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Beneficial and Adverse Effects of Sorafenib Drug on Hepatocellular Carcinoma-bearing Rats: Histopathological and Molecular Evidence

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ABSTRACT

Hepatocellular carcinoma (HCC) is a primary liver cancer commonly found in adults. Globally, HCC is the sixth most prevalent cancer and the fourth most common cause of cancer death. In terms of drug treatment options, sorafenib (Nexavar) is the only Food and Drug Administration-approved drug to treat unresectable HCC patients. However, several side effects in said patients were noted after receiving treatment with sorafenib. Therefore, this study aimed to evaluate the inhibitory effect of HCC in sorafenib-treated HCC-bearing rats. The results showed that sorafenib treatment induced high levels of liver enzymes (AST and ALT) in the HCC rat liver. In addition, even though sorafenib-treated rats did not show any side effects during the treatment, inflamed hepatocytes, ballooning degeneration, and microvesicular steatosis were observed in rat liver tissues. Moreover, as revealed by qPCR and immunohistochemical staining, sorafenib enhances higher expression of Bax mRNA and protein in HCC tissues. Thus, this study suggests that sorafenib can inhibit tumor growth through promotion of apoptosis but has the adverse effect of inducing liver injury. Further studies are needed to investigate whether or not HCC cells play a role in how sorafenib exhibits adverse effects on the liver.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor in the liver and the fourth most common cause of cancer-related mortality in humans [1]. The incidence rate of HCC are highest in Asia and Africa, with poor prognosis worldwide [2]. However, advanced-stage patients can prolong their lives with appropriate treatment and care.

The first orally administered drug approved to target multiple kinases was sorafenib (Nexavar, BAY 43-9006). It was approved for advanced-stage HCC [3]. Sorafenib exhibits anti-proliferative and anti-angiogenic effects by inhibiting serine/threonine kinases [4,5]. In addition, it has been shown to inhibit receptor tyrosine kinases, including vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR) [6,7].

Apoptosis is one of the processes that stimulate cell death when cells sustain DNA damage. Resistance to cell death is one of the recurrent hallmarks of several cancers, including HCC [8]. The intrinsic/mitochondrial apoptotic pathway is controlled by members of the Bcl-2 family of proteins and can be regulated by the tumor suppressor protein p53 [9]. Bcl-2 family members are overexpressed in many types of cancer cells [10,11]. The resulting imbalance in the expression of pro-and anti-apoptotic proteins enhances tumor development. For example, overexpression of Bcl-2 and down-regulation of Bax favor cell survival [11-13]. p53 is an important apoptosis-regulating gene modulating apoptosis through the p53 mediated apoptotic pathway [14,15]. p53 can induce the expression of pro-apoptotic Bcl-2 proteins such as Bax [14] and also the downregulation of anti-apoptotic protein Bcl-2 [16]. Modulating apoptosis in cancer, therefore, may be useful for HCC therapy.

It has been documented that sorafenib exhibits a potential effect on apoptosis induction in prostate cancer cells by inhibiting the androgen receptor and Akt signaling pathways [17]. Also, in liver cancer cell lines, sorafenib suppresses cell proliferation and induces apoptosis [4,18,19]. It had the ability to inhibit the protein Bcl-2 and induced caspase3 activation in liver cancer [20,21]. However, HCC patients have shown several side effects when treated with sorafenib such as fatigue, hypertension, diarrhea, and hand-foot skin reaction [22-24]. Furthermore, several reports of sorafenib treatment leading to fatal hepatotoxicity in patients can be found [25-26]. Therefore, this present study aimed to investigate sorafenib's effect in HCC-bearing rats and its effect on apoptotic protein expression.

METHODOLOGY

Animal experiment

All animal procedures were followed and approved by the Animal Ethics Committee of the Faculty of Medicine, Srinakharinwirot University

(Bangkok, Thailand; approval no. 2/2559). A total of 18 male Wistar rats (*Rattus norvegicus*) weighing between 200–250 g was obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The rats were divided into three groups, each consisting of six rats: control rat, untreated HCC rat, and sorafenib-treated HCC groups. For HCC induction, rats were intraperitoneally injected with a single dose of 200 mg/kg diethylnitrosamine (DEN; Sigma-Aldrich, St. Louis, MO, USA). Two weeks later, the rats were intraperitoneally injected with 300 mg/kg of thioacetamide (TAA; Sigma-Aldrich, St. Louis, MO, USA) three times per week for four consecutive weeks. After two weeks, rats were received 30 mg/kg of sorafenib orally daily for eight weeks. At the end of the experiments, blood and liver tissues were collected.

Liver function test

Blood samples were collected by heart puncture. The samples were centrifuged at 3,000 rpm for 15 minutes then the supernatant was collected. The liver serum enzymes, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured in a standard clinical lab (Bangkok R.I.A. Laboratory, Bangkok, Thailand) using commercially available standard enzymatic reagents (Abbott Pharmaceutical Co. Ltd.).

Histopathological and immunohistological study

Liver tissues were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde. The specimens were dehydrated in a graded series of ethanol, embedded in paraffin, and then sectioned at 5 μm by microtome. The sections were deparaffinized in xylene and then rehydrated in a descending series of ethanol and stained with hematoxylin and eosin (H&E) for general histopathological study. The immunohistochemistry for Bax expression was performed using ImmunoCruz® rabbit ABC Staining System (sc-2018, Santa Cruz, CA, USA). Briefly, the liver sections were incubated with 10 mM sodium citrate at 120°C for 10 minutes to perform antigen retrieval then block endogenous peroxidase with 3% hydrogen peroxide in methanol. The slides were then washed with PBS and incubated with 1.5% blocking serum for 1.5 hours. The sections were incubated with rabbit anti-Bax antibody (1:250, ab32503, Abcam, Cambridge, MA, USA) and rabbit anti-Bcl-2 antibody (1:100, ab196495, Abcam, Cambridge, MA, USA) overnight at 4°C in a humidified chamber. After washing with PBS, the sections were incubated with biotinylated secondary antibody (1:100, sc-2018, Santa Cruz, CA, USA) for 1.5 hours. Tissue sections were incubated for 30 minutes with avidin and biotinylated horseradish peroxidase (AB reagents) followed by peroxidase substrate incubation. Mayer's hematoxylin (Bio-Optica, Milano, Italy) was incubated for 1 minute for counterstaining. The cancer area was observed as previously described [27].

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The assay was performed using the TUNEL assay kit - horseradish peroxidase (HRP)- 3,3′-diaminobenzidine (DAB) (cat. no. ab206386; Abcam, Cambridge, UK) according to the manufacturer's instructions.

Light microscope (LM) observation

All tissue slides were mounted and photographed under a light microscope (Olympus, Tokyo, Japan) or scanned and captured with a panoramic digital slide scanner program (3Dhistech, Hungary).

Quantitative real-time PCR analysis

Total RNA was extracted from frozen rat liver using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, 50 mg of the liver sample were homogenized with a tissue homogenizer (Omni international, USA) for 10 seconds in 1 mL of TRIzol reagent and then incubated at room temperature for 5 minutes before adding 0.2 mL of chloroform, then mixed vigorously by hand for 15 seconds, incubated at room temperature for 3 minutes, and centrifuged at 12,000 g for 15 minutes at 4°C. After centrifugation, the RNA in the upper aqueous phase was transferred to a fresh tube and then precipitated with 0.5 mL of isopropanol, mixed gently by vortex, and incubated at room temperature for 10 minutes. The pellet of total RNA was collected by centrifugation at 12,000 g for 10 minutes. The supernatant was discarded**.** The RNA pellet was washed with 1 mL of cold 75**%** ethanol and centrifuged at 7,500 g for 5 minutes. Finally, the RNA was dissolved in nuclease-free water, and the RNA concentrations were determined using a NanoDrop® spectrophotometer (Thermo Scientific, Waltham, MA, USA). The reverse transcription was performed using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized and run on a thermal cycler (Eppendorf Mastercycler® personal; Eppendorf AG, Hamburg, Germany) at 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes. Real-time PCR was carried out in CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) with commercial PrimePCR primers in Table 1. Rat beta-actin (*Actb*) mRNA was assayed as an internal control for relative quantification. The thermal cycler protocol consisted of 2 minutes activation at 95°C followed by 40 cycles of denaturation at 95°C for 5 seconds, and then annealing/extension at 60°C for 30 seconds. Analysis of the mRNA expression levels was performed by Bio-Rad CFX manager™ software version 1.3.1 (Hercules, CA) and quantification of relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ (Livak) method of relative quantification [28].

Table 1. PrimePCR primers designed for SYBR® Green gene expression.

Gene symbol	Unique Assay ID	GenBank accession No
TP53	qRnoCEP0025896	NM_030989
Bax	qRnoCED0002625	Not Available
Bcl2	qRnoCED0006419	NM_016993
Actb	qRnoCID0056984	NM_031144

RESULTS AND DISCUSSION

In HCC-bearing rats, the cancer area was reduced by about 10% in the sorafenib treatment group. The liver serum enzyme AST and ALT, however, were significant higher in the sorafenib group compared to the untreated HCC group (Table 2). These abnormal liver enzyme levels may indicate liver damage [29], suggesting that sorafenib treatment promotes the leakage of liver enzymes into the bloodstream. Moreover, there was no significant difference in the AST and ALT level between the control and HCC groups, indicating that the rise in AST and ALT level is caused by sorafenib, which suggests liver injury being present in the sorafenib group, but not in the untreated HCC group. A case report also found a high level of AST and ALT in a patient after two months of sorafenib treatment [30].

With regard to histopathology, the liver sections stained with H&E shows that sorafenib-treated HCC group has numerous cancer nodules (Figure 1A). Outside the cancer nodules, inflamed hepatocytes (Figure 1B), hydropic degeneration (cellular swelling), and microvesicular steatosis can be observed in liver tissues of sorafenib-treated HCC rats whereas these changes were not found in non-tumor area of untreated HCC group (Figure 1C and D). Moreover, sorafenib has been reported to

Table 2. Percentage area of cancer cells and serum hepatic enzymes (mean ± SEM.). The data were adapted from the previous study [27].

Groups	Cancer area (%)	AST (U/L)	ALT (U/L)
Control	-	80.6 ± 7.2 **	40.4 ± 3.9 ***
HCC	19.6 ± 2.5	105.3 ± 9.9 *	48.4 ± 3.2 ***
Sorafenib-treated HCC	2.1 ± 0.4	160.4 ±17.4	90.1 ± 6.2

*p<0.05 compared to sorafenib-treated HCC group, **p<0.01 compared to sorafenib-treated HCC group, ***p<0.001 compared to sorafenib-treated HCC group.

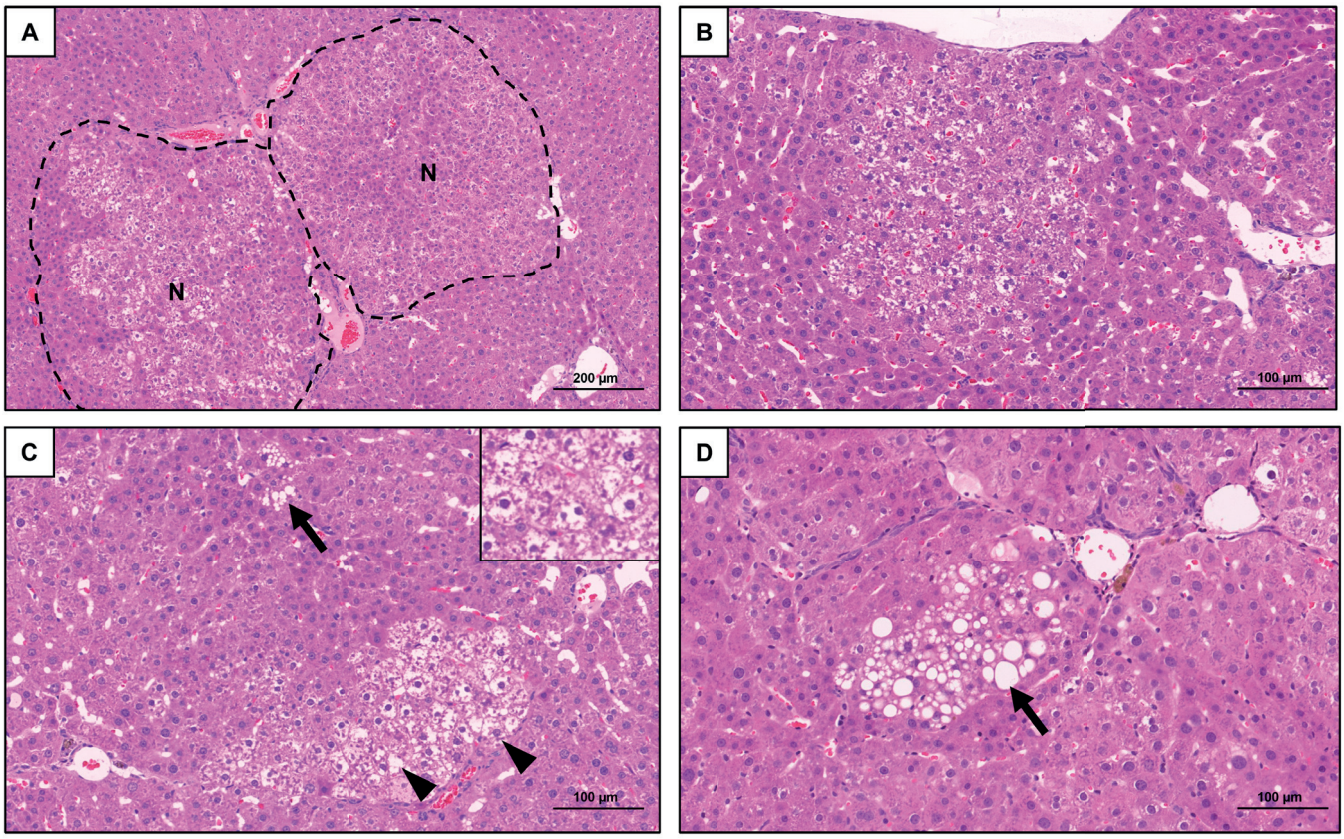

Figure 1. Histopathological observation of rat liver tissue in sorafenib-treated HCC group by H&E staining (black arrow = microvesicular steatosis, arrowhead = hydropic degeneration empty space around nuclei represents hydropic degeneration, and N = cancer nodule).

promote hydropic (ballooning) degeneration and necrosis in hepatocytes [30-32]. The hydropic degeneration in hepatocytes following sorafenib treatment suggest increased accumulation of intracellular fluids in hepatocytes, which is a precursor morphological feature to cell death.

To investigate the mechanisms of sorafenib, we further observed the apoptotic markers in rat liver tissues. In Figure 2, the qPCR results showed that sorafenib could induce *TP53* and *Bax* mRNA expression. High levels of Bax is associated with increase in apoptosis and response to chemotherapy, and improved survival in HCC patients [33]. Resistance to apoptosis can promote tumor development and tumor progression of the liver [34]. An alteration or mutation of molecular mechanism, such as, the expression of p53 tumor suppressor gene (*TP53*), is found frequently in HCC [35,36]. In this study, the expression of *TP53* and *Bax* were significantly higher in the sorafenib-treated HCC group, indicating the up-regulation of p53 mediated Bax, resulting in the promotion of apoptosis in HCC-bearing rats. The apoptotic effect was confirmed by immunohistochemistry and TUNEL assay. Immunohistochemical staining showed that sorafenib treatment caused up-regulation of Bax in both tumor and non-tumor areas compared to untreated HCC group. Moreover, TUNEL results showed that sorafenib induces DNA fragmentation of cancer cells (brown-positive cells) in the sorafenib-treated group (Figure 3 and 4).

Several mechanisms of sorafenib have been revealed, for example, crosstalk involving PI3K/Akt and JAK-STAT pathways, hypoxia-inducible pathways activation, and epithelial-mesenchymal transition [37]. The therapeutic effects of sorafenib does not affect solely tumor cells and hepatocytes, but also extends to other liver cells. Regarding liver fibrosis, the major consequence of liver inflammation and cancer, sorafenib has been reported to exhibit anti-fibrotic effects. Sorafenib has been found to inhibit TGF-β/Smad signaling pathway, STAT3 phosphorylation, and apoptosis in hepatocytes [38,39]. Moreover, the apoptotic effects of sorafenib also extends to hepatic stellate cells [40].

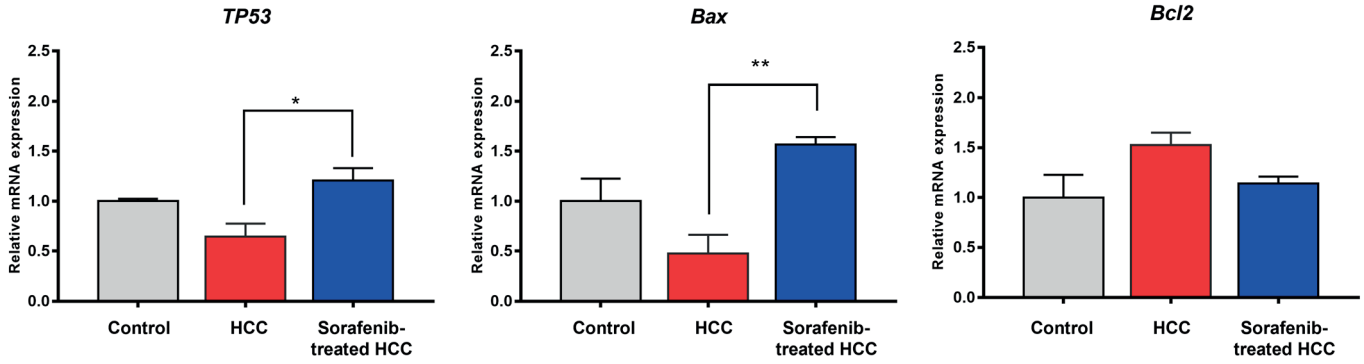

Figure 2. Diagram showing the relative mRNA expression of *TP53, Bax,* and *Bcl-2* by real-time PCR (*p<0.05, **p<0.01).

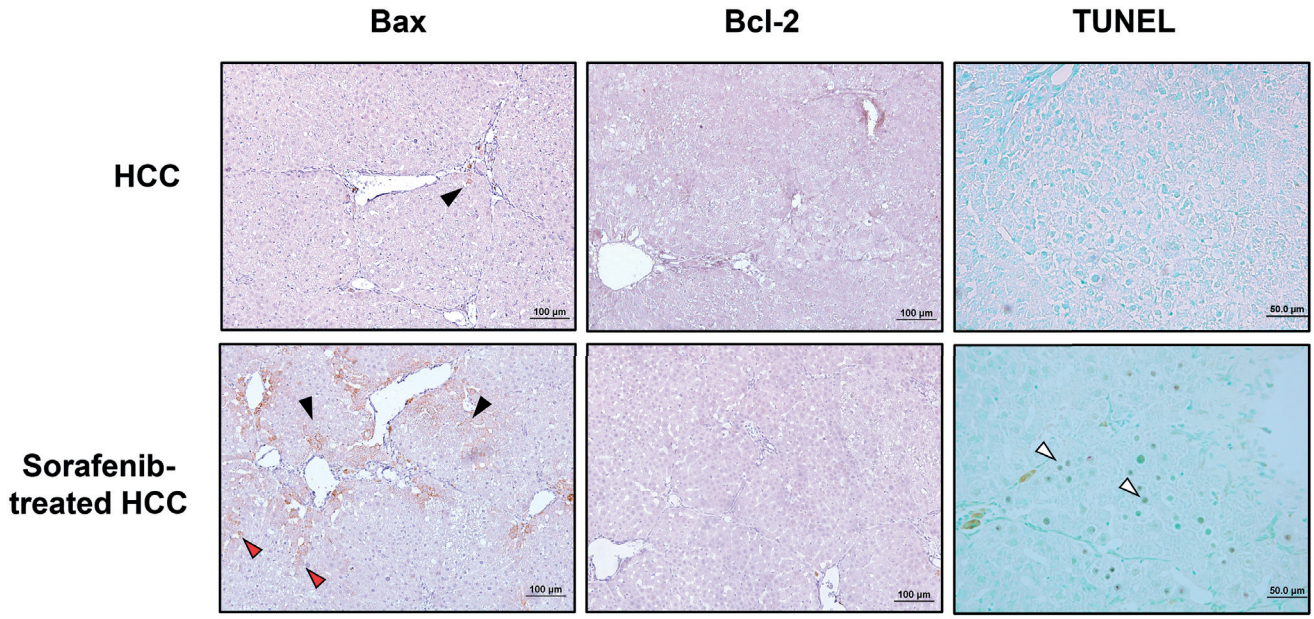

Figure 3. Immunohistochemical staining of apoptotic markers and TUNEL assay (black arrowhead = Bax-positive cells in tumor area, red arrowhead = Bax-positive cells in non-tumor area, and white arrowhead = TUNEL-positive cells).

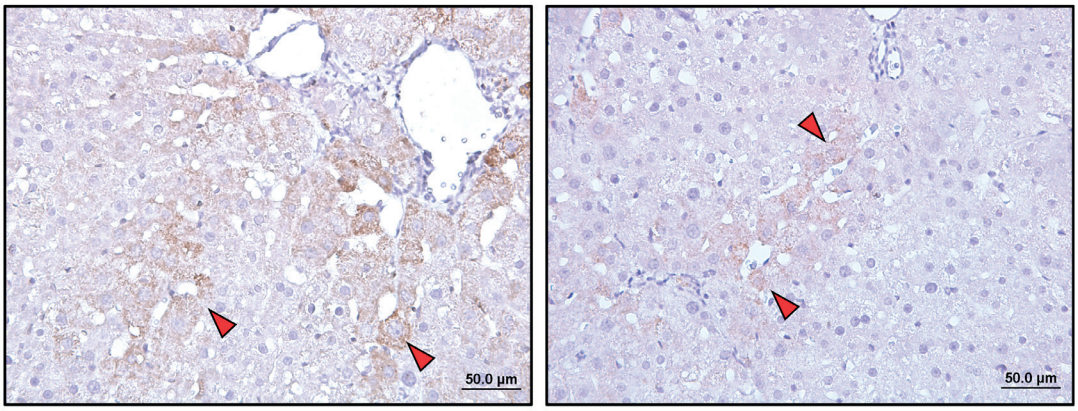

Figure 4. Immunohistochemical staining of Bax in the sorafenib-treated group at high magnification (red arrowhead = Bax-positive cells in healthy liver cells).

A study suggested that sorafenib selectively induces apoptosis of cancer cells, but not in healthy liver cells [39]. Sorafenib-induced liver injury can be often reversible by removing the drug from the system. The duration of amelioration of the liver injury matched sorafenib's elimination half-life of 2 weeks [41]. However, the Bax-positive cells were also seen outside cancer nodules, among normal liver cells, which potentially suggests increased death of normal liver cells. Further studies are needed to investigate sorafenib's effect on all liver cell types.

CONCLUSION

This present study suggests that even though sorafenib exhibit anti-cancer effects by promoting apoptosis in HCC-bearing rats, it has adverse side effects of inducing liver injury.

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