

• Basic Research •

Schedule-dependent effects of sorafenib in combination with paclitaxel on human hepatocellular carcinoma cell line BEL-7402

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[Abstract] **Background and Objective:** Sorafenib is a multi-targeted antitumor drug. The monotherapy efficacy of sorafenib is relatively low. This study was to evaluate the schedule-dependent effect of sorafenib in combination with paclitaxel (TAX) on human hepatocellular carcinoma cell BEL-7402, and explore the underlying mechanism. **Methods:** BEL-7402 cells were treated with sorafenib or paclitaxel alone or in three different schedules: sorafenib was give prior to, after, or simultaneously with paclitaxel. The half maximal inhibitory concentration (IC_{50}) of sorafenib and paclitaxel was estimated by MTT. Alteration of cell cycle and apoptosis were analyzed by flow cytometry. The protein level of Bcl-2 in BEL-7402 cells was measured by western blot. **Results:** At 48 h, the IC_{50} of sorafenib and paclitaxel for BEL-7402 cells was $(2.43 \pm 0.32) \mu\text{g/mL}$ and $(1.89 \pm 0.72) \mu\text{g/mL}$, respectively. Sorafenib caused cell cycle arrest at S phase, while paclitaxel blocked cells at G_2/M phase. S and G_2/M phases were extended and a higher apoptotic rate $(36.43 \pm 2.29)\%$ was induced when sorafenib was given after paclitaxel in comparision with other groups ($P < 0.01$). The protein level of Bcl-2 was the lowest in BEL-7402 cells treated with sorafenib after paclitaxel. **Conclusions:** Administration of sorafenib after paclitaxel induces a higher apoptotic rate in BEL-7402 cells than administration before or simutanously with paclitaxel. This is probably due to that the two drugs act on different cell cycle phases and the expression of Bcl-2 might be involved.

Kye words: hepatocellular carcinoma, sorafenib, paclitaxel, schedule-dependent, Bcl-2

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China. Surgery is the basic therapy for this disease. However, it is so difficult to diagnose HCC at the early stage, so that most patients do not meet the criteria for radical surgery. The five year survival rate of patients after surgery is only 15%.^{1,2} Moreover, the recurrent rate is very high, around 50% in two years after surgery and most of them do not have a chance for the second radical surgery. Therefore, drug therapy has become one of the most important strategies. However, HCC cells are less sensitive to chemotherapy, and patients with advanced liver cancer suffer from cirrhosis. Disorder of the liver functions is an obstacle for chemotherapy. Molecular targeted therapy has gradually become a hot topic with more and further studies on molecular biology of

HCC.

Sorafenib is the first multi-targeted anti-tumor small molecule drug for oral use, which inhibits both tumor growth and vascularization. SHARP was a phase III trial of Sorafenib for patients with primary HCC, published in ASCO annual meeting in 2007. It showed that compared to control, the overall survival time and median progression-free survival in treatment group were prolonged by 2.8 months and 2.7 months, respectively.³

Small molecule targeted anti-tumor drugs cause some response in patients. The response rate is only 15% ~19% for monotherapy, resulting from its inhibitory effect of tumor cell growth, but not the eradication effect.⁴ Therefore, it is necessary to find out the effect of molecular targeted anti-tumor therapy combined with chemotherapy, and explain its mechanism and establish the most appropriate strategy for clinical use.

Our study aimed to evaluate the schedule-dependent effect of Sorafenib in combination with paclitaxel (TAX) on human HCC cell line BEL-7402, explore the underlying mechanisms and provide basic theoretical evidence for comprehensive treatment of HCC.

Materials and Methods

Materials. Human HCC cell line BEL-7402 was obtained from cell tank of Chinese Academy of Sciences and preserved by the Second Affiliated Hospital of Dalian Medical University Laboratory Center. RPMI-1640 was purchased from Hyclone Inc. Newborn bovine serum was purchased from Hangzhou Sijiqing Biotechnology Companies. Trypsin was purchased from Sigma. Sorafenib was synthesized by Bayer. Paclitaxel (TAX) was purchased from Tai Chi Group Sichuan Pharmaceutical Co., Ltd. Oxaliplatin and Irinotecan were purchased from Chinese Medicine, Jiangsu Henry AG. Nedaplatin was purchased from Qilu Pharmaceutical Co., Ltd. Mouse anti-human Bcl-2 monoclonal antibody was purchased from Santa Cruz.

Methods. MTT assay. To estimate the half

maximal inhibitory concentration (IC_{50}) of sorafenib, paclitaxel oxaliplatin, irinotecan or nedaplatin, cells in the logarithmic phase of growth were plated into 96-well plates at the density of 5×10^4 cells / mL, 100 μ L /well. Triplicate experiments were set for each treatment, 100 μ L of drugs at different concentrations were added into each well. The cells were then incubated in 37 $^{\circ}$ C, 5% CO_2 , saturated humidity environment. Four hours prior to the end of treatments, 5 mg/mL MTT was added into each well and incubated for 4 hours. DMSO (150 μ L) was added into each well. The plates were gently shaken on a micro-oscillator for 5-10 min. A blank control was adjusted to 0. Absorbance (A) at 570 nm wavelength of each well was detected using an automatic microplate reader. Cell inhibition rate = (A value of experimental group - A value of blank control) / (A value of normal control group - A value of blank control) \times 100%. IC_{50} was calculated. Steps mentioned above were repeated for three times. $IC_{50} < 20 \mu$ g/mL was considered to be highly sensitive. Drugs with high or moderate sensitivity were chosen as the major drugs for study.

Flow cytometry. BEL-7402 cells in the logarithmic phase of growth were cultured in 100 mL culture flasks at the density of 5×10^5 cells/mL. There were six groups: 1) control group, cells were cultured for 48 hours without treatment; 2) TAX group, cells were cultured with TAX (0.80 μ g/mL) for 48 hours; 3) Sorafenib group, cells were cultured with Sorafenib for 48 hours (1.83 μ g/mL); 4) (T \rightarrow S) group, cells were cultured with TAX for 24 h then Sorafenib for the next 24 hours; 5) (S \rightarrow T) group, cells were cultured with Sorafenib for 24 hours then TAX for the next 24 hours; 6) (T+S) group, cells were treated with TAX and Sorafenib simultaneously for 24 h. Cell suspension was harvested after digestion by 0.25% trypsin. Then, cells were treated according to the instruction of the flow cytometry kit. All the data generated by flow cytometry was input into MACINTISH650 computer, and the distribution of cell cycle was analyzed by Modifit1.0 software. Three independent experiments were conducted.

Western blot. The treatments were the same as described in flow cytometry. β -actin was used as a internal reference. Protein samples were loaded in SDS-PAGE Gels and transferred to NC membranes after electrophoresis Membranes were blocked with non-fat milk and incubated in primary antibody at 4° C overnight, incubated with second antibody with horseradish peroxidase after wash for three times, then developed by DAB. When clear specific lanes were observed, the reaction was ended by washing with water.

Statistical analysis. SPSS 13.0 software package was used. All the data were expressed as mean \pm SD (). Cell growth inhibition rate was analyzed by the logistic regression. The comparison among different groups was analyzed by variance analysis. The criterion for significance was α =0.05.

Results

IC₅₀ of different kinds of drugs. The IC₅₀ of sorafenib and paclitaxel for BEL-7402 cells was (2.43 \pm 0.32) μ g/mL and (1.89 \pm 0.72) μ g/mL, respectively, while the IC₅₀ of oxaliplatin, irinotecan, nedaplatin was (10.43 \pm 1.12) μ g/mL, (20.42 \pm 0.82) μ g/mL, (23.66 \pm 1.49) μ g/mL.

Effects of sorafenib at different concentrations and different time course on proliferation of BEL-7402 cells are shown in Table 1. BEL-7402 was strongly inhibited by sorafenib in dose- and time-dependent manner. (F = 95.733, p < 0.01).

The effect on cell cycle and apoptosis in BEL-7402 induced by sorafenib or TAX alone or in combination in different orders of administration. The results of flow cytometry are shown in Table 2. Sorafenib caused cell cycle arrest at S phase, while paclitaxel blocked cells at G₂/M phase. S and G₂/M phases were extended and a higher apoptotic rate (36.43 \pm 2.29)% was induced when sorafenib was given after paclitaxel (T→S group) in comparison with other groups (p < 0.01), while apoptotic rate (9.74 \pm 1.59) % in S→T group was much lower than T→S group, but there was no significant difference compared to other groups (p > 0.05).

Bcl-2 protein expression induced by

Table 1 Effect of sorafenib on proliferation of BEL-7402 cells

Sorafenib concentration (μ g/mL)	Inhibition ratio(%)		
	24 h	48 h	72 h
0.20	6.01 \pm 1.95	12.23 \pm 1.47	30.73 \pm 2.73
0.39	9.63 \pm 1.40	29.48 \pm 0.68	49.35 \pm 2.41
0.78	26.52 \pm 2.12	37.36 \pm 1.46	59.77 \pm 0.37
1.56	31.26 \pm 2.50	51.54 \pm 1.70	71.49 \pm 1.83
3.13	40.01 \pm 2.84	58.01 \pm 2.67	75.19 \pm 3.34
6.25	44.01 \pm 3.12	62.07 \pm 2.97	80.78 \pm 1.57
12.50	53.01 \pm 1.90	65.25 \pm 1.28	88.73 \pm 0.97
25.00	61.79 \pm 0.60	80.38 \pm 2.93	92.56 \pm 0.92
IC ₅₀	9.17 \pm 0.90	2.43 \pm 0.32	0.52 \pm 0.61

sorafenib or TAX alone and in combination in different orders of administration. The results of western blot are displayed in Fig. 2. The protein expression level of Bcl-2 in sorafenib group was reduced compared to that in the negative control group, while lower in T→S group than S→T, T+S group. There was no significant difference among other groups (p>0.05).

Discussion

Chemotherapy is one of the most important treatments for advanced HCC. At present, drugs widely used in the clinical practice are as follows: adriamycin, cisplatin, mitomycin, 5-FU, as well as gemcitabine, irinotecan which are approved by SFDA in recent years. Besides, increasingly more pre-clinical studies have been conducted to explore the therapeutic effect of oxaliplatin and paclitaxel on hepatocellular carcinoma. Our study found that BEL-7402 cell is relatively sensitive to paclitaxel and oxaliplatin, indicating those two drugs may become new choices for hepatocellular carcinoma in the future.

Although chemotherapy can be used in treatment of primary hepatocellular carcinoma, there is insufficient proof from evidence-based medicine to support the benefits from one certain chemotherapeutic drug. With the results of SHARP published in ASCO annual meeting in 2007, sorafenib is considered to usher treatment of hepatocellular carcinoma into a new era.

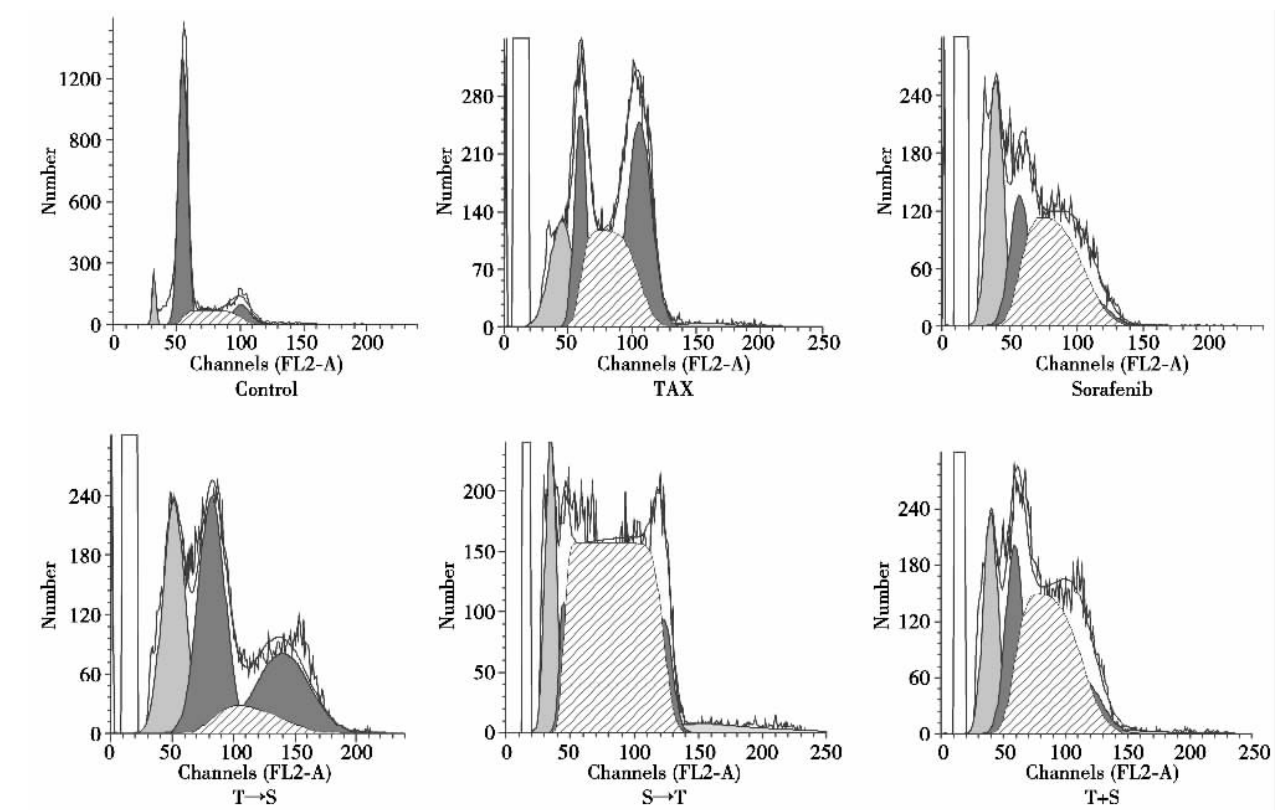


Figure 1 Cell cycle distribution and apoptosis of BEL-7402 cells treated by sorafenib and paclitaxel alone or in different schedules T→S: sorafenib was given after paclitaxel; S→T: sorafenib was given before paclitaxel; T+S: sorafenib was given simultaneously with paclitaxel. ^a*P*<0.01.

Table 2 Cell cycle distribution and apoptosis of BEL-7402 cells treated by sorafenib and paclitaxel alone and in different schedules

Group	G ₀ -G ₁ (%)	S(%)	G ₂ /M(%)	Apoptosis(%)
Control	70.63±2.38	19.36±1.49	10.01±0.95	1.37±0.53 ^a
TAX	20.72±2.30	30.75±2.70	48.53±0.76	11.43±1.52
Sorfenib	24.22±3.77	60.49±4.36	15.29±0.98	10.68±1.52
T→S	42.37±2.67	26.28±1.86	31.35±4.52	36.43±2.29 ^a
S→T	5.60±1.22	83.94±3.48	10.46±2.27	9.74±1.59
T+S	29.28±2.71	45.20±3.16	25.52±0.50	17.40±0.54 ^a

However, targeted drugs only inhibit tumor cells not eradicating them; targeted drugs in combination with traditional chemotherapy might enhance anti-tumor effect. Further studies are needed to identify the additive effect of combination of those two different therapies and establish an optimal protocol.

Nowadays, EGFR-TKIs such as IMC-C225, Iressa and OSI-774 have become hot topics in the field of combination treatment. Studies have

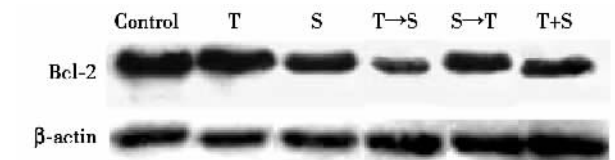


Figure 2 Expression of Bcl-2 in BEL-7402 cells treated by sorafenib or paclitaxel alone or in combination in different schedules

shown that tumor cells with high EGFR expression are relatively sensitive to chemotherapy, while anti-drug resistance of tumor cells is related to declination of cell growth signal.⁵⁻⁸ If targeted therapy is given before chemotherapy or both two treatments are conducted simultaneously, the effect of chemotherapy would be reduced. On the contrary, if multi-cycle chemotherapy was applied before targeted therapy, EGFR expression will increase, which enhances the effect of targeted therapy.^{9,10}

There were two large-scaled multi-center phase III clinic trials: one studied on Iressa in combination with carboplatin and paclitaxel in advanced non-small cell lung cancer, the other studied on Iressa in combination with cisplatin and gemcitabine in advanced non-small cell lung cancer. Neither of those studies showed that the combination therapy is beneficial.^{11,12} Therefore, the combination of targeted drugs and chemotherapy does not simply have additive effect. Researchers from 307 Hospital of Academy of Military Medical Science and Italian National Cancer Institute had a discussion about the mechanism of Iressa in combination with different chemotherapeutic drugs in the terms of molecular pharmacology and first raised a theory that the best effects of Iressa in combination with chemotherapeutic drugs can be achieved with the most appropriate protocol and administration time.¹³ They considered effective induction chemotherapy followed by a sequential monotherapy of Iressa as the best strategy.

So far, there is no report on the most appropriate protocol and administration order for sorafenib in combination with chemotherapeutic drugs, which is similar with EGFR-TKIs. We analyzed the effect of cell cycle and apoptosis in BEL-7402 induced by different drug administration order using flow cytometry. We found that different administration order induced different effects. The apoptotic rate of $S \rightarrow T$ group was the same as that in TAX group. The apoptotic rate in $T+S$ group was higher than TAX group, but the highest apoptotic rate was observed in $T \rightarrow S$ group. Our study suggests that similar to EGFR-TKIs, there probably was an appropriate protocol and administration order for sorafenib combined with chemotherapeutic drugs. Induction chemotherapy followed by sorafenib might be a better choice.

Results of cell cycle analysis showed that S phases were shortened and G_2/M phase were extended induced by sorafenib in BEL-7402 cells, which was consistent with the effect of sorafenib in HepG₂ cells reported by Liu etc^[14]. Our study indicates that sorafenib causes cell cycle arrest at S phase and block the transition to the following phases in most cells, resulting in inhibition of cell

proliferation and induction of apoptosis. Furthermore, paclitaxel is a cell cycle specific drug, which can inhibit normal reorganization of microtubule network and block cells at G_2/M phase^[15]. Our study showed that TAX alone can extend G_2/M phase in BEL-7402 cells, inhibited cell growth and induced apoptosis.

In $S \rightarrow T$, $T \rightarrow S$ and $T+S$ groups, we found that G_0-G_1 phase was shortened to different extent. In $T \rightarrow S$ group, G_2-M phases were extended, no obvious change was observed in S phase, while apoptotic rate increased apparently. In $S \rightarrow T$ group, S phase was distinctively extended (83.94 ± 3.48 %), while no obvious change was observed in G_2-M phases and apoptotic rate is much lower than $T \rightarrow S$ group. In $T+S$ group, S phase and G_2-M phases were extended; apoptotic rate was between other two groups. The reason why apoptotic rate in $S \rightarrow T$ group was much lower than any other groups is sorafenib and TAX has distinctive effects on different cell phases. Sorafenib causes cell arrest at S phases, blocks transition to into G_2-M phase, thus abolishes the sensitivity of cells to cell cycle specific drug TAX, resulting in reduced apoptotic rate. Therefore TAX followed by sorafenib might be the best administration order.

Subbaramaiah et al.¹⁶ and Lieu et al.¹⁷ reported TAX promotes the activity of ERK in several cell lines and blockade of the MEK/ERK pathway would inhibit the apoptosis effect induced by TAX. Okano et al.¹⁸ found that TAX can inhibited cell growth of hepatocellular carcinoma and block cell cycle at the G_2/M phase by the ERK pathway, while blockade of the ERK pathway may abolish these effect induced by paclitaxel. Keila et al.¹⁹ reported that Raf-1 can cause cell arrest at G_2-M phases, indicating it may play a very important role in mitosis. They also suggested that there are two possible mechanisms by which TAX induces apoptosis: (1) At the concentration of lower than 9 nmol/L, TAX-induced apoptosis is not related to Raf-1 pathway.(2) At the concentration of no less than 9 nmol/L, TAX induces apoptosis via Raf-1 pathway. In our studies, the concentration of TAX was 937 nmol/L ($0.8 \mu g/mL$), indicating apoptosis induced by TAX may involve Raf-1

pathway. However sorafenib administrated before TAX which blocks the RAF/MEK/ERK pathway, may reduce the sensitivity to TAX. Details must be tested and verified in further experiments.

On the contrary, Okano et al.²⁰ reported that MEK/ERK pathway is not involved in TAX cytotoxicity in human esophageal squamous cancer cell lines. The same drugs and the same cell signal transduction pathways may work in totally different ways and mechanisms in different cell lines, which should be verified in further related experiments.

Bcl-2 is an important anti-apoptosis gene. Bcl-2 gene and its protein can suppress apoptosis of various tissues and cells and prolong cell life, which is called "surviving gene". It regulates apoptosis through self-dimerization or formation of heterodimers with Bax etc. Evidences²¹ indicate that Bcl-2 phosphorylation is relevant to cell accumulation in the G₂/M phases, which delays cells transition from M phase to G₀/G₁ phase. Microtubule-damaging agent TAX can induce Bcl-2 phosphorylation in the cells at G₂/M phases.²²

Blalock et al.²³ and Moye et al.²⁴ showed that the RAF/MEK/ERK signaling transduction pathway is relevant to Bcl-2 expression in hepatocellular carcinoma. The mRNA expression of Bcl-2 increases in Raf-1-activated cells, suggesting that RAF/MEK/ERK signal transduction pathway regulates Bcl-2 expression, which may related to expression of cAMP response element binding protein mediated by ERK. Sorafenib can down-regulate Bcl-2 expression through blocking RAF/MEK/ERK signal transduction pathway. In our study, we found sorafenib down-regulated Bcl-2 expression, which may result in apoptosis.

Bcl-2 expression was obviously decreased in T→S group, but remains unchanged in S→T and T+S groups, indicating that TAX followed by sorafenib can apparently reduce Bcl-2 expression and thus promote cell apoptosis. However, details should be verified in the further experiments.

In conclusion, we studied the effects of Sorafenib alone and Sorafenib in combination with TAX on human hepatocellular carcinoma

cell lines using MTT assay, flow cytometry and western blot. We found that sorafenib can induce apoptosis in BEL-7402 cells, and administration of sorafenib after paclitaxel induces a higher apoptotic rate than administration before or simultaneously with paclitaxel. Therefore, among all comprehensive therapy for primary hepatocellular carcinoma, administration of targeted drugs after chemotherapeutic drugs may achieve better anti-tumor effects. Chemotherapeutic drugs in combination with targeted drugs are one of the future directions of clinical application. Rigorous preclinical researches must be conducted based on the features of various targeted drugs and chemotherapeutic drugs to provide basic evidences for clinical practice.

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