JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Investigation of new treatment option for hepatocellular carcinoma: a combination of sorafenib with usnic acid

Beste Yurdacan[a], Unal Egeli[b], Gamze Guney Eskiler[c], Isil Ezgi Eryilmaz[b], Gulsah Cecener[b] and Berrin Tunca[b]

[a]Pharmacology Department, Faculty of Pharmacy, Ege University, Izmir, Turkey, [b]Medical Biology Department, Faculty of Medicine, Bursa Uludag University, Bursa, Turkey and [c]Medical Biology Department, Faculty of Medicine, Sakarya University, Sakarya, Turkey

Keywords
combination treatment; hepatocellular carcinoma; sorafenib; usnic acid

Correspondence
Unal Egeli, Medical Biology Department, Faculty of Medicine, Bursa Uludag University, 16059, Gorukle, Bursa, Turkey.
E-mail: egeli@uludag.edu.tr

Received November 19, 2018
Accepted March 24, 2019

doi: 10.1111/jphp.13097

Abstract

Objectives Sorafenib (SOR) is an orally administered molecular targeted agent in the systemic chemotherapy of hepatocellular carcinoma (HCC). However, the partial response of SOR is limited due to its adverse side effect and high heterogeneity and resistant phenotype of HCC. In the current study, we investigated synergistic effects of SOR and usnic acid (UA) on HCC cell lines including HepG2 and SNU-449, and a normal cell line, HUVEC.

Methods The antiproliferative and apoptotic effects of combination therapy and SOR alone were analysed by WST-1 and Annexin V analysis, respectively. Furthermore, cell cycle, gene expression analysis of SOR-targeted kinases and acridine orange–ethidium bromide staining were also performed in combined treatments.

Key findings Our results demonstrated that SOR and UA combination indicated a strong synergism in HCC cell lines and reduced SOR toxicity in HUVEC cells. Additionally, the combination treatment SOR and UA significantly induced much more apoptotic cell death and G0/G1 arrest through downregulation of SOR-targeted kinases.

Conclusions Consequently, SOR and UA combination could be a new therapeutic strategy for HCC treatment.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the fifth most common cause of cancer-related death.[1–3] Chronic hepatitis C and chronic hepatitis B, alcoholic cirrhosis and non-alcoholic steatohepatitis are the main causes in HCC. In patients diagnosed with HCC, surgical treatment options have been limited due to liver transplantation and hepatic resection and an advanced stage with intra- or extrahepatic metastasis. Therefore, chemotherapy is a main treatment option for the patients who cannot be treated surgically. More strikingly, HCC is a highly heterogeneous and resistant cancer type because each primary liver cancer from patients exhibits different altered genotype.[3–5]

Sorafenib (SOR) is a multikinase inhibitor approved by the FDA for the treatment of advanced HCC patients who are unable to undergo surgical intervention in order to prolong survival and prevent disease progression. When examined molecular mechanism, SOR inhibits the growth and progression of the tumour by blocking cell surface kinases (VEGFR 1, 2 and 3, PDGFR, KIT, FLT3, FGFR1 and RET) which are involved in the development of cancer phenotype. Additionally, SOR also inhibits RAF/MEK/ERK signalling pathways including CRAF and BRAF kinases causing HCC development.[6] However, the efficacy of SOR is limited in patients with HCC due to SOR-related side effects including dermatological toxicity, diarrhoea, hypertension and nausea.[7] Thus, combination of drugs or molecules targeting different pathways is a promising strategy to increase drug efficacy and reduce adverse side effects in this disease.

Natural products isolated from plants have provided a rich source of therapeutic agents for the treatment of cancer due to low toxicity.[8,9] It is well known that natural product-derived anticancer drugs have clinically used or in clinical trials as anticancer agents. Additionally, natural

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023

products have a synergistic effect with different chemotherapeutic drugs in cancer treatment.[10]

Lichens are symbiotic organisms that produce unique substances, and thus, lichen compounds have drawn attention for pharmaceutical drug development.[11] As a lichen secondary metabolite, usnic acid (UA) has become the most extensively studied substance for cancer therapy due to its unique properties. The results including our previous one indicate that UA has antitumour activity in a variety of cancer cell types by inhibiting the proliferation of cancer cells dose- and time-dependently and inducing apoptosis and autophagy, *in vitro* and *in vivo*.[6,9,10,12–18]

For this purpose, we investigated, for the first time, a potentially synergistic effect of SOR and UA combination in HCC (HepG2 and SNU-449) and human umbilical vein endothelial cell (HUVEC) lines in the current study.

Materials and Methods

Materials

Sorafenib (BAY 43-9006) (≥98% purity) was purchased from BioVision, Inc. (Milpitas, CA, USA). UA (≥98% purity), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, dimethyl sulfoxide (DMSO) and acridine orange were obtained from Sigma-Aldrich (St Louis, MO, USA). RPMI 1640 with L-glutamine was purchased from Lonza (Basel, Switzerland). The Muse® Annexin V Dead Cell Assay Kit, Cell Cycle Assay Kit and Autophagy LC3-antibody-based Kit were supplied by Millipore (Darmstadt, Germany). The WST-1 Cell Proliferation Kit was purchased from Boster Bio (Pleasanton, CA, USA). Other chemicals used in the test procedures; 0.25% trypsin EDTA (Sigma-Aldrich) was used to collect the appropriate saturation cells from culture flasks. Phosphate-buffered saline (PBS; Lonza Basel, Switzerland) was used for washing, ethanol (Merck Millipore) and 4% paraformaldehyde solution (Merck Millipore) for fixation, and acridine orange/ethidium bromide (AO/Et-Br; Sigma-Aldrich) was used to determine morphological changes.

Cell culture

The HepG2 (HB-8065™) and SNU-449 (CRL-2234™) cell lines were supplied from Professor Mehmet Ozturk Izmir Biomedicine and Genome Center and verified by the expression of hepatitis B virus (HBV) DNA (Figure S2). HUVEC cell line (CRL-1730™) was purchased from the American Type Culture Collection. HepG2 and HUVEC cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Carlsbad, CA, USA) and penicillin–streptomycin (Sigma-Aldrich).[13] Similarly, SNU-449 cells were cultured in Roswell Park Memorial Institute (RPMI; Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco, Invitrogen, USA) and penicillin–streptomycin (Sigma-Aldrich).[14] Cell lines were produced in media at 37 °C in 5% CO_2, in a humidified incubator.

Determination of cytotoxicity

A 25 mg SOR in 1 ml DMSO kept in the dark at −20 °C and UA was prepared 0.0145 M (0.1 mg) stock solution with DMSO. For the cell proliferation analysis, Ready-to-use Cell Proliferation Colorimetric Reagent WST-1 was purchased from BioVision. Briefly, the HepG2 cells were seeded into 96-well plates at a density of 3×10^4 cells/well, while SNU-449 and HUVECs were seeded at 2×10^4 cells/well and incubated overnight. These cells were treated with UA alone (12.5–100 μM) and SOR alone (0.1–10 μM) for 12, 24 and 48 h. For combination experiment, the cells were treated with the lowest minimal inhibitory concentrations (12.5 and 25 μM) of UA, based on our previous results,[9] and SOR (0.1 and 0.5 μM) for 12, 24 and 48 h as shown in Table 1. Then, 10 μl WST-1 reagent was added to each well. After incubation for 4 h, absorbance was measured at wavelength of 450 nm with multimicroplate reader (Berthold Technologies, Bad Wildbad, Germany). Synergism between UA and SOR was calculated according to WST-1 results using the Chou–Talalay combination index method.[15] Based on the analysis result, CI < 1, CI = 1 and CI > 1 mean synergism, additivity and antagonism, respectively.

Annexin V and cell cycle analysis

Apoptotic effects induced by SOR and SOR + UA combination drugs were detected using a The Muse Annexin V &

Table 1 Combined treatment of sorafenib (SOR) and usnic acid (UA) groups. Each number represented a different combination of UA with SOR in HepG2, SNU-449 and HUVEC cells

Number	Combined group
0	Control
1	12.5 μM UA + 0.1 μM SOR
2	25 μM UA + 0.1 μM SOR
3	50 μM UA + 0.1 μM SOR
4	100 μM UA + 0.1 μM SOR
5	12.5 μM UA + 0.5 μM SOR
6	25 μM UA + 0.5 μM SOR
7	50 μM UA + 0.5 μM SOR
8	100 μM UA + 0.5 μM SOR
9	12.5 μM UA + 1 μM SOR
10	25 μM UA + 1 μM SOR
11	50 μM UA + 1 μM SOR
12	100 μM UA + 1 μM SOR

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023

Dead Cell Assay (Millipore) according to the manufacturer's instructions. Briefly, HepG2, SNU-449 and HUVEC cells were treated with SOR alone and UA (12.5 and 25 μM) and SOR (0.1 and 0.5 μM) combination for 48 h. After, the cells were collected at 2000 *g* for 5 min, washed twice with cold PBS and stained with Annexin V and Dead Cell Assay Kit for 30 min in the dark and room temperature. Finally, the cells were analysed using a Muse® Cell Analyzer (Millipore).

To detect cell cycle arrest, The Muse Cell Cycle Kit (Millipore) was used. Briefly, the cells (5×10^5 cells/well) were treated with SOR alone and UA (12.5 and 25 μM) and SOR (0.1 and 0.5 μM) combination for 48 h. Afterwards, the cells were fixed in 70% ethyl alcohol (EtOH) and stored at −20 °C for 3 h. The fixed-cell pellets were washed with ice-cold PBS, collected by centrifugation (2000 *g* for 5 min) and were stained with Muse® Cell Cycle Assay Kit (Millipore) for 30 min in dark. Finally, the cells were assessed with a Muse® Cell Analyzer (Millipore).

Determination of morphological changes and acidic vesicular organelles

To investigate morphological changes, HepG2, SNU-449 and HUVEC cells were seeded in six-well plates (5×10^5 cells/well) and treated with SOR alone and UA (12.5 and 25 μM) and SOR (0.1 and 0.5 μM) combination for 48 h. Then, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then stained with AO/EB (100 mg/ml AO and 100 mg/ml EB) for 30 min in the dark. After washing with PBS three times, images were analysed with an EVOS FL Cell Imaging System (Thermo Fisher Scientific, Waltham, MA, USA).

Furthermore, we detected the formation of acidic vesicular organelles (AVO) by AO staining in HCC cell lines because we previously reported that UA induced autophagy in HCC cell lines.[9] Briefly, HepG2, SNU449 and HUVECs were seeded into 6-well plates and then treated with UA (12.5 and 25 μM) and SOR (0.1 and 0.5 μM) combination for 12, 24, 36 and 48 h. After washing, the cells were stained with 1 mg/ml AO for 15 min, washed with PBS and examined using an EVOS FL Cell Imaging System (Thermo Fisher Scientific, Waltham, MA, USA).

RNA isolation and RT-qPCR analysis

Total RNA materials were isolated from HCC cells using E.Z.N.A.® Total RNA Kit I (Omega Bio-tek, Inc., Norcross, GA, USA) after treatment with SOR alone and UA + SOR combination at the relevant times. All RNAs were controlled for quality and quantity determination with a spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA). Approximately 500 ng RNA sample was used for cDNA synthesis according to High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) protocol. Then, RT-qPCR was performed by using TaqMan™ Gene Expression Assays specific to each gene in ABI StepOne+™ Real-Time PCR System (Applied Biosystems). Expression results were normalized to expression of a housekeeping gene, GAPDH.

Statistical analysis

GraphPad Prism 6 (La Jolla, CA, USA) was performed for the statistical analysis. All experiments were analysed at least three times. The groups were analysed using one-way analysis of variance (ANOVA) with a post hoc test. Combination index (CI) was determined by using CompuSyn software (ComboSyn Inc, Paramus, NJ, USA).[16]. Additionally, statistics and relative expression of the genes compared to the non-treated group were performed using web-based data analysis program, https://www.qiagen.com/tr/shop/genes-and-pathways/data-analysis-center-overview page/other-real-time-pcr-probes-or-primers-data-analysis-center/

Results

The cytotoxicity of SOR and UA alone

The cytotoxic effects of UA and SOR alone on HepG2, SNU-449 and HUVEC cells were analysed by WST-1 analysis, and the results were shown in Figure 1a,b. The viability of HepG2, SNU-449 and HUVEC was reduced to 91.0%, 40.6% and 95.5%, respectively, at 12.5 μM, while 61.3%, 70.1% and 2.6% inhibition were detected in the growth of these cells, respectively, at 25 μM for 48 h ($P < 0.01$, Figure 1a). The cell viability of HepG2, SNU-449 and HUVEC cells reduced to 76.4%, 41.8% and 31.7% at 0.1 μM, whereas the viability of these cells decreased to 26.1%, 16.7% and 31.7% at 10 μM, respectively, for 48 h ($P < 0.01$, Figure 1b). Therefore, SOR significantly inhibited HCC and HUVEC cells viability in dose- and time-dependently.

Combination treatment UA with SOR

In order to reduce the cytotoxic effect of SOR in HUVEC cells and to determine new treatment options, the impact of combination therapy (SOR and UA) on cell proliferation was analysed by WST-1 assay in HepG2, SNU-449 and HUVEC cell lines (Figure 1c). Different combinations of UA and SOR concentrations were analysed and were summarized in Table 1. The HepG2 cell viability remarkably reduced to 26.7%, 27.0%, 28.8% and 26.3% ($P < 0.01$), whereas the percentage of SNU-449 viability considerably decreased to 21.5%, 19.34%, 24.2% and 18.0% in

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023

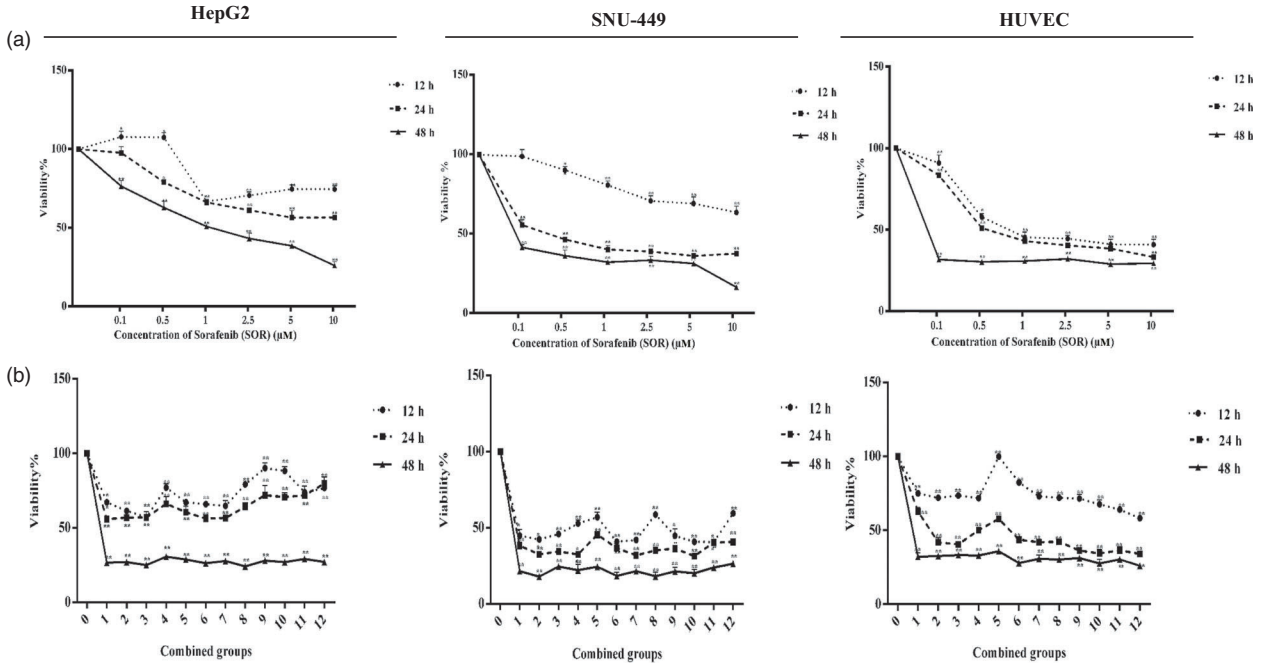

Figure 1 WST-1 proliferation assay of HepG2, SNU-449 and HUVEC cells. The cells treated with different concentrations of (a) usnic acid and (b) sorafenib as a single agent for 12, 24 and 48 h. (c) The effect of sorafenib combined with usnic acid treatment on cell viability for 12, 24 and 48 h (*$P < 0.05$, **$P < 0.01$).

combination treatment 12.5 μM UA + 0.1 μM SOR, 12.5 μM UA + 0.5 μM SOR, 25 μM UA + 0.1 μM SOR, 25 μM UA + 0.5 μM SOR, respectively, for 48 h ($P < 0.01$). On the other hand, the viability of HUVEC cells was significantly reduced to 32.2%, 32.6%, 35.9% and 27.9% at 12.5 μM UA + 0.1 μM SOR, 12.5 μM UA + 0.5 μM SOR, 25 μM UA + 0.1 μM SOR, 25 μM UA + 0.5 μM SOR, respectively, for 48 h ($P < 0.01$). Additionally, combination index (CI) was computed for each combination, and the findings were summarized in Table 2. As a result, combination of UA and SOR was more effective than SOR and UA alone. Especially, a strong synergistic interaction (CI < 1) between SOR and UA was detected at lower doses of SOR (0.1 and 0.5 μM) and UA (12.5 and 25 μM) in HCC cell lines. Thus, HepG2 and SNU-449 cell proliferations were significantly reduced in combination with UA and SOR without the application of higher concentrations of SOR. However, combined treatment with SOR + UA had toxic effect on HUVEC cells.

Table 2 Synergistic interaction with sorafenib (SOR) and usnic acid (UA) in HepG2, SNU-449 and HUVEC cells for 48 h as evaluated by CI values

	UA	CI at 0.1 μM SOR	CI at 0.5 μM SOR	CI at 1 μM SOR
HepG2	12.5 μM	0.151	0.214	0.264
	25 μM	0.297	0.327	0.389
	50 μM	0.539	0.645	0.748
	100 μM	1.320	1.068	1.259
SNU-449	12.5 μM	0.160	0.262	0.181
	25 μM	0.192	0.210	0.280
	50 μM	0.903	0.633	0.893
	100 μM	1.336	0.802	2.334
HUVEC	12.5 μM	0.912	1.960	2.736
	25 μM	2.976	0.036	0.068
	50 μM	2.981	1.376	0.812
	100 μM	2.991	0.418	0.017

Single SOR and combined effects of SOR and UA on apoptosis in HCC

To investigate apoptotic cell death, we performed the Annexin V analysis in HepG2, SNU-449 and HUVEC cells (Figure 2). In combination experiments, we selected the strong synergistic combinations between SOR and UA for 48 h according to WST-1 result and CI values. Our results showed that the rate of total apoptotic cells was 63.55%, 53.41% and 68.10% in HepG2, SNU-449 and HUVEC cells, respectively, at 5 μM SOR for 48 h (Figure 2a). Besides, the rate of apoptotic death in HepG2 and SNU-449 cells treated by SOR + UA was considerably increased. The percentage of total apoptotic cells significantly increased to 75.89% and 83.35% in HepG2 and SNU-449, respectively, at 25 μM UA + 0.5 μM SOR. However, significant apoptotic cell death was analysed in HUVEC cells (54.65%) at 25 μM

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023

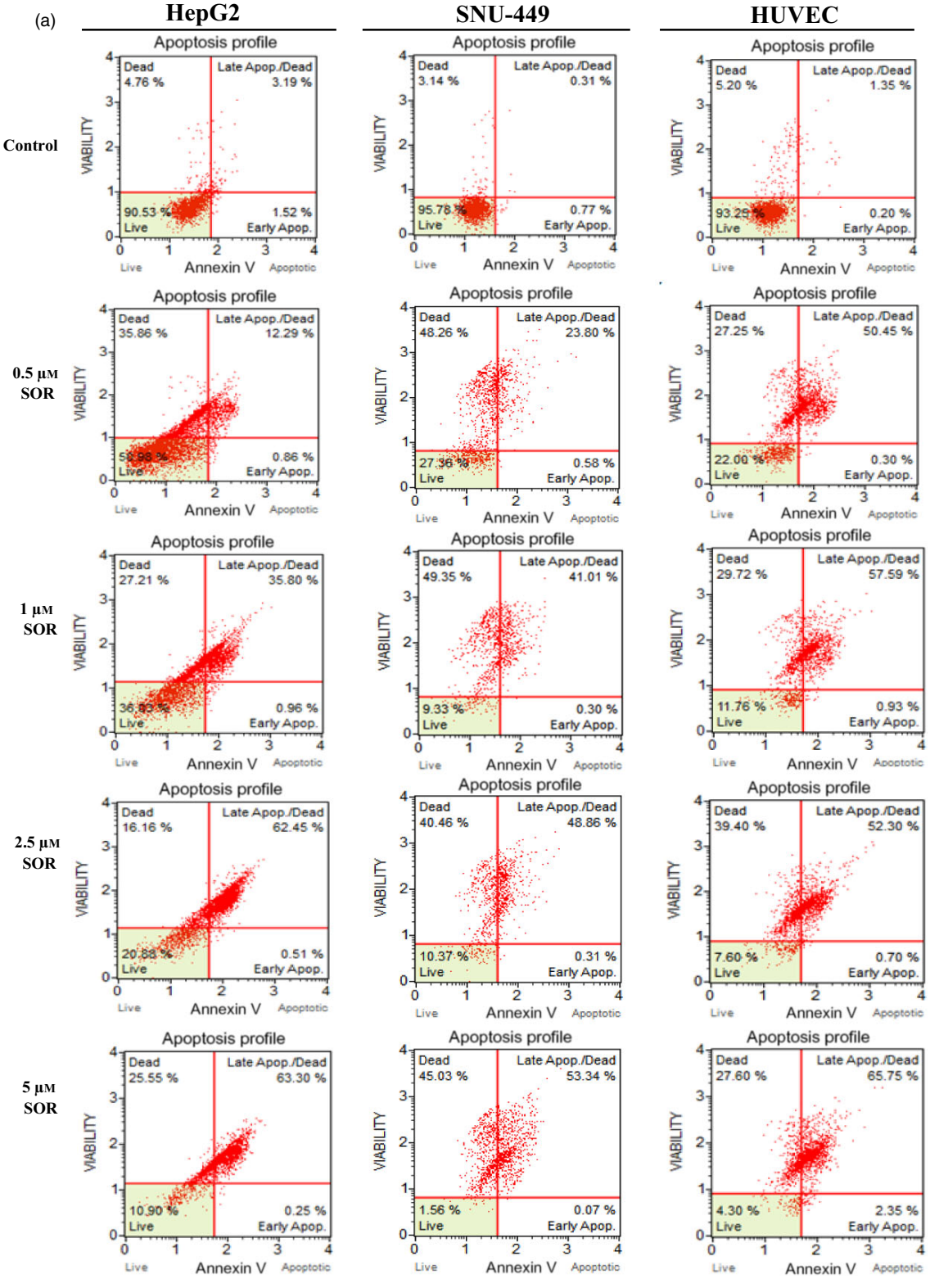

Figure 2 Annexin V analysis of HCC cells after treatment with sorafenib alone and combined with usnic acid. (a) The cells treated with 0.5, 1, 2.5 and 5 μM concentrations of sorafenib as a single agent for 48 h. (b) The cells treated with sorafenib combined with usnic acid for 48 h. [Colour figure can be viewed at wileyonlinelibrary.com]

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023

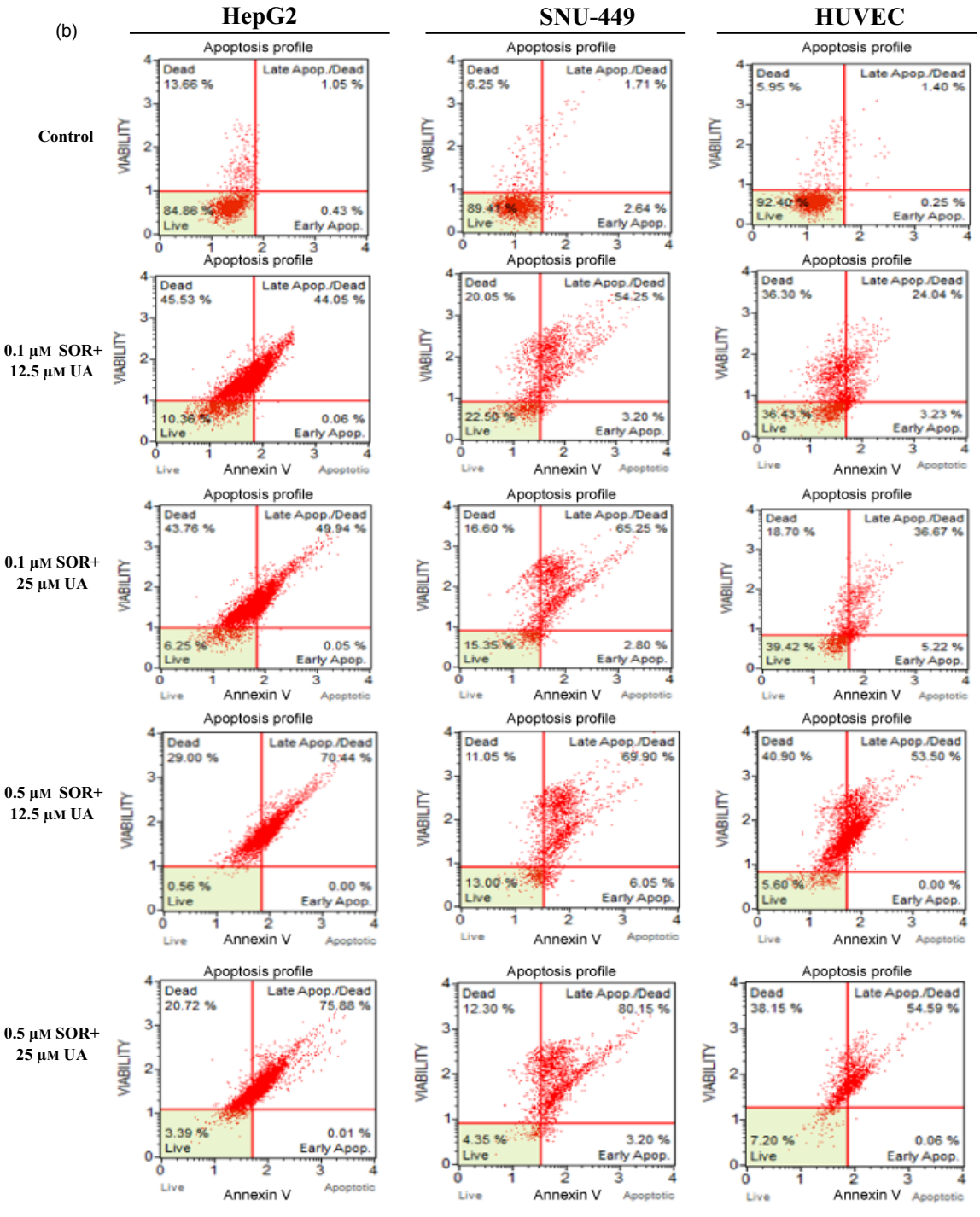

Figure 2 continued.

UA + 0.5 μM SOR for 48 h (Figure 2b) According to these results, more apoptotic cell death was observed in combination of SOR and UA in HCC cell lines compared with SOR alone. Additionally, the apoptotic death rate was decreased in combined treatment compared to SOR alone in HUVEC control cells.

Effects of single SOR and SOR + UA on cell cycle

In this study, the cell cycle analysis was performed to explore apoptotic cell death caused by SOR and SOR + UA (Figure 3). The percentage of cells in G0/G1 phase

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023

significantly increased from 32.3% and 28.9% in control cells to 80.2% and 39.2% at 5 μM SOR for HepG2 and HUVEC cells, respectively. However, the proportion of SNU-449 cells in G2/M phase increased from 28.9% in control cell to 39.7% (Figure 3a). Furthermore, combined treatment with 0.5 μM SOR and 25 μM UA caused a significant increase from 40.0%, 67.5% and 29.7% in control cells to 94.4%, 71.5% and 61.1% in G0/G1 phase for HepG2, SNU-449 and HUVEC cells, respectively (Figure 3b). However, the combination of SOR + UA caused different cell cycle arrest in SNU-449 cells. As a result, SOR and UA combined treatment led to G0/G1 arrest in HCC and control cell lines.

Effect of single SOR and SOR + UA combination with UA on cell morphology

AO/Et-Br staining was used to observe different morphological changes in HepG2, SNU-449 and HUVEC cells after 48-h treatment with single SOR and SOR + UA, and the findings were summarized in Figure 4. Particularly, 2.5 and 5 μM SOR treatment induced apoptotic cell death including cell shrinkage, apoptotic blebbing and formation of cytoplasmic vacuoles in HepG2, SNU-449 and HUVEC cells compared with each control group. Additionally, some necrotic cells were observed after treatment with higher concentrations (2.5 and 5 μM) of SOR in HUVEC cells (Figure 4a). However, the combination of SOR and UA induced apoptosis more than drug alone in HCC cell lines. We observed nuclear fragmentation, shrinkage of cells, blebbing of cytoplasm and vacuolar cytoplasm in HCC cells (Figure 4b). On the other hand, the formation of vacuoles and chromatin condensation were observed in HUVEC cells treated with SOR + UA (0.5 μM SOR + 12.5 μM UA and 0.5 μM SOR + 25 μM UA). Therefore, the combination of UA and SOR was more effective in HCC cell lines than drug alone. However, combination treatment also induced apoptotic cell death in control cells.

Autophagy profiling of cells treated with SOR + UA

To verify the formation of vacuoles, acridine orange staining of the live cells was carried out as shown in Figure S1. Based on our previous results, UA increased accumulation of autophagic vacuoles in HCC cell lines.[9] In this study, we observed less accumulation of autophagic vacuoles in HepG2 and SNU-449 cells treated with the combination of SOR and UA. Thus, SOR treatment in combination with UA more significant attenuation of the apoptotic cell death than UA and SOR alone.

Expression analysis of tyrosine kinases in HCC cells treated with SOR alone and UA + SOR combinations

We analysed the mRNA levels of PDGFR-mediated MAPK pathway proteins including MAPK1, MAPK3 and MAP2K3 to evaluate the protein kinase inhibitory effect of UA + SOR combination compared to SOR alone in HCC cells as shown in Figure 5. Based on expression results, we detected that tyrosine kinase and serine/threonine kinase inhibitory effect of SOR were significantly increased in both cell lines treated with UA + SOR compared to the control group ($P < 0.05$). *PDGFRβ* was downregulated in HepG2 and SNU-449 cells treated with all doses of UA + SOR combination, especially at 0.1 μM SOR + 25 μM UA treatment in SNU-449 cells ($P < 0.01$). The *MAPK1* expression was found to be increased 1.22- and 1.39- ($P < 0.01$) fold in HepG2 and SNU-449 cells treated with 0.5 μM SOR alone, respectively. However, at combined dose of 0.5 μM SOR with 12.5 μM UA, *MAPK1* was 2.23- ($P < 0.05$) and 3.36- ($P < 0.001$) fold downregulated in HepG2 and SNU-449 cells compared to the control group, respectively. Similarly, only SOR treatment did not result in reducing *MAPK3* expression in both HCC cells. However, especially at 0.1 SOR μM + 2.5 μM UA combination, *MAPK3* expression was detected to be significantly decreased 2.19- ($P < 0.001$) and 1.42- ($P < 0.01$) fold in HepG2 and SNU-449 cells compared to the control group, respectively. Finally, we analysed *MAP2K3* expression level in HCC cells treated with only SOR and UA + SOR combinations. Unlike the alterations in other MAPKs' regulation, *MAP2K3* expression was not decreased in UA + SOR-treated HepG2 cells. However, in SNU-449 cells, *MAP2K3* expression was determined to be decreased in different levels in UA + SOR treatments compared to those only SOR. Additionally, at maximum combined dose, 0.5 SOR μM + 25 μM UA, *MAP2K3* was determined 2.04- ($P < 0.05$) fold downregulated in treated SNU-449 cells compared to the control group.

Discussion

In this study, we investigated the antitumour effect of SOR alone and in combination SOR with UA on HCC cell lines and our results suggested that SOR + UA may be a potential treatment approach in the treatment of HCC.

Sorafenib is the only approved therapy in patients with advanced patients with HCC. Despite the success of treatment in HCC, SOR has adverse side effects in a significant proportion of patients.[16] Additionally, HCC is highly

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023

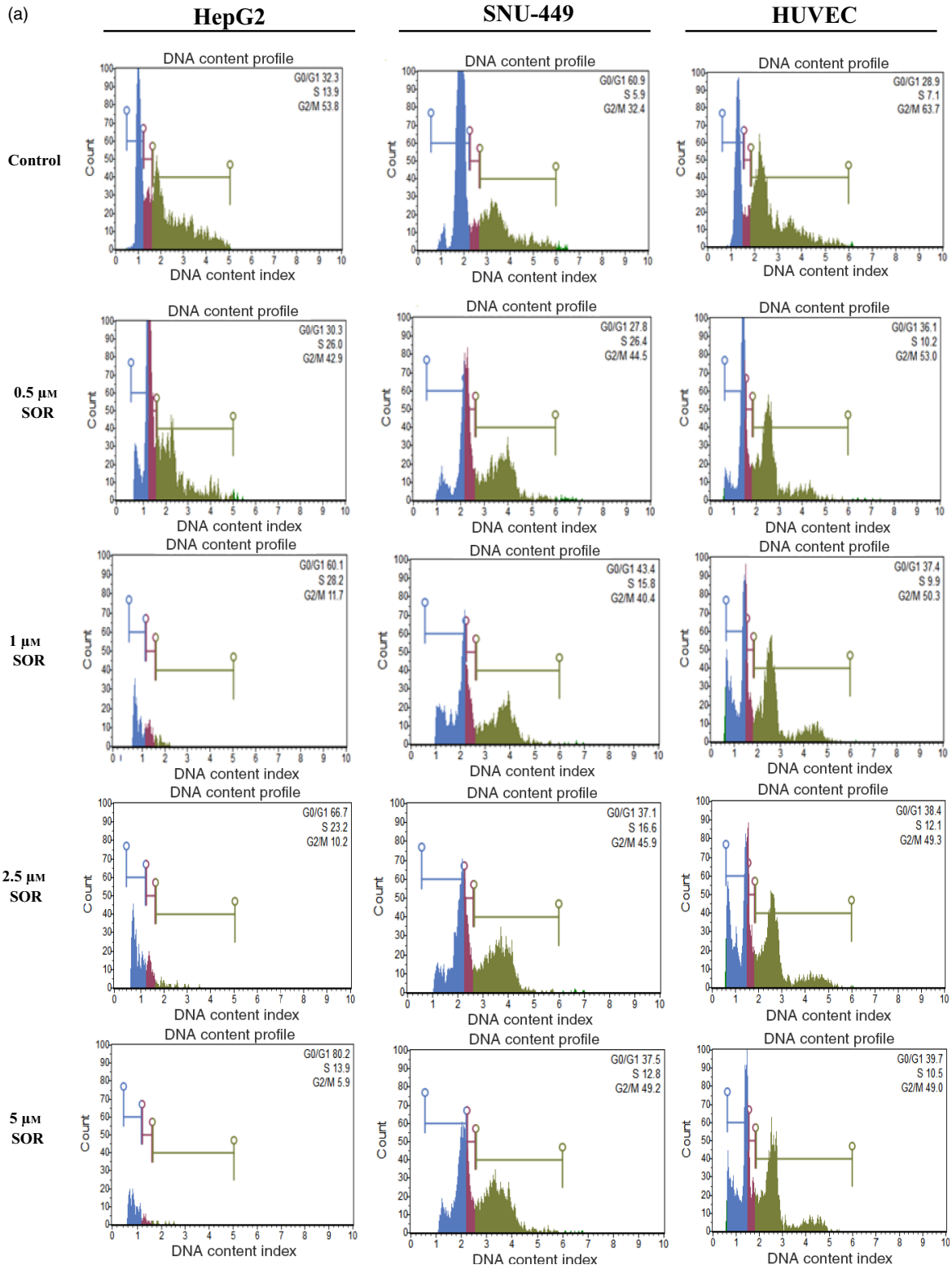

Figure 3 Cell cycle analysis of hepatocellular carcinoma cells sorafenib alone and combined with usnic acid. (a) The cells treated with 0.5, 1, 2.5 and 5 μM concentrations of sorafenib as a single agent for 48 h. (b) The cells treated with sorafenib combined with usnic acid for 48 h. [Colour figure can be viewed at wileyonlinelibrary.com]

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023

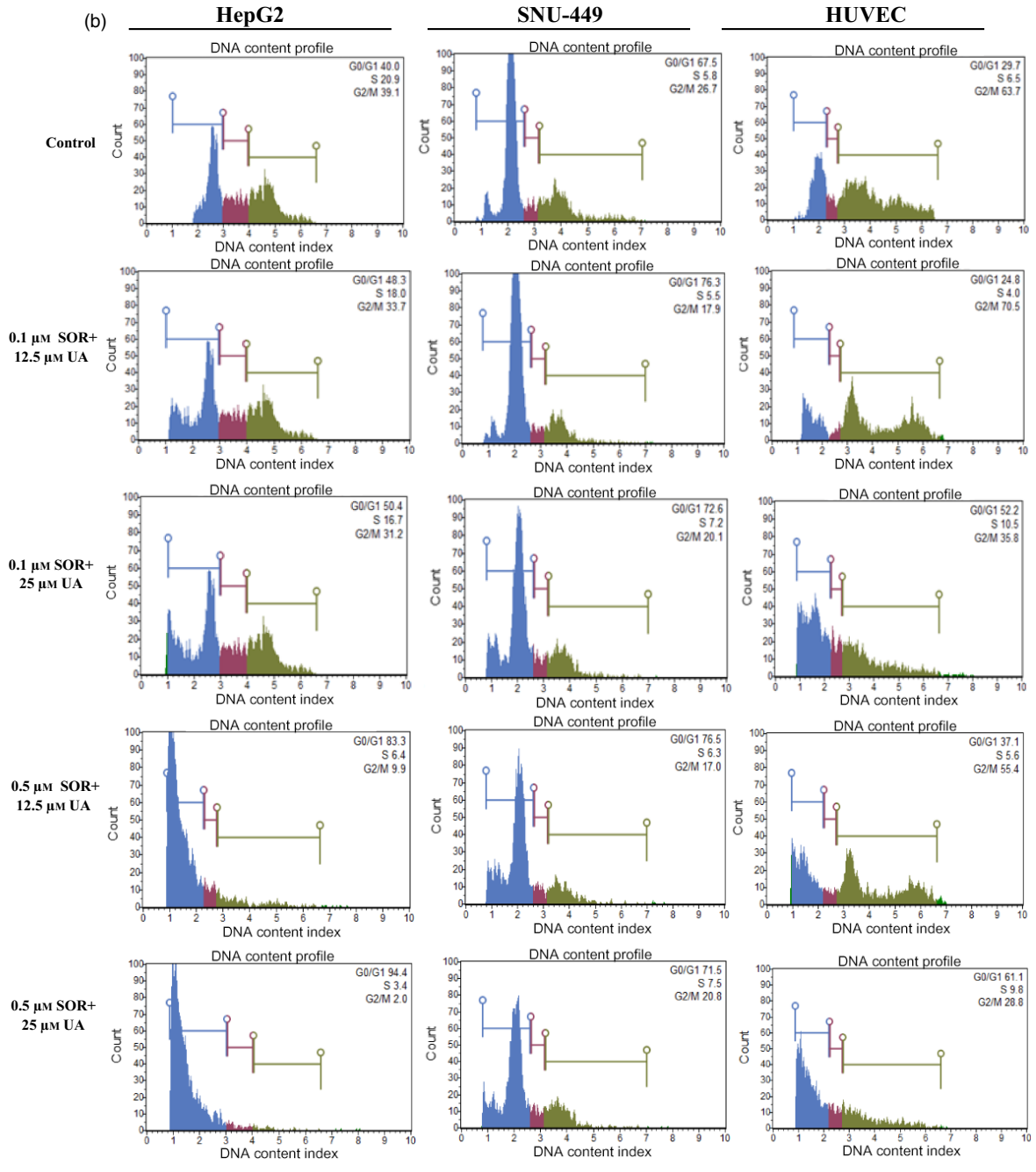

Figure 3 Continued.

resistant to chemotherapeutic treatment and it is extremely complex and heterogeneous. Thus, the development of novel combined therapy strategies is urgently needed.[6,17] Combination therapy is based on the combination of two or more therapeutic agents, and thus, it is essential for cancer treatments.[18] Several studies have shown that the combination of SOR with other drug (celecoxib) and different products (matrine, tetrandrine) has synergistic anticancer effects in HCC.[19–21] To meet this need and to reduce toxicity and to increase the efficacy of SOR, we investigated potentially a synergistic effect of SOR and UA in HCC cell lines in the current study.

As the secondary metabolite of lichen, UA has attracted much attention in cancer therapy.[22,23] The anticancer effects of UA have been explored in different cancer cells (Bcap-37, U87MG, CaCo2, HeLa, H460, H1650 and

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023

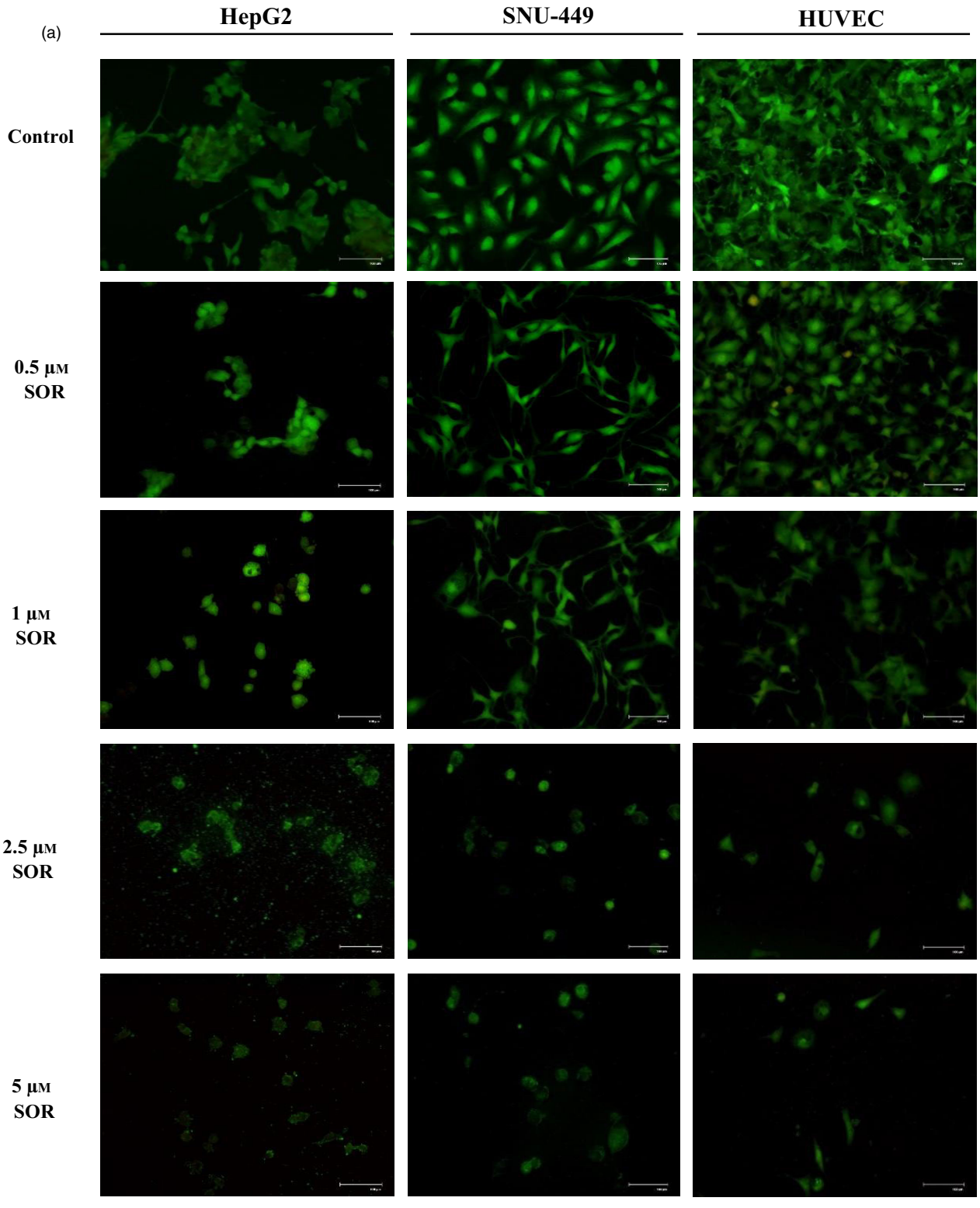

Figure 4 Effects of sorafenib alone and in combination with usnic acid on cell morphology. (a) The cells treated with 0.5, 1, 2.5 and 5 μM concentrations of sorafenib as a single agent for 48 h. (b) The cells treated with sorafenib combined with usnic acid for 48 h. [Colour figure can be viewed at wileyonlinelibrary.com]

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023

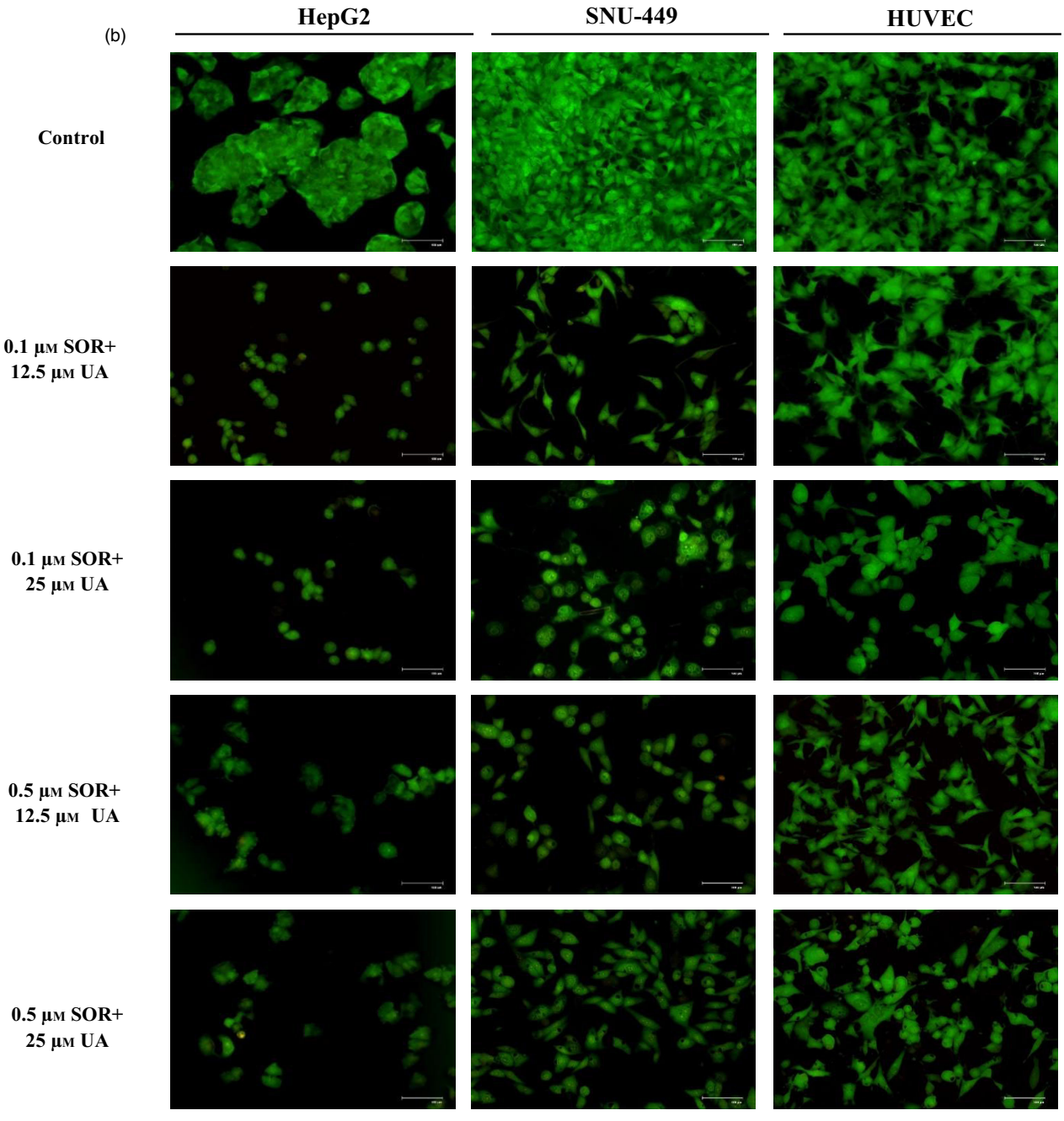

Figure 4 Continued

H197).[24–27] In our previous study, we indicated that UA induced autophagy and apoptosis in HCC (SNU-449 and HepG2) and breast and prostate cancer cells (MCF7 and LNCaP), respectively.[9,10]

In this study, our results demonstrated that SOR and UA had significantly synergistic antitumour effect on HCC cell lines. Compared with SOR alone, the combination of SOR and UA caused much more cytotoxic effect on HCC cells and induced apoptotic cell death. However, we also detected necrotic cell death after treatment with SOR alone or SOR and UA combination treatment. In the literature, some combined treatment strategies (α-mangostin and apigenin, ergosterol and amphotericin B) induced the necrotic cell death in cancer cells (breast and hepatocellular carcinoma cells).[28,29] Thus, combined therapy could enhance the therapeutic effect of different chemotherapeutic agents by inducing different

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023

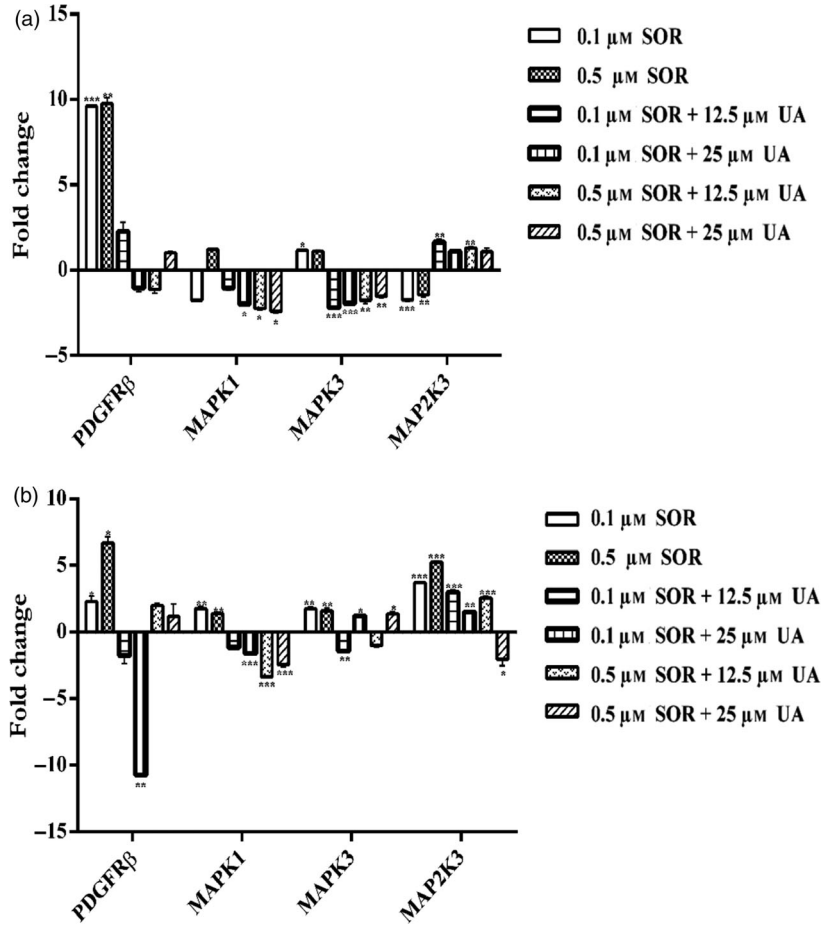

Figure 5 Altered expressions of sorafenib-targeted genes in (a) HepG2 and (b) SNU-449 cell treated with sorafenib alone and usnic acid + sorafenib combinations. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

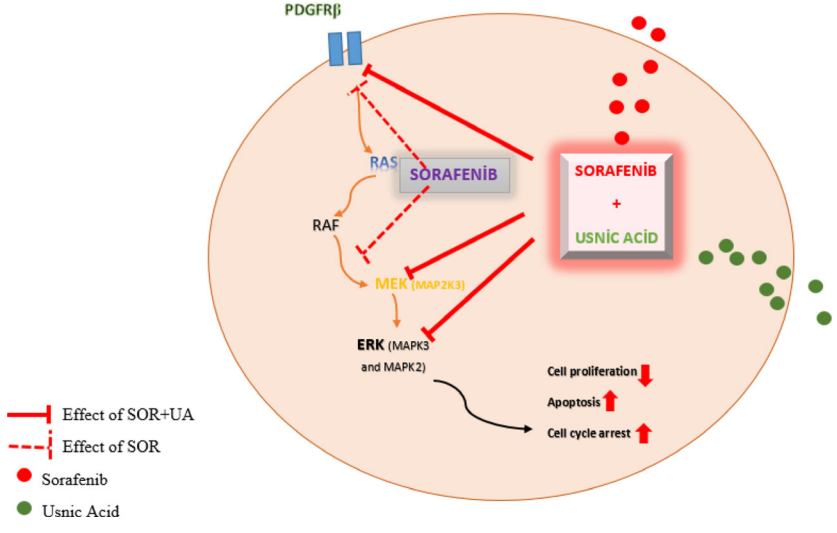

Figure 6 Schematic drawing of the probable mechanism of synergy between sorafenib and usnic acid. RAS/RAF/MAPK pathways are responsible for the control of proliferation and apoptosis. According to the results of the analysis, sorafenib + usnic acid has a more effect on the treatment pathway. [Colour figure can be viewed at wileyonlinelibrary.com]

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023

death pathways (apoptosis, necrosis and autophagy). In this context, further detailed studies are needed to elucidate the underlying mechanism of UA- and SOR-induced apoptotic and necrotic cell death in HCC cell lines.

Sorafenib and UA combination therapy showed less toxic effect on HUVEC cells than SOR alone. Thus, combination therapy reduced the toxic effect of SOR on normal cells. In cell cycle analysis, SOR and UA combined treatment caused G0/G1 cell cycle arrest HepG2 and SNU-449 cells. Interestingly, combination therapy induced G0/G1 phase arrest in SNU-449 cells despite the mutation in the *CDK2A* gene. The *CDKN2A* gene encodes *p16* and *p14*, and this protein inhibits the CDK4/6-cyclin D1 complexes. P16 and p14 proteins regulate G0/G1 cell cycle.[30] However, *CDKN2A* gene is mutated in SNU-449 cell line and p16 protein cannot be produced due to the mutation in this gene.[31] In this way, blocking damaged cells in the G0/G1-phase allows to prevent the proliferation of irreparably damaged cells. Therefore, the underlying molecular mechanism of G0/G1cell cycle arrest should be elucidated for SNU-449 cells. Furthermore, HCC cell lines were more prone to apoptotic cell death than autophagy after treatment with UA and SOR combination. Further studies are necessary to explore how death pathways are interlinked and the molecular mechanism of apoptosis.

Sorafenib inhibits cell proliferation, angiogenesis migration, metastasis effective on membrane tyrosine kinases (RAF/MEK/ERK pathway).[32] SOR targets multiple kinases that mainly involved in HCC progression and angiogenesis. The main targets include serine/threonine kinases such as members of the mitogen-activated protein kinase (MAPK) pathway and receptor tyrosine kinases responsible for signal transduction on the cell surface, especially vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR).[33,34] Recent studies have also showed that UA has potential inhibitory effect of MAPK and ERK1/2 signalling pathways by altering miRNA expression and angiogenesis via suppressing VEGFR in breast cancer.[23,35] In our study, we investigated the effect of combined therapy by UA + SOR on inhibiting SOR targets, *PDGFRβ* and *MAPK* members such as *MAPK1*, *MAPK3* and *MAP2K3 at* mRNA level comparing this effect with SOR treatment alone. Based on gene expression results, 0.1 and 0.5 μM application of SOR alone was not enough to inhibit the expressions of the genes at RNA level. However, when the same SOR doses were combined with UA, *MAPKs* and *PDGFRβ* were detected as downregulated for all combined doses in HCC cell lines. The combination of UA + SOR in the inhibition of kinases at the RNA level was found to be more effective than SOR alone at the same doses as summarized in Figure 6. Thus, we concluded that UA increased the potential of SOR to inhibit the expressions of its target genes at the lowest doses, 0.1 and 0.5 μM.

Conclusion

In conclusion, our accumulating evidence demonstrated that a lower concentration of SOR (0.1 and 0.5 μM) in combination with UA synergistically inhibited HCC cell proliferation by apoptotic pathways and combination treatment could reduce side effects of SOR on control cells. Thus, SOR in combination with UA may have alternative therapeutic options in HCC treatment. However, further studies are required to explore the mechanism underlying the anticancer effect of drug combination *in vitro* and *in vivo*.

Declarations

Conflict of interest

The Authors declare that they have no conflicts of interest to disclose.

Acknowledgements

We thank Dr. Harun Ağca from Uludag University, Microbiology Department, for the technical support to analyse HBV infection in HCC cell lines used in this study.

References

1. Pinter M *et al.* P1280 epidemiological trends of hepatocellular carcinoma in Austria. *J Hepatol* 2014; 60: S516.
2. Maxwell Parkin D *et al.* Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001; 94: 153–156.
3. Siegel RL *et al.* Cancer statistics, 2017. *CA Cancer J Clin* 2017; 67: 7–30.
4. Nguyen VT *et al.* Hepatitis B-related hepatocellular carcinoma: epidemiological characteristics and disease burden. *J Viral Hepatitis* 2009; 16: 453–463.
5. Gao JJ *et al.* Sorafenib-based combined molecule targeting in treatment of hepatocellular carcinoma. *World J Gastroenterol* 2015; 21: 12059–12070.
6. Keating GM, Santoro A. Sorafenib: a guide to its use in advanced hepatocellular carcinoma. *Drugs Ther Perspect* 2009; 25: 5–8.
7. Morisaki T *et al.* Combining celecoxib with sorafenib synergistically inhibits hepatocellular carcinoma cells in vitro. *Anticancer Res* 2013; 33: 1387–1395.
8. Bahman AA. Sequence-dependent effect of sorafenib in combination with natural phenolic compounds on hepatic cancer cells and the possible

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023

mechanism of action. *Int J Mol Med* 2018; 42: 1695–1715.
9. Yurdacan B *et al.* The role of usnic acid-induced apoptosis and autophagy in hepatocellular carcinoma. *Hum Exp Toxicol* 2018; 38: 201–215.
10. Eryilmaz IE *et al.* In vitro cytotoxic and antiproliferative effects of usnic acid on hormone-dependent breast and prostate cancer cells. *J Biochem Mol Toxicol* 2018; 32: e22208.
11. Miao V *et al.* Genetic approaches to harvesting lichen products. *Trends Biotechnol* 2001; 19: 349–355.
12. Zhou B, Chen X. Usnic acid induces cycle arrest, apoptosis, and autophagy in gastric cancer cells in vitro and in vivo. *Med Sci Monit* 2018; 24: 556–566.
13. Xue F *et al.* eIF5A2 is an alternative pathway for cell proliferation in cetuximab-treated epithelial hepatocellular carcinoma. *Am J Transl Res* 2016; 8: 4670–4681.
14. Zhu X *et al.* TMEM2 inhibits hepatitis B virus infection in HepG2 and HepG2.2.15 cells by activating the JAK-STAT signaling pathway. *Cell Death Dis* 2016; 7: e2239.
15. Chou TC, Talalay P. Generalized equations for the analysis of inhibitions of Michaelis-Menten and higher-order kinetic systems with two or more mutually exclusive and nonexclusive inhibitors. *Eur J Biochem* 1981; 115: 207–216.
16. Beljanski V. Combined anticancer effects of sphingosine kinase inhibitors and sorafenib. *Invest New Drugs* 2010; 29: 1132–1142.
17. Llovet JM *et al.* Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; 359: 378–390.
18. Kojima-Yuasa A *et al.* Synergistic anticancer activities of natural substances in human hepatocellular carcinoma. *Diseases* 2015; 3: 260–281.
19. Mokhtari RB. Combination therapy in combating cancer. *Oncotarget* 2015; 8: 38022–38043.
20. Lin Y *et al.* Combination of matrine and sorafenib decreases the aggressive phenotypes of hepatocellular carcinoma cells. *Chemotherapy* 2014; 60: 112–118.
21. Wan J *et al.* Synergistic antitumour activity of sorafenib in combination with tetrandrine is mediated by reactive oxygen species (ROS)/Akt signaling. *Br J Cancer* 2013; 109: 342–350.
22. Li Y *et al.* Combination of metformin and sorafenib suppresses proliferation and induces autophagy of hepatocellular carcinoma via targeting the mTOR pathway. *Int J Oncol* 2017; 50: 297–309.
23. Song Y *et al.* Usnic acid inhibits breast tumor angiogenesis and growth by suppressing VEGFR2-mediated AKT and ERK1/2 signaling pathways. *Angiogenesis* 2012; 15: 421–432.
24. Oettl L *et al.* HHS public access. *Neuron* 2017; 90: 609–621.
25. Emsen B *et al.* The anti-cancer efficacies of diffractaic, lobaric, and usnic acid: in vitro inhibition of glioma. *J Cancer Res Ther* 2018; 14: 941.
26. Dinçsoy AB, Cansaran Duman D. Changes in apoptosis-related gene expression profiles in cancer cell lines exposed to usnic acid lichen secondary metabolite. *Turk J Biol* 2017; 41: 484–493.
27. Kiliç N *et al.* Investigation of synergistic effect of tamoxifen and usnic acid on breast cancer cell line. *JSM Biol* 2016; 1: 1006.
28. Ittiudomrak T *et al.* α-Mangostin and apigenin induced the necrotic death of BT474 breast cancer cells with autophagy and inflammation. *Asian Pac J Trop Biomed* 2018; 8: 519.
29. Lin YC *et al.* Combination treatment of ergosterol followed by amphotericin B induces necrotic cell death in human hepatocellular carcinoma cells. *Oncotarget* 2017; 8: 72727.
30. Foulkes WD *et al.* The CDKN2A (p16) gene and human cancer. *Mol Med* 1997; 3: 5–20.
31. Baek MJ *et al.* P16 is a major inactivation target in hepatocellular carcinoma. *Cancer* 2000; 89: 60–68.
32. Wilhelm SM *et al.* BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* 2004; 64: 7099–7109.
33. Adnane L *et al.* Sorafenib (BAY 43-9006, Nexavar®), a dual-action inhibitor that targets RAF/MEK/ERK pathway in tumor cells and tyrosine kinases VEGFR/PDGFR in tumor vasculature. *Methods Enzymol* 2005; 407: 597–612.
34. Murphy DA *et al.* Inhibition of tumor endothelial ERK activation, angiogenesis, and tumor growth by sorafenib (BAY43-9006). *Am J Pathol* 2006; 169: 1875–1885.
35. Kiliç N *et al.* Determination of usnic acid responsive miRNAs in breast cancer cell lines. *Anticancer Agents Med Chem* 2018; 18: 1. https://doi.org/10.2174/1871520618666181112120142 [Epub ahead of print].

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Live cell imaging analysis of the UA combination therapy of SOR in (A) HepG2, (B) SNU-449 (C) HUVEC cells for 12, 24, 36, and 48 h.

Figure S2. The HBV DNA expression results of the cell lines used in our study (A) SNU- 449 which is positive for HBV DNA (B) HepG2 which is negative for HBV DNA. The assay was performed by using Abbott RealT*ime* HBV kit. The blue peak is indicated internal control and the red peak as shown in (A) is indicated positive results for HBV.

Downloaded from https://academic.oup.com/jpp/article/71/7/1119/6122173 by Bursa Uludag University user on 14 June 2023