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Sorafenib-irinotecan sequential therapy augmented the anti-tumor efficacy of monotherapy in hepatocellular carcinoma cells HepG2

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The current study aimed to evaluate the efficacy of sorafenib-based combined therapy against hepatocellular carcinoma (HCC).

HepG2 cells were exposed to sorafenib, irinotecan, and oxaliplatin and then subjected to MTT assay to determine chemosensitivity. Flow cytometry was used to examine cell cycle distribution and cell apoptosis. Levels of cleaved caspase-8, -3, and PARP were determined by Western blot. Real-time PCR and Western blot were used to determine p53 expression, respectively. The efficacy of combined therapy were verified in nude mice bearing HepG2 xenografts.

HepG2 cells used in the current study were sensitive to sorafenib, irinotecan, and oxaliplatin. Sorafenib arrested cell cycle in S phase and the peak effect appeared at 30 h post treatment. Sorafenib exposure for 30 h followed by irinotecan exposure for 48 h synergistically induced cell apoptosis in HepG2 cells. On the other hand, sorafenib-oxaliplatin sequential exposure for the same time only acted an additive effect in soliciting cell apoptosis. Sorafenib and irinotecan sequential treatment significantly increased the levels of cleaved caspase-8, -3, and PARP in HepG2 cells. Sorafenib suppressed p53 expression at both mRNA and protein levels, which might contribute to cell cycle arrest and sensitize tumor cells to irinotecan. Sorafenib and irinotecan sequential therapy was obviously superior to monotherapy in suppressing the growth of HepG2 xenografts.

Sorafenib-irinotecan sequential treatment augmented the efficacy of either drug used alone in soliciting HepG2 cells apoptosis *in vitro* and in suppressing the growth of HepG2 xenografts *in vivo*.

Key words: hepatocellular carcinoma, irinotecan, sorafenib, synergistic effect

Liver cancer is the second most common cause of death from cancer worldwide, estimated to be responsible for nearly 746,000 deaths in 2012 according to the statistics published by World Health Organization [1]. Among the diverse, histologically distinct primary hepatic neoplasms, hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for 83% of all cases [2]. The prevalence of HCC is especially severe in East Asian countries such as China and Japan due to high rate of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection in the population [3]. Curative treatments including liver transplantation and hepatic resection are suitable only for fewer than 20% of HCC patients because most cases have progressed to an advanced stage with intra- or extrahepatic metastasis when the disease is diagnosed [4,5]. As a palliative treatment, chemotherapy is a highly needed means for the patients with unresectable and metastatic HCC.

Sorafenib is the first and only approved molecule-targeting agent to date that has been applied for systemic chemotherapy in HCC patients with metastatic disease or transcatheter arterial chemoembolization (TACE)-refractory disease who are not suitable candidates for local treatments [6]. Although clinical studies demonstrated the beneficial effects of sorafenib on the time-to-progression (TTP) and overall survival (OS), its efficacy against HCC remains moderate. The TTP demonstrated to be 5.5 months for sorafenib and 2.8 months for placebo, and the median OS was 10.7 months for sorafenib and 7.9 months for placebo [6]. Enhancement of the efficacy of sorafenib by its use in combination regimens is a rational

strategy for further prolonging the survival of HCC patients. A randomized phase II study of sorafenib plus doxorubicin in treatment of advanced HCC showed encouraging outcomes, with a median TTP of 8.5 months and median OS of 14.0 months [7]. Because of the diverse drug susceptibility of HCC cells, studies on sorafenib combined other chemotherapeutic agents with different mechanisms of action is expected in HCC treatment.

HCC is generally recognized as a chemo-resistant tumor. Selection of effective drugs according to the result of drug sensitivity tests is of great importance for both monotherapy and combined therapy. In a previous study, Chen *et al.* investigated the efficacies of cytotoxic agents against HCC cells isolated from 50 human HCC samples and reported that 44% and 6% of samples were sensitive to irinotecan and oxaliplatin, respectively [8]. The safety and efficacy of sorafenib plus irinotecan or platinum compounds in cancer treatment were investigated in previous clinical studies. Results demonstrated that these combined therapies were well-tolerated and achieved encouraging response rates in several malignances including colorectal, ovarian, and pancreatic cancers [9-12]. In light of these achievements, we aim to investigate the efficacy of sorafenib combined with irinotecan or oxaliplatin in treatment of HCC in the current study.

Concomitant administration of sorafenib increased the exposure of irinotecan and its active metabolite SN38 in a clinical pharmacokinetic research [9]. In order to avoid the potential metabolism based drug-drug interaction and the additional toxicities of combined therapy, sorafenib-irinotecan or sorafenib-oxaliplatin sequential therapies were designed and their efficacy was tested in HCC cells HepG2 both *in vitro* and *in vivo*. The underlying mechanisms were also investigated to clarify the potent anti-HCC effects of sorafenib-based combination therapy in this study.

Materials and methods

Chemicals and agents. Sorafenib, irinotecan, and oxaliplatin were obtained from the Bayer Schering Pharma AG (Leverkusen, Germany), Jiangsu Hengrui Medicine Co., Ltd. (Lianyungang, Jiangsu, China), and Sanofi-Aventis France (Paris, France), respectively. Sorafenib was firstly dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and further diluted in cell culture medium at 3.08 mg/mL as stock solution. Irinotecan and oxaliplatin were initially dissolved in 0.9% chloride sodium, and further diluted in cell culture medium at 3.08 mg/mL and 2.00 mg/mL as stock solutions, respectively. The drug concentration of each stock solution is designed as 400-fold of human peak plasma concentration (PPC, 7.7, 7.7, and 5.0 μg/mL for sorafenib, irinotecan, and oxaliplatin, respectively) [13-15].

Cell line and cell culture. HCC cell line $HepG_2$ was purchased from China Cell Bank, Shanghai, China. The cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Evergreen Biotechnology, Hangzhou, Zhejiang, China) at 37°C in a humid atmosphere (5% CO_2–95% air). Cells were harvested by brief incubation in 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS).

Cell proliferation assay. HepG2 cells (1×10^4 per well) seeded in 96-well plates were exposed to various concentrations of sorafenib, irinotecan, or oxaliplatin (10, 5, 1, 0.5, 0.1, 0.05, and 0.01 × PPC, respectively) for 48 h. Then the medium was removed and the wells were washed with PBS. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μl of MTT (5 mg/mL, Sigma, USA) for 4 h. Light absorbance of the solution was measured at 570 nm on a microplate reader (Perkin-Elmer, USA).

Cell cycle analysis. $HepG_2$ cells seeded in 25-cm^2 culture flasks (1.5×10^5 cells per mL; 6 mL per flask) were synchronized by 24 h of growth in serum free medium, and then were exposed to 10% serum medium containing sorafenib for 48 h. Cells were harvested every 6 h and fixed in cold 70% ethanol overnight. Cells were then suspended in propidium iodide (PI) solution for 30 min. Cell cycle distribution was analyzed by using a FACScan flow cytometer (Becton Dickinson and Company, Franklin Lakes, NJ, USA). The percentages of cells in G0/G1, S, and G2/M phases were determined using ModFit LT software 3.0 (Varity Software House, Topsham, USA).

Annexin V/FITC/PI staining assay. The apoptotic cells were estimated by determining the levels of phosphatidylserine on cell surface [16]. HepG2 cells seeded in 25-cm^2 culture flasks (1.5×10^5 cells per mL; 6 mL per flask) were exposed to sorafenib, irinotecan, oxaliplatin, or their combinations. The levels of phosphatidylserine were determined by using Annexin-V/FITC and PI kit (Labtek, Dalian, Liaoning, China). The experiment was performed on a FACScan flow cytometry. The population of apoptotic cells was estimated by comparing to the vehicle control. The following equation is used to calculate the interaction index (Q) for a combination of drugs A and B: $Q = E_{a+b} / (E_a + E_b - E_a \times E_b)$, where E_{a+b} correspond to the proportion of apoptotic cells induced by combination of A and B, and E_a and E_b correspond to the proportions of apoptotic cells induced by A and B alone, respectively. The interaction effect was determined to be antagonistic (Q = 0.55-0.85), additive (Q = 0.85-1.15), or synergistic (Q = 1.15-2.00) based on the Q value [17].

Real-time PCR. Cells (3×10^5 per well) seeded in 6-well plates were exposed to sorafenib, irinotecan, or their combination for specified time period. Total cellular RNA samples were isolated using Trizol reagent (Bioer Scientific, Hangzhou, China), dissolved in DEPC buffer (Sigma-Aldrich), and then stored at −20°C for further use. cDNA was synthesized through reverse transcription using First Strand cDNA Synthesis kit (Toyobo, Japan) according to the manufacturer's protocol. SYBR green real-time RT-PCR amplification was performed using LightCycler®480 II (Roche, Germany) to examine the

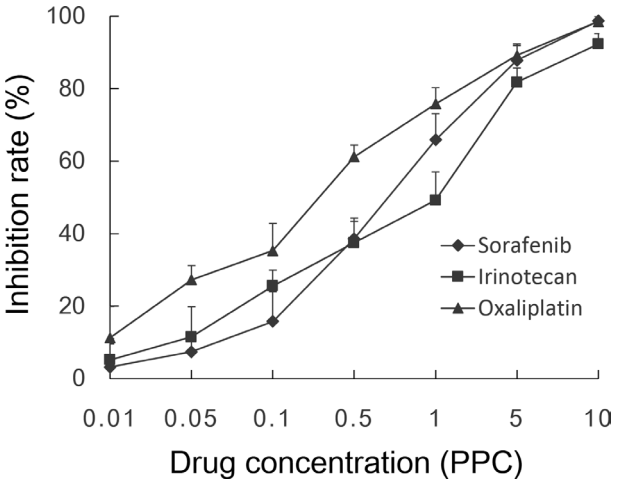

Figure 1. Sorafenib, irinotecan, and oxaliplatin dose-dependently inhibited the proliferation of HepG2 cells. Cells were incubated with 10, 5, 1, 0.5, 0.1, 0.05, and 0.01 PPC for 48 h and then subjected to MTT assay.

level of candidate gene expression. The expressions of genes were normalized against that of a housekeeping gene, GAPDH, and plotted as relative change in the expression with respect to control. The Primers used for the real-time RT-PCR were as follows: p53, forward 5'- GTTCCGAGAGCTGAATGAGG-3' and reverse 5'-TTATGGCGGGAGGTAGACTG-3' (product size of 121 bp, Tm = 60°C); GAPDH, forward 5'- GACAACGGCTCCGGCATGTGCA-3' and reverse 5'-TGAGGATGCCTCTCTTGCTCTG-3' (product size of 530 bp, Tm = 60°C).

Western blot analysis. Cells (3.0×10^5 per well) seeded in 6-well plates were exposed to sorafenib, irinotecan, or their combination for specified time period. Cells were harvested and cell lysates (30 μg of protein per lane) were fractionated by 10% SDS-PAGE. The proteins were electro-transferred onto nitrocellulose membrane and the protein levels were detected using the primary antibodies against p53, active caspase-3 and -8, cleaved PARP, and β-actin (Santa Cruz, Dallas, TX, USA) with appropriate dilution. The bound antibodies were visualized using an enhanced chemiluminescence reagent and quantified by densitometry using ChemiDoc XRS+ image analyzer (Bio-Rad, Hercules, CA, USA). Densitometric analyses of bands were adjusted with β-actin as loading control. The percentages of increase or decrease of protein were estimated by comparison to the vehicle control (100%).

Table 1. Inhibition rates of sorafenib, irinotecan, and oxaliplatin on the proliferation of HepG2 cells at 10, 1, and 0.1 PPC

Drug concentration	Growth inhibition rate		
	Sorafenib	Irinotecan	Oxaliplatin
10 × PPC	98.7%	92.3%	98.5%
1.0 × PPC	65.9%	49.2%	75.8%
0.1 × PPC	15.8%	25.6%	35.3%

PPC: peak plasma concentration. The PPC for sorafenib, irinotecan, and oxaliplatin were recorded as 7.7, 7.7, and 5.0 μg/mL, respectively (*Ref. 13-15*).

***In vivo* inhibition of tumor growth.** The *in vivo* efficacy of sorafenib-irinotecan sequential therapy was assessed in a HepG2 xenograft mouse model. Balb/c athymic (nu+/nu+) female mice, 4–6 weeks of age, were purchased from the Animal Center of China Academy of Medical Sciences (Beijing, China). The animals were housed under pathogen-free conditions. The research protocol was in accordance with the institutional guidelines of the Animal Care and Use Committee at Liaoning Cancer Hospital.

Tumors were generated by harvesting HepG2 cells from mid-log phase cultures using trypsin-EDTA (Life Technologies, Carlsbad, CA, USA). Cells were then pelleted and resuspended in PBS to a final cell count of 5×10^7/mL. A volume of 0.2 mL of the cell suspension was injected s.c. in the right flank of each mouse. After 7 days, when the tumor volume had reached approximately 0.1 cm^3, all the mice were divided into 4 groups ($n = 6$) and then subjected to treatment. Group 1: mice were injected with 0.2 mL normal saline *via* tail vein. Group 2: sorafenib was administered p.o. at 10 mg/kg. Group 3: irinotecan was administered i.v. at 10 mg/kg. Administration of vehicle, sorafenib, or irinotecan in the above three groups was performed daily for 21 days. For combination studies, mice in group 4 received sorafenib p.o. at 10 mg/kg on day 1, followed by irinotecan i.v. at 10 mg/kg on day 2 and 3, respectively. Seven cycles of this treatment regimen was consecutively repeated. Drug doses in this animal study were determined with reference to previous studies [18,19]. Tumors were harvested at the end of experiment and tumor growth inhibition rates were defined as a percentage of the control tumor weight.

Statistical analysis. Data were expressed as mean ± S.D. for three different determinations. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests. $p < 0.05$ was considered as statistically significant. Statistical analysis was performed using the SPSS/Win 12.0 software (SPSS, Inc, Chicago, IL, USA).

Results

Inhibition of cell proliferation. Since HCC is usually resistant to chemotherapy, we first performed chemosensitivity assay to determine whether HCC cells HepG2 used in our study are susceptible to sorafenib, irinotecan, and oxaliplatin. HepG2 cells were exposed to these drugs for 48 h and then subjected to MTT assay. Results showed that all the drugs strongly inhibited cell proliferation in a dose-dependent manner (Figure 1). Statistical analysis indicated that the median inhibitory concentrations (IC_{50}) of sorafenib, irinotecan, and oxaliplatin were 4.79, 7.84, and 1.0 μg/mL, respectively, and the 20%-inhibitory concentra-

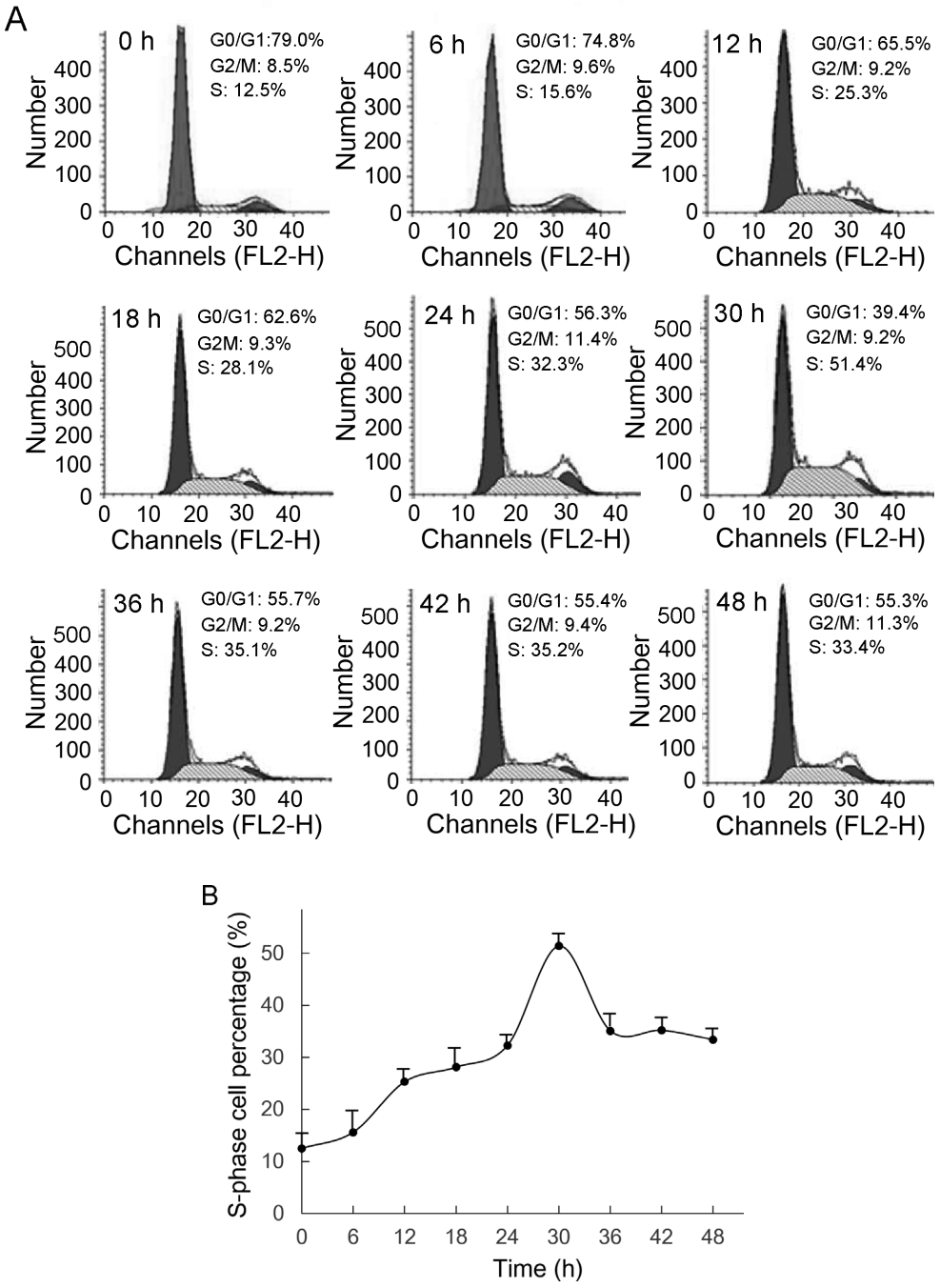

Figure 2. Sorafenib arrested cell cycle in the S phase. HepG2 cells were incubated with sorafenib (4.79 μg/mL) for a total of 48 h. Cells were harvested, stained with PI, and subjected to flow cytometry at an interval of 6 h (A). Triplicate experiments were performed with triplicate samples and percentages of S-phase cells were presented (B). The bars indicate means ± S.D.

tions (IC_{20}) were determined as 1.62, 0.50, and 0.12 μg/mL, respectively.

The growth inhibition rates of sorafenib, irinotecan, and oxaliplatin at 10, 1.0, and 0.1× PPC are listed in Table 1. Sorafenib, irinotecan, and oxaliplatin at the concentration of 1× PPC inhibited the growth of HepG2 cells by 65.9%, 49.2%, and 75.8%, respectively. The inhibition rates of these three drugs are more than 90% at 10 × PPC. According to the assessment criteria that the drug is defined to be sensitive when its growth inhibition rates are more than 30% at 1× PPC and 50% at 10× PPC, respec-

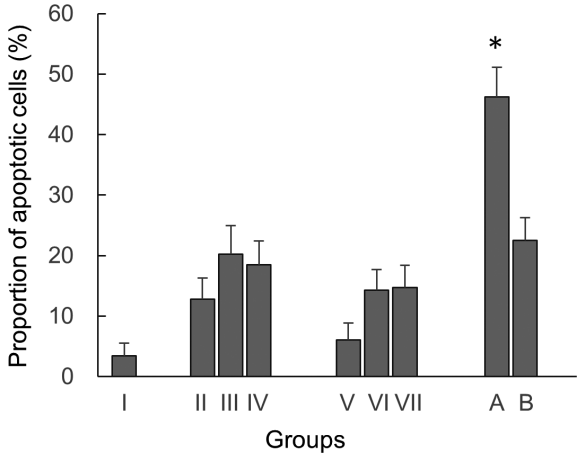

Figure 3. Effect of sorafenib, irinotecan, oxaliplatin, and drug combinations on cell apoptosis. HepG2 cells were exposed to I) vehicle for 78 h, II) sorafenib for 78 h, III) irinotecan for 78 h, IV) oxaliplatin for 78 h, V) sorafenib for 30 h followed by vehicle for 48h, and VI) vehicle for 30 h followed by irinotecan for 48 h, VII) vehicle for 30 h followed by oxaliplatin for 48 h, A) sorafenib for 30 h followed by irinotecan for 48 h, and B) sorafenib for 30 h followed by oxaliplatin for 48 h, respectively. Flow cytometry analyzed the percentage of apoptotic cells in each group. * p < 0.05.

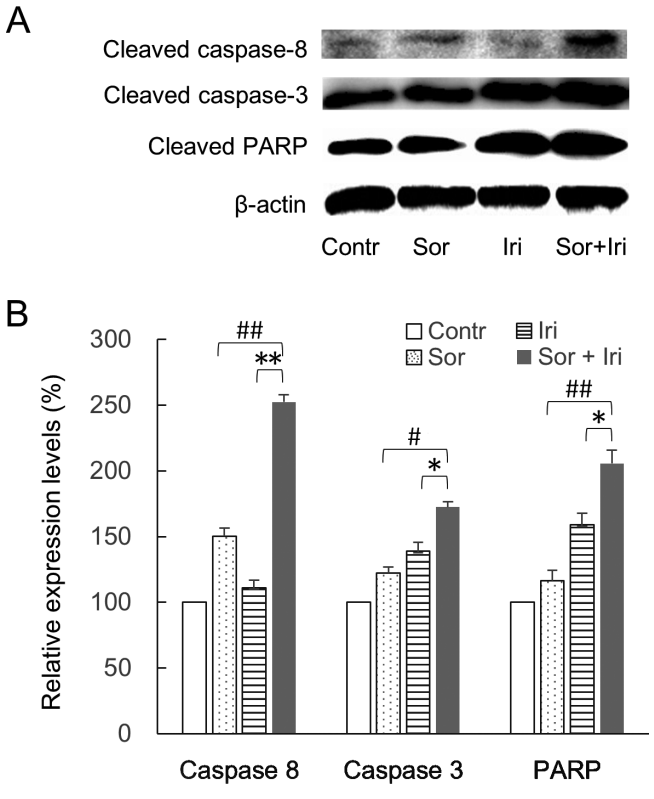

Figure 4. (A) Western blotting analyzed the changes of apoptotic proteins including cleaved caspase-8, -3, and PARP in HepG2 cells. (B) Contr, negative control; Sor, sorafenib alone treatment; Iri, irinotecan alone treatment; Sor + Iri, sequential treatment of sorafenib and irinotecan. * $p < 0.05$, ** $p < 0.01$, combined treatment *vs.* irinotecan alone treatment; # $p < 0.05$, ## $p < 0.01$, combined treatment *vs.* sorafenib alone treatment.

tively [20,21], HepG2 cells used in the current study is regarded to be sensitive to sorafenib, irinotecan, and oxaliplatin.

Cell cycle arrest. Cell cycle interference is an important feature of anticancer drugs and is commonly used in the design of sequential chemotherapy. It is known that irinotecan is an S-phase specific cytotoxic agent while oxaliplatin, without selectivity, is capable to kill cancer cells located at all cell cycle phases. In order to design rational combined therapies, we examined the effect of sorafenib on cell cycle distribution in HepG2 cells. The cells incubated with 4.79 μg/mL sorafenib were subjected to flow cytometry every 6 h. Results showed that HepG2 cells were arrested in the S phase after exposure to sorafenib and this effect was related to drug exposure time (Figure 2A). To be specific, the proportion of cell population located in S phase was increased from 12.5% before exposure to 15.6% after 6 h treatment and continuously increased to a maximum of 51.4% at 30 h. Then, the S-phase cell percentage decreased to 33.4% at 48 h (Figure 2B). These results provide reference and direction for the design of sorafenib-based sequential therapies.

Induction of cell apoptosis. Two sequential therapies were designed: **A**) Cells were treated with sorafenib (4.79 μg/mL, IC_{50}) for 30 h followed by irinotecan (0.50 μg/mL, IC_{20}) exposure for 48 h; **B**) Cells were treated with sorafenib (4.79 μg/mL, IC_{50}) for 30 h followed by oxaliplatin (0.12 μg/mL, IC_{20}) exposure for 48 h. Control groups were subjected to the following treatments respectively: I) vehicle for 78 h; II) sorafenib at 4.79 μg/mL for 78 h; III) irinotecan at 0.50 μg/mL for 78 h; IV) oxaliplatin at 0.12 μg/mL for 78 h; V) sorafenib at 4.79 μg/mL for 30 h followed by vehicle for 48 h; VI) vehicle for 30 h followed by irinotecan at 0.50 μg/mL for 48 h; and VII) vehicle for 30 h followed by oxaliplatin at 0.12 μg/mL for 48 h. Apoptosis index was analyzed using flow cytometry after treatment (Figure 3). The apoptotic cells in vehicle-treated group (Group I) occupied 3.4%. Sorafenib, irinotecan, and oxaliplatin treatment alone for 78 h resulted in cell apoptotic rates of 12.8% (Group II), 20.2% (Group III), and 18.5% (Group IV), respectively. When exposure to sorafenib for 30 h and vehicle for the followed 48 h, the percentage of apoptotic cells was determined to be 6.1% (Group V). When exposure to vehicle for 30 h and irinotecan or oxaliplatin for the subsequent 48 h, cell apoptotic rates were measured to be 14.3% (Group VI) and 14.7% (Group VII), respectively. The apoptotic cell percentages in groups treated by sequential therapies A and B were 46.2% and 22.5%, respectively. These results indicated that sorafenib-irinotecan sequential therapy was obviously superior to the monotherapy in soliciting HepG2 cells apoptosis ($p < 0.05$, Group A vs. Group II or III). However, no statistical difference in cell apoptotic rate was found between sorafenib-oxaliplatin sequential therapy and the monotherapy ($p > 0.05$, Group B vs. Group II or IV). The Q values of therapies A and B that reflect the interaction effects between two drugs were calculated to be 2.36 and 1.13, respectively. The results suggested that sorafenib-irinotecan sequential therapy exhibited a synergistic effect in soliciting

apoptosis of HepG2 cells, while sorafenib-oxaliplatin therapy showed an additive effect in inducing cell apoptosis.

We further examined cell apoptosis by measuring the levels of apoptosis related proteins including cleaved caspase-8, -3, and PARP in HepG2 cells. Western blot suggested that sequential treatment of sorafenib and irinotecan had more potent effect than either drug used alone in regulation of apoptotic proteins (Figure 4A). As shown in Figure 4B, sorafenib at 4.79 μg/mL for 78 h exposure, the levels of the cleaved caspase-8, -3, and PARP were increased by 50.2%, 22.2%, and 16.5%, respectively. Irinotecan at 0.50 μg/mL for 78 h exposure, the levels of cleaved caspase-8, -3, and PARP were increased by 11.1%, 38.9%, and 58.9%, respectively. For the combination studies, exposure of sorafenib at 4.79 μg/mL for 30 h followed by irinotecan at 0.50 μg/mL for 48 h resulted in upregulation of cleaved caspase-8, -3, and PARP by 152.5%, 72.5%, and 105.6%, respectively. Significant differences existed between sequential treatment and either drug alone in the levels of active caspase-3, -8, and PARP (Figure 4B).

Influence on p53 expression. We next determined the effects of sorafenib, irinotecan, and their sequential combination on p53 expression in HepG2 cells at both mRNA and protein levels. Real-time RT-PCR showed that sorafenib at 4.79 μg/mL for 30 h exposure significantly decreased the p53 mRNA expression ($p < 0.01$ *vs.* vehicle) (Figure 5A). On the other hand, irinotecan at 0.50 μg/mL for 48 h exposure obviously increased the level of p53 mRNA ($p < 0.05$ *vs.* vehicle). The expression of p53 mRNA in sequential treatment group was demonstrated to be lower than that in control and irinotecan group ($p < 0.05$ *vs.* vehicle; $p < 0.01$ *vs.* irinotecan). Similar with mRNA expression profile, Western blot assay showed that the expression of p53 protein in HepG2 cells was significantly reduced by sorafenib but increased by irinotecan (Figure 5B and 5C). The level of p53 protein in sequential treatment group was less than that in control and irinotecan group ($p < 0.05$ *vs.* vehicle; $p < 0.01$ *vs.* irinotecan).

Suppression of tumor growth *in vivo*. The efficacy of sorafenib-irinotecan sequential therapy was evaluated in nude mice bearing HepG2 xenografts. For monotherapy, administration of sorafenib at dose of 10 mg/kg (*p.o.*) and irinotecan at 10 mg/kg (*i.v.*) significantly delayed the growth of HepG2 xenografts by 19.5% and 24.6%, respectively ($p < 0.05$, *vs.* vehicle, Figure 6A). With regard to combined therapy, alternate use of sorafenib and irinotecan at the above doses effectively inhibited tumor growth by 49.2% ($p < 0.01$, *vs.* vehicle), which was obviously higher than the inhibition rates generated by monotherapy ($p < 0.05$). Oral sorafenib was generally well tolerated by mice with no significant loss of body weight ($p > 0.05$, *vs.* vehicle, Figure 6B). Irinotecan injection resulted in a significant reduction in body weight ($p < 0.05$, *vs.* vehicle). Similarly, a significant loss of body weight was also observed in mice receiving sequential therapy. However, this toxic effect was decreased in combined treatment group compared with irinotecan alone treatment group, although no statistical difference was observed.

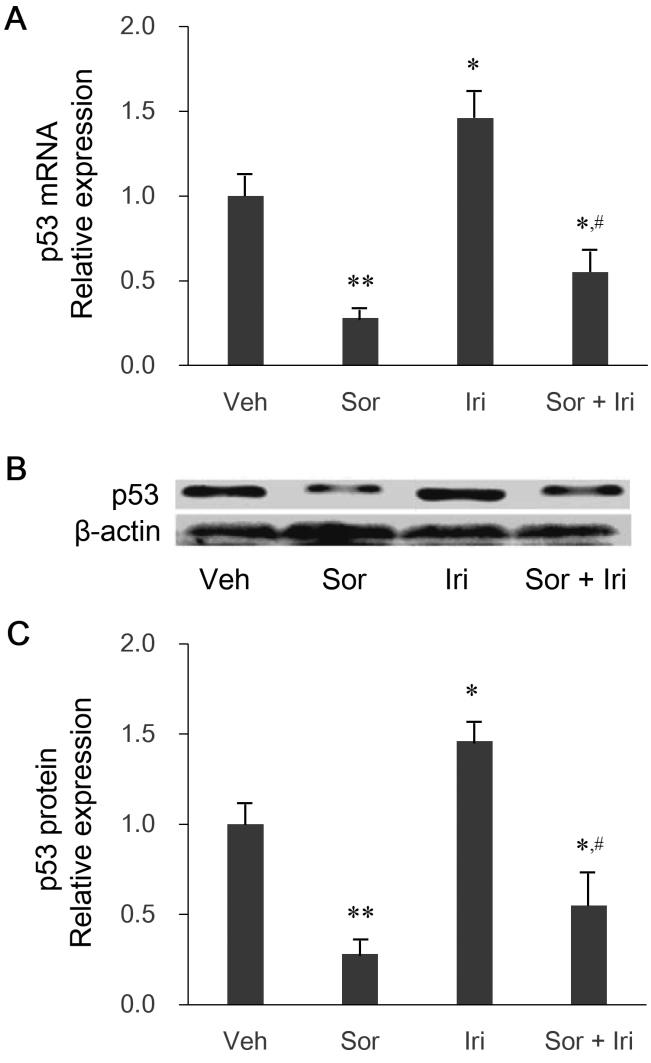

Figure 5. Effect of sorafenib, irinotecan, and drug combination on p53 expression. HepG2 cells were incubated with *i*) sorafenib for 30 h, *ii*) irinotecan for 48 h, and *iii*) sorafenib for 30 h followed by irinotecan for 48 h, respectively. Real-time PCR and Western blot analyzed p53 mRNA (A) and protein (B and C) expression in each group. Triplicate experiments were performed with triplicate samples. The bars indicate means ± S.D. * $p < 0.05$, ** $p < 0.01$ *vs.* vehicle; # $p < 0.01$ *vs.* irinotecan. Veh, vehicle; Sor, sorafenib; Iri, irinotecan.

Discussion

In order to overcome the shortcomings of sorafenib monotherapy for HCC, we investigated the efficacy of sorafenib based combined therapy in the present study. HCC cells HepG2 used in the current study is sensitive to sorafenib, irinotecan, and oxaliplatin. Cell cycle analysis revealed that sorafenib arrested cell cycle in S phase in HepG2 cells and the peak effect appeared at 30 h post treatment. We next determined the apoptosis induction effects of sequential combination of sorafenib with an S-phase specific agent irinotecan or a cell cycle nonspecific agent

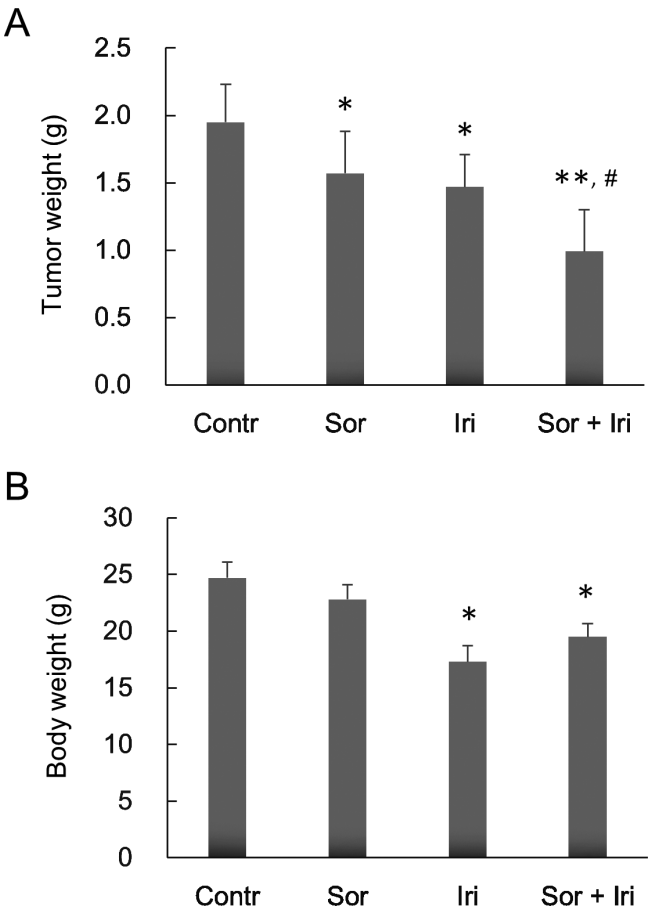

Figure 6. The inhibitory effects of sorafenib, irinotecan, and the combined treatment on the growth of HepG2 xenografts in nude mice. Drug administration methods were described in "Materials and Methods". Tumor weight (A) and body weight (B) were measured after the mice were sacrificed at the end of the experiment. * $p < 0.05$, ** $p < 0.01$, *vs.* vehicle; # $p < 0.01$, *vs.* sorafenib or irinotecan treatment alone.

oxaliplatin in HepG2 cells. It was demonstrated that sorafenib for 30 h exposure and irinotecan for subsequent 48 h exposure induced cell apoptosis in a synergistic manner, while sequential treatment of sorafenib for 30 h and oxaliplatin for 48 h acted an additive effect in soliciting cell apoptosis. Further studies revealed that sorafenib-irinotecan sequential treatment had more potent effect than either drug treatment alone in upregulating the levels of caspase-8, -3, and PARP in HepG2 cells. *In vivo* studies demonstrated that sorafenib-irinotecan sequential therapy was obviously superior to monotherapy in suppressing the growth of HepG2 xenografts in nude mice. These results suggested the potential value of sorafenib-irinotecan sequential therapy in HCC treatment. The molecular mechanism underlying the synergy of sorafenib-irinotecan sequential therapy in inducing cell apoptosis might be associated with the effect of sorafenib on p53 expression.

We found that sorafenib for 30 h exposure obviously reduced p53 expression at both mRNA and protein levels in HepG2 cells. As an oral multi-target kinase inhibitor, sorafenib can potently inhibit RAS/RAF/MEK/ERK signaling pathway that is usually overactivated in HCC cells [22,23]. Since overexpression of RAS can activate p53 expression in $HepG_2$ cells *via* ARF signaling [24], inhibition of RAS signaling by sorafenib may result in down-regulation of wild-type p53 expression. It is known that p53 is capable of holding cell cycle in G1 phase through checking G1/S regulation point [25]. Thus, the increased S-phase fraction following sorafenib treatment may be ascribed to the down-regulation of p53 expression, which drives cells to bypass G1/S checkpoint. SN-38, the main active metabolite of irinotecan, is an inhibitor of topoisomerase I. Its suppression of topoisomerase I leads to inhibition of both DNA replication and transcription, which eventually causes cell apoptosis [26]. Since the cytotoxic mechanism of SN-38 is largely S phase-dependent [27], pretreatment of sorafenib enhanced the apoptosis induction effects of irinotecan in HepG2 cells as observed in the present study *via* increasing the number of S-phase cells. On the other hand, because the antitumor action of oxaliplatin is cell cycle independent, it is theoretically less affected by changes of cell cycle distribution induced by sorafenib, which may explain that only additive effect was achieved in the sequential treatment.

The synergistic effect of sorafenib-irinotecan sequential therapy may also be directly related to the activity of sorafenib in decreasing p53 expression in HepG2 cells because p53 could cause resistance of cancer cells to topoisomerase I inhibitors. Kaina and co-workers revealed that p53 mediates the repair of topoisomerase I-cleavable complex which leads to resistance of cells to topotecan, an inhibitor of topoisomerase I [28]. They showed that p53-deficient mouse embryonic fibroblasts (MEF) and p53 mutated glioblastoma cells U138 were significantly more sensitive to the apoptotic activity of topotecan than the p53-proficient and p53 wild-type counterparts. Their results suggested that tumor response to topoisomerase I inhibitors is dependent on the p53 level in cancer cells. In the present study, we observed reduced expression of p53 in HepG2 cells after sorafenib treatment, which may sensitize HepG2 cells to the subsequent irinotecan treatment. Although exposure to irinotecan increased p53 expression, the p53 level in combined treatment group was still significantly lower than that in irinotecan alone treatment group. Thus, down-regulating p53 expression by sorafenib may be responsible for the synergistic effect of sorafenib-irinotecan sequential therapy in soliciting cell apoptosis in HepG2 cells.

In conclusion, sorafenib suppressed p53 expression and arrested cell cycle in S phase in HCC cells HepG2, causing tumor cells more sensitive to irinotecan. Sorafenib-irinotecan sequential exposure synergistically induced cell apoptosis in HepG2 cells *in vitro* and augmented the growth inhibitory effect of each drug used alone *in vivo*. This study provided evidence for usage of sorafenib-irinotecan therapy in HCC treatment in clinics. The efficacy and tolerance of this therapy in treating HCC warrant further preclinical and clinical research in the future.

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