Gene 737 (2020) 144428

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Investigation of possible effects of apigenin, sorafenib and combined applications on apoptosis and cell cycle in hepatocellular cancer cells

Nazlı Şirin, Levent Elmas, Mücahit Seçme, Yavuz Dodurga*

Department of Medical Biology, Faculty of Medicine, Pamukkale University, Denizli, Turkey

ARTICLE INFO

Keywords:
HCC
Hepatocellular carcinoma
Sorafenib
Apigenin
Combined effects
HepG2 cells

ABSTRACT

Hepatocellular carcinoma (HCC) is the most common type of liver tumors. There is only one chemodrug for treatment called sorafenib that is an effective multikinase inhibitor. However, most of the patients gain resistance to sorafenib treatment in six months. Thus, there is a limitation for treatment of HCC. Apigenin is a natural flavonoid that has been used for many years as an antioxidant and anti-inflammatory agent. The aim of this study is to investigate the combined therapeutic effects of sorafenib and apigenin upon apoptosis and cell cycle on HepG2 cell line. Cytotoxic effects of sorafenib and apigenin on HepG2 cells were determined by XTT assay. Effects of single and combined treatment on cell migration, invasion and colony formation were analysed by wound healing, transwell matrigel invasion assay and colony formation assay, respectively. TUNEL assay was performed for analyse apoptosis rates. Expression changes of genes related with apoptosis and cell cycle were analysed by quantitative real-time PCR. Combined treatment of sorafenib and apigenin has more decreasing effects on cell viability than single treatment groups. Also, combination group caused significant increase of apoptotic cells. Migration and invasion capability of cells in combined treatment group are decreased. Lastly, quantitative real-time PCR results showed that combination of both drugs arrested cell cycle and increased apoptotic gene expressions more than single treatment groups. This is the first study that investigating the combined treatment of sorafenib and apigenin on HCC *in vitro*. By combined treatment, apigenin potentiates sorafenib cytotoxicity on HepG2 cells. Effects of combined treatment on migration, invasion, apoptosis and gene expressions showed that may sorafenib and apigenin have synergistic effect.

1. Introduction

Hepatocellular carcinoma (HCC) which is major forms of liver cancers (70–80%) is seventh most common cancer type and third cancer related death around the world, also its incidence and mortality rates are getting increase (Venook et al., 2010; Yang and Roberts, 2010). About 600.000 deaths worldwide annually are related to HCC (Chen et al., 1997; Lin et al., 2012). Most common risk factor for development of HCC is cirrhosis and hepatitis B or C virus infection. 80% of HCC patients have chronic hepatitis B and/or hepatitis C virus infections (Chang et al., 1997; Nguyen et al., 2009). HCC development risk of chronic hepatitis B virus carrier is 100 times more than in other people that have no infection. Therefore, it is known that HCC is the most common virus-related cancer type (Arbuthnot and Kew, 2001; Beasley, 1988). Its incidence depending on geographical location and regions that have endemic HBV infection like Asia, Africa are the most seen places. More than one million new cases worldwide annually are diagnosed HCC. Unfortunately, survival rate is less than 3% in 5 years (Tang et al., 1999; Tang, 2001).

Regulation faults of cell cycle in protooncogenes and tumor suppressor genes are caused uncontrolled cell growth leading to cancer (Bos, 1989). In various cancer types, tumor suppressor genes have low level of expression while CDK's have high level of expression (Zhang, 2007). This changes at protein expressions are the reasons of uncontrolled growth of cancer cells. In cancer cells, cell cycle check points are not functional or cancer cells can skip these points straight away.

Abbreviations: Bax, Bcl-2 related X protein; Bcl-2, BCL-2 apoptosis regulator; BID, BH3 interacting domain death agonist; Bim, BCL2 like 11; CDK, cyclin-dependent kinase; cDNA, complementer DNA; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; ERK1/2, extracellular regulated MAP kinase; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; MAPK, mitogen activated kinase-like protein; MDM2, transformed mouse 3T3 cell double minute 2; PDGFR, platelet derived growth factor receptor; PI3K, phosphatidylinositol 3-kinase; PCR, polimerase chain reaction; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TRAIL, TNF-related apoptosis inducing ligand; VEGF, vascular endothelial growth factor

* Corresponding author.
E-mail address: yavuzdodurga@gmail.com (Y. Dodurga).

https://doi.org/10.1016/j.gene.2020.144428
Received 27 November 2019; Received in revised form 16 January 2020; Accepted 30 January 2020
Available online 08 February 2020

Thus, damaged DNA inherited to new cells continuously and mutations are getting increased. For instance, damages skipped from G2 checkpoint caused chromosomal breaks and genomic instability eventually (Pecorino, 2015; Stein, 1998).

The dynamic balance between cell death and cell proliferation is crucial for protection of homeostasis. DNA damage is detected and repaired with DNA repair mechanisms. If it can not be repaired, cell destroyed and controlled by genetic control mechanisms (Lozano and Elledge, 2000). When some damage occurs in this control mechanisms, cell can not be destroyed and proliferate fastly that triggering neoplastic tissue and cancer eventually. Curtin and Cotter found that survival related genes like *Bcl-2* and *c-myc* are overexpressed in cancer cells while death trigger gene expressions are decreased (Curtin and Cotter, 2003). Damaged cells are destroyed by apoptosis to prevent the transfer of mutations to new generations. Thus, genetic mutation number that triggered cancer decreased for organism (Klug and Cummings, 2003). For this reason, researchers were focused on new agents for induce apoptosis in recent years (Fesik, 2005).

Today, sorafenib is the only chemotherapeutic agent used for HCC treatment. Sorafenib represses growth of tumor cells and angiogenesis by inhibiting different oncogenetic pathways (Gong et al., 2017). Sorafenib is a multikinase inhibitor has specific cellular targets and orally used in HCC treatment. c-Raf, B-Raf, VEGFR2, VEGFR3, PDGFR, FLT3, Ret and c-kit are some kind of kinases that sorafenib inhibits at nanomolar concentration levels (Gedaly et al., 2010; Wei et al., 2010). and angiogenesis Vascularization level is dramatically high in HCC and proteins like in Ras/Raf/MAPK pathways are significantly more expressed than non-tumorigenic tissues. Therefore, repression of cell proliferation in HCC treatment with sorafenib occurs by inhibition of Ras/Raf/MAPK and VEGFR-PDGFR pathways, respectively.

Tumor tissues have heterogeneous structure because of increasing genomic instability. Treatment success rate is fairly low because cancer cells have high proliferation capability, resistance to apoptosis, high metastasis, invasion and angiogenesis potential. In this case, combine treatments are more attracted than single treatment methods lately (Emil Frei and Eder, 2003). Single or combined treatment effects of different agents to cell cycle and apoptosis on various cancer types are investigated until today. However, there is no research about apigenin and sorafenib combination treatment, especially in hepatocellular carcinoma in literature.

Apigenin (4′,5,7-trihydroxyflavone) is a natural flavonoid. It is abundant in fruits (orange, apple, cherry, grape), vegetables (onion, parsley, broccoli, sweet pepper, celery, barley, tomato) and drinks (tea, wine) (Yan et al., 2017). Unlike other flavonoids apigenin has low cytotoxicity on normal cells (Gupta et al., 2001). *In vitro* and *in vivo* studies in mammals showed that apigenin has anti-oxidant activity as a free radical scavenger and also it has anti-mutagenic, anti-inflammatory, anti-viral and purgative effects (Ozçelik et al., 2011). Additionally, studies showed that apigenin induce apoptosis and cell cycle arrest and inhibit cell growth in prostate, breast, colon, leukemia and lung cancer cells (Angulo et al., 2017; Huang et al., 2016; Kim and Kim, 2012; Zhao et al., 2017).

The aim of this study is to investigate the combined therapeutic effects of sorafenib and apigenin upon apoptosis and cell cycle on HepG2 cell line and offer a new approach for HCC treatment, by increasing the anticancer activity of sorafenib combined with apigenin.

2. Material and methods

2.1. Cell culture and reagents

In this study, experiments were performed on hepatocellular carcinoma cell line HepG2 (ATCC® HB-8065™) provided from our stock. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, penicillin (20 units/mL), streptomycin (20 μg/mL) and 10% (vol/vol) heat-inactivated fetal calf serum at 37 °C in a humidified, 5% CO_2 atmosphere. Collagenated petri dishes were used for culturing. Cells were passaged along the experiments at 80–90% confluency.

Table 1
Real TimePCR forward and reverse primer sequences.

Genes	Forward and reverse primers
ACTB	F 5′-CACCATTGGCAATGAGCGGTTC-3′
	R 5′-AGGTCTTTGCGGATGTCCACGT-3′
BAX	F 5′-AGAGGATGATTGCCGCCGT-3′
	R 5′-CAACCACCCTGGTCTTGGA-3′
BCL-2	F 5′-CAGGGCGATGTTGTCCACC-3′
	R 5′-GGGGAGGATTGTGGCCTTC-3′
CASP-3	F 5′-CATGGAAGCGAATCAATGGACT-3′
	R 5′-CTGTACCAGACCGAGATGTCA-3′
CASP-8	F 5′-ACACCAGGCAGGGCTCAAAT-3′
	R 5′-GCAGGTTCATGTCATCATCCAGTT-3′
CASP-9	F 5′-CTTCGTTTCTGCGAACTAACAGG-3′
	R 5′-GCACCACTGGGGTAAGGTTT-3′
CASP-10	F 5′-TAGGATTGGTCCCCAACAAGA-3′
	R 5′-GAGAAACCCTTTGTCGGGTGG-3′
BID	F 5′-CCTACCCTAGAGACATGGAGAA-3′
	R 5′-TTTCTGGCTAAGCTCCTCACG-3′
P53	F 5′- ATCTACAAGCAGTCACAGCACA-3′
	R 5′- GTGGTACAGTCAGAGCCAACC-3′
P21	F 5′-TGGAGACTCTCAGGGTCGAAA-3′
	R 5′- GGCGTTGGAGTGGTAGAAATC-3′
P16	F 5′-CAGTAACCATGCCCGCATAGA-3′
	R 5′- AAGTTTCCCGAGGTTTCTCAGA-3′
CDK-6	F 5′-AGACCCAAGAAGCAGTGTGG-3′
	R 5′-AAGGAGCAAGAGCATTCAGC-3′
CYCLIN-D1	F 5′-AGCTCCTGTGCTGCGAAGTGGA-3′
	R 5′-AGTGTTCAATGAAATCGTGCGG-3′
MDM2	F 5′-GGATTTCGGACGGCTCTCGC-3′
	R 5′- CGCGCAGCGTTCACACTAGTG-3′
RB	F 5′-TGTAAAACGACGGCCAGT-3′
	R 5′-CAGGAAACAGCTATGACC-3′
PUMA	F 5′-GACCTCAACGCACAGTACGAG-3′
	R 5′-AGGAGTCCCATGATGAGATTGT-3′

Sorafenib (Nexavar, USA) and apigenin (Selleckchem, USA) were dissolved in dimethyl sulfoxide (DMSO) and prepared stock concentration of 100 mM. The concentrations of sorafenib and apigenin ranging from 1 μM to 40 μM and 10 μM to 1000 μM respectively were applied to HepG2 cells for 24, 48 and 72 h.

2.2. Cell viability

HepG2 cells were seeded at a density of 1×10^4 cells/well in collagenated-96 well plates according to trypan blue dye cell counting method. Apigenin was prepared at 8 different concentrations ranging between 10 and 100 μM and sorafenib was prepared at 10 different concentrations ranging between 1 and 40 μM. Different apigenin and sorafenib concentrations were applied after 24 h incubation. Afterward 24 h incubation, cells were exposed with these apigenin and sorafenib concentrations for 24, 48, and 72 h. Concentrations of sorafenib and apigenin that decreased the cell viability by 50% (IC_{50}) were determined with cell proliferation assay with XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2h-tetrazolium-5 carboxanilide) Reagent-Cell Proliferation Kit (Cat # 20-300-1000-Biological Industries, USA) based on the manufacturer's protocol and results was spectrophotometrically measured at 450 nm (reference wavelength, 630 nm) using a microplate reader (BioTek, USA).

2.3. Colony formation analysis

HepG2 cells were seeded at a density of $1x10^3$ cells/well in collagenated-6-well plates according to trypan blue dye cell counting method. Afterward 24 h incubation, cells were exposed with single and combined doses of apigenin and sorafenib concentrations for 48 h.

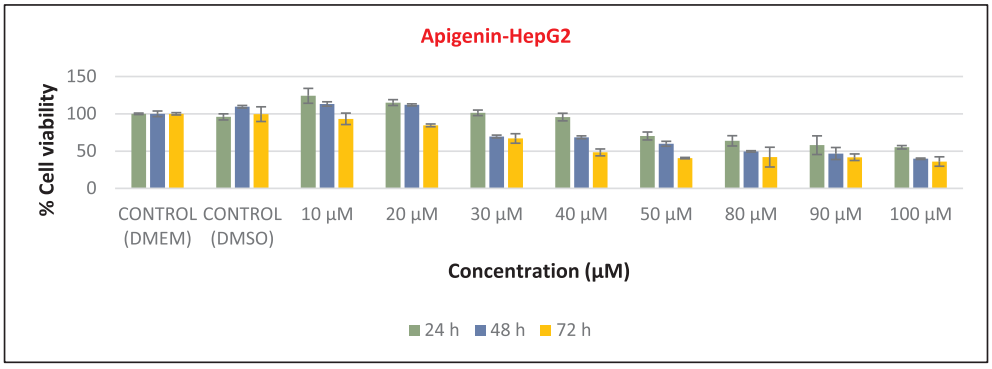

Fig. 1. Percentage cell viability of HepG2 cells induced by apigenin in different concentrations calculated with XTT assay.

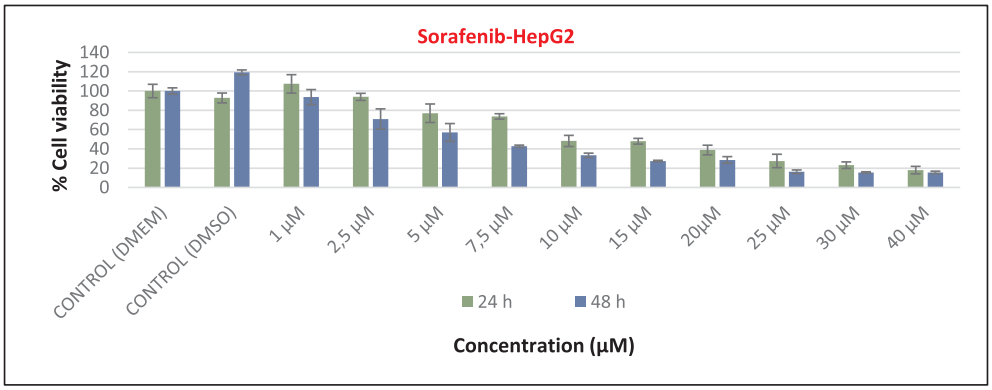

Fig. 2. Percentage cell viability of HepG2 cells induced by sorafenib in different concentrations calculated with XTT assay.

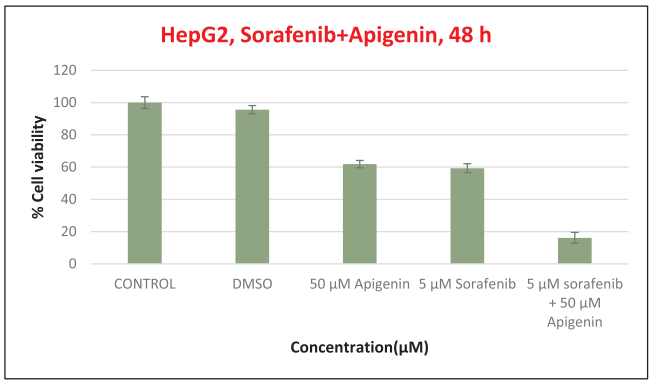

Fig. 3. Percentage cell viability of HepG2 cells induced by apigenin and sorafenib combined treatment calculated with XTT assay.

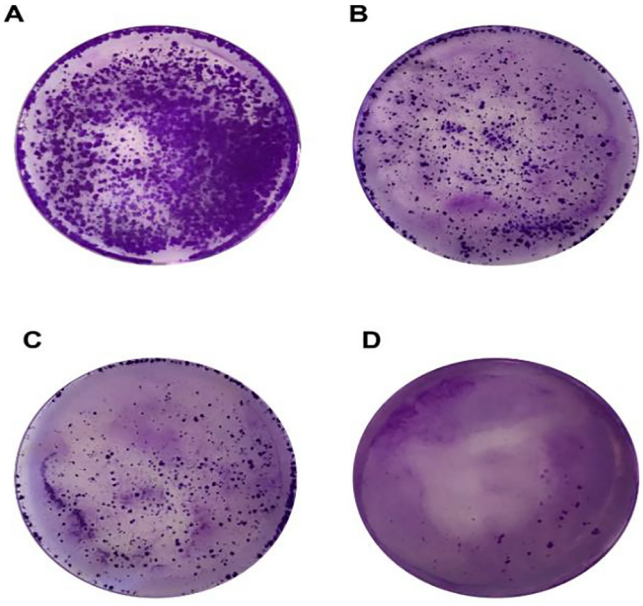

Fig. 4. Representative images for colony formation after 48 h **A)** Control group **B)** 50 µM apigenin treated **C)** 5 µM sorafenib treated group **D)** 50 µM apigenin + 5 µM sorafenib combined group.

Subsequently, cells were incubated for 14 days at 37 °C in a humidified, 5% CO_2 atmosphere and only mediums were changed in every 2 days in a roll. At the end of 14 days, mediums were removed and cells were fixed by incubation with methanol at −20 °C for 10 min. After the fixation, colonies were stained with crystal violet dye and counted. Results were evaluated for control and dose groups.

2.4. Wound-healing migration analysis

Effects of apigenin, sorafenib and combine treatments on migration of cells were determined with wound-healing migration assay. HepG2 cells were seeded at a density of $2x10^5$ cells/well in collagenated-6-well plates. When the confluency was about 90%, mediums were removed and plates were washed with serum-free DMEM. 6-well dishes were scratched straightly with 200 µl sterile pipette tip. After scratching, cells were washed 3 times with serum-free DMEM and cell scraps were removed. Cells were treated with apigenin, sorafenib and combined dose groups and DMEM supplemented with 10% serum was used for control group. Cells were incubated for 48 h at 37 °C in a humidified, 5% CO_2 atmosphere and photographed at 0, 16th, 24th and 48th hours for comparison of cell movements at control and dose groups.

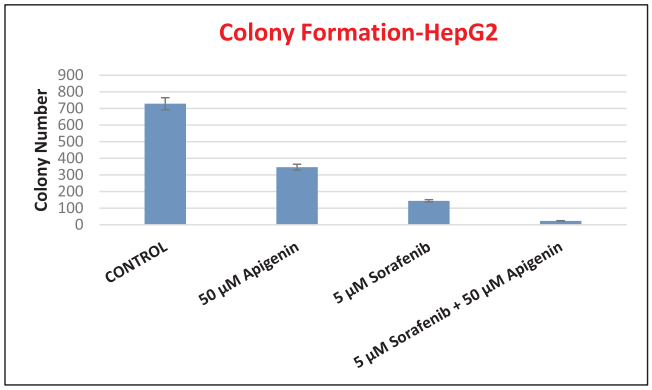

Fig. 5. Comparison of colony formation rates of HepG2 cells cultured with 50 µM apigenin, 5 µM sorafenib, 50 µM apigenin + 5 µM sorafenib.

2.5. Trans-well matrigel invasion analysis

Invasion capacity of cells were measured using "trans-well matrigel invasion chamber" 24-well invasion dishes. HepG2 cells were seeded at a density of $5x10^5$cells/well on matrigel membrane with 8 µm pores and incubated in serum-free medium overnight. Simultaneously, DMEM supplemented with 10% serum was put in 24-well plate dishes out of the wells with membrane and outside of the wells became chemoattractant for cells by this way. After the overnight incubation apigenin, sorafenib and combined doses were applied with serum free medium and incubated for 48 h at 37 °C in a humidified, 5% CO_2 atmosphere. At the end of the incubation, cells passed through the membrane were fixed with methanol, stained with crystal violet dye and counted.

2.6. TUNEL assay apoptosis analysis

TUNEL kit (AAT bioquest, CA) was used for apoptosis assay. HepG2 cells were seeded at a density of $5x10^3$ cells/chamber on culture slides with 8 chambers (BD Falcon, USA). After 24 h, Apigenin, sorafenib and combined doses were treated to chambers and incubated for 48 h. After incubation, mediums were removed and 100 µl formaldehyde (4%) were added fixative buffer in each chamber and incubated for 20–30 min. Fixative buffer was removed and chambers were washed with PBS for 2–3 times. Reaction mix was prepared according to manufacturer's protocol. Reaction mixture was allocated as 50 µl on each chamber and incubated at 37 °C for 60 min. After that reaction mixture was removed and cells were washed with 200 µl PBS for 3–5 times. For visualization of nucleus, 20 µl Hoechst (1X) was used for each chamber and analysed with fluorescence microscope (Ex/Em = 350/460 nm).

2.7. Real-Time PCR analysis

HepG2 cells were seeded on collagenated six-well plates at a concentration of 3×10^5 cells/well. After 24 h incubation, the IC_{50} doses of sorafenib, apigenin and combination were applied to each well excluding the control well and incubated for 48 h. Total RNA was isolated according to the Trizol reagent protocol. Complementary DNA (cDNA) synthesis was conducted using Transcriptor High Fidelity cDNA Synthesis kit (Roche, Germany) as per the manufacturer's instructions.

ACTB (*Actin Beta*), *BAX* (*BCL2 associated X*), *BCL-2*, *Caspase-3* (*CASP-3*), *Caspase-8* (*CASP-8*), *Caspase-9* (*CASP-9*), *Caspase-10* (*CASP-10*), *BID* (*BH3 interacting domain death agonist*), *p53*, *p21*, *p16*, *CDK6* (*cyclin dependent kinase 6*), *CYCLIN-D1*, *MDM2* (*MDM2 proto-oncogene*), *RB* (*Retinoblastoma*), *PUMA* gene expression analyses were performed using the StepOnePlus quantitative real-time PCR system (Applied-Biosystems, USA) with respect to SYBR Green (Thermo-Scientific, USA) method. Forward and reverse primer sequences of these genes were shown at Table 1. The real-time PCR analysis was performed using specific primers for each gene. To identify gene expressions, the results of the selected gene's expressions were normalized to the *beta-actin* housekeeping gene.

2.8. Statistical analysis

The analyses of real-time PCR results were performed using computer program according to ΔΔCT method. The groups were compared using Volcano plot analyses from RT^2 Profiles™ PCR Array Data Analysis with Student *t* test and p value of < 0.05 value was considered significant.

3. Results

3.1. Cytotoxic activity

HepG2 cell death upon treatment with apigenin, sorafenib and combined treatment was assessed using the XTT assay. Time and dose-dependent decrease patterns were found in the viability of HepG2 cells. In this study, IC_{50} dose of apigenin and sorafenib was found to be 40 µM for 72 h (Fig. 1), 7,5 µM for 48 h (Fig. 2), respectively. While 5 µM sorafenib treatment for 48 h was killed 43% of cells, 50 µM apigenin treatment was killed 41% of cells. But the combined treatment of 5 µM sorafenib and 50 µM apigenin for 48 h was killed 84% of cells (Fig. 3).

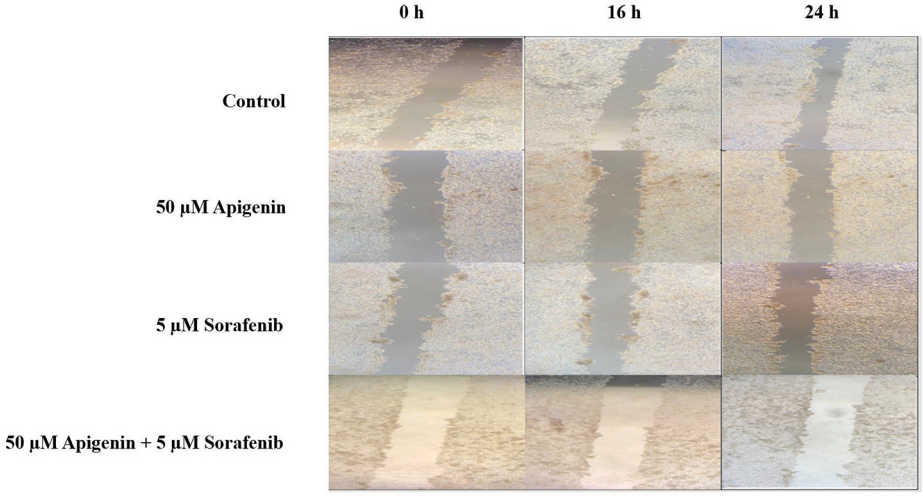

Fig. 6. Wound healing images of control, 50 µM apigenin, 5 µM sorafenib and 50 µM apigenin + 5 µM sorafenib treated groups at 0, 16, 24 h.

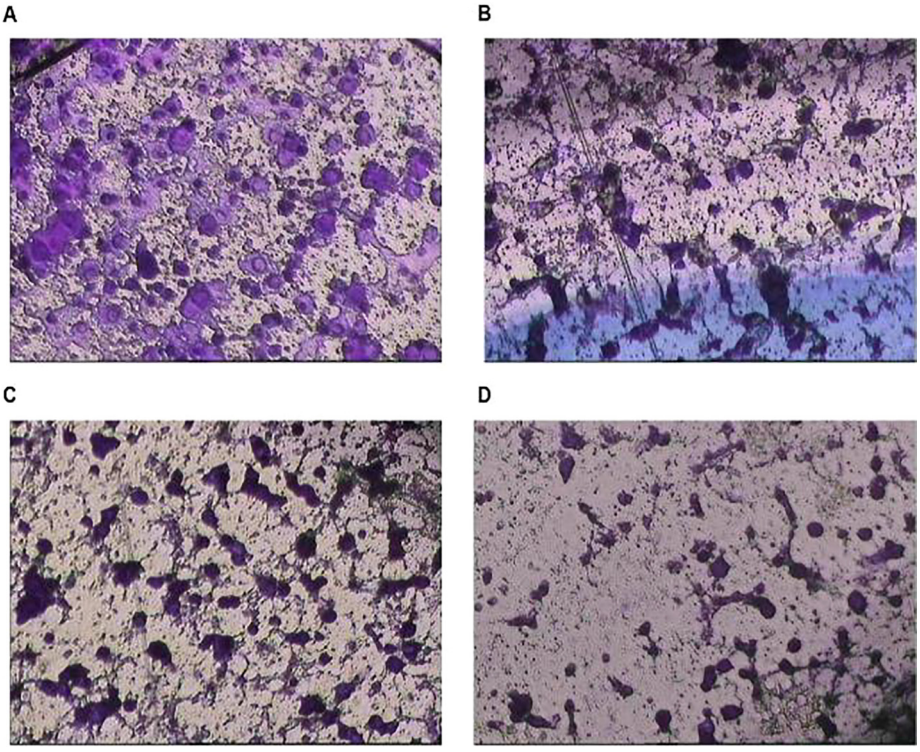

Fig. 7. Invased cells in **A)** Control **B)** 50 μM apigenin **C)** 5 μM sorafenib **D)** 50 μM apigenin + 5 μM sorafenib treated groups.

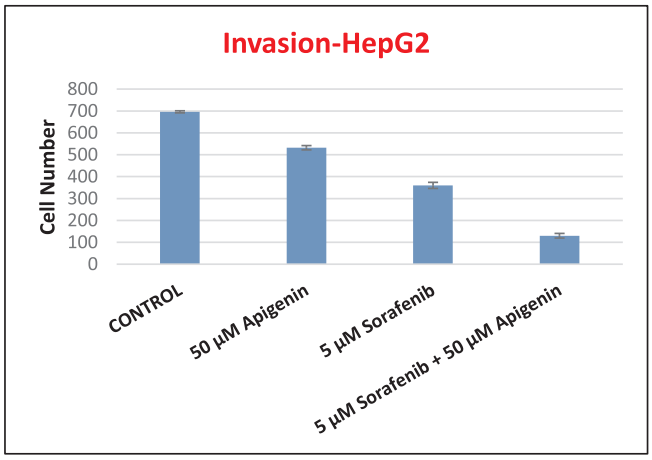

Fig. 8. Invased cell numbers in control, 50 μM apigenin, 5 μM sorafenib and 50 μM apigenin + 5 μM sorafenib treated groups.

So, 5 μM sorafenib + 50 μM apigenin was used for combine treatment groups for next experiments.

3.2. Colony formation analysis

Colonies of HepG2 cells were formed along 14 days after being cultured with 5 μM sorafenib, 50 μM apigenin and 5 μM sorafenib + 50 μM apigenin combination treatment. Colonies were counted and evaluated. 728, 347, 144 and 24 numbers of colonies have counted in control, 50 μM apigenin, 5 μM sorafenib and 5 μM sorafenib + 50 μM apigenin groups, respectively (Fig. 4). When these numbers compared with control group, 27% decrease was observed in 50 μM apigenin treatment group and 80% decrease was observed in 5 μM sorafenib treatment group. In combined treatment group, colony formation rate was 96% decreased compared with control group. These numbers showed that treatment with apigenin and sorafenib combination remarkably repressed colony formation in HepG2 cells more than single treatment groups (Fig. 5).

3.3. Wound healing migration analysis

Migration ability of HepG2 cells in control and dose groups were compared by wound healing migration assay. As corelated with cytotoxicity and colony formation analysis, migration ability of cells in 50 μM apigenin + 5 μM sorafenib group was significantly repressed (Fig. 6).

3.4. Matrigel invasion analysis

696, 532, 360, 130 cells were counted in control, 50 μM apigenin, 5 μM sorafenib, 50 μM apigenin + 5 μM sorafenib groups, respectively (Fig. 7). According to these results, invasion ability of cells in 50 μM apigenin + 5 μM sorafenib combined group was critically decreased (Fig. 8).

3.5. TUNEL apoptosis analysis

Apoptotic cells have some morphological changes such as cell condensation and shrinkage, disintegration of cell skeleton, nucleus membrane and DNA. In this assay, Hoechst dye was used to see apoptotic cells under fluorescence microscope by take advantage of these morphological changes. Control and dose groups have seen as Fig. 9. Five different areas were counted for each control and dose groups.

According to these numbers, apoptotic cells were proportioned with living cells and apoptosis percentage calculated for each group (Fig. 10).

3.6. Real-time PCR analysis

Real-time PCR analyses were demonstrated significant changes in mRNA levels of some genes related with cell cycle and apoptosis as shown in Table 2. In 50 μM apigenin treated group, *caspase-3*, *caspase-8*,

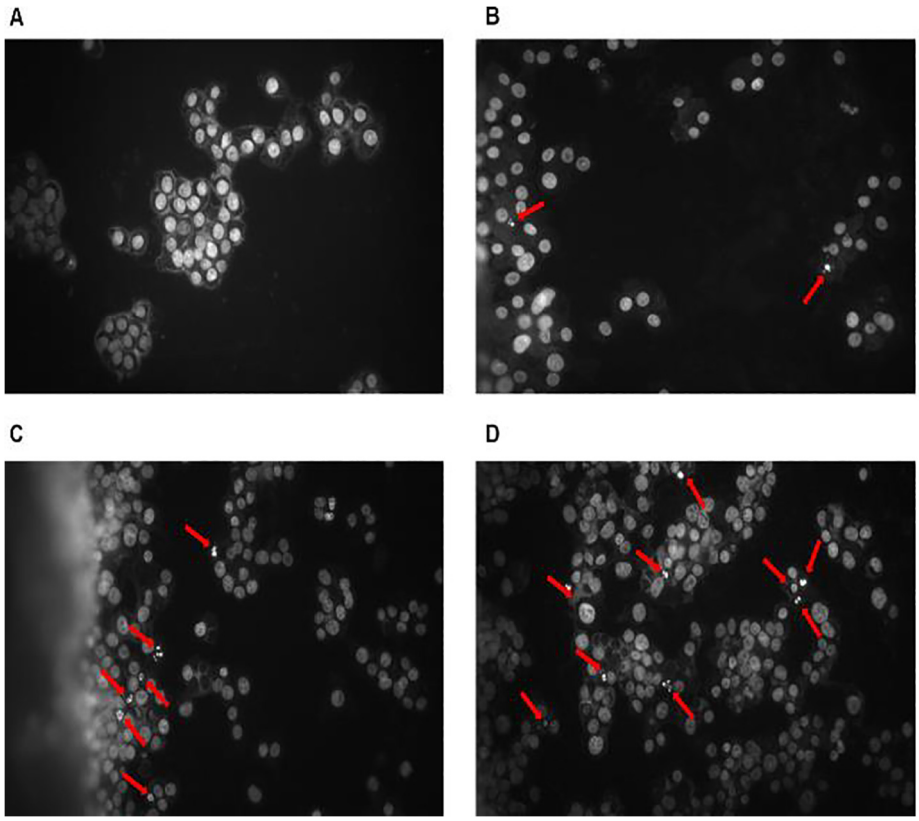

Fig. 9. A) Control **B)** 50 μM apigenin **C)** 5 μM sorafenib **D)** 50 μM apigenin + 5 μM sorafenib treated HepG2 cells under fluorescence microscope (20X). Apoptotic cells were pointed with red arrows.

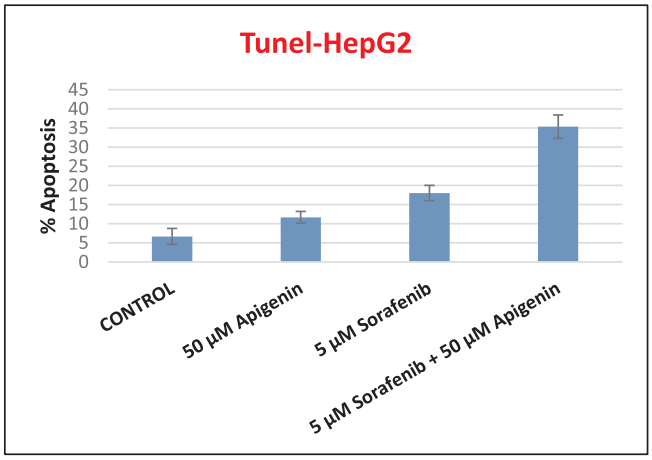

Fig. 10. Apoptosis percentage in control, 50 μM apigenin, 5 μM sorafenib and 50 μM apigenin + 5 μM sorafenib treated groups.

BID, *p53*, *p21* and *p16* expression levels were significantly increased. These changes are showed that single treatment of apigenin induce apoptosis in HepG2 cells. On the other hand, mRNA expression levels of *caspase-8*, *caspase-10* and *BID* were significantly increased in the 5 μM sorafenib treated group. In 50 μM apigenin + 5 μM sorafenib combine group, expression levels of *caspase-3, caspase-8 and caspase-10 were* significantly increased compared with single treatment groups. Also, expression levels of *BID, p21* and *p16 were* significantly increased (Table 2). In this respect, combine treatment with apigenin and sorafenib can induced extrinsic pathway of apoptosis in HepG2 cells.

4. Discussion

Hepatocellular carcinoma is the fifth most common cancer types in the world and about 600,000 deaths worldwide annually are related to this cancer (Llovet et al., 2003). Unfortunately, the fact that HCC diagnosis can only be made in the advanced stage causes the patients can't get benefit from the potential treatment methods. 5-year survival rate of these patients is about 7% (Bosch et al., 2004). Also, the development of resistance to chemotherapeutic agents used in HCC as in other cancers, angiogenesis and metastasis ability of tumor, and severe side effects of chemotherapeutic agents adversely affect the success of the treatment (Emil Frei and Eder, 2003; Zhang et al., 2012). For this reason, researchers have recently focused on herbal extracts or active substances for use in combination with chemotherapeutic agents in the treatment of HCC.

Sorafenib is an orally used multiple kinase inhibitor. Raf-1 and B-Raf Serine-Threonin kinases, Vascular Endothelial Growth Factor (VEGF) receptors 1, 2 and 3, Platelet Derived Growth Factor (PDGF), KIT, RET and FLT-3 are some of these kinases that related with vital functions such as survival, growth, proliferation, apoptosis (Wilhelm et al., 2004). Neoplastic processes such as tumor development, proliferation, metastasis and suppression of apoptosis are controlled by pathways induced by active tyrosine kinase receptors.

Cancer cells produce high levels of growth factors and cytokines such as PDGF-β and VEGF. By binding these molecules to receptors on endothelial cells, Raf/MEK/ERK pathways are activated, which is one of the factors that trigger angiogenesis (Hood et al., 2002). Therefore, Raf kinases, VEGF and PDGF-β are potential therapeutic targets for inhibition of angiogenesis and cancer treatment (Furuse, 2008). Studies have shown that sorafenib inhibits cell proliferation and angiogenesis and also accelerates apoptosis in various cancer types (Liu et al., 2006). It has been prolonged survival of advanced HCC and renal carcinoma patients (Escudier et al., 2007; Ratain et al., 2006).

Table 2
mRNA expression levels in 50 μM apigenin, 5 μM sorafenib and 50 μM apigenin + 5 μM sorafenib treated groups ($p < 0,05$).

50 μM Apigenin			5 μM Sorafenib			50 μM Apigenin + 5 μM Sorafenib		
Gene	Change	p-Value	Gene	Change	p-Value	Gene	Change	p-Value
ACTB	1	0	***ACTB***	1	0	***ACTB***	1	0
CASP-3	25,8772	0,004373	***CASP-3***	7,6452	0,263194	***CASP-3***	38,3339	0,000383
CASP-8	9,2679	0,009727	***CASP-8***	7,1014	0,019322	***CASP-8***	35,7977	0,00002
CASP-9	1,6372	0,243546	***CASP-9***	1,4048	0,516756	***CASP-9***	1,5123	0,37111
CASP-10	1,3635	0,59007	***CASP-10***	2,6379	0,016314	***CASP-10***	5,6785	0,003432
BID	5,4378	0,007372	***BID***	3,3969	0,015453	***BID***	4,0826	0,001222
P53	2,74	0,011864	***P53***	2,0882	0,076615	***P53***	2,1042	0,056283
P21	66,0504	0,029612	***P21***	5,7746	0,131687	***P21***	15,7327	0,001871
P16	2,3975	0,0301	***P16***	1,6386	0,318869	***P16***	2,7531	0,013047
PUMA	1,9648	0,088869	***PUMA***	2,0327	0,07139	***PUMA***	1,4447	0,466551

Liu et al. investigated the effect of sorafenib on cell viability in two different HCC cell lines, HepG2 and PLC/PRF/5 cells by CellTiter Glo analysis. IC_{50} dose in PLC/PRF/5 cells was 6.3 mol/L and IC_{50} dose in HepG2 cells was 4.5 mol/L for 72 h (Liu et al., 2006). In our study, the IC_{50} dose of sorafenib in HepG2 cells was found 7.5 μM for 48 h using XTT analysis. It is thought that the IC_{50} dose differences between our results and literature results may arising from different cell viability analysing methods.

As a flavonoid, apigenin has many different properties beside its low toxicity on healthy cells. Studies have shown that single and combine treatments of apigenin have very strong anti-cancer activity on human cancers. Apigenin inhibits the proliferation of cancer cells by inducing apoptosis and autophagy, regulates cell cycle, suppresses migration and invasion by limiting the mobility of cancer cells, and also demonstrated its anti-cancer effects by stimulating the immune system.

Tseng et al. reported that 40 μM apigenin was upregulated p21 expression while suppressing the expression of cyclin A, cyclin B, and CDK1 in MDA-MD-231 breast cancer cell line and causing the cell cycle arrest in the G2/M phase. It was also found that HDAC activity was inhibited and histone H3 acetylation was induced (Tseng et al., 2017). In our study, according to the results of RT-PCR analysis, there was a significant increase in *p21* expression in the 50 μM apigenin treated group, whereas no significant change in *p21* expression was observed in the 5 μM sorafenib treated group due to possible methods, kits or hand errors. However, there was a statistically significant increase in *p21* expression level in the combined group treated with 50 μM apigenin + 5 μM sorafenib. In this case, it can be said that the combine treatment of sorafenib and apigenin at determined doses inhibits proliferation by inhibiting cell cycle through p21, i.e. synergistic effect, but further studies required to determine the molecular targets and mechanisms in the pathways associated with the cell cycle.

Ruela-de-Sousa et al. reported that 50 μM apigenin was inhibited the JAK/STAT pathway and causing the cell cycle arrest in HL60 leukemia cell line (Ruela-De-Sousa et al., 2010). In the same cell line, Wang et al. found that 60 μM apigenin induces apoptosis by increasing caspase-9 and caspase-3 activation (Wang et al., 1999). In our study, a significant increase in *caspase-3* expression was observed in the 50 μM apigenin group as well.

Combined *in vivo* and *in vitro* studies have shown that the use of apigenin with other agents accelerates the anti-cancer effects. Anticancer drugs such as cisplatin and paclitaxel, which are used today, significantly increase the survival of patients. However, their toxic effects on healthy cells are a cause for concern for clinicians and patients. Combination of agents with apigenin to accelerate their anti-cancer effects, reduce their limitations and minimize their side effects have been studied in several types of cancer.

One of the problems in cancer treatment is the resistance to the existing drug which adversely affects the success of the treatment. Combined therapies also have an effective and promising potential to break the resistance to the drug used. In a study performed on SKOV3 ovarian cancer cell lines, it was observed that 40 μM apigenin in taxol resistant SKOV3 cells downregulated Axl and Tyro3 receptor tyrosine kinases and exterminated taxol resistance and accelerated anti-cancer activity (Suh et al., 2015). One of the biggest problems in the treatment of HCC is that resistance development to sorafenib within 6 months and the treatment fails. Based on these studies, it is thought that apigenin may be effective in exterminating sorafenib resistance and accelerating the anticancer activity of sorafenib. Our study is expected to illuminate on long-term and advanced studies related to overcoming sorafenib resistance.

In vitro and *in vivo* studies were performed in SK-Hep-1 and BEL-7402 HCC cell lines and xenograft model athymic nude mice. Apigenin and 5-FU combined dose in SK-Hep-1 and BEL-7402 cells significantly reduced cell viability compared to control and single dose groups. Also, the number of apoptotic cells was significantly increased compared to control and single dose groups. On the other hand, TUNEL test performed with tumor sections from mice showed that DNA damage was higher in the combined group than control and single treatment groups (Hu et al., 2015). Johnson et al. were found that apigenin combination with 5-FU increased the anti-proliferative effect of 5-FU through decreased NF-kB and p65 in BxPC-3 pancreatic cancer cell line (Johnson and Gonzalez de Mejia, 2013). In the present study, combine treatment of sorafenib and apigenin doses selected under their single IC_{50} doses was accelerated the cytotoxic activity of sorafenib compared to control and single dose groups, and induced apoptosis. Combined treatment of sorafenib and apigenin at low doses is thought to induce apoptosis in cells. Also, it is thought that the minimization of the cytotoxic effect of sorafenib on normal cells may be possible as a result of combined application with apigenin, but further studies are required.

The most common problem in the treatment with sorafenib is that patients develop resistance to sorafenib in a short period of time. According to the information obtained from the literature reviews, it has been reported that apigenin overcomes the resistance against the drugs currently used and increases the anti-cancer properties of the drug by combined treatment. It is thought that apigenin is a promising flavonoid to overcome sorafenib resistance and increase the anticancer activity of sorafenib in HCC treatment and further *in vitro* and *in vivo* studies are needed. Therefore, in order to be able to use apigenin in clinical treatment effectively, appropriate dosage and durations for different types of cancer, molecular effects and direct targets on apoptosis and cell cycle can be determined.

5. Conclusion

In the scope of this study, it was determined that the combined treatment of sorafenib and apigenin in HepG2 human HCC cell line at low doses and time statistically decreased the viability of tumor cells more than single dose groups and also induced apoptosis. Sorafenib is the only chemotherapeutic agent currently used for HCC treatment. However, sorafenib has a cytotoxic effect on health cells either and

patients develop resistance to sorafenib in a short period of time, which reduces the success of treatment. Apigenin is a flavonoid with low cytotoxicity on healthy cells as well as anti-cancer properties. We found that apigenin accelerates and increases the anti-cancer effect of sorafenib as corelated with literature. In addition, it is thought that apigenin may be a promising compound to overcome sorafenib resistance and increase its efficacy. Further researches and studies are needed to determine the appropriate dose and treatment methods for apigenin to be included in routine sorafenib treatment. In this regard, the present study is unique for combined treatment of sorafenib and apigenin in the HepG2 cell line and has been tried for the first time. Therefore, it can be a source by providing a basis for further researches.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by Pamukkale University Scientific Research Projects Coordination Unit through project number: 2017SABE002.

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