Original Article

Combined effect of honokiol and rosiglitazone on cell growth inhibition through enhanced G0/G1 phase arrest in hepatoma cells

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Abstract

Background: Honokiol, a derivative extracted from the stem and bark of *Magnolia officinalis*, has been reported to have anticancer effects in hepatoma cells. Recently, it was found that honokiol acted as not only a retinoid X receptor (RXR) agonist but also as a peroxisome proliferator-activated receptor gamma (PPARγ) agonist. Additionally, honokiol is capable of activating PPARγ/RXR heterodimers synergistically in the presence of rosiglitazone in 3T3-L1 adipocyte and HLE human hepatoma cells. Furthermore, synthetic PPARγ agonist thiazolidinediones exhibited growth inhibition effects in hepatoma cells through PPARγ-dependent and PPARγ-independent pathways. However, the combined effects of treatment with honokiol and PPARγ agonist are unclear in hepatoma cells.
Methods: In this study, sulforhodamine B assay, flow cytometry, and Western blot analysis were used to examine the combined effects of honokiol and PPARγ agonist (rosiglitazone) treatment on growth inhibition in SK-Hep1 and Mahlavu hepatoma cells.
Results: Honokiol or rosiglitazone treatment in hepatoma cells induced growth inhibition at high dose by sulforhodamine B assay. Moreover, we found that combined treatment with honokiol and rosiglitazone showed more effective growth inhibition in hepatoma cells than treatment with honokiol or rosiglitazone alone. Also, treatment with honokiol and rosiglitazone induced cell cycle arrest in the G0/G1 phase; increased p21; and decreased cyclin D1, cyclin E1, and Rb expression in SK-Hep1 hepatoma cells.
Conclusion: Honokiol combined with rosiglitazone showed more effective growth inhibition in hepatoma cells mediated through the regulation of G0/G1 phase-related proteins p21, cyclin D1, cyclin E1, and Rb and cell cycle progression.

1. Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death in men and the sixth in women.[1] Currently,

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surgical resection is the best treatment for HCC, especially for patients with a tumor ≤5 cm in diameter. Recurrence and metastasis are the major causes of increased mortality after treatments such as transcatheter arterial chemoembolization, chemotherapy, and radiotherapy.[2] Therefore, the development of new therapeutic agents or strategies for HCC is urgently needed.

Peroxisome proliferator-activated receptor gamma (PPARγ) belongs to the nuclear hormone receptors subfamily, whose members are ligand-dependent transcriptional factors.[3] Most studies have shown that PPARγ agonists present antitumoral

activity in cell lines and animal models.[4] Thiazolidinediones (TZDs) serve as PPARγ agonists and are used as antidiabetic drugs including pioglitazone, rosiglitazone, ciglitazone, and troglitazone.[5] TZDs induce cell growth inhibition, cell cycle arrest, or apoptosis of tumor cells. In particular, troglitazone may induce cytotoxicity and apoptosis in human hepatoma cells.[6] Moreover, a study indicated that treatment of hepatoma cells with troglitazone led to cell growth arrest, which was associated with the suppression of cyclin D1 expression, and the apoptotic process may be related to the PI3K/Akt signaling pathway.[7] Additionally, the downregulation of COX-2 and Bcl-2, and upregulation of Bax and Bak were observed in HepG2 hepatoma cells after troglitazone treatment.[8] A recent study showed that rosiglitazone had an antimetastatic effect in MHCC97L and BEL-7404 human hepatoma cell lines.[9] Taken together, it seems that PPARγ could be a potential therapeutic target to fight HCC.[10] However, the exact working mechanism of TZDs and the role of PPARγ activation in HCC are still unclear and thus worthy of further investigation.

Honokiol is a derivative extracted from the stem and bark of *Magnolia officinalis* and is reported to exhibit many pharmacological effects, such as suppression of oxidative stress via regulating the NF-κB (nuclear factor-kappa B) signaling pathway in human umbilical vein endothelial cells,[11,12] antithrombosis activity,[13] and anti-inflammatory effects through inhibiting signal transducer and activator of transcription-3 (STAT3).[14,15] Moreover, honokiol induced antiproliferation and apoptosis through inhibiting the STAT3 signaling pathway by regulating tyrosine phosphatase SHP-1 in HCC cells.[16] Furthermore, honokiol activated the p38 mitogen-activated protein kinase and the caspase-3 pathway to induce apoptosis in human hepatoma cell lines.[17] Therefore, honokiol may be a potential therapeutic agent in cancer treatment.

It was found that honokiol performs as a natural rexinoid and thus is able to serve as an retinoid X receptor (RXR) agonist.[18,19] Additionally, it has been reported that honokiol is able to synergistically activate the PPARγ/RXR heterodimers in the presence of rosiglitazone in 3T3-L1 adipocyte and HLE human hepatoma cells.[19] However, the combined effects and detailed mechanism of treatment with honokiol and PPARγ agonist remain unclear in hepatoma cells. In this study, sulforhodamine B assay, flow cytometry, and Western blot analysis were used to examine the combined effects of honokiol and PPARγ agonist (rosiglitazone) treatment on growth inhibition in SK-Hep1 and Mahlavu human hepatoma cells.

2. Methods

2.1. Cell culture

Mahlavu and SK-Hep1 human heptoma cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin—streptomycin amphotericin B solution, 1% L-glutamate, and 1% nonessential amino acids in a 37°C incubator containing 5% CO$_2$. All of the reagents mentioned above were obtained from Gibco (Gaithersburg, MD, USA) and Biological Industries (Beit Haemek, Israel).

2.2. Drug treatment

Cells were seeded on 96-well plates with a density of 4000 cells/well and treated with different doses of honokiol (0μM, 10μM, 20μM, and 40μM; Sigma-Aldrich, St. Louis, MO, USA) and/or rosiglitazone (0μM, 10μM, 20μM, 50μM, and 100μM; Cayman, Ann Arbor, MI, USA) and incubated for 24 hours, 48 hours, and 72 hours. Both honokiol and rosiglitazone were dissolved in dimethyl sulfoxide.

2.3. Sulforhodamine B assay

Sulforhodamine B (SRB) assay was used to determine cell viability. Cells were seeded on 96-well plates with a density of 4000 cells/well. After the cells were treated with drugs and incubated for 24 hours, 48 hours, and 72 hours, 50% trichloroacetic acid was added to the wells immediately and the plate was incubated at 4°C for 1 hour to fix the cells. After washing the cells with ddH$_2$O four times and air-drying them, 0.057% SRB solution was added to the wells and then the plate was left at room temperature for 30 minutes away from light. Unincorporated SRB was washed away with 1% acetic acid four times, and the plate was left to air-dry. Finally, 10mM Tris base was added to the wells to solubilize the SRB solution, and the absorbance was measured at the wavelength of 510 nm. Reagents mentioned above were purchased from Sigma-Aldrich.

2.4. Flow cytometry

Cells were seeded in six-well plates with a density of 20,000 cells/well. After drug treatment for different time durations, cells were washed with phosphate buffered saline (PBS; Gibco) and collected from the six-well plates via treatment with trypsin EDTA (Biological Industries). The supernatant was discarded after centrifugation (270g, 5 minutes), and the cell pellet was washed with cold PBS and centrifuged again. PBS was added to the cell pellet, and then the sample was transferred to a 5-mL round-bottom tube. The sample was fixed with 70% cold ethanol and stored at 4°C for at least 1 hour. Next, the sample was centrifuged and washed with PBS in order to remove the 70% ethanol. Propidium iodide (PI) solution, composed of 0.1% Triton X-100, 20 μg/mL PI, and 0.2 mg/mL RNase A, dissolved in PBS, was added to the sample without light. After staining for 1 hour in the dark, samples were filtered using a 5-mL round-bottom tube with cell-strainer cap and analyzed by flow cytometry. Afterward, cell cycle distribution was quantified using Cell-quest software (Becton Dickinson (San Jose, CA, USA)), and the detailed percentage of the cell cycle was analyzed using ModFit LT software (Verity Software House (Topsham, ME, USA)). Reagents for PI staining were purchased from Sigma-Aldrich, and the *FACSCalibur* flow cytometer was obtained from Becton Dickinson (San Jose, CA, USA).

2.5. Western blot

Cells were resuspended with RIPA (radioimmunoprecipitation assay) buffer [150mM NaCl, 50mM Tris—HCl, 0.25% sodium

deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS)] supplemented with protease inhibitor cocktail (Calbiochem, San Diego, CA, USA) and incubated for 30 minutes on ice. After centrifugation (13,000g, 10 minutes), the supernatant containing the whole-cell lysate was obtained. Protein concentration was measured using the BCA (bicinchoninic acid) assay. Protein lysates were separated in 12% SDS-PAGE (polyacrylamide gel electrophoresis) in running buffer (25mM Tris pH 8.8, 192mM glycine, 0.1% SDS). The separated proteins were transferred onto a polyvinylidene difluoride membrane in transfer buffer (25mM Tris pH 8.8, 192mM glycine, 20% methonal). After blocking the membrane in TBST buffer (Tris-buffered saline, 0.1% Tween 20) with 5% milk at room temperature for 1 hour, primary antibodies (p21, cyclin D1, cyclin E1, and Rb) were used to probe the proteins on the membrane at 4°C overnight. After incubation with horseradish peroxidase-conjugated secondary antibody, the probed proteins were detected by an enhanced chemiluminescence system (Thermo, Waltham, MA, USA).

2.6. Statistical analysis

The means and standard deviation were calculated from three independent experiments. Student t test was used for comparing the means of the two treatment groups. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Honokiol and/or rosiglitazone induced growth inhibition in hepatoma cell lines

The SRB assay was used to examine the cytotoxic effects of honokiol and/or rosiglitazone treatment in SK-Hep1 and Mahlavu hepatoma cell lines. Different doses of honokiol (0μM, 10μM, 20μM, and 40μM) and rosiglitazone (0μM, 10μM, 20μM, 50μM, and 100μM) were used to treat hepatoma cells for 24 hours, 48 hours, and 72 hours. Treatment of SK-Hep1 and Mahlavu cells with honokiol (40μM) for 48 hours led to growth inhibition (Figs. 1A and 1D). For the effects of rosiglitazone, SK-Hep1 cells showed growth inhibition when treated by rosiglitazone (50μM) for 72 hours, whereas there was no significant effect in Mahlavu cells under this treatment (Figs. 1B and 1E). Also, it is observed that combined treatment with honokiol (20μM) and rosiglitazone (50μM and 100μM) induced growth inhibition in SK-Hep1 hepatoma cells but not in Mahlavu cells (Figs. 1C and 1F).

3.2. Honokiol and/or rosiglitazone treatment resulted in cell cycle arrest in hepatoma cell lines

After examining the growth inhibition caused by honokiol and/or rosiglitazone treatment in SK-Hep1 and Mahlavu cells, we further investigated whether these treatments would have any impact on the cell cycle distribution of hepatoma cells. Analysis with flow cytometry revealed that treatment with honokiol (40μM) for 48 hours or 72 hours induced a G0/G1 phase arrest in SK-Hep1 cells (Fig. 2A). We also found

increased percentage of the G0/G1 phase in SK-Hep1 hepatoma cells after treatment with honokiol (20μM) and rosiglitazone (50μM and 100μM) for 48 hours (Fig. 2B). The cell cycle distribution is shown in Figs. 2C and 2D. Moreover, it was found that cotreatment with honokiol and rosiglitazone in SK-Hep1 cells for 24 hours did not increase the percentage of the G0/G1 phase, which was consistent with the results of the SRB assay (data not shown).

3.3. Expression of cell cycle-related proteins after honokiol and/or rosiglitazone treatment in SK-Hep1 hepatoma cells

After examining the effects of honokiol and/or rosiglitazone treatment on cell cycle distribution of SK-Hep1 hepatoma cells, we found that these treatments increased the percentage of the G0/G1 phase. We further investigated the expression of G0/G1 phase-related proteins under these drug treatments. We found increased p21 and decreased cyclin D1, cyclin E1, and Rb levels after treatment with 40μM honokiol for 24 hours. The same phenomena were observed at 48 hours except for the expression of cyclin E1 (Fig. 3A). In addition, combined treatment with honokiol (20μM) and rosiglitazone (50μM and 100μM) for 24 hours and 48 hours were observed with the same results. However, the expression of Rb was increased after rosiglitazone treatment for both 24 hours and 48 hours. For the combined treatment groups, the expression of Rb was decreased at 24 hours but increased at 48 hours (Fig. 3B). Our results indicated that honokiol combined with rosiglitazone had a more effective impact on growth inhibition in SK-Hep1 hepatoma cells by regulating the expression of cyclin D1, cyclin E1, Rb, and p21.

4. Discussion

In this study, we focused on the interaction between honokiol and PPARγ activation and further investigated the combined effects of honokiol and PPARγ agonist (rosiglitazone) on antiproliferative effects in human hepatoma cell lines. In our observation, honokiol or rosiglitazone treatment on SK-Hep1 (Figs. 1A–1C) and Mahlavu (Figs. 1D–1F) cells exhibited growth inhibition effects under certain conditions. The results showed that honokiol reduced the cell viability in a dose- and time-dependent manner in human hepatoma cells (Figs. 1A and 1D), which was consistent with previous studies.[16,17] However, the growth of rosiglitazone-treated Mahlavu cells did not show significant changes even when the concentration reached up to 100μM (Fig. 1E). A previous study showed that the cell viability of human heptoma cells (BEL-7402 and Huh7) was decreased after treatment with 30μM rosiglitazone.[20] Different responses induced by the same drug may result from the different characteristics of the cell lines. These results showed that SK-Hep1 cells are more sensitive to honokiol or rosiglitazone than are Mahlavu cells. Additionally, a low dose of honokiol (20μM) combined with the higher doses of rosiglitazone (50μM or 100μM) showed more efficient growth inhibition in SK-Hep1 cells compared to either drug alone.
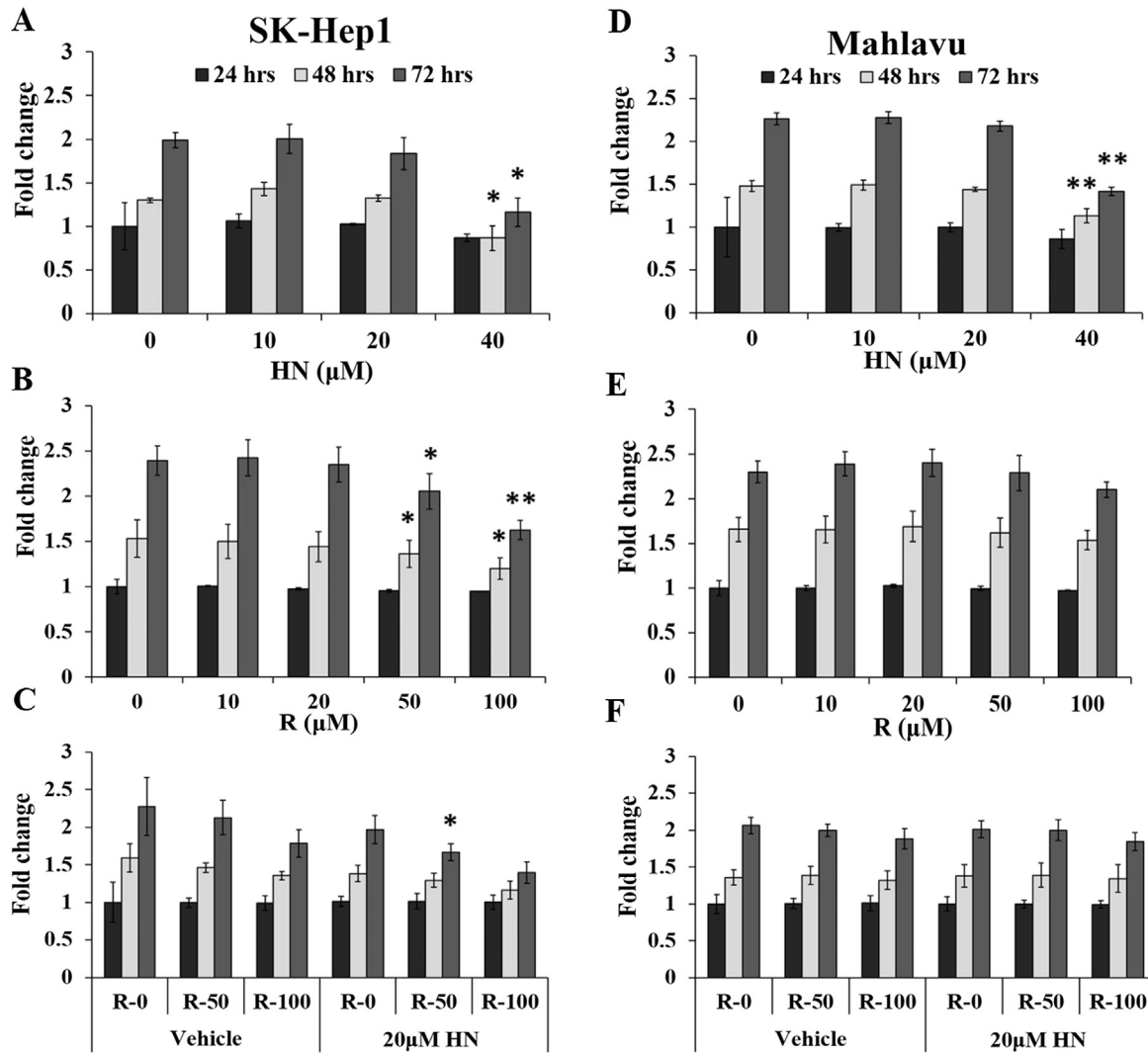
Fig. 1. Effects of honokiol (HN) and/or rosiglitazone (R) treatment on cell growth in SK-Hep1 and Mahlavu hepatoma cells. SRB assay was used to measure the growth of hepatoma cells after treatment with (A, D) HN (0μM, 10μM, 20μM, 40μM), (B, E) R (0μM, 20μM, 50μM, 100μM), (C, F) HN (0μM, 20μM) and/or R (0μM, 50μM, 100μM) for 24 hours, 48 hours, and 72 hours in (A−C) SK-Hep1, and (D−F) Mahlavu hepatoma cells, respectively. Data are expressed as mean ± standard deviation (SD) from three independent experiments. *$p < 0.05$; **$p < 0.01$, significantly different compared to respective control group. SRB = sulforhodamine B.

Analysis of cell cycle distribution by flow cytometry indicated that honokiol (40μM) treatment in SK-Hep1 cells resulted in G0/G1 arrest (Fig. 2A). However, our results are different from those of a previous study that indicated that honokiol (50μM) induced the accumulation of the sub-G$_0$ phase in HepG2 hepatoma cells.[16] The difference may result from the differentiation status of hepatoma cell lines because HepG2 are well differentiated and SK-Hep1 are poorly differentiated hepatoma cells.[21] Honokiol treatment in well-differentiated hepatoma cells may show more effective anti-tumor effects. Also, when we examined the combined effects of honokiol and rosiglitazone on growth inhibition in SK-Hep1 cells, we observed that the percentage of the G0/G1 phase was enhanced in SK-Hep1 cells after the combined treatment of honokiol and rosiglitazone for 48 hours (Fig. 2B). These results together indicated that honokiol potentiated the growth inhibition effect via regulating cell progression.

Previous studies showed that honokiol and PPARγ agonists induced cell cycle arrest and upregulated cell cycle arrest-related proteins expressions.[22,23] Because we had also found G0/G1 phase arrest after drug treatment (Fig. 2A), we further examined the expression of G0/G1 phase-related proteins after drug treatments in SK-Hep1 cells. We observed that p21 was upregulated and cyclin D1, cyclin E1, and Rb were down-regulated after honokiol treatment for 24 hours or 48 hours and thus increased the percentage of the G0/G1 phase in SK-Hep1 hepatoma cells (Fig. 3A). In addition, we found that combined treatment with honokiol and rosiglitazone induced more effective growth inhibition in SK-Hep1 hepatoma cells mediated through regulating the expression of p21, cyclin D1, cyclin E1, and Rb and cell cycle progression (Fig. 3B). Our results are consistent with those of recent studies, which reported that honokiol induced growth inhibition by down-regulating the expression of cyclin D1 in HepG2 cells.[16]
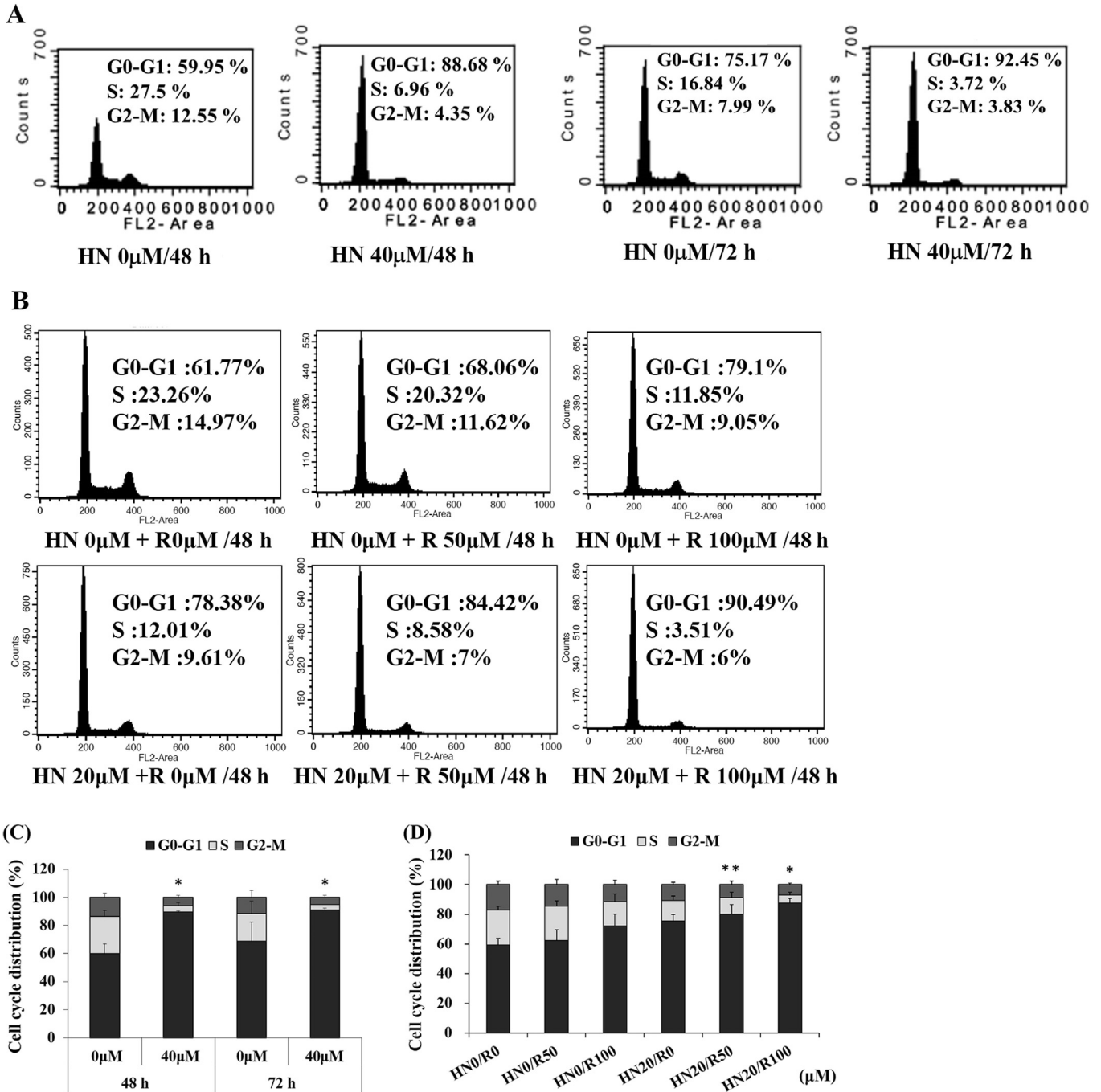
Fig. 2. Effects of honokiol (HN) and/or rosiglitazone (R) treatment on cell cycle phase distribution in SK-Hep1 hepatoma cells. Flow cytometry was used to analyze the distribution of cell cycle phases in SK-Hep1 hepatoma cells after drug treatments. (A) The representative cell cycle profile of SK-Hep1 cells after treatment with HN (0μM, 40μM) for 48 hours and 72 hours, respectively. (B) Representative cell cycle profile of SK-Hep1 cells after HN (0μM, 20μM) and/or R (0μM, 50μM, 100μM) treatment for 48 hours. (C, D) Stacked bar graphs were the quantification results for panels A and B. Data are expressed as mean ± standard deviation (SD) from three independent experiments. *$p < 0.05$; **$p < 0.01$, significantly different compared to respective control group.

Decreased cyclin D1 and increased p21 levels could also be observed in MiaPaCa and Panc1 pancreatic cancer cells after honokiol treatment.[24] In another recent study, it was claimed that honokiol synergistically potentiated the activation of PPARγ/RXR heterodimers in the combination of rosiglitazone in 3T3-L1 adipocytes and HLE human hepatoma cells to increase glucose uptake.[19] Importantly, our study demonstrates that treatment with honokiol and PPARγ agonist had synergistic effects on growth inhibition in SK-Hep1 cells. Moreover, our pilot study has found that the expression of PPARγ and RXRα was decreased after treatment with honokiol and/or rosiglitazone at 24 hours and 48 hours (data not shown). This finding was consistent with results in previous studies in lung cancer cells and gastric cancer cells.[25,26]
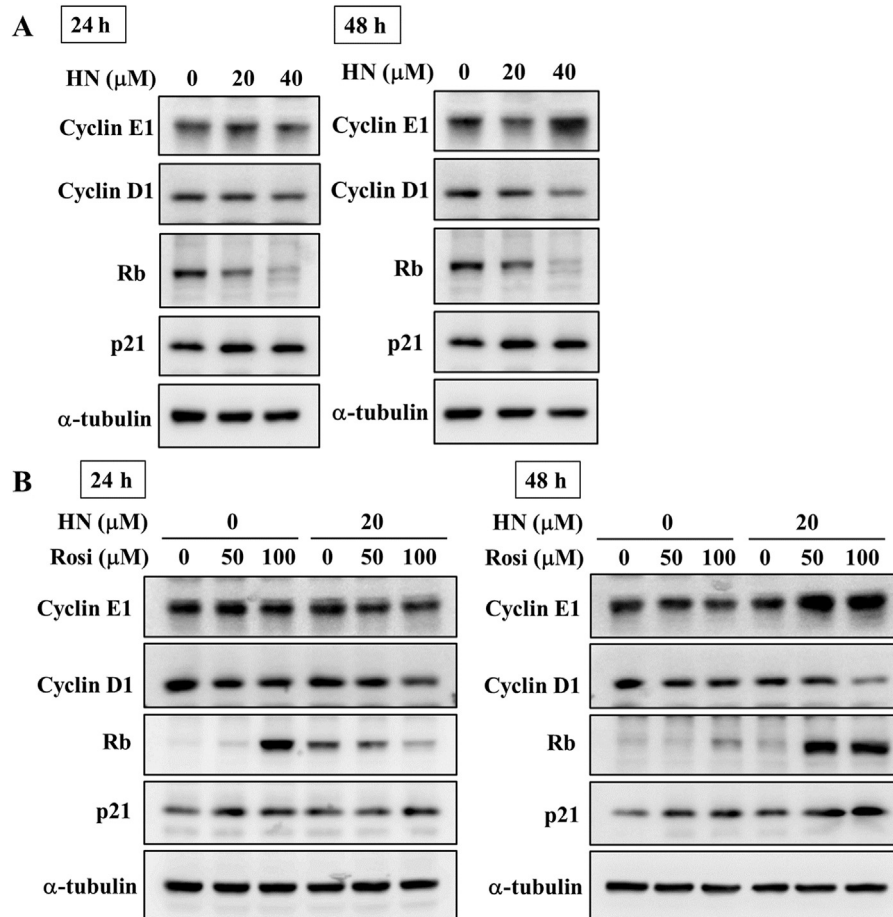
Fig. 3. Effect of honokiol (HN) and/or rosiglitazone (R) treatment on cell cycle-related proteins in SK-Hep1 hepatoma cells. Western blot analysis was used to detect the expression of cyclin D1, cyclin E1, Rb, and p21 after (A) HN treatment (0μM, 20μM, 40μM) and (B) HN (0μM, 20μM) and/or rosiglitazone (0μM, 50μM, 100μM) treatment for 24 hours and 48 hours in SK-Hep1 hepatoma cells. α-Tubulin was used as loading control.

In conclusion, we have shown the effects of honokiol and/or rosiglitazone on growth inhibition in human hepatoma cells. We found that combined treatment with honokiol and rosiglitazone induced more effective growth inhibition than treatment with honokiol alone in SK-Hep1 cells. We suggest that the growth inhibition is mediated through regulating the expression of G0/G1 phase-related proteins and may be involved in the PPARγ-related signaling pathway.

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