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Sorafenib-Inhibited Signaling: Emerging Evidence of RAF-Independent Pathways as Potential Therapeutic Targets in Hepatocellular Carcinoma

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Additional information is available at the end of the chapter

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide [Serag et al., 2007, Liovet et al., 2003, Yang et al., 2010]. More than 500,000 people are diagnosed with HCC every year, and it remains the leading cause of death among patients with hepatitis B virus (HBV), hepatitis C virus (HCV) and alcohol-induced liver cirrhosis. One of the main obstacles for treating HCC is late diagnosis of patients. Many unresectable HCC patients are treated with loco-regional therapies such as radiofrequency ablation and transarterial chemo-embolization (TACE), but the prognosis remains poor [Bruix et al., 2005]. A recent study in multiple clinical facilities in Japan reported that 5-year survival of patients treated with TACE was less than 30% [Takayasu et al., 2006]. Moreover, HCC is poorly responsive to chemotherapeutic drugs and radiotherapy [Arii et al., 2000, Kuwahara et al., 2009]; thus, effective therapeutic tools for HCC are long-awaited.

Sorafenib (Nexavar, BAY 43-9006, Bayer HealthCare Pharmaceuticals) is a new type of drug designed to target RAF signaling, and represents a new era of HCC treatment. However, accumulating evidence has revealed the limited effect of sorafenib, and many clinical trials of sorafenib-based combination therapy are now underway. It should be noted that, while sorafenib was originally designed to target RAF-mediated signaling, recent studies have strongly indicated that its effect is closely involved in various types of non-RAF signaling [Matsuda et al., 2011]. To explore safe and effective therapies combined with sorafenib, full understanding of the functional mechanism of sorafenib is necessary. Herein, we review recent findings from studies of sorafenib-mediated inhibition of RAF and non-

RAF signaling pathways. We also discuss the possibility of administering sorafenib with other drugs in combination therapy, which might become a promising approach in the treatment of advanced HCC.

2. Clinical perspectives of sorafenib

2.1. The effects and limitations of sorafenib

Sorafenib is an orally bioavailable inhibitor of multiple kinases including RAF, vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor (PDGF) receptor, and the v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene (*KIT*) and fms-like tyrosine kinase 3 (*FLT-3*) oncogene [Wilhelm et al., 2004, Hochhaus et al., 2011]. At present, sorafenib is the only oral drug shown to improve the survival of unresectable HCC. The SHARP trial (the Sorafenib HCC Assessment Randomized Protocol), a multicenter double-blind phase III trial in Europe, North America, South America, and Australasia conducted in 2008, reported overall survival in the sorafenib-treated group was significantly longer than in the placebo group (10.7 *vs.* 7.9 months) [Llovet et al., 2008]. An Asia-Pacific study, which was conducted in China, South Korea, and Taiwan in 2009, also reported that the median overall survival in the sorafenib-treated group was improved (6.5 *vs.* 4.2 months) [Cheng et al., 2009], suggesting that the effect of sorafenib is universal among different ethnic backgrounds. Unfortunately however, subsequent clinical studies have highlighted several issues. First, sorafenib treatment rarely results in tumor shrinkage [Jubb et al., 2010]. A partial tumor response was seen in only 2% and 3.3% of the SHARP and the Asia-Pacific studies, respectively, and there have been few reported cases that achieved complete remission after sorafenib treatment [SO et al., 2008, Yeganeh et al., 2009, Wang et al., 2010, Sacco et al., 2011]. Second, sorafenib is less effective when the patients are affected with medium to severe liver dysfunction (Child-Pugh class B and C) [Pinter et al., 2009, Schütte et al., 2011]. The reason for the influence of liver function on the efficacy of sorafenib should be determined in the near future. Because liver cirrhosis is a unique condition in which excessive inflammatory cytokines is produced, it is plausible that cancer microenvironment in liver disease might affect the sorafenib efficacy (Fig. 1). Third, sorafenib causes many side-effects, including diarrhea, skin eruption, and bone marrow dysfunction. All these lines of evidence strongly suggest that safer and more effective sorafenib therapy should be established for HCC patients.

2.2. Clinical trial of sorafenib-based combination therapy

To improve the limited efficacy of sorafenib, many clinical trials of sorafenib-based combination treatment have been undertaken. For example, a phase II multicenter study in Italy reported that the combination of sorafenib and long-acting octreotide (an analogue of somatostatin) resulted in a better survival rate as compared with sorafenib monotherapy [Prete et al., 2010]. This report suggests a possible synergic tumor killing effect by sorafenib, because octreotide monotherapy has been regarded as less effective in advanced HCC [Becker et al.,
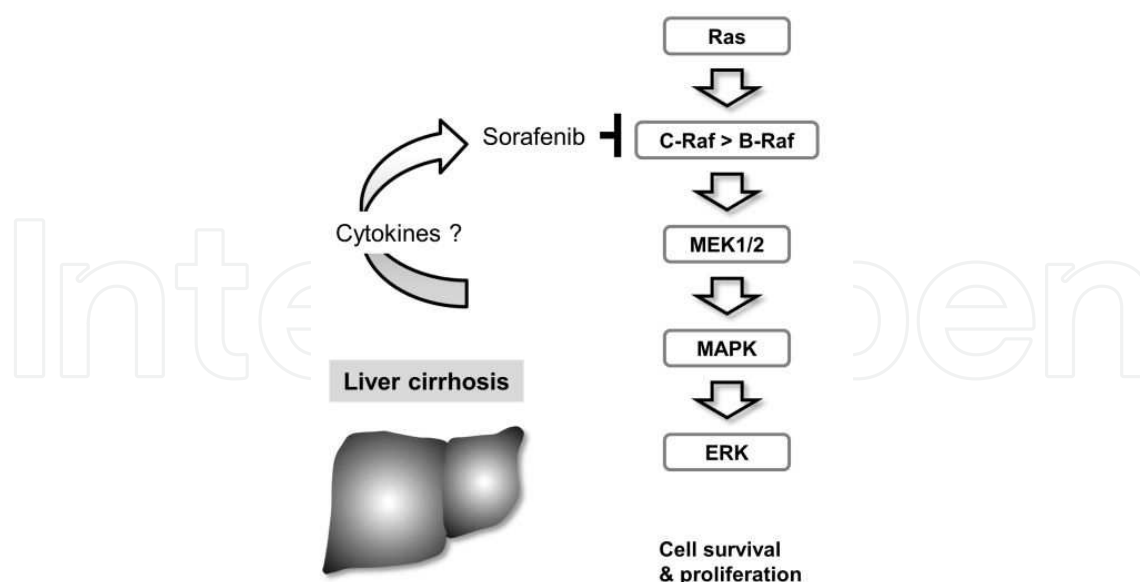
Figure 1. Sorafenib and Raf signaling. Sorafenib inhibits C-Raf rather than B-Raf, and attenuates the activation of MAPK/ERK signaling. However, the effect of sorafenib might be influenced by the cancer microenvironment in liver cirrhosis. MEK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.

2007]. Furthermore, combination of sorafenib and the chemotherapeutic drug doxorubicin was found to be effective in HCC [Abou-Alfa et al., 2010].

Basic studies have now evaluated the preclinical protocols of the combination of sorafenib with other therapeutic agents. The most prospective method for treating HCC is a combination of sorafenib with rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR) pathway. mTOR is known to be activated in many types of cancer cells, and around half of human HCC cases showed aberrant mTOR signaling [Villanueva et al., 2008]. Several studies using a human HCC xenograft mouse model have reported that the combination of rapamycin and sorafenib synergistically enhanced the anti-tumor effect, and resulted in tumor shrinkage [Wang et al., 2008, Huynh et al., 2009, Newell et al., 2009]. Thus far, a phase I trial of the combination therapy of sorafenib and temsirolimus (a rapamycin analog) is in progress for treating advanced HCC [Kelley et al., 2010].

3. Sorafenib affects both RAF and non-RAF signaling pathways

3.1. RAF signaling

3.1.1. RAF signaling and HCC

The *RAS* oncogene encodes a small guanosine triphosphate-binding protein (GTPase) that plays a central role in promoting the cell proliferation, survival and transformation [Karnoub

et al., 2008]. Four proteins, including H-RAS, N-RAS, K-RASA and K-RASB are encoded by the *RAS* gene, and all of these mutant forms have been known to lead to increased GTP-bound RAS (RAS-GTP). Of these, K-RAS is frequently activated by gene mutation in many types of cancer cells [Karnoub et al., 2008], indicating that *RAS* is a common oncogene in various cell types. Several growth factors such as epidermal growth factor (EGF), insulin-like growth factor-I (IGF-1) and PDGF induce cell proliferation through enhanced exchange of guanine nucleotides on RAS. RAS has a guanosine diphosphate (GDP) binding domain, and GDP-bound RAS (RAS-GDP) is activated when converted into RAS-GTP. Downstream mediators of RAS (RAF, PI3K-bound RAL-GEF) bind RAS-GTP with higher affinity than RAS-GDP [Herrmann et al., 1995]. RAS recruits members of the RAF serine/threonine kinase family to the plasma membrane, whereupon they are activated by phosphorylation. The RAF kinase family is composed of three members: A-RAF, B-RAF, and RAF-1 (also termed C-RAF). Of these, many studies have suggested that RAF-1 plays a critical role in the early step of carcinogenesis. It has now been widely accepted that RAF signaling exerts a critical role on the progression in many of the incurable diseases. Good example might be an autosomal dominant polycystic kidney disease (ADPKD). Recent studies have unveiled that Ras/Raf signaling is hyper-activated in cyst epithelial cells in this disease, and both sorafenib and a novel Raf kinase inhibitor PLX5568 can attenuate the proliferation of ADPKD cyst epithelial cells [Yamaguchi T et al., 2010, Buchholz et al., 2011].

In the case of HCC, point mutations of *RAS* have been reported to be infrequent in HCC [Challen et al., 1992]. However, it has been revealed that its downstream signaling is frequently activated during hepatocarcinogenesis. Hepatitis B virus X protein (HBx) and HCV core protein, both of which have been considered as strong promoters of hepatocarcinogenesis, increase the kinase activity of RAF-1 [Aoki et al., 2000, Chen et al., 2007]. It has been also reported that the C-terminal of HCV-encoded nonstructural protein 5A (NS5A) binds to and activates RAF-1 [Bürckstümmer et al., 2006]. More importantly, activated RAF-1 has been found in around 90% of liver cirrhosis cases and in 100% of HCC cases [Hwang et al., 2004].

3.1.2. RAF signaling and sorafenib

RAF-1 is a mitogen-activated protein kinase (MAPK) kinase, which phosphorylates and activates the serine/threonine-specific extracellular signal-regulated protein kinases ERK1 and ERK2 [Avruch et al., 1994]. In the nucleus, phosphorylated ERK activates transcription factors such as ELK-1 and c-JUN, leading to cell proliferation and survival. RAF-1 has been also reported to form a complex with Cdc25 and activates the cyclin E-Cdk2 complex, leading to progression through the G1-S phase transition [Kerkhoff et al., 1998, Hindley et al., 2002]. When activated, RAF members form homologous and heterologous complexes, leading to activation of the MEK/ERK pathway. It should be noted that the kinase activity of RAF complexes is defined by the type of RAF constituents. It has been reported that the kinase activity of B-RAF and c-RAF heterodimers is higher than that of B-RAF or C-RAF homodimers [Rushworth et al., 2006]. Moreover, when B-RAF is mutated at V600E (B-RAFV 600E), as observed in some types of cancer cells, it acquires strong kinase activity and can directly stimulate the MEK/ERK pathway [Wan et al., 2004, Garnett et al., 2005]. In turn, the kinase activity of a heterologous

complex consisting of C-RAF and B-RAFV600E becomes decreased as compared with C-RAF and non-mutated B-RAF heterodimers [Garnett et al., 2005].

Intriguingly, the difference in the kinase activities of each RAF complex affects the therapeutic effect of sorafenib. Sorafenib inhibits C-RAF at low doses, while it inhibits wild-type and mutated B-RAF at high doses [Wilhelm et al., 2004]. Therefore, high doses of sorafenib can inhibit the activities of both C-RAF and B-RAF (either wild-type or V600E), while at lower doses, sorafenib only inhibits C-RAF, resulting in disinhibition of B-RAF [Garnett et al., 2005]. More importantly, low doses of sorafenib has the unique ability in that it induces the formation of heterologous complexes between B-RAFV600E and wild-type B-RAF, leading to the enhancement of the kinase ability of B-RAFV600E [Garnett et al., 2005]. It has been recently reported that cells expressing oncogenic *RAS* are selectively inhibited, B-RAF, B-RAF-C-RAF heterodimers are induced, and RAF/MEK/ERK signaling is activated [Heidorn et al., 2010]. It has been also shown that B-RAF-ERK signaling and C-RAF signaling play dominant roles in the regulation of proliferation of lung cancer cells with wild-type or mutant *KRAS*, respectively. Intriguingly however, sorafenib can inhibit both cell types by targeting B-RAF-mediated ERK phosphorylation in cells with wild-type *KRAS*, and by targeting C-RAF in the cells with mutant *KRAS* [Takezawa et al., 2009]. These lines of evidence strongly suggest that clinicians should decide the dosage of sorafenib in reference to the level of each RAF kinase in the tumors.

3.1.3. Apoptotic pathways and sorafenib

Recently, several studies have reported that sorafenib has a significant effect on non-RAF-signaling pathways, as well as RAF-mediated signaling, particularly caspase-mediated apoptotic signaling. Apoptosis is mainly regulated by two major pathways; (1) tumor necrosis factor- alpha receptors (TNFRs) or the Fas-mediated caspase-8 signaling pathway, and (2) BCL-2 family members-regulated caspase-9 pathway [Ashkenazi, 2008, Leber et al., 2010]. It has been recently reported that sorafenib kills tumor cells by regulating MCL-1, which is a member of the BCL2 protein family [Akgul, 2009, Thomas et al., 2010]. MCL-1 is a repressor of apoptotic cell death via its interactions with the cell death inducer BAX, and overproduction of MCL-1 inhibits cell apoptosis induced by growth factor withdrawal, MYC overexpression, or cytotoxic agents. MCL-1 has been known to be overexpressed in many types of malignancies, and many studies have suggested that the levels of MCL-1 expression may determine the therapeutic efficacy of anti-tumor agents.

Recent studies have led to the suggestion that MCL-1 might be one of the main targets for MEK/ERK-independent mechanisms of action of sorafenib. Sorafenib reduces MCL-1 in various types of cancer cells by proteasome-mediated degradation [Yo et al., 2005], and sorafenib-mediated MCL-1 downregulation is associated with MCL-1-translation and cytochrome-c release into the cytosol. [Rahmani et al., 2005] Intriguingly, MCL-1 might be a promising biomarker of therapeutic efficacy, because it was found to be upregulated in sorafenib-resistant cells [Ulivi et al., 2009]. It has also been reported that HCV can increase therapeutic response to sorafenib by miR-193b-dependent modulation of MCL-1 [Braconi et al., 2010], suggesting that MCL-1 might be involved in the virus-associated drug response in cancer cells.

3.1.4. Endoplasmic reticulum stress and sorafenib

Another RAF-independent mechanism involved in sorafenib-inhibited signaling is endoplasmic reticulum (ER) stress [Rahmani et al., 2007]. The endoplasmic reticulum (ER) is a central organelle in each eukaryotic cell that serves many general functions, including lipid synthesis, protein folding and protein maturation, transportation of synthesized proteins, and activation of chaperone proteins. When cells are exposed to various types of stress such as hypoxia, oxidative stress, hypoglycemia and viral infection, unfolded protein aggregates (unfolded protein response, UPR) accumulate to interfere the function of ER, which is generally called ER stress [Tsukada et al., 1993, Kim et al., 2008]. ER stress causes decreased protein translation to prevent further accumulation of unfolded proteins. It should be noted, however, that the function of ER stress is complex because it can induce either cell survival or autophagy-related cell death [Schleicher et al., 2010]. Several UPR-involving signaling molecules have been identified; the PKR-like kinase (PERK) is an important inhibitor of protein translation through phosphorylation of eukaryotic initiation factor 2 (eIF2α). The kinase activity of PERK is induced by ER stress, and phosphorylation of PERK at Thr980 is regarded as a marker for ER stress. Endoplasmic oxidoreductin-1 (Ero1) is an ER membrane-associated N-glycoprotein that provides oxidizing potential and protein folding. Inositol requiring-1 (IRE1) and activating transcription factor-6 (ATF6) induce calcium-dependent protein chaperones such as GRP78/BiP to maintain correct protein folding [Kim et al., 2008, McConkey et al., 2008]. Calnexin is a calcium-binding protein that retains the synthesized glycoproteins inside the ER. CAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) is a dominant-negative inhibitor of C/EBP and LAP, which plays a role in cell cycle arrest during G1 to S phase. During ER stress, the level of CHOP is increased to induce the activation of GADD34, a downstream protein of P53 tumor suppressor that causes DNA excision repair and cell arrest, and ERO-1 expression. ERO-1 promotes oxidative stress inside the ER, leading to programmed cell death.

Several studies have reported that sorafenib strongly induces ER stress. Sorafenib results in the phosphorylation of PERK and eIF2α, leading to decreases in protein synthesis [Tsukada, 1993]. ERK cannot rescue this cellular reaction, indicating that sorafenib-induced ER stress is RAF-independent [Rahmani et al., 2007]. It has been also reported that sorafenib-induced apoptosis is associated with the increase in the level of CHOP expression [Niessner et al., 2011]; therefore ER stress might be another important mechanism of sorafenib efficacy.

3.1.5. Oxidative stress and sorafenib

It is well known that reactive oxygen species (ROS) is an important player in the process of various types of cellular process. ROS is unique in its dual role; and it plays a critical role in maintaining cancer phenotype, cell proliferation and genetic instability [Radisky et al., 2005, Chen et al., 2005]. In turn, when produced at high concentrations, it activates the caspases to induce apoptosis. Although the functional mechanisms of these opposing effects of ROS is unknown, recent studies have revealed that the effect of cytotoxic anti-tumor agents is exclusively caused by elevated ROS production. Recently it was revealed that sorafenib induces mitochondria-dependent ROS production to induce hepatoma cell death. Chiou et al. reported that ROS could be generated just 30 minutes after cells were treated with sorafenib,

suggesting that sorafenib-induced oxidative stress might not be the secondary phenomenon during cell death [Chiou et al., 2009]. Chiou et al. also reported that glutathione (GSH), an intracellular non-protein-thiol antioxidant, was decreased after treatment with sorafenib. Currently it is not known that why sorafenib results in the dysregulated balance of oxidants and anti-oxidants. Park et al. reported that low doses of sorafenib and vorinostat, a histone deacetylase inhibitor (HDACI) that has shown preclinical evidence of anti-tumor activity against hepatoma, rapidly increase ROS, Ca (2+), and ceramide levels in gastrointestinal tumor cells [Park et al., 2010]. In turn, Banerjee et al. reported that the anti-oxidant enzyme heme oxygenase-1 (HO-1) protected apoptosis of cells treated with sorafenib [Banerjee et al., 2012]. HO-1 was found to induce the expression of anti-apoptotic BCL-$_\chi$L and decreased the expression of autophagic proteins Beclin-1 and LC3B-II, indicating that ROS might determine the therapeutic efficacy of sorafenib. To improve the efficacy of sorafenib, further investigation of the relationship between ROS and sorafenib should be performed.

4. Future perspectives of combination treatment with sorafenib

Because accumulating evidence strongly indicated that the anti-tumor effect of sorafenib is mediated by both RAF and non-RAF signaling, recent studies have investigated the usefulness of sorafenib-based combination therapy via targeting of non-RAF signaling pathways [Peck-Radosavljevic et al., 2010, Shen et al., 2010, Kudo et al., 2010]. Some studies have reported that targeting non-RAF signaling such as TNF-related apoptosis-inducing ligand (TRAIL) [Meng et al., 2007, Rosato et al., 2007, Ricci et al., 2007, Kudo et al., 2010], histone deacetylase [Dasmahapatra et al., 2007] and BCL-2 [Lin et al., 2007] might be effective when combined with sorafenib. Moreover, several clinically available agents such as sulforaphane (SF) [Rausch et al., 2010], zoledronic acid [Zhang et al., 2010], and vitamin K1, K2, and K5 [Wei et al., 2010a, 2010b] have been also reported to be useful for combination treatment. Recently we found that caffeine, which is a well-known inhibitor of DNA damage-response kinase ataxia telangiectasia mutated (ATM), can effectively enhance the effect of sorafenib [Fujimaki et al., 2012]. ATM is a widely known DNA damage-stimulated serine/threonine kinase that phosphorylates several of the DNA damage checkpoint molecules [Lavin, 2008]. We found that ATM is activated by sorafenib-mediated non-genotoxic ROS production, resulting in the sorafenib-induced reciprocal activation of AKT signaling to help the cells to acquire drug resistance [Fujimaki et al., 2012]. Interestingly, it has been recently reported that intra-arterial local administration of caffeine could potentiate cisplatin-based chemotherapy, without severe side-effects [Takeuchi et al., 2007]. Together with our finding, it would be intriguing to investigate if caffeine could enhance the effect of sorafenib in HCC patients.

5. Conclusions

At present, sorafenib is the only molecular targeting agent proven to have a significant effect on the survival of patients with advanced HCC. Although its tumor-killing effect has been

found to be limited, recent basic studies have revealed that this new agent acts upon multiple non-RAF signaling pathways as well as RAF-mediated signaling. More interestingly, recent studies have unveiled that sorafenib might also act as preventive agents against liver fibrosis. Wang et al. reported that sorafenib treatment attenuated liver fibrosis in rat liver fibrosis model, possibly due to its inhibitory role on the cell proliferation of hepatic satellite cells [Wang at al., 2010]. Thus, to further identify an efficient protocol for sorafenib treatment, clinicians should pay more attention to non-RAF signaling in cancer cells.

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