantly inhibits HepG2-stimulated HUVEC proliferation, migration and tube formation, accompanied by the downregulation of VEGF and MMP-2 expressions. We also reveal
that Rh2 inhibits HCC growth through the downregulation of glypican-3-mediated activation of the Wnt/β-catenin pathway. We observe a dose-dependent inhibition of proliferation and induction of apoptosis in HepG2 cells upon Rh2 treatment, which is mediated by the inhibition of glypican-3/Wnt/β-catenin signaling. Moreover, downregulation of glypican-3 expression enhances the effects of Rh2 on the glypican-3/Wnt/β-catenin signaling pathway, resulting in greater suppression of tumor growth in HepG2 cells. Collectively, our findings shed light on the molecular mechanisms through which Rh2 modulates HCC growth, which involve the regulation of angiogenesis and the glypican-3/Wnt/β-catenin pathway. These insights may pave the way for the development of novel therapeutic strategies targeting these pathways for the treatment of HCC.

Key words: 20(S)-Rh2, angiogenesis, hepatocellular carcinoma, GPC3/Wnt/β-catenin

Introduction

Hepatocellular carcinoma (HCC) is a prevalent and highly lethal tumor characterized by rapid deterioration, poor overall prognosis, and a high recurrence rate [1]. Patients who suffer from chronic liver diseases, such as cirrhosis and hepatitis, are particularly susceptible to developing HCC [1]. The incidence of HCC in younger individuals is increasing, primarily due to the increasing incidence of obesity and fatty liver disease [2]. Early detection and appropriate treatment of HCC are crucial for achieving favorable outcomes and improving the overall quality of life of patients.

Angiogenesis is a pivotal process in the progression of solid tumors [3]. In particular, HCC relies heavily on a well-established blood supply to fuel its growth and enable metastasis [4]. Central to the regulation of angiogenesis are vascular endothelial growth factors (VEGFs) and their corresponding receptors, which play critical roles in this intricate process [5]. Notably, overexpression of VEGF in HCC significantly augments tumor expansion and metastatic potential, leading to a dismal prognosis and increased aggressiveness [6]. In concert with VEGF, matrix metalloproteinases (MMPs) contribute to angiogenesis by degrading the extracellular matrix (ECM) and activating pro-angiogenic factors [7]. MMPs are excessively expressed in HCC, further facilitating tumor development [8]. Consequently, targeting angiogenesis is considered a highly promising treatment avenue for HCC.

Glypican-3 (GPC3), a cell-surface glycoprotein, is prominently upregulated in HCC.
Its elevated expression triggers Wnt/β-catenin activation, thereby promoting HCC proliferation, invasion, and metastasis [9,10]. Abnormal activation of the Wnt/β-catenin pathway is widely recognized as a hallmark of cancer, where it drives growth, migration, and invasion [11,12]. Therefore, GPC3 holds great promise as a potential therapeutic target, and interventions aimed at modulating the GPC3/Wnt/β-catenin pathway present novel prospects for cancer treatment.

Ginseng, a medicinal and edible plant that has been popular in China and other Asian countries for centuries, is known to promote longevity and enhance body functions [13]. Ginsenosides are the primary bioactive ingredients in ginseng roots. To date, more than 100 types of ginsenosides have been identified. Among them, the ginsenoside Rh2, a protopanaxadiol-type ginsenoside, exhibits various effects, including anti-oxidant, anti-inflammatory, and anti-tumor activities [14]. In particular, the broad application of Rh2 in cancer treatment has been reported, mainly because it inhibits proliferation, invasion, and metastasis [15–17]. However, the potential effects of Rh2 on HCC angiogenesis and GPC3 targeting remain poorly understood.

Here, we investigated the effect of Rh2 on angiogenesis and GPC3/Wnt/β-catenin signaling, which leads to the repression of HCC growth. Our results indicated that Rh2 inhibited HCC angiogenesis via the suppression of VEGF and MMP-2 expression. Furthermore, Rh2 treatment decreased GPC3 expression by downregulating the Wnt/β-catenin pathway. Our findings demonstrated the anti-HCC effect of Rh2 via inhibiting angiogenesis and the GPC3/Wnt/β-catenin signaling pathway.

**Materials and Methods**

**Reagents and cell lines**

HepG2 cells (HCC cell line, Cat. CL-0103) were obtained from Procell Life Science & Technology (Wuhan, China). HUVECs (human umbilical vein endothelial cells, Cat. CRL-4053) were purchased from ATCC (Manassas, USA). 20(S)-Ginsenoside Rh2 with a purity > 98% was acquired from Solarbio Biotechnology (Beijing, China) and was dissolved in dimethyl sulfoxide. Rh2 (0, 2, 5, 10, or 20 mg/L) was added to HepG2 cells and cultured in DMEM medium for 24 h. Next, the cell culture medium was harvested and filtered as conditioned medium (CM), and the resulting CM-Rh2 was stored at −80°C.

**Cell counting kit-8 (CCK8) assay**
Cells were seeded in 96-well plates and treated with various doses of drugs (Rh2 or CM-Rh2 for HUVECs, Rh2 for HepG2 cells) for 24 h. Then, 10 μL of CCK-8 solution (SparkJade Biotechnology Co., Ltd., Jinan, China) was added to each well, and the absorbance was measured at 450 nm after 2 h.

**Flow cytometric analysis**
Cells were plated in a six-well plate at 2×10^5 cells/well and cultured overnight, and then incubated with Rh2 for another 24 h. After that, the cells were collected and treated with an ANNEXIN V-FITC/PI apoptosis detection kit (Solarbio). Flow cytometry and FlowJo software (v 10.8; Becton Dickinson, Franklin Lakes, USA) were utilized to analyze the samples. For cell cycle analysis, the cells were collected following the apoptosis procedure. Then, the collected cells were fixed, stained with a DNA quantitation assay kit (Solarbio) and analyzed by flow cytometry.

**JC-1 staining assay**
HUVECs were seeded in 6-well plates and treated with Rh2 for 24 h. Then, JC-1 staining solution (Solarbio) was added into each well. Following a 20-min incubation at 37°C, the cells were washed twice with JC-1 staining buffer (Solarbio). Next, 1640 culture medium was added into cells, and observed under a fluorescence microscope.

**Wound healing assay**
HUVECs were seeded in a 6-well plate and incubated until they reached 90~100% confluence. A sterile yellow tip was used to create a wound on the bottom of the plate. Subsequently, HUVECs were incubated with CM-Rh2, and images of the same angular scratches were taken at two days by an inverted microscope.

**Tube formation assay**
Fifty-five microliters/well of cold Matrigel was added to a 96-well plate, which was then incubated at 37°C for solidification. HUVECs (3×10^4 cells/well) were seeded onto Matrigel with various concentrations (0, 2.5, 5, 10, or 20 mg/L) of CM-Rh2. After 6 h, tube formation was observed using an inverted microscope.

**Cell transfection**
siRNAs targeting GPC3 (siGPC3) and a negative control siRNA (siNC) were
synthesized by Sangon Biotech (Shanghai, China) (Supplementary Table S1). HepG2 cells (4 × 10^5 cells/well) were seeded in six-well plates and reached ~50% confluence after 24 h. Next, the siRNA or siNC (2 µL) was diluted with 190 µL of Opti-MEM, followed by the addition of 8 µL of Hieff Trans™ siRNA transfection reagent (YEASEN, Shanghai, China) immediately. Following a 10-min incubation at room temperature, the transfection complex was added to HepG2 cells directly. After 24 h of incubation, the media were discarded, and different doses of Rh2 were added and incubated for another 24 h. Finally, the cells were harvested for PCR and western blot (WB) analysis.

**Real-time PCR**
An Evo M-MLV RT mix kit (Accurate Biotechnology Co. Ltd., Changsha, China) was used to extract total RNA, which was reverse transcribed into cDNA. Primer design and synthesis were carried out by Sangon Biotech (Supplementary Table S2). Next, real-time PCR was performed using a qPCR SYBR Green master mix kit (YEASEN) on a real-time fluorescence quantitative PCR instrument (Bio-Rad, Hercules, USA). qPCR data were analyzed using the 2-ΔΔCt method.

**Western blot analysis**
First, the cells were lysed in RIPA buffer supplemented with protease inhibitors (KeyGen BioTECH Corp., Nanjing, China). After centrifugation, the protein level in the supernatant was determined using a BCA kit. Protein samples (approximately 20 µg) were separated via 10% SDS–PAGE and then transferred to PVDF membranes. The membranes were incubated successively with specific primary antibodies and corresponding secondary antibodies (Supplementary Table S3). Finally, the protein bands were visualized on a ChemiDoc™ MP imaging system (Bio-Rad) and quantified by ImageJ software.

**Molecular docking**
We prepared the ligand structure of Rh2 in mol2 format using ChemBioDraw 14.0 software. The crystal structures of VEGFA (1cz8), MMP-2 (8h78), and GPC3 (7azw) were downloaded from the RCSB Protein Data Bank in PDB format. AutoDock Vina 1.1.2 was used to conduct molecular docking, employing a Monte Carlo global optimization algorithm. The Vina docking score was used to search for the best-fit
docking poses. Visual analysis was performed using PyMOL software.

**Statistical analysis**

The experiments were conducted at least three times, and the data are presented as the means ±SD. Two-tailed Student’s *t* tests were used to determine the statistical significance. *P* < 0.05 was considered statistically significant.

**Results**

**Rh2 inhibited cell viability of HUVECs**

As depicted in Figure 1A, Rh2 significantly decreased the viability of HUVECs at concentrations up to 20 and 40 μg/mL. However, when HUVECs were treated with CM-Rh2 at 20 μg/mL, there was no observable impact on cell viability, which indicated that the cytotoxicity of Rh2 diminished after a 24-h incubation with HepG2 cells (Figure 1B). Overall, we concluded that CM-Rh2 (0 to 20 μg/mL) could be used in subsequent experiments to avoid any potential toxicity effects.

**Rh2 induced cell apoptosis and influenced the cell cycle of HUVECs**

The Annexin-V-FITC/PI double-staining method is highly sensitive and specific for detecting cellular apoptosis [18]. Thus, we employed double-staining flow cytometry to evaluate the apoptotic potential of Rh2. As shown in Figure 1C, Rh2 dose-dependently induced apoptosis after incubation for 24 h, and the apoptosis rates at 10 and 20 μg/mL Rh2 were 12.6% and 66.8%, respectively. Notably, compared with those at 10 μg/mL Rh2, the early and late death rates at 20 μg/mL Rh2 significantly increased.

The early stage of the intrinsic apoptosis pathway is characterized by disturbance of the mitochondrial membrane potential [19]. According to the JC-1 staining results, Rh2 administration reduced the red-to-green fluorescence ratio, indicating a reduction in the mitochondrial membrane potential (Figure 1D). This could initiate a series of cellular damage and death responses, such as apoptosis and necrosis. These results further confirmed the ability of Rh2 to induce apoptosis in HUVECs.

Moreover, the cell cycle distribution was assessed after Rh2 treatment. As depicted in Figure 1E, compared with control treatment, Rh2 treatment dose-dependently increased the number of cells in the G0/G1 phase, accompanied by a decrease in the proportion of cells in the S phase. These results provide evidence that Rh2 can arrest cells in the G0/G1 phase, effectively preventing cells from entering the DNA replication
phase. This finding suggested that Rh2 inhibits cell proliferation by disrupting normal cell cycle progression.

**Rh2 inhibited HepG2-stimulated migration and tube formation of HUVECs**

The correlation between endothelial cells and cancer cells is essential for tumor angiogenesis and metastasis [20,21]. Thus, we assessed the impact of Rh2 on the HepG2-stimulated migration and tube formation of HUVECs. First, a wound-healing assay was employed to investigate the impact of CM-Rh2 on the migratory behavior of HUVECs. Compared with that in the serum-free medium group, the migration rate in the CM-control group was significantly greater, indicating that CM from HepG2 cells stimulated HUVEC migration. Furthermore, CM-Rh2 treatment significantly inhibited this stimulation in a dose-dependent manner, which suggested that Rh2 suppressed the pro-migration effect of HepG2 cells on HUVECs (Figure 2A,B). The *in vitro* tube formation assay is a simple, rapid, and quantitative evaluation model used to assess the effects of natural products on angiogenesis [22]. The tubular network is formed by HUVECs grown on Matrigel, which mimics the process of endothelial cells forming capillaries *in vivo*. HUVECs were subjected to varying doses of CM-Rh2, and the visualized tube formation networks are shown in Figure 2C–E. Notably, CM from HepG2 cells stimulated HUVEC tube formation compared to that in the serum-free group. Moreover, CM-Rh2 dose-dependently suppressed tube formation, and there was virtually no observable tube formation at 20 μg/mL. Quantitative analysis revealed a significant decrease in both the branch point and tube length rate, with reductions of 74% and 71% at 20 μg/mL, respectively. These findings provide compelling evidence that CM-Rh2 potently inhibits the HepG2-stimulated motility and tube formation of HUVECs, indicating its anti-angiogenesis effect.

**Rh2 inhibited VEGF and MMP-2 expression in HUVECs**

Rh2 dose-dependently down-regulated the *VEGF* mRNA level, while *MMP-2* mRNA level was significantly decreased by Rh2 only at 20 μg/mL (Figure 3A). Furthermore, the protein levels of VEGF and MMP-2 were also evaluated. Rh2 treatment at 20 μg/mL markedly suppressed the protein expressions of both VEGF and MMP-2. The inhibition rates were determined to be 30% for VEGF and 49% for MMP-2 (Figure 3B,C).

**Rh2 inhibited HCC growth via suppression of GPC3 expression and Wnt/β-
catenin signaling in HepG2 cells

CCK-8 and flow cytometry assays results showed that Rh2 treatment significantly inhibited cell proliferation at 20 and 40 μg/mL, and the inhibitory rates were 16% and 74%, respectively (Figure 4A). Furthermore, flow cytometry assays indicated that Rh2 dose-dependently caused cell death after 24 h of treatment, and the rates of induced apoptosis at 10 and 20 μg/mL Rh2 were 12% and 28%, respectively (Figure 4B). Cell death was associated with cell cycle arrest, and the results indicated that Rh2 caused cell cycle arrest in the G0/G1 phase, as indicated by a significant increase in the population of cells in this phase (Figure 4C). These findings suggest that Rh2 stimulation has a pro-apoptotic effect on HCC cells.

To investigate the impact of Rh2 on GPC3 expression, HepG2 cells were incubated with various doses of Rh2 for 24 h. As depicted in Figure 4D,E, Rh2 treatment markedly decreased GPC3 expression at both the mRNA and protein levels. Moreover, as the concentration of Rh2 increased, the protein levels of β-catenin, c-myc, and cyclin D1 significantly decreased. These findings indicated that Rh2 treatment effectively downregulated the expressions of these key proteins involved in GPC3-mediated Wnt/β-catenin signaling.

GPC3 silencing enhanced Rh2-induced HCC inhibition by suppressing GPC3/Wnt/β-catenin signaling

To further investigate the role of GPC3 in Rh2-induced anti-HCC effects, a GPC3-targeted siRNA transfection experiment was conducted in HepG2 cells. Successful siGPC3 transfection was indicated by a decrease in GPC3 expression levels according to qPCR (Figure 5D). Furthermore, combined treatment with siGPC3 and Rh2 resulted in even lower GPC3 mRNA expression levels than treatment with siGPC3 or Rh2 alone.

CCK-8 results demonstrated that siGPC3 transfection significantly suppressed the viability of HepG2 cells (Figure 5A). Moreover, GPC3 silencing significantly enhanced the suppressive effect of Rh2 on HepG2 proliferation. The inhibitory rates of Rh2 at 20 and 40 μg/mL were 50% and 90%, respectively, which were notably greater than those observed with Rh2 treatment alone (Figure 4A and 5A).

Moreover, flow cytometry assay demonstrated that siGPC3 transfection significantly increased cell death, with an induced apoptosis rate of 22% (Figure 5B). Additionally, a greater increase in the rate of apoptosis was observed after combined treatment with siGPC3 and Rh2 (Figure 5B). Similarly, GPC3 silencing affected the regulation of cell
cycle progression, specifically leading to increased arrest at the G0/G1 phase (Figure 5C).

Our study also demonstrated that silencing of GPC3 substantially decreased the levels of β-catenin, c-myc and cyclin D1 (Figure 5E). To determine whether the impact of Rh2 on Wnt signaling relies on GPC3, we conducted further experiments. The results showed that combined treatment with siGPC3 and Rh2 more notably inhibited the expressions of GPC3, β-catenin, c-myc and cyclin D1 than treatment with siGPC3 or Rh2 alone (Figure 5E).

**Rh2 showed strong binding with VEGF, MMP-2, and GPC3 receptors**

Molecular docking is a valuable tool for predicting the affinity and orientation of small molecules within the binding sites of macromolecules, thereby assisting in drug discovery and design [23]. In this study, we conducted molecular docking analyses of Rh2 with the receptor molecules VEGF, MMP-2, and GPC3 (Figure 6). The resulting binding scores are listed in Supplementary Table S4. Molecular docking between Rh2 and VEGFA revealed favorable binding scores, with the formation of 6 hydrogen bonds. Notably, a low binding score (−9.0 kJ/mol) indicated a strong binding interaction between Rh2 and MMP-2, accompanied by the formation of 5 hydrogen bonds. Similarly, the docking between Rh2 and GPC3 demonstrated good binding, as indicated by the observation of 5 hydrogen bonds. These molecular docking results provide insights into how Rh2 interacts with the corresponding proteins, although the precise mechanism of action requires further investigation.

**Discussion**

HCC is a prevalent malignant tumor that severely endangers human well-being. Recently, traditional Chinese medicines have shown promising therapeutic effects in the clinical treatment of tumors. Ginsenoside Rh2, derived from ginseng, is an essential bioactive ingredient known for its diverse pharmacological properties, including anti-tumor, anti-oxidation, anti-inflammation, and immunomodulation effects [24–26]. However, despite these known effects, the specific molecular targets and mechanisms of action of Rh2 are still largely unknown. Our current research has shed light on the potential mechanisms of Rh2 in HCC. We found that Rh2 not only inhibited angiogenesis by downregulating VEGF and MMP-2 expressions but also targeted GPC3 by downregulating the Wnt/β-catenin signaling pathway in HCC cells.
Tumor angiogenesis drives subsequent growth, invasion, and metastasis and has gradually become a focal point in tumor treatment in recent years [27]. HCC is a hypervascular solid tumor distinguished by abnormal vascularization and angiogenesis, with HCC endothelial cells exhibiting significant heterogeneity compared to normal endothelial cells, making them more prone to forming new blood vessels in situ and supporting metastasis [28–30]. Therefore, targeting endothelial cell function has emerged as a potential therapeutic strategy. Our study demonstrated the effective anti-proliferative, pro-apoptotic, and cell cycle-modulating properties of Rh2 in HUVECs. Earlier investigations reported that HepG2 cells could stimulate HUVEC differentiation, remodeling, migration, and angiogenesis [31]. Pan et al. [32] demonstrated that gold nanoparticles could inhibit HepG2-stimulated proliferation and migration of HUVECs by reducing VEGF activity. Similarly, in our investigation, Rh2 dose-dependently suppressed HepG2-stimulated migration and tube formation of HUVECs. Angiogenesis is tightly regulated by VEGF and its receptors, which activate signaling pathways such as the PI3K/AKT pathway, leading to cell division, proliferation, and migration, thus promoting new blood vessel formation within tumors [33–35]. Zhang et al. [36] demonstrated that Rh2 inhibited VEGF-induced corneal neovascularization. Furthermore, elevated level of MMP-2 in HUVECs can lead to matrix degradation, further promoting angiogenesis, cell migration, and invasion [37]. Our study demonstrated that Rh2 treatment dramatically down-regulated both VEGF and MMP-2 expressions in HUVECs. This indicates that Rh2 inhibits angiogenesis by effectively suppressing VEGF and MMP-2.

GPC3, a member of the glypican family, is specifically overexpressed in HCC patients, making it a novel biomarker with high diagnostic sensitivity [38]. Previous studies have indicated that GPC3 promotes HCC growth by activating diverse signaling pathways, especially the Wnt/β-catenin signaling pathway. GPC3 has the potential to activate Wnt pathway by forming stable complexes with Wnt ligands and its receptor Frizzled [39]. Additionally, under the influence of sulfatase-2, the heparan sulfate side chain of GPC3 can directly bind to Frizzled, further facilitating complex formation [40]. Wnt/β-catenin signaling can be hyperactivated in HCC, which is reflected through the expression levels of β-catenin and its downstream target gene cyclin D1 [41,42]. Moreover, c-Myc, a transcription factor, directly regulates GPC3 transcription, leading to increased GPC3 expression [43,44]. Ma et al. [45] demonstrated that Rh2 inhibited the progression of non-small cell lung cancer by inactivating Wnt/β-catenin signaling.
In addition, Rh2 inhibits HCC growth by coordinating β-catenin and autophagy [46]. Therefore, developing drugs that target the GPC3/Wnt/β-catenin pathway could offer new opportunities for HCC treatment.

In this context, our study on Rh2 aligns with previous findings, as we observed significant anti-proliferative, pro-apoptotic, and cell cycle-modulating effects in HepG2 cells following Rh2 administration [46,47]. Furthermore, Rh2 treatment led to downregulation of GPC3, β-catenin, c-myc, and cyclin D1, suggesting the potential involvement of GPC3 and the Wnt/β-catenin signaling pathway in the inhibitory effects of Rh2. The observed inhibition of HCC cell migration and invasion following GPC3 knockdown implies that GPC3 acts as a key factor in HCC progression [48]. Our results indicated that GPC3 silencing promoted Rh2-induced anti-proliferative and pro-apoptotic effects in HepG2 cells, concomitant with the downregulation of β-catenin, c-myc and cyclin D1. These observations suggest that Rh2 inhibits HCC progression by suppressing GPC3-mediated activation of the Wnt/β-catenin pathway.

In summary, our research offers novel perspectives on the inhibitory effect of Rh2 on HCC, which is specifically attributed to its anti-angiogenic activity and downregulation of GPC3/Wnt/β-catenin signaling. Rh2 effectively suppresses HepG2-induced proliferation, migration, and tube formation of HUVECs by reducing VEGF and MMP-2 expressions. Moreover, Rh2 inhibits HCC growth by downregulating GPC3-mediated Wnt/β-catenin activation. Silencing of GPC3 further enhances the effects of Rh2 on HCC growth by suppressing the Wnt/β-catenin pathway. The above discoveries provide insights into the multifaceted actions of Rh2 and may pave the way for its prospective use in HCC treatment. Further in vivo research is required to confirm the mechanisms of Rh2.

**Supplementary Data**

Supplementary data is available at *Acta Biochimica et Biphysica Sinica* online.

**Funding**

This work was supported by grants from the National Natural Science Foundation of China (No. 32101029), the Natural Science Foundation of Shandong Province (No. ZR2020QB164), the PhD Scientific Research Foundation (No. 2021BKQ09), the Clinical Medical Research Foundation (No. 2021wyfylcyj04) and the Special Funds for Yuandu Scholars.
Conflicts of Interest

The authors declare that they have no conflict of interest.

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inactivating Wnt/β-catenin signaling. *Cancer Med* 2023, 12: 12653-12667


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affects the biological behavior of human hepatocellular carcinoma cells. *Mol Med
Rep* 2014, 10: 3177-3184

Figure Legends

**Figure 1. The anti-proliferative, pro-apoptotic, and cell cycle-modulating effects of Rh2 in HUVECs.**
(A,B) Rh2 (A) and Rh2-treated HepG2 conditioned medium (CM-Rh2) (B) inhibited the viability of HUVECs. HUVECs were treated with various concentrations of Rh2 or CM-Rh2 for 24 h, and then subjected to CCK-8 assay. (C) Flow cytometric analysis of Rh2-induced early and late apoptosis in HUVECs. (D) Mitochondrial membrane potential analysis by JC-1 staining after Rh2 treatment in
HUVECs. Scale bar: 50 μm. (E) Flow cytometric analysis of cell cycle parameters in HUVECs after incubation with Rh2. Data are presented as the mean ± SD, n≥3. *P<0.05 and **P<0.01 vs the control group.

Figure 2. Rh2 inhibited HepG2-stimulated migration and tube formation of HUVECs  (A,B) The migration of HUVECs treated with different doses of CM-Rh2 was assessed by wound healing assay. (C,D) Tube formation of HUVECs on Matrigel after 6 h of treatment with different doses of CM-Rh2 in a 96-well plate. Data are presented as the mean ± SD, n≥3. *P<0.05 and **P<0.01 vs CM-control group. #P<0.05 and ##P<0.01 vs serum-free medium control group.

Figure 3. Rh2 downregulated VEGF and MMP-2 expressions in HUVECs  (A) HUVECs were treated with Rh2 for 24 h. The relative mRNA expressions of VEGF and MMP-2 determined by RT-qPCR. (B,C) Western blot analysis of VEGF and MMP-2 levels in HUVECs. Data are presented as the mean ± SD, n≥3. *P<0.05 and **P<0.01 vs the control group.

Figure 4. Rh2 inhibited HCC growth via suppression of GPC3 expression and Wnt/β-catenin signaling in HepG2 cells  (A-C) Rh2 inhibited cell viability (A), induced cell apoptosis (B), and influenced the cell cycle (C) in HepG2 cells. Rh2 inhibited GPC3 expression and Wnt/β-catenin signaling in HepG2 cells. (D) The relative mRNA expression of GPC3 determined by RT-qPCR after Rh2 treatment for 24 h. (E) Western blot analysis of GPC3 expression and Wnt/β-catenin signaling. Data are presented as the mean ± SD, n≥3. *P<0.05 and **P<0.01 vs the control group.

Figure 5. GPC3 silencing enhanced Rh2-induced HCC inhibition by suppressing GPC3/Wnt/β-catenin signaling  (A-C) GPC3 silencing enhanced Rh2-induced HepG2 cell proliferation inhibition (A) and cell apoptosis (B), and influenced the cell cycle (C). GPC3 silencing inhibited Wnt/β-catenin signaling in HepG2 cells. HepG2 cells were transfected with 20 nM siRNA for 24 h and then incubated with or without Rh2 for another 24 h. (D) The relative mRNA expression of GPC3 determined by RT-qPCR. (E) Western blot analysis of GPC3 expression and Wnt/β-catenin signaling. Data are presented as the mean ± SD, n≥3. *P<0.05 and **P<0.01 vs siNC group, ##P<0.01 vs siGPC3 group.
Figure 6. Molecular docking analysis between Rh2 and VEGFA, MMP-2 and GPC3. All analyses were performed with the AutoDock Vina algorithm.
**Highlight**

20(S)-ginsenoside Rh2 has significant anti-tumor effects in human hepatocellular carcinoma (HCC). Here, we show that Rh2 not only inhibits angiogenesis by downregulating VEGF and MMP-2 expression, but also suppresses GPC3-mediated activation of Wnt/β-catenin pathway in HCC cells.

- Rh2 significantly inhibits HepG2-induced proliferation, migration and tube formation of HUVECs by reducing VEGF and MMP-2 expressions.
- Rh2 inhibits HCC growth by downregulating GPC3-mediated Wnt/β-catenin activation.
- *GPC3* silencing enhances the effects of Rh2 on HCC growth by increasing the suppression of Wnt/β-catenin pathway.
### Supplementary Table S1. Sequences of siRNA used for cell transfection

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### Supplementary Table S2. Sequences of primers used for RT-qPCR analysis

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### Supplementary Table S3. Antibodies used for western blot analysis

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<th>Mol weight</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPC3</td>
<td>ab207080</td>
<td>Abcam</td>
<td>67 kDa</td>
<td>1:1000</td>
</tr>
<tr>
<td>β-catenin</td>
<td>66379-1-lg</td>
<td>Proteintech</td>
<td>92 kDa</td>
<td>1:1000</td>
</tr>
<tr>
<td>c-Myc</td>
<td>67447-1-lg</td>
<td>Proteintech</td>
<td>57 kDa</td>
<td>1:2000</td>
</tr>
<tr>
<td>CyclinD1</td>
<td>26939-1-AP</td>
<td>Proteintech</td>
<td>34 kDa</td>
<td>1:1000</td>
</tr>
<tr>
<td>GAPDH</td>
<td>30201ES20</td>
<td>YEASEN</td>
<td>36 kDa</td>
<td>1:1000</td>
</tr>
<tr>
<td>VEGF</td>
<td>ab69479</td>
<td>Abcam</td>
<td>23k Da</td>
<td>1:1000</td>
</tr>
<tr>
<td>MMP2</td>
<td>K001437M</td>
<td>Solarbio</td>
<td>35 kDa</td>
<td>1:1000</td>
</tr>
<tr>
<td>β-actin</td>
<td>66009-1-lg</td>
<td>Proteintech</td>
<td>42 kDa</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

### Supplementary Table S4. Binding score and the number of hydrogen bonds between 20(S)-Rh2 and VEGFA, MMP-2 and GPC3

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Binding score(kJ/mol)</th>
<th>Number of hydrogen bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Value</td>
<td>Rank</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>VEGFA</td>
<td>-8.3</td>
<td>6</td>
</tr>
<tr>
<td>MMP-2</td>
<td>-9.2</td>
<td>2</td>
</tr>
<tr>
<td>GPC3</td>
<td>-7.6</td>
<td>5</td>
</tr>
</tbody>
</table>
160x191mm (300 x 300 DPI)
A

Relative mRNA expression

<table>
<thead>
<tr>
<th></th>
<th>0 µg/mL</th>
<th>10 µg/mL</th>
<th>20 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>1.0</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>MMP2</td>
<td>1.5</td>
<td>1.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

B

VEGF
MMP-2
β-actin

Rh2 (µg/mL) 0 10 20

C

Relative intensity (band to p-actin)

<table>
<thead>
<tr>
<th></th>
<th>0 µg/mL</th>
<th>10 µg/mL</th>
<th>20 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>1.0</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>MMP2</td>
<td>1.5</td>
<td>1.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>
160x151mm (300 x 300 DPI)
A  

Cell viability

sIGPC3: - + + + + +

B  

Apoptosis rate (%)

sIGPC3: siNC, siGPC3, Rh2+siGPC3

C  

Cell cycle distribution [%]

sIGPC3: sIGPC3, Rh2+siGPC3

D  

Relative GPC3 mRNA

sIGPC3: siNC, siGPC3, Rh2, Rh2+siGPC3

E  

Relative protein activity

sIGPC3: siNC, siGPC3, Rh2, Rh2+siGPC3

160x139mm (300 x 300 DPI)