LY3214996 relieves acquired resistance to sorafenib in hepatocellular carcinoma cells

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Primary research

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Abstract

Background: Due to the reactivation of ERK signaling in sorafenib-resistant hepatocellular carcinoma (HCC) cells, in this study, the anti-cancer effect of LY3214996 (selective ERK1/2 inhibitor) combined with sorafenib on HCC cells was evaluated.

Methods: Phosphorylation of the key kinases in the Ras/Raf/MAPK and PI3K/Akt pathways were detected using Western blot. Cells proliferation, migration, cell cycle and apoptosis were evaluated in Huh7 and Huh7 R cells.

Results: LY3214996 significantly reduced phosphorylation levels of the tested kinases of Ras/Raf/MAPK and PI3K/Akt pathways including p-c-Raf, p-P90RSK, p-S6K and p-eIF4EBP1 activated by sorafenib, despite increased p-ERK1/2 levels. It was found that LY3214996 enhanced the anti-proliferation, anti-migration, blocking cell cycle progression and pro-apoptotic effects of sorafenib on Huh7 R cells.

Conclusions: The reactivation of ERK1/2 might be highly related to molecular mechanism of acquired drug resistance. LY3214996 combined with sorafenib enhanced anti-tumor effects in HCC. Consequently, combined treatment of LY3214996 and sorafenib provides a second-line therapy for acquired resistant in advanced HCC.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the fourth leading cause of cancer-related death in the world reported by the International Agency for Research on Cancer (IARC), representing approximately 90% of all cases of primary liver cancer occurring usually in the setting of chronic liver disease and cirrhosis [1-2]. The curative treatment options accepted are surgical resection, liver transplantation, and local ablative therapies which are effective for early stage HCC patients. Due to lack of early effective diagnosis, around 80% patients are diagnosed with advanced HCC, which limits treatment options. But the prognosis of advanced HCC is poor, the median overall survival (OS) time is less than one year [3]. Currently, systemic therapy is a more effective treatment for extending overall survival time.

Sorafenib, an oral multi-target, multi-kinase inhibitor, was the most widely used systemic chemotherapy approved as a first-line agent for unresectable or advanced HCC by the United States Food and Drug Administration (FDA) in 2007 [4-5]. On the one hand, it inhibited the receptor tyrosine kinases c-Kit, Flt-3 [6] and the serine/threonine kinases including Raf kinases involved in the Raf/MEK/ERK pathway [7], which leads to significantly inhibit tumor cell proliferation; On the other hand, it also inhibits the receptor tyrosine kinase including vascular endothelial growth factor receptor (VEGFR-2/3) and platelet-derived growth factor receptor (PDGF-R), which notably inhibit tumor angiogenesis [8]. Despite encouraging progress was acquired, treatments for patients with advanced HCC are still very limited owing to acquired sorafenib resistance, and further development of new treatment options is necessary. In addition, the low response rate of sorafenib is a worrying phenomenon and epithelial-mesenchymal transition (EMT),

Ras/Raf/MAPK, PI3K/Akt, JAK/STAT pathways, hypoxia, and many other mechanisms were considered to be the main obstacles that led to the failure of sorafenib in HCC patients [9-11].

Recently, biological studies have pointed to aberrant rapidly accelerated fibrosarcoma (Raf)/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway activation as being central for cancer growth, survival and motility, as well as for targeted therapy resistance mechanisms [12-14]. Ras/Raf/MAPK cascade played a variety of roles in cell cycle regulation, apoptosis and cell differentiation [15]. In addition, cascade plays an important role in the apoptosis process by phosphorylating various apoptosis regulating factors, such as Bad, Bim, Mcl-1, caspase-9 and the controversial Bcl-2 [16]. Therefore, this pathway has promoted the development of multiple drugs to inhibit Ras/Raf/MAPK signals in cancer. Raf and MEK inhibitors were approved and used clinically [17-18]. In particular, the combination of Raf /MEK inhibition seemed to improve progression-free survival compared to Raf or MEK monotherapy [19]. Despite the success of these treatments, almost all patients developed resistance to these drugs [20]. Many studies on resistance mechanisms to Raf/MEK inhibition have been described and activating MAPK pathways through multiple methods, including alternate splicing BRAF, NRAS or MEK1/2 mutations, MAPK38 upregulation or receptor tyrosine kinase signaling, are the most common clinical drug resistance mechanisms [21-22]. Since the pathway from Raf to MEK to ERK was linear, ERK1/2 itself was regarded as a target and all these changes were focused on the continuous activation of ERK, the clinical development of small molecule ERK inhibitors had aroused great interest. The treatment of LY3214996, a potent, selective, ATP competitive ERK inhibitor, inhibited the pharmacodynamic biomarker, phospho-P90RSK, in cells and tumors, and was related to the exposures and antitumor activities of LY3214996 [23].

In this study, we investigated the mechanism of acquired resistance to sorafenib in HCC, and we combined LY3214996 with sorafenib to restore chemosensitivity of drug-resistant cells. our results provide the theoretical basis for the combination therapy of sorafenib and ERK1/2 inhibitors in HCC.

Methods

Reagents and antibodies

Sorafenib was obtained from MedChem Express (Monmouth Junction, NJ, USA). ERK1/2 inhibitor (LY3214996) was purchased from Selleckchem (Houston, TX, USA). Antibodies against cyclin D1 and phospho-Rb were purchased from Abcam Biological Technology (USA). Phospho-ERK1/2 Kit (#9911) and antibodies to ERK1/2, Caspase-3, Cleaved Caspase-3, Caspase-9, Cleaved Caspase-9, PARP, Cleaved RARP, PI3K110 β , Akt, phospho-Akt (Ser473), mTOR, phospho-mTOR (Ser2481), Bim, Bad, Bak, Bax, P70S6K, S6K, β -actin, phospho-S6K, phospho-P70S6K and secondary horse radish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse antibodies were purchased from Cell Signal Technology (Danvers, MA, USA).

Cell lines and cell culture

Huh7, one of human HCC cell lines, was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Huh7^R, an acquired sorafenib-resistant HCC cell line, was established from Huh7 cells as follows: When the cells were in the logarithmic growth phase, changed the culture medium, added a lower concentration of sorafenib to it for 24 h, then performed cell inheritance, and repeatedly stimulated with this concentration until it was stable. When the sorafenib concentration reached 4-5 times the IC₅₀ value of the sensitive strain, the resistant strain was obtained. Two cells were cultured in RPMI-1640 (Hyclone, Salt Lake City, UT, USA), supplemented with 15% fetal bovine serum (FBS) (Sijiqing Bioengineering Materials, Hangzhou, China) and incubated at 37 °C with 5% CO₂.

Cell viability assay

Cells (10,000 cells per well) were seeded into 96-well plates, allowed to adhere overnight, and exposed to a range of different drug concentrations. After 12, 24, 48 and 72 h, 10 µL of 5 mg/ml MTT dissolved in PBS was added to each well and incubated for 4 h at 37 °C. The medium was aspirated, and 100 µL of DMSO was added and shaken for 15 minutes to each well. Absorbance was measured at 490 nm. IC₅₀ value was calculated by GraphPad Prism Version 5.0 software.

Colony-formation assay

Cells were cultured into six-well plates at a density of 2×10^3 cells per well for 24 h and drug experiments previously designed were carried out. After two weeks, when cellular clones were clearly visible, they were fixed in 4% paraformaldehyde for 15 min, and then stained with 0.1% crystal violet for 30 min. Subsequently, 6-well plates were thoroughly washed with water to count the number of clones. Colony formation rate reflects two important characteristics of cell population dependence and proliferation ability. All experiments were done in triplicate.

Wound-Healing assay

Cells (2×10^5 cells/well) were plated overnight in 6-well plates with 15% FBS medium. All wells covered with cells were emerged scratched areas with a 10 µL tip. PBS was used to remove injured cells. Serum-free medium with various drug were added to corresponding well and incubated at 37 °C in a humidified atmosphere with 5% CO₂. Pictures with a light microscope were used to record the width of the scratched area after 24 and 48 h.

Cell cycle assay

Cells were digested by 0.25% trypsin-EDTA after incubated with different drug combinations for 24h, washed by PBS three times and fixed through 70% precooled ethanol. Before testing, cells were rewashed by PBS, added 50 µg/ml propidium iodide and 100 µg/ml RNase A in the dark for 30 min at 37 °C followed by flow cytometry analysis (BD FACSCalibur, USA). The distribution of cells at specific cell cycle stages was assessed with ModFit Version 3.0 software (Verity Software House, Topsham, ME).

Apoptosis assay

After drug treatment for 24h, cells from different experimental groups were collected. After washed with cold PBS, cells were resuspended in 500 μ L binding buffer containing 5 μ L PI and 10 μ L FITC-labelled Annexin-V and incubated for 15 min in the dark at room temperature. Cell apoptosis was detected using Flow cytometry analysis (BD Biosciences), and FlowJo Version 7.6.1 software (FlowJo, Ashland, OR) was operated to analyze apoptotic rate.

Western blot

Whole cell lysates were obtained from the combination of Radio Immunoprecipitation Assay (RIPA) buffer (Beyotime, Jiangsu, China) and a mixture of protease and phosphatase inhibitor (Beyotime, Jiangsu, China). The BCA protein assay kit (Biosharp, Hefei, China) was applied to determine protein concentration. Supernatants per sample were aliquoted, mixed with loading buffer, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene Fluoride (PVDF) membrane. After blocked with 5% skim-milk, corresponding primary antibodies were employed to react at 4 °C for 24 h, then the secondary antibody binding reaction time was set to 1 h at 37 °C. Clarity Western ECL substrate (Bio-Rad, Hercules, CA) was assisted to visualize the signals. We quantified protein expression using Image J Version 1.48 software (NIH, Bethesda, MD) and regarded β -actin as a loading control. Relative expression of indicated proteins were normalized to β -actin.

Statistical analysis

All in vitro assay results represent three replicates of three independent experiments performed under the same conditions. All data were expressed as mean values $\pm$ standard deviation. Statistical tests were performed on the paired values using the Student's t-test ($p < 0.05$) was considered statistically significant).

Results

PI3K/Akt and Ras/Raf/MAPK pathways were stably activated in Huh7R cells

To learn about the activation status of PI3K/Akt and Ras/Raf/MAPK pathways in Huh7^R and Huh7 cell, we analyzed the phosphorylation levels of the main kinases of the related pathways in the two cells. WB results showed that the expression of p-Akt (Ser473), p-mTOR (Ser2481), p-S6K, p-eIF4EBP1, p-c-Raf (Ser289), p-MEK (Ser217/221), p-ERK (Thr202/Tyr204) and p-P90RSK (Ser380) in Huh7^R cells were higher than Huh7 cells (Fig. 1a and 1b), which suggested that the above pathways were further activated in Huh7^R cells. Next, Huh7^R cells was treated with sorafenib for 0 h, 12 h, 24 h, 36 h, 48 h and 72 h at the same concentration to investigated the activated form of the two pathways. WB results showed that the phosphorylation levels of major kinases in the two pathways were decreased at 12 h compared with 0 h,

but reached the lowest level at 24 h, and then they were in a stable activation from 24 h to 72 h (Fig. 1c and 1d).

LY3214996 enhanced sorafenib anti-proliferative effects in Huh7R cells

To detect the inhibitory effects on Huh7^R cells treated with sorafenib, LY3214996 or a combination of two, we detected cell viabilities by MTT assays. IC₅₀s of sorafenib on Huh7 and Huh7^R cells were obtained from results (Fig. 2a). There was a dose- and time-dependent reduction in the cell viability of Huh7^R cells treated with sorafenib, LY3214996 and the combination of two drugs (Fig. 2b and 2c). Compared with sorafenib, LY3214996 monotherapy, the combination of two drugs had a stronger inhibiting effect on cell viability according to IC₅₀ values. The results of colony formation assays also showed that sorafenib plus LY3214996 had stronger inhibitory effect on clone formation compared with the control group and any single agent treatment (Fig. 2d and 2e), which confirmed that LY3214996 enhanced the effects of sorafenib to suppress cell viability in Huh7^R cells.

LY3214996 strengthened sorafenib inhibition of Huh7R cellular migration

To verify whether LY3214996 enhanced the inhibitory effect of sorafenib on Huh7^R cell migration, Huh7^R cells were treated with sorafenib, LY3214996 and a combination of the two for 12 h and 24 h using wound healing test, respectively. Compared to the control group, the wound size of sorafenib, LY3214996 were 92.37% and 72.11% (12 h); 67.30% and 61.39% (24 h) respectively ($p<0.05$), while the migration inhibition rates of LY3214996 combined with sorafenib were 94.14% (12 h) and 93.86% (24 h) (Fig. 3a and 3b), which was significantly higher than that of any single agent group ($p<0.05$). WB results revealed that after treatment with sorafenib combined with LY3214996, the expressions of MMP-2 and MMP-9 in Huh7^R cells were significantly reduced compared with the monotherapy group (Fig. 3c and 3d) ($p<0.05$). These results indicated that the LY3214996 strengthened sorafenib inhibition of Huh7^R cell migration.

LY3214996 combined with sorafenib induced cell cycle arrest in the G0/G1 phase

Abnormal cell cycle progression causes unrestricted cell division, leading to continuous proliferation, and represents a key driver of carcinogenesis [24]. Next, we detected the effects of each treatment group on the cell cycle distribution by flow cytometry analysis. After drugs treatment for 24 h, The G0/G1 phase cell number percentages of sorafenib plus LY3214996 (61.95%) ($p<0.05$) was higher than that the sorafenib (45.37%), LY3214996 (50.03%) and control group (40.00%) (Fig. 4a and 4b). Moreover, the combined treatment significantly increased the percentage of sub-G1 phase cells than that in cells treated with either drug alone, which suggested that the combination of two drugs inhibited cells proliferation and promoted apoptosis.

WB results showed that the expression of Cyclin D1 and p-Rb, two of the critical proteins promoting cell cycle progression from G1 to S phase, were suppressed in LY3214996 treated Huh7^R cells. Furthermore, sorafenib combined with LY3214996 treatment led to further decreased levels of Cyclin D1 and p-Rb in

the Huh7^R cells. Besides, the expression level of upstream protein p-GSK-3 β was decreased after the combined treatment (Fig. 4c and 4d). All these data indicated that combined sorafenib and LY3214996 treatment resulted in a more pronounced cell cycle arrest G0/G1 phase.

LY3214996 enhanced sorafenib-induced apoptosis in Huh7R cells

To investigate the effect of combination treatment on Huh7^R apoptosis, Huh7^R cells were incubated with sorafenib (12 μ M), LY3214996 (1 μ M), or the combination, harvested cells after 24 h and analyzed using flow cytometry. As showed in Figure 5, compare to control group, the apoptosis rates of sorafenib and LY3214996 were 5.8% and 11% ($p < 0.05$), respectively. As expected, the apoptosis rate of combined drug treatment was increased to 17.72% (Fig. 5a and 5b) ($p < 0.05$), which was higher than that of any single agent treatment and control group.

Accordingly, immunoblotting analysis was performed to detect apoptosis-related molecules for understanding the mechanism of mitochondrial apoptotic pathway. The results revealed that levels of anti-apoptotic protein (Bcl-xL) in the combined treatment group were significantly down-regulated ($p < 0.05$), while the key death effector proteins (Bax and Bak), BH3-only proteins (Bad, Bim) and downstream proteins (caspase-3, -7, -9 and PARP) were significantly elevated (Fig. 5c and 5d). These suggested that sorafenib displayed an efficient pro-apoptotic effect when co-treated in the presence of LY3214996 in Huh7^R cells.

LY3214996 enhanced anti-tumor effect by blocking Ras/Raf/MAPK pathway in Huh7R cells

Next, we further explored the anti-tumor mechanism of LY3214996 combined with sorafenib in Huh7^R cells. Western Blot was performed to detect the major proteins levels of Ras/Raf/MAPK pathway in Huh7^R cells with LY3214996 and sorafenib monotherapy or in combination treatment for 24 h. The results revealed that sorafenib treatment increased levels of p-Akt, p-mTOR, p-c-Raf, p-ERK1/2, p-S6K, p-eIF4EBP1 and p-P90RSK compared to control group. However, combination of sorafenib and LY3214996 led to suppression of p-Akt, p-mTOR, p-S6K, p-c-Raf and p-P90RSK compared to control, sorafenib and LY3214996 treatment alone. Interestingly, p-ERK1/2 and p-MEK1/2 had an opposite result (Fig. 6a and 6b). Reactivation of p-ERK1/2 mediated by LY3214996 may be due to effective inhibition of p-c-Raf (S289/S296/S301), which are phosphorylation sites that inhibit feedback activation of ERK, indicating that the increase in p-ERK1/2 is due to the activation of c-Raf [25]. These results suggested that LY3214996 enhanced anti-tumor effect by blocking Ras/Raf/MAPK pathway and interfering crosstalk of PI3K/Akt pathway.

Discussion

Chemotherapy is an important option for patients with advanced HCC, while the treatment effect of available chemotherapeutic drugs is limited and accompanied by side effects [26]. Sorafenib, as an oral multi-targeted kinase inhibitor, is well tolerated and significantly prolongs the survival, which makes it

become the first choice for patients with advanced HCC [27]. However, in many clinical studies and practices, acquired resistance of sorafenib results in a low response rate and limited clinical efficacy after a few treatment cycles [28]. Therefore, it is necessary to identify its resistant origins and explore potential protocols that can overcome the resistance and improve the anti-tumor effect of sorafenib.

Many studies have showed that the activation of PI3K/Akt pathway was highly related to sorafenib resistance in HCC [29-30]. Sorafenib exerted anti-tumor effect mainly by inhibiting Ras/Raf/MAPK pathway but crosstalk activated PI3K/Akt pathway during this process [31]. Despite the feedback activation of p-ERK1/2, LY3214996 still shows continuous inhibition of downstream signals of ERK signaling [32]. In this study, we found that the levels of p-Akt and p-ERK1/2 in Huh7^R cells were higher than that in Huh7 cells, while p-Akt and p-P90RSK were downregulated in a combination treatment of LY3214996 and sorafenib, which revealed that PI3K/Akt pathway was activated when sorafenib treatment and inhibited ERK1/2 could suppress Akt activation. Due to ERK or RSK activation directly mediated PI3K/Akt/mTOR pathway [33-34], leading to compensated proliferation and anti-apoptotic effects, blocking the up-regulation of PI3K/Akt/mTOR pathway induced by ERK activation is an effective strategy to relieve sorafenib resistance and improve its efficacy.

Some studies have shown that sorafenib-resistant HCC cells have enhanced migration and invasion ability by activating EMT after long-term sorafenib treatment [35-36]. MMP2 and MMP9 are participated in angiogenesis by destroying basal layer molecules and remodeling ECM during angiogenesis [37]. The MMPs present in the extracellular matrix (ECM) have been participated in the acquisition of migration characteristics, and made it easier for tumor cells to invade surrounding tissues and metastasize to secondary sites [38]. It has been reported that ERK1/2 can regulate MMP-2 and MMP-9 expression [39]. The similar results were shown in our study and when Huh7^R exposed to the combination of LY3214996 and sorafenib, the wound size measured by the wound healing assays was significantly increased than sorafenib or LY3214996 monotherapy, and the expression levels of MMP-2 and MMP-9 were correspondingly reduced.

PI3K/Akt signaling is closely related to mitochondrial apoptosis pathway. P-Akt phosphorylated Bad, a member of Bcl-2 family, and followed initiating Bcl-xL induced anti-apoptosis [40]. We found that LY3214996 plus sorafenib treatment inhibited levels of p-Akt and p-P90RSK, but upregulated Bad, Bim and induced a cascade of mitochondrial apoptotic proteins, caspase-9, caspase-3 and caspase-7, which indicating sorafenib and LY3214996 inhibited Ras/Raf/MAPK and PI3K/Akt pathways, leading to activating the mitochondrial apoptotic pathway. The above evidences showed that LY3214996 enhanced the anti-tumor effect of sorafenib by inhibiting the Ras/Raf/MAPK pathway to promote the mitochondrial apoptotic pathway.

LY3214996 was shown to induce G1 cell cycle arrest in some cell lines of melanoma, colorectal cancer, pancreatic cancer, and NSCLC [32]. In addition, our results revealed that combined treatment of LY3214996 and sorafenib suppressed the expression of Cyclin D1 and p-Rb then increased the proportion

of cells in the G0/G1 phase to enhance the anti-proliferative effect of sorafenib on Huh7^R cells, which is consistent with previous research results [28].

Overall, we found that acquired resistance to sorafenib in Huh7^R cells resulted in limited effectiveness in treatment. One of the most important mechanisms of this result was the activation of PI3K/Akt and Ras/Raf/MAPK pathways. The strategy of ERK inhibition simultaneously blocked the downstream of the MAPK pathway and crosstalk with PI3K/Akt, which provided a theoretical basis for overcoming sorafenib resistance in patients with advanced HCC. Since the experiment was limited to *in vitro* studies, our future work will focus on *in vivo* researches and more signaling pathways will be validated. We believe that the results of sorafenib combined with LY3214996 are promising strategy in the treatment of sorafenib-resistant HCC.

Conclusion

The present study demonstrated that PI3K/Akt and MAPK/ERK pathways were abnormally activated in sorafenib-resistant HCC cells. sorafenib combined with LY3214996 inhibited these two pathways to enhance anti-tumor effects by inducing apoptosis as well as inhibiting cell proliferation. These results suggest that combining LY3214996 with approved sorafenib treatment standards may be an attractive strategy for treating advanced hepatocellular carcinoma.

List Of Abbreviations

HCC: Hepatocellular carcinoma

SFB: Sorafenib

LY: LY3214996

IARC: International Agency for Research on Cancer

OS: Overall survival

FDA: the United States Food and Drug Administration

VEGFR: Vascular endothelial growth factor receptor

PDGFR: Platelet-derived growth factor receptor

EMT: Epithelial-mesenchymal transition

Raf: Rapidly accelerated fibrosarcoma

MEK: Mitogen-activated protein kinase kinase

ERK: Extracellular signal-regulated kinase

HRP: Horse radish peroxidase

ATCC: American Type Culture Collection

FBS: Fetal bovine serum

RPMI: Roswell Park Memorial Institute

MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide

DMSO: Dimethyl sulfoxide

IC50: 50% inhibitory concentration

PBS: Phosphate Buffered Saline

RIPA: Radio Immunoprecipitation Assay

BCA: Bicinchoninic acid

FITC: Fluorescein isothiocyanate

PI: Propidium iodide

PVDF: Polyvinylidene Fluoride

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

ECM: Extracellular matrix

NSCLC: Non-small cell lung cancer

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The content of this manuscript has not been previously published and is not under consideration for publication elsewhere and it has been read and approved by all the co-authors.

Availability of data and materials

The data bracing the results of this survey can be acquired from the corresponding author as required.

Competing interests

All authors have no competing interests.

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Authors' contributions

YFM and RYX conceived and designed the study. XKL and YCZ carried out the study and analyzed the data. YFM wrote the manuscript. All authors participated in discussing and revising the manuscript. All authors read and approved the final manuscript.

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Figures

Fig.1

Figure 1

PI3K/Akt and Ras/Raf/MAPK pathways were stably activated in Huh7R cells. a, b Major kinase levels of PI3K/Akt and Ras/Raf/MAPK pathways in Huh7 and Huh7R cells. c, d Kinase expression levels in related pathways in Huh7R cells treated with sorafenib at different times. Data expressed as mean $\pm$ SD of three independent experiments (* p <0.05; ** p <0.01; *** p <0.001).

Fig.2

Figure 2

LY3214996 enhanced sorafenib anti-proliferative effects in Huh7R cells. a Cell viability of Huh7 and Huh7R cells incubated with different concentrations of sorafenib for 24 h. b Cell viability of Huh7R cells treated with sorafenib, LY3214996 and the combination after 24 h. c Cell viability of Huh7R cells treated with sorafenib, LY3214996 and the combination for 12 h, 24 h and 48 h. d, e Colony formation assays of Huh7R cells treated with sorafenib, LY3214996 or a combination of two. Data expressed as mean $\pm$ SD of three independent experiments (* p <0.05; ** p <0.01; *** p <0.001).

Fig.3

Figure 3

LY3214996 strengthened sorafenib inhibition of Huh7R cell migration. a, b Wound healing assays were carried out to assess cell migration of Huh7R cells treated with sorafenib LY3214996 or the combination of the two for 12 h and 24 h. c, d Related proteins of tumor cell migration (MMP-2 and MMP-9) were detected by WB. e A proposed working model of how LY3214996 acts on the Ras/Raf/MAPK pathway about cell migration in Huh7R cells. Data expressed as mean $\pm$ SD of three independent experiments (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Fig.4

Figure 4

LY3214996 combined with sorafenib induced cell cycle arrest in the G0/G1 phase. a, b Huh7R cells accumulated at the G0/G1 phase after exposed to sorafenib (12 μ M), LY3214996(1 μ M) and the combination (sorafenib12 μ M, LY3214996 1 μ M) for 24 h measured by flow cytometry. c, d Cell cycle proteins expression (p-GSK3 β , Cyclin D1 and p-Rb) were quantified by Western blot. Data expressed as mean $\pm$ SD of three independent experiments (*p<0.05; **p<0.01; ***p<0.001).

Fig.5

Figure 5

LY3214996 enhanced sorafenib-induced apoptosis in Huh7R cells. a, b Cells were treated with sorafenib (12 μ M), LY3214996 (1 μ M), or in combination treatment for 24 h. Apoptosis of Huh7R cells were detected by flow cytometry with Annexin V-FITC/PI staining. c, d Apoptosis-related proteins expression of Bcl-xL, Bax, Bak, Bad, Bim, caspase-3, -7, -9 and PARP were measured using western blot. e Signal mechanism model of LY3214996 inducing mitochondrial apoptosis in Huh7R cells. Data expressed as mean $\pm$ SD of three independent experiments (* p <0.05; ** p <0.01; *** p <0.001).

Fig.6

Figure 6

LY3214996 enhanced anti-tumor effect by blocking Ras/Raf/MAPK pathway in Huh7R cells. a, b After incubated with sorafenib (12 μ M), LY3214996 (1 μ M), or in combination for 24 h, cells were lysed and detected designed proteins in Ras/Raf/MAPK and PI3K/Akt pathways by Western blot analysis. Data expressed as mean $\pm$ SD of three independent experiments (* p <0.05; ** p <0.01; *** p <0.001).