By inhibiting PFKFB3, aspirin overcomes sorafenib resistance in hepatocellular carcinoma

Sainan Li 1, Weiqi Dai 1, Wenhui Mo 2, Jienging Li 1, Jiao Feng 1, Liwei Wu 1, Tong Liu 1, Qiang Yu 3, Shizan Xu 3, Wenwen Wang 1, Xiya Li 1, Qingshu Zhang 4, Kan Chen 1, Yijing Xia 1, Jie Lu 1, Yingqun Zhou 1, Xiaoming Fan 5, Ling Xu 6 and Chuanyong Guo 1

1 Department of Gastroenterology, Shanghai Tenth People’s Hospital, Tongji University School of Medicine, Shanghai, China
2 Department of Gastroenterology, Minhang Hospital, Fudan University, Shanghai, China
3 Department of Gastroenterology, Shanghai Tenth Hospital, School of Clinical Medicine of Nanjing Medical University, Shanghai, China
4 Department of Clinical Laboratory, Kunshan First People’s Hospital Affiliated to Jiangsu University, Kunshan, JiangSu, China
5 Department of Gastroenterology, Jinshan Hospital of Fudan University, Jinshan, Shanghai, China
6 Department of Gastroenterology, Shanghai Tongren Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Hepatocellular carcinoma (HCC) is one of the few cancers with a continuous increase in incidence and mortality. Drug resistance is a major problem in the treatment of HCC. In this study, two sorafenib-resistant HCC cell lines and a nude mouse subcutaneously tumor model were used to explore the possible mechanisms leading to sorafenib resistance, and to investigate whether aspirin could increase the sensitivity of hepatoma cells to sorafenib. The combination of aspirin and sorafenib resulted in a synergistic antitumor effect against liver tumors both in vitro and in vivo. High glycolysis and PFKFB3 overexpression occupied a dominant position in sorafenib resistance, and can be targeted and overcome by aspirin. Aspirin plus sorafenib induced apoptosis in tumors without inducing weight loss, hepatotoxicity or inflammation. Our results suggest that aspirin overcomes sorafenib resistance and their combination may be an effective treatment approach for HCC.

According to data from the latest cancer statistics, in contrast to declining trends for the three major cancers (lung, prostate and colorectum), the incidence and death rates for liver cancer continue to increase rapidly. In the two randomized controlled trials, the Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) 2 and the Asia-Pacific trials, 3 sorafenib was demonstrated to improve the survival of patients with advanced hepatocellular carcinoma (HCC). Sorafenib, as the first-line oral multi-kinase inhibitor of HCC, has been shown to exert an inhibitory effect on tumor growth for two main reasons: first, it blocks the Raf/MEK/ERK signaling pathway to inhibit tumor cell proliferation directly; second, it also targets the vascular endothelial growth factor receptor and platelet-derived growth factor receptor to prevent angiogenesis. 4

Key words: combination treatment, cell metabolism, glycolysis, mitochondrial apoptosis

Abbreviations: 3PO: 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one; AIF: apoptosis-inducing factor; AMPK: AMP-activated protein kinase; ASA: aspirin/acylsalicylic acid; CE: combination index; CLD: chronic liver disease; COX-2: cyclooxygenase-2; Cyto c: cytochrome c; DMEM-h: high glucose Dulbecco’s modified Eagle’s medium; DRI: dose reduction index; F2,6BP: fructose 2,6-bisphosphate; Fa: fraction affected; GLUT: glucose transporter; HCC: hepatocellular carcinoma; HK: hexokinase; Huh7-R: sorafenib-resistant Huh7 cell line; IC50: half maximal inhibitory concentration; LDHA: lactate dehydrogenase-A; NSAIDs: nonsteroidal anti-inflammatory drugs; OXPHOS: oxidative phosphorylation; PARP: poly ADP-ribose polymerase; PCNA: proliferating cell nuclear antigen; PE/7-AAD: phycoerythrin-Annexin V/7-amino-actinomycin; PFK2: 6-phosphofructo-1-kinase 2; PFKFB3: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; RIP: radio-immunoprecipitation assay; SHARP: sorafenib hepatocellular carcinoma assessment randomized protocol; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; VEGF: vascular endothelial growth factor; ΔΨm: mitochondrial inner membrane potential.

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

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Correspondence to: Ling Xu, Department of Gastroenterology, Shanghai Tongren Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, E-mail: xiaoling05@126.com (or) Chuanyong Guo, Department of Gastroenterology, Shanghai Tenth People’s Hospital, Tongji University School of Medicine, Shanghai 200072, China, E-mail: guochuanyong@hotmail.com

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Sorafenib, a kinase inhibitor, is one of the most effective drugs available for the treatment of hepatocellular carcinoma (HCC). Its use, however, is limited by acquired resistance. The present study shows that the expression of PFKFB3, a gene involved in glycolytic flux that encodes 6-phosphofructo-2-kinase (PFK2), is strongly associated with sorafenib resistance in HCC cells. PFK is a suspected target of aspirin, a drug associated with reduced HCC risk. Experiments in cells and animals reveal the existence of a synergistic antitumor effect between aspirin and sorafenib, suggesting that sorafenib-resistant HCC patients may benefit from combined treatment with aspirin.
instructions. The early apoptotic cells displayed PE-A+/7-AAD− staining and the late apoptotic cells displayed PE-A+/7-AAD+ staining.

**JC-1 staining**
JC-1 (BD Pharmingen) was used to measure mitochondrial membrane potential. Briefly, cells were cultured in six-well plates and then stained with 10 μg/mL JC-1 for 15 min. Cells were washed twice with PBS and then analyzed using a flow cytometer (Beckman Coulter, Villepinte, France).

**2-DG uptake, lactate production and O2 consumption**
Cells were washed with uptake buffer twice, cultured in 1 μCi/mL [3H]-2-DG at 37°C for 30 min, and solubilized with 0.1% sodium dodecyl sulfate. The radioactivity was calculated and was normalized to the protein content and corrected for the zero-time uptake per mg protein. Lactate levels were measured using a fluorometric assay (BioVision, Milpitas, CA) according to the manufacturer’s protocol. O2 consumption was tested using the 110 Fiber optic oxygen monitor (Instech, Plymouth Meeting, PA), and results were expressed as nmol O2/million cells/min.

**Reverse transcription (RT)-PCR and quantitative real time (qRT)-PCR**
TRizol reagent was used to extract total RNA. cDNA was synthesized using SuperScript II reverse transcriptase with Oligo (dT) (Invitrogen, Carlsbad, CA). The real-time PCR experiment was performed following the protocol of the real-time PCR kit (Takara, Dalian, China). The levels of the target genes were normalized to β-actin.

**Protein extraction and western blotting**
The cytosolic and mitochondrial fractions were separated and purified using a Mitochondrial Isolation Kit (Pierce, Rockford, IL) according to the manufacturer’s protocol. Total cellular proteins were extracted using radio-immunoprecipitation assay buffer (Sigma-Aldrich) containing protease inhibitors.

The samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinyl difluoride membranes. The membranes were sequentially blocked in PBS containing 0.1% Tween 20 (PBST) with 5% non-fat milk for 1 hr and probed with primary antibodies (Supporting Information Table S1). Membranes were then washed with PBST three times and incubated with the appropriate secondary antibodies for 1 hr at room temperature. Finally, the membranes were washed again and scanned using the Odyssey two-color infrared laser imaging system (fluorescence detection). HSP-70 and β-actin were used as an internal control.

**Plasmid construction, lentivirus packaging and infection**
A full-length cDNA encoding the PFKFB3 sequence was amplified from 293 T cDNA and then cloned into the pCDH-CMV-MCS-EF1-GFP vector (System Biosciences, Mountain View, CA). Empty lentiviral vector was used as control. All plasmid sequences were confirmed by DNA sequencing. Target cells were infected with empty vector or PFKFB3 in the presence of 8 μg/mL polybrene (Sigma-Aldrich) overnight. The transduction efficiency was measured by real-time PCR and western blotting.

For PFKFB3 siRNA knockdown, PFKFB3 expression in Huh7-R cells was ablated with small interfering RNAs (siRNAs), and PFKFB3 scramble siRNA (scRNA) was used as control. All plasmid sequences were confirmed by DNA sequencing. The siRNAs were transfected into cells using Lipofectamine™2000 following the manufacturer’s instructions. The transduction efficiency was measured by real-time PCR. The sorted cells were then characterized and used in further assays.

**Animal experiments**
Four-week-old male athymic BALB/C nu/nu mice with free access to water and food were housed in a standard animal laboratory with a 12-hr light-dark cycle and constant environmental conditions. All experiments were performed in accordance with ethical standards and in compliance with the Declaration of Helsinki, and according to national and international guidelines. The study was approved by the Animal Care and Use Committee of Shanghai Tongji University. Serum-free culture medium (200 μL) containing HCC-LM3 cells (5 × 10⁴) was subcutaneously injected into the upper flank region of 54 mice. When the tumor volume was ~100 mm³, the animals were randomly divided into nine groups: NC, ASA alone (20, 50 and 100 mg/kg), sorafenib alone (10 and 20 mg/kg) and combination treatment (sorafenib 10 mg/kg + ASA 20 mg/kg, sorafenib 10 mg/kg + ASA 50 mg/kg and sorafenib 10 mg/kg + ASA 100 mg/kg). Both sorafenib and ASA were given by oral gavage once a day for 30 days. Tumor volume was calculated using the following formula: volume (mm³) = (width)² × length/2. Body weight of the mice was measured every 5 days. Mice were euthanized 24 hr after the last treatment. Tumors were resected and imaged using a high-definition digital camera. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was used to measure the degree of tumor cell apoptosis. The percentage of TUNEL-positive tumor cells was determined by counting the average cell number in each of four high-power fields in each sample.

**Immunohistochemistry**
Slices (3 μm) were dewaxed, rehydrated and treated with 3% H₂O₂. The nonspecific sites were blocked with 10% goat serum for 30 min at room temperature. Then, slices were incubated overnight with primary antibody. On the following day, after incubation with secondary antibody, the slides were counterstained with hematoxylin and imaged under a light microscope.
Statistical analysis
All results are expressed as means ± standard deviation. Statistical analysis was performed using a two-tailed unpaired Student’s t test and SPSS 17.0 software (IBM, Armonk, NY). Quantitative data are representative of at least three independent experiments. Values of *p < 0.05, **p < 0.01 and ***p < 0.001 were considered statistically significant.

Results
Metabolic characterization change of HCC cells is associated with their resistance to sorafenib
The five sorafenib-naïve HCC cell lines tested showed different half maximal inhibitory concentration (IC50 values) for sorafenib (range: 4.79–26.10 μM; Fig. 1a). Among them, HCC-LM3 displayed the highest IC50 for sorafenib, and was regarded as a typical innate sorafenib-resistant HCC cell line, which was consistent with the conclusions of previous reports.29,30 The IC50 of the acquired sorafenib-resistant Huh7-R cells was 26.10 ± 1.73 μM, much higher than for sorafenib-naïve Huh7 cells (p < 0.001). The prominent sorafenib resistance of HCC-LM3 and Huh7-R cells was also confirmed by flow cytometry compared with Huh7 cells when treated with 10 μM sorafenib (apoptosis rate of Huh7-R vs. Huh7: 1.31 ± 0.47 vs. 35.58 ± 2.71, p < 0.001; apoptosis rate of HCC-LM3 vs. Huh7: 8.35 ± 1.62 vs. 35.58 ± 2.71, p < 0.01; Fig. 1b). Based on these results, HCC-LM3 and the selected Huh7-R cells were used as sorafenib-resistant HCC cells for further experiments.

Since previous studies indicated that a bioenergetic propensity for using glycolysis is highly associated with resistance of HCC cells to sorafenib,25 the metabolic characteristics, including glucose uptake and lactate production, along with O2 consumption and byproducts of OXPHOS of two sorafenib-resistant HCC cell lines were compared with the sorafenib-sensitive Huh7 cells. When cultured under normoxic conditions for 24 hr, glucose uptake and lactate production in Huh7-R cells were approximately five times more than Huh7 cells (Fig. 1c), and those in HCC-LM3 cells were increased ~4-fold over Huh7, demonstrating a higher rate of glycolysis. In opposite to high glycolytic flux, energy generation from oxidative phosphorylation (OXPHOS) was impaired, evidenced by decreased oxygen consumption and disrupted OXPHOS capacity. Our data showed the low efficiency of O2 consumption and down-regulated expression of OXPHOS metabolism-correlated proteins (complexes I/II/III/IV/V in the electron transport chain) in Huh7-R and HCC-LM3 cells (Figs. 1d and 1e). Based on these results, there is a shunt directed toward glycolysis from oxidative phosphorylation in the two sorafenib-resistant HCC cell lines.

To examine the mechanism underlying the effect of elevated glycolysis in sorafenib-resistant HCC cells, we measured the expression of several key glycolytic enzymes, including GLUT1/2/3/4, HK1/2, PFK1, PFKFB3, PKM2 and LDH-A by qRT-PCR. Among them, the PFKFB3 mRNA level was increased the most in both Huh7-R and HCC-LM3 cells, followed by GLUT1 and HK2 (Fig. 1f). Results of western blotting further confirmed the up-regulated expression of PFKFB3, GLUT1 and HK2 in untreated resistant cells (Fig. 1e). The tremendous heterogeneity suggests a positive correlation between enhanced glycolysis, especially the ascendant expression of PFKFB3, GLUT1 and HK2, and resistance to sorafenib.

Combination treatment with aspirin and sorafenib in vitro increased cell inhibition and apoptosis rate in sorafenib-resistant HCC cells
Previous studies indicated ASA could induce cancer cell death through inhibition of glycolysis.27 We hypothesized that ASA could reverse the sorafenib-resistance induced by elevated glycolysis in HCC cells. Huh7-R and HCC-LM3 cells were treated with ASA or sorafenib alone, or ASA plus sorafenib, for 24 hr, and the inhibitory effect on cell viability with combination treatment was found to be drastically boosted compared with either ASA or sorafenib alone (Supporting Information Fig. S1).

Fraction affected (Fa) values (the fraction of cells inhibited with drug exposure) were obtained after exposure of HCC cells to a series of drug concentrations. To indicate the effects at different Fa values, the CI and DRI values were calculated for each Fa. Fa-CI plots illustrate the effects of ASA and sorafenib at different fixed drug ratios, and demonstrate synergism (CI < 1) at Fa > 0.5 in both cell lines (Fig. 2a). As expected, synergism corresponding to CI < 1 always yields a favorable DRI (> 1) for both drugs (Fig. 2b), indicating that dosage of sorafenib can be significantly reduced when combined with ASA. We then treated Huh7-R cells with constant drug ratios, and the results also indicated synergistic interactions, evidenced by CI < 1 (Fig. 2c) and DRI > 1 (Fig. 2d). In addition, the drug ratio of option to exhibit the best synergistic effect is 1: 500 (sorafenib: ASA), followed by 1: 200 (sorafenib: ASA) in both Huh7-R and HCC-LM3 cells (Figs. 2a and 2b). Thus, sorafenib at 10 μM and ASA at 5 mM was chosen for the following in vitro study.

Next, the combination effect of ASA and sorafenib on apoptosis was evaluated by flow cytometry. The results showed that apoptosis with combination therapy increased significantly in two sorafenib-resistant HCC cell lines (Fig. 2e). Western blot analysis showed that combination treatment significantly decreased the expression of PCNA, and induced the activation of caspase 3 and 9 and cleavage of PARP (Fig. 2f), which was not obviously seen when cells were treated with sorafenib alone.

To explore the safety of the drug dose, two normal hepatic cell lines, QSG-770121 and LO2,32 were treated with ASA or sorafenib alone, or ASA plus sorafenib for 24 hr. The IC50 values of both ASA and sorafenib in two normal hepatic cell lines were evidently higher than in the sorafenib-resistant Huh7-R cells (Supporting Information Fig. 3). The two normal hepatic cell lines were evidently higher than in the sorafenib-resistant Huh7-R cells (Supporting Information Fig. 3).
Meanwhile the combination treatment, at the same dose that significantly increase, the apoptosis rate in two sorafenib-resistant HCC cell lines, had no obvious pro-apoptosis effect on QSG-7701 and LO2 cells (Supporting Information Fig. S2C), indicating a rather safe situation for the combination chemotherapy.

Figure 1. Enhanced glycolysis is correlated with sorafenib resistance of HCC cells.(a) IC50 values of sorafenib in a panel of sorafenib-naive HCC cells and one acquired sorafenib-resistant Huh7-R cell line at 24 hr. (b) Huh7, Huh7-R and HCC-LM3 cells were treated with or without 10 μM sorafenib for 24 hr, and the apoptosis rate was examined by flow cytometry. (c) Normalized lactate production and 2-DG uptake in Huh7, Huh7-R and HCC-LM3 cells within 24 hr of culture under normoxic conditions. The lactate production was normalized to the level of Huh7 cells. (d) O2 consumption in the indicated cell lines (nmol O2/million cells/min) was tested by a Clark-type oxygen electrode, which detected the concentration of dissolved oxygen in a closed chamber over time. (e) Western blot analysis of oxidative phosphorylation (OXPHOS) and glycolysis enzymes (GLUT1, GLUT4, HK2, PFKFB3, PK1 and LDHA) in total cell extracts from HCC cells. β-actin was used as a loading control. (f) qRT-PCR analysis of the expression of genes associated with glycolysis in HCC cells. Plotted values represent the mean ± standard error of three independent experiments (n = 3; *p < 0.05, **p < 0.01, ***p < 0.001). [Color figure can be viewed at wileyonlinelibrary.com]
Figure 2. Combination treatment of sorafenib-resistant HCC cells with ASA or/and sorafenib in vitro. (a, b) Illustrative Fa-CI (a) and Fa-DRI (b) plots for the combination of sorafenib and ASA using different constant drug ratios in Huh7-R and HCC-LM3 cells. (c, d) Fa-CI (c) and Fa-DRI (d) plots for the combination of sorafenib and ASA using inconstant drug ratios (fixed sorafenib and various ASA concentrations; fixed ASA and various sorafenib concentrations) in Huh7-R cells. CI < 1 and DRI > 1 denotes synergistic interactions. (e) Huh7-R and HCC-LM3 cells were treated with sorafenib (10 μM) or/and ASA (5 mM) for 24 hr, then apoptosis was monitored by flow cytometry. (f) Huh7-R and HCC-LM3 cells were treated with sorafenib (10 μM) or/and ASA (5 mM) for 24 hr, then the expression of PCNA, caspase 9, caspase 3 and PARP was monitored by western blot analysis. Results are the means of three experiments. [Color figure can be viewed at wileyonlinelibrary.com]
ASA sensitized sorafenib-resistant HCC cells to sorafenib in vivo

To investigate the effects of the combination of ASA and sorafenib in vivo, we established a mouse xenograft model using HCC-LM3 cells. Saline, sorafenib (10 or 20 mg/kg BW per day) alone, or combination treatment with sorafenib (10 mg/kg BW per day) and ASA (20, 50 and 100 mg/kg BW per day) for 30 days. At the time points indicated, diameters of tumors were measured.

Mice treated with ASA or sorafenib alone showed a relatively smaller tumor size than untreated mice after 30 days of treatment (Figs. 3a and 3b). Alternatively, sorafenib combined with ASA at 50 and 100 mg/kg significantly suppressed tumor size compared with sorafenib alone (0.145 ± 0.025, 0.024 ± 0.008 vs. 0.308 ± 0.022, p = 0.0027, p < 0.0001, respectively; Figs. 3a and 3b). Although the combination of ASA at 20 mg/kg with sorafenib showed no significant difference compared with sorafenib alone, there was a tendency towards a reduction in tumor size (0.166 ± 0.078 vs. 0.308 ± 0.022, p = 0.1316). It is notable that, tumor size in the sorafenib (10 mg/kg) + ASA (100 mg/kg) group was smaller than in the sorafenib (20 mg/kg) alone Group (0.024 ± 0.008 vs. 0.209 ± 0.053, p = 0.0139). These findings indicate that with the addition of ASA, the antitumor effect of sorafenib can still be maintained, or even improved, even at a reduced dose.

The results of dynamic observations of the antitumor effects over 30 days of treatment showed the same pattern (data not shown), while no significant loss of body weight, or...
elevation of serum AST or ALT was observed over the 30 days of treatment (Supporting Information Fig. S3), demonstrating no obvious deleterious effect on body weight or hepatic function in mice treated with ASA or sorafenib alone or in combination.

Compared with treatment with sorafenib alone, combination treatment with ASA and sorafenib significantly increased the rate of apoptosis in vivo (65.97 ± 4.52% vs. 32.20 ± 5.95%, p = 0.0107; Figs. 3c and 3d). These data further confirmed the hypothesis that ASA could sensitize cells to sorafenib in sorafenib-resistant HCC cells in a xenograft mouse model.

**PFKFB3 is essential for combination treatment-inhibition of glycolysis and proliferation in HCC cells both in vitro and in vivo**

Huh7-R cells treated with ASA (5 mM) showed significantly lower (p = 0.023) glucose uptake (514.3 pmol/mg/min) than the untreated controls (1029.0 pmol/mg/min). Combination treatment (sorafenib 10 μM + ASA 5 mM) also led to markedly lower (p = 0.0198) glucose uptake (402.7 pmol/mg/min) compared with sorafenib alone (627.3 pmol/mg/min; Fig. 4a). Lactate production showed a similar pattern to that of glucose uptake (Fig. 4a), indicating a reduced glycolytic rate in sorafenib-resistant HCC cells with combination treatment.

To examine the mechanism underlying the effect of ASA plus sorafenib on inhibition of glycolysis, the effects of combination treatment on the expression of key glycolytic enzymes were assessed in Huh7-R cells using qRT-PCR. Among these, PFKFB3 was the most down-regulated enzyme at the mRNA level (Fig. 4b). Western blot analysis also indicated a significant decrease in PFKFB3 and PFK1 protein expression with combination treatment (Fig. 4c). In addition, analysis of the PFKFB3 immunohistochemical changes in xenograft mouse tumors confirmed these results in vivo (IOD of combination treatment vs. sorafenib: 37.00 ± 3.61 vs. 71.33 ± 2.03, P = 0.0012; Figs. 4d and 4e). Taking these data together, we can infer that PFKFB3 plays a vital role in combination treatment-inhibition of HCC cells glycolysis and proliferation.

To verify the effect on PFKFB3, 3–(3-pyridinyl)-1–(4-pyridinyl)-2-propen-1-one (3PO), a specific PFKFB3 inhibitor was used as a positive control. The reduced glycolysis and cell proliferation and elevated apoptosis effect induced by the combination treatment were similar to those induced by the PFKFB3 inhibitor (Figs. 4f/h). These results indicate that both treatments may function through the same mechanism: inhibition of PFKFB3.

**Overexpression of PFKFB3 mimics the sorafenib resistance effect in Huh7 cells**

To further prove the status of PFKFB3, lentivirus was used to infect the sorafenib-naïve Huh7 cells, causing them to overexpress PFKFB3 (Supporting Information Fig. S4). The increased glucose uptake and lactate production in PFKFB3-overexpressing Huh7 cells indicated the ascendency of PFKFB3 in the glycolysis pathway (Fig. 5a). In addition, the IC50 of PFKFB3-overexpressing Huh7 cells was higher than the empty virus-infected Huh7 cells (11.67 ± 0.78 vs. 4.97 ± 0.49, p = 0.0019; Fig. 5b). Sorafenib at a concentration of 10 μM combined with Lenti-PFKFB3 significantly attenuated sorafenib-induced apoptosis in Huh7 cells (24.10% for combination vs. 37.03% for sorafenib alone; Fig. 5c) by inhibiting the activation of caspases 3 and 9 (Fig. 5d). These results indicated a sorafenib-resistance effect in PFKFB3-overexpressing Huh7 cells, showing that PFKFB3 is essential for both glycolysis and sorafenib resistance.

In addition, ASA was able to sensitize PFKFB3-overexpressing Huh7 cells to sorafenib, as evidenced by the synergism (CI < 1 and DRI > 1) at Fa > 0.5 in cell growth inhibition (Figs. 5e and 5f) and apoptosis elevation (51.62% vs. 35.38% for sorafenib alone; Fig. 5g).

Furthermore, the siRNA-induced knockdown of PFKFB3 in Huh7-R cells also restored their sensitivity to sorafenib (Supporting Information Fig. 5). Taken together, the loss of PFKFB3 in the sorafenib-resistant cells restored their sensitivity, while the over-expression of PFKFB3 in sorafenib-sensitive cells created sorafenib-resistance, indicating the dominant status of PFKFB3 in the sorafenib-resistance mechanism of HCC cells. ASA combined with sorafenib showed synergism in PFKFB3-overexpressing Huh7 cells, indicating the cell death promoting effect of the combination treatment is targeting PFKFB3.

**PFKFB3 was suppressed through inhibition of HIF-1α in HCC cells**

To determine a possible mechanism by which combination treatment induced apoptosis and reduced glycolysis in HCC cells, real-time PCR was used to test mRNA levels of 27 apoptosis- and glycolysis-related molecules after Huh7-R cells were treated with ASA plus sorafenib for 24 hr. The values of log relative mRNA compared with the negative control group are shown in Supporting Information Fig. S6A. Among those, HIF-1α mRNA level altered the most, with an 85% reduction in combination-treated cells (p < 0.001), followed by the tumor suppressor PTEN and P53, which increased by 4.4- and 4.9-fold respectively (p < 0.001 for PTEN; p < 0.01 for P53). Expression of COX-2 was also down-regulated, while AMPKα1/2 and AMPKβ1/2 was up-regulated with the combination treatment (Supporting Information Fig. S6A).

To test the possible connection between AMPK and PFKFB3, Compound C, a potent AMPK inhibitor, was used to test glycolysis and PFKFB3 alteration. As is shown in Supporting Information Fig. S6B, effects of ASA plus sorafenib on the expression of PFKFB3 and glycolytic rate were not altered with the inhibited AMPK, suggesting that the effects of PFKFB3 on HCC cell lines is AMPK-independent. Meanwhile, roxadustat (FG-4592), a prolyl-4-hydroxylases inhibitor and HIF-1α stabilizer, stabilized HIF-1α and reversed the glycolysis-reducing effect induced by ASA combined with sorafenib through up-regulating the expression of PFKFB3 and PFK1 (Supporting Information Fig. S6B,C). These data show that, ASA plus sorafenib reduce glycolysis via inhibition of HIF-1α/PFKFB3.
Figure 4. PFKFB3 is essential for combination treatment-inhibited Huh7-R cells glycolysis and proliferation both in vitro and in vivo. (a) Relative lactate production and 2-DG uptake were measured in Huh7-R cells treated with sorafenib (10 μM) or/and ASA (5 mM) for 24 hr under normoxic conditions. (b) qRT-PCR analysis of the effect of combination treatment (sorafenib 10 μM and ASA 5 mM) on the expression of genes associated with glycolysis in Huh7-R cells. (c) Western blot analysis of sorafenib (10 μM) or/and ASA (5 mM) on the protein expression of PFKFB3 and PFK1 in Huh7-R cells. β-actin was used as the loading control. (d, e) Immunohistochemical analysis of PFKFB3 expression was performed in a xenograft mouse model (original magnification, ×400). Mice were treated with ASA (100 mg/kg BW per day) or/and sorafenib (10 mg/kg BW per day) for 30 days. (f, g) Relative lactate production, 2-DG uptake and cell viability were measured in Huh7-R cells treated with combination treatment (sorafenib 10 μM and ASA 5 mM) or/and 3PO (10 μM) for 24 hr under normoxic conditions. (h) Huh7-R cells were treated with combination treatment (sorafenib 10 μM and ASA 5 mM) or/and 3PO (10 μM) for 24 hr, then the expression of caspase 3 and 9 was monitored by western blot analysis. Results are the means of three experiments. Plotted values represent the mean ± standard error of three independent experiments (n = 3; *p < 0.05, **p < 0.01, ***p < 0.001). [Color figure can be viewed at wileyonlinelibrary.com]
The mitochondrial apoptosis pathway is involved in combination treatment-induced apoptosis and is partly caspase-dependent.

Mitochondria play a pivotal role in two integral components of cellular transformation, cellular metabolism and apoptosis. The major form of apoptosis in most cancer cells is the mitochondrial pathway, defined by a pivotal event in the process: mitochondrial outer membrane permeabilization, which can be detected as mitochondrial inner transmembrane potential (ΔΨm). The loss of ΔΨm in combination treatment provides evidence to this particular mechanism (Fig. 6a).

During the process of mitochondrial apoptosis, caspase activation is closely linked to mitochondrial outer membrane permeabilization. To test whether the combination treatment-induced
Figure 6. Apoptosis regulatory effects of combination treatment in Huh7-R cells. (a) To measure changes in the Δψm, Huh7-R cells (5 × 10^4) treated with sorafenib (10 μM) or/and ASA (5 mM) for 24 hr were stained with JC-1 (10 μg/mL) and analyzed by flow cytometry. (b) Huh7-R cells were treated with combination treatment (sorafenib 10 μM and ASA 5 mM) or/and Z-VAD-FMK (100 μM) for 24 hr, and the apoptosis rate was examined by flow cytometry. (c) The expression of mitochondrial apoptotic regulatory proteins in cytoplasm or mitochondria was monitored by western blot analysis in Huh7-R cells treated with sorafenib (10 μM) or/and ASA (5 mM) for 24 hr. Actin and hsp-70 served as loading controls. Results are the means of three experiments. (d) Mode of aspirin reverses sorafenib resistance in HCC cells: With aspirin treatment, high PFKFB3 expression in sorafenib-resistant HCC cells is suppressed, leading to restrained aerobic glycolysis by PFK1, resulting in the suppression of ATP production. This leads to mitochondrial membrane potential breakdown and causes direct caspases activation, resulting in cell death. In addition, the inhibition of PFKFB3 induces the suppression of PFK1, thus activating Bad by dephosphorylation, which interacts with the anti-apoptotic Bcl-2 family proteins Bcl-2 and Bcl-xl to relieve their inhibition of pro-apoptotic proteins Bax and Bak. Oligomerization and activation of Bax and Bak leads to the increase of mitochondrial outer membrane permeabilization, and releases apoptogenic substrates from the mitochondria, such as Cyto c into the cytoplasm and AIF to the nucleus, activating caspases, resulting in nuclear DNA fragmentation and cell apoptosis, and restoring the sensitivity to sorafenib. [Color figure can be viewed at wileyonlinelibrary.com]
apoptosis is caspase-dependent, a caspase inhibitor z-VAD-fmk (100 μM) was used in flow cytometry. Combination treatment-induced apoptosis was effectively blocked by z-VAD-fmk (21.91% for Sorafenib + ASA + z-VAD-fmk vs. 52.25% for sorafenib + ASA; Fig. 6b), demonstrating that caspase serves as a near obligate mediator in the process.

One link between apoptosis inhibition and metabolic reprogramming may be provided by the association of PFK with the proapoptotic protein Bad. As shown in Figures 6c and 6d, Bad was dephosphorylated in response to the combination treatment, and its de-phosphorylation encouraged the translocation of Bax and Bak from the cytosol to the mitochondria and of Bcl-2 and Bcl-xl from mitochondria, leading to the release of apoptogenic substrates from the mitochondria, such as cytochrome c (Cyto c) and apoptosis-inducing factor (AIF). Taken together, these data show that, Bcl-2 family proteins are involved in the regulation of combination treatment-induced mitochondrial apoptosis.

**Discussion**

HCC is one of the most common malignancies worldwide, with an incidence and mortality that are increasing yearly. Most modern medicines currently available for treating HCC are expensive, toxic, and not effective enough. The recent approval of sorafenib as the first effective oral drug for HCC marks a significant milestone. However, sorafenib has been proven to limit survival benefits with very low response rates because of drug resistance. The Warburg effect is reported to be closely associated with either inherent or acquired sorafenib resistance. Glycolysis in sorafenib resistant HCC cells increases to generate macromolecules such as lactate, etc., and the consequent usage of which is required for cancer cell growth. This is clearly observed in our constructed sorafenib-resistant Huh7 cell line (Huh7-R) and in the innate sorafenib-resistant HCC-LM3 cells compared with the parental sensitive cells (Fig. 1c), indicating that drug resistance in HCC cells is directly linked to an increase in glycolysis. Further exploration of the key glycolysis enzymes reveals that upregulated PKFB3 is strongly associated with sorafenib resistance in HCC cells. This is proven by three key lines of evidence: first, the expression of PKFB3 is higher in sorafenib-resistant cells compared to the parental sensitive cells (Figs. 1e and 1f); second, overexpression of PKFB3 in sorafenib-sensitive Huh7 cells mimics the sorafenib resistance effect (Figs. 5a); and third, siRNA-knockdown of PKFB3 in sorafenib-resistant Huh7-R cells restores its sensitivity to sorafenib (Supporting Information Fig. S5). All these imply that bioenergetic changes toward increased PKFB3 and glycolysis are linked to sorafenib-resistance. Therefore, in the search for a combination of chemotherapeutic drugs with tumor glycolysis inhibitors, inhibitors of PKFB3 in particular may represent a promising strategy to overcome sorafenib resistance.

Recently, the preventive effects of ASA on cancers have been studied extensively, including in prostate cancer, colorectal cancer, pancreatic cancer and HCC. However, most laboratory experiments and clinical trials were carried out to explore its preventive effects on tumorigenesis; its therapeutic effect has rarely been mentioned. This study illustrated the therapeutic effect of aspirin in HCC cells (Figs. 2e and 2f and Supporting Information Fig. S1) and on tumor size in an established-mouse subcutaneous tumor model (Figs. 3a and 3b). In addition, our research also demonstrated that high glycolysis and PKFB3 overexpression in sorafenib-resistant HCC cells could be overcome by aspirin (Figs. 4ae and 4g), which explained the possible molecular mechanism of its cancer-suppressing effects.

Given the characteristics of aspirin, in the present study, two sorafenib-resistant HCC cell lines (Huh7-R and HCC-LM3 cells) and a nude mouse subcutaneous tumor model were used to determine whether the combination of aspirin and sorafenib could enhance the sorafenib sensitivity of HCC cells. Results showed that the combination therapy had a synergistic antitumor effect against liver tumors, evidenced by CI < 1 in Chou-Talalay median effect analysis (Figs. 2a and 2c); and the DRI > 1 (Figs. 2b and 2d) illustrated that to reach the same therapeutic effect, both sorafenib dose and aspirin dose could be significantly reduced with combination treatment, but especially the dose of sorafenib; besides, the best drug ratio is explored: 1: 500 for sorafenib vs. aspirin in vitro. These finding have also been confirmed in vivo: tumor size in the sorafenib (10 mg/kg) + ASA (100 mg/kg) group was smaller than in the sorafenib Group (20 mg/kg; Figs. 3a and 3b), further indicating that with the addition of aspirin, although sorafenib dosage was reduced by half, the antitumor effect was still maintained, or even improved. A similar synergistic effect was also observed in the PKFB3 overexpression-induced sorafenib-resistant Huh7 cells (Figs. 5b). Analysis of apoptosis in vitro and in vivo also confirmed the synergistic antitumor effect, as evidenced by alterations in the number of positive apoptotic cells (Fig. 2e), expression of proteins that regulate apoptosis (Figs. 2f and 6c) and positive TUNEL cells (Figs. 3c and 3d) after combination treatment.

The synergistic antitumor effect of aspirin combined with sorafenib provides several advantages for clinical HCC patients: first, through combining with aspirin, the sensitivity of sorafenib is increased. Sorafenib, with its gifted and unique advantages in HCC, can benefit the increasing number of HCC patients that were originally sorafenib resistant; second, since there is a subgroup of HCC patients who suffer from deleterious side effects of sorafenib, after an initial satisfactory response, who then have to reduce the drug dosage or even terminate the therapy. With combination treatment, aspirin ameliorates the side effects of sorafenib, allowing more patients who could not tolerate sorafenib previously to re-gain the benefits; third, sorafenib is an expensive oral medicine, it costs about $US 4,079 per month and about $US 40,639 ± $US 3,052 over a patient’s lifetime according to research by Carr et al. in Canada in 2010. This is a huge economic burden for most families. Through combination
with aspirin, the dosage of sorafenib can be reduced, which may provide a financial opportunity for more patients to try the treatment, since aspirin is relatively cheap. In addition, the safety of combination treatment has been proven both in vitro and in vivo, given the fact that the drug dose shown to be effective in cancer inhibition has no obvious influence on normal hepatic cells in vitro (Supporting Information Fig. S2), or on hepatic function or body weight in vivo (Supporting Information Fig. S3), indicating its practicable application in the clinic.

In addition, the possible mechanism of aspirin combined with sorafenib was explored, and results showed that both aspirin alone and the combination treatment decreased the expression of activated PFKFB3 as well as glycolysis in sorafenib-resistant HCC cells (Fig. 4), proving our hypothesis that inhibition of the tumorigenic/proliferative ability of HCC by aspirin was linked to sensitization of HCC cells to sorafenib, through reversing the overexpression of PFKFB3 and glycolysis. Further exploration of the possible pathways, which regulate glycolysis and apoptosis showed that HIF-1α changed the most obviously with the combination treatment (Supporting Information Fig. S6A). After HIF-1α was stabilized by roxadustad (FG-4592), inhibition of PFKFB3 expression and glycolysis by combination treatment was attenuated (Supporting Information Fig. S6B,C). Taken together, these data suggest that the combination treatment acts through the HIF-1α/PFKFB3/PFK1 pathway to regulate glycolysis and apoptosis.

The mechanism of cross-talk between glycolysis and apoptosis is still unclear. Through analyzing the results of our research, we proposed that the inhibition of glycolysis by aspirin increased mitochondrial outer membrane permeabilization, and eventually activated downstream mitochondrial apoptotic signaling, leading to the death of sorafenib-resistant cancer cells. This may occur through two approaches: first, the decrease in glycolysis would reduce the production of metabolites such as lactate, which provides advantages for cancer cell growth and metastasis, that eventually leading to apoptosis; second, PFK1, which is activated by PFKFB3, was recently identified as a novel Bad-associated proapoptotic protein. The interaction of PFK1/Bad also contributes to the process of apoptosis. In this study, results showed that the increase in mitochondrial permeabilization and expression of caspase proteins play a decisive part in combination treatment-induced apoptosis, which is regulated by the phosphorylation of Bad (Fig. 6).

In addition to PFKFB3, other glycolysis-regulating enzymes such as glucose transporter 1 (GLUT1), hexokinase 2 (HK2) and lactate dehydrogenase-A (LDHA) are also activated in sorafenib-resistant HCC cells (Figs. 1e and 1f). And agents that target glycolysis such as resveratrol,29 inhibitors of HK2, and epigallocatechin-3-gallate,30 inhibitors of PFK, have also shown promising efficacy in overcoming drug resistance in several in vitro models. What’s more, expression of MAP kinase signaling, VEGF receptor and PDGF receptor is positively correlated with sorafenib resistance (Supporting Information Fig. S7). Therefore, further in vitro and in vivo studies identifying the specific molecular targets, signaling and metabolic pathways that affect sorafenib sensitivity, the therapeutic effect of aspirin and other selective NSAIIds alone or combined with sorafenib are required.

In conclusion, this study is the first to demonstrate that PFKFB3 overexpression occupied a dominant position in sorafenib resistance, and can be targeted and overcome by aspirin. In addition, our research is the first to exhibit that aspirin could enhance the effect of conventional cancer therapies through synergistic effects as well as through amelioration of the deleterious side effects of sorafenib, providing effective treatment strategies for HCC patients.

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