Activation of Phosphatidylinositol 3-Kinase/Akt Signaling Pathway Mediates Acquired Resistance to Sorafenib in Hepatocellular Carcinoma Cells

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common potentially lethal human malignancies worldwide. Sorafenib, a tyrosine kinase inhibitor, was recently approved by the United States Food and Drug Administration for HCC. In this study, we established two sorafenib-resistant HCC cell lines from Huh7, a human HCC cell line, by long-term exposure of cells to sorafenib. Sorafenib induced significant apoptosis in Huh7 cells; however, Huh7-R1 and Huh7-R2 showed significant resistance to sorafenib-induced apoptosis at the clinical relevant concentrations (up to 10 μM). Thorough comparisons of the molecular changes between Huh7 and resistant cells showed that the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway played a significant role in mediating acquired resistance to sorafenib in Huh7-R1 and Huh7-R2 cells. Phospho-Akt and p85 (a regulatory subunit of PI3K) were up-regulated, whereas tumor suppressor phosphatase and tensin homolog were down-regulated in these resistant cells. In addition, ectopic expression of constitutive Akt in Huh7 demonstrated similar resistance to sorafenib. The knockdown of Akt by RNA interference reversed resistance to sorafenib in Huh7-R1 cells, indicating the importance of Akt in drug sensitivity. Furthermore, the combination of 8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-1,2,4-triazolo[3,4-f][1,6]naphthyridin-3(2H)-one dihydrochloride (MK-2206), a novel allosteric Akt inhibitor, and sorafenib restored the sensitivity of resistant cells to sorafenib-induced apoptosis. In conclusion, activation of PI3K/Akt signaling pathway mediates acquired resistance to sorafenib in HCC, and the combination of sorafenib and MK-2206, an Akt inhibitor, overcomes the resistance at clinical achievable concentrations.

Introduction

Human hepatocellular carcinoma (HCC) is one of the most prevalent malignant tumors worldwide (Bruix and Llovet, 2003; Huo et al., 2005). Surgical resection and traditional chemotherapy are typical forms of treatment for patients with HCC (Bioulac-Sage et al., 2009; Di Maio et al., 2009; Fransvea et al., 2009); however, targeted anticancer therapies using small molecules provide significant benefits in patients with HCC who do not respond well to traditional treatment (Thomas, 2009; Huynh, 2010; Midorikawa et al., 2010; Villanueva et al., 2010; Vitale et al., 2010).

Sorafenib (Nexavar), a multiple kinase inhibitor, is the first and only drug that is clinically approved for patients with advanced HCC (Palmer, 2008; Di Maio et al., 2009; Johnson and Billingham, 2009; Liu et al., 2009; Scanga and Kowdley, 2009). The major target of sorafenib is the serine-threonine kinase Raf-1, which is involved in the Ras/Raf/MEK/mitogen-activated protein kinase signaling cascade (Wilhelm et al., 2004; Panka et al., 2006). In vitro kinase assay, sorafenib could efficiently inhibit the activity of Raf-1 at a very low dose (IC50 of 6 nM) (Adnane et al., 2006; Wilhelm et al., 2006). Other receptor tyrosine kinases are

ABBREVIATIONS: HCC, hepatocellular carcinoma; PP2A, protein phosphatase 2A; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; MK-2206, 8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-1,2,4-triazolo[3,4-f][1,6]naphthyridin-3(2H)-one dihydrochloride PARP, poly(ADP-ribose) polymerase; MEK, mitogen-activated protein kinase kinase; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; siRNA, small interference RNA; PRAS40, 40-kDa proline-rich Akt substrate.
also suppressed by sorafenib, including vascular endothelial growth factor receptors 1, 2, and 3, platelet-derived growth factor receptor, and fibroblast growth factor receptor (Wilhelm et al., 2006; Zhang et al., 2008). Although sorafenib showed survival benefits in large randomized phase III studies, the response rate of sorafenib is actually quite low (2–3%) (Llovet et al., 2008; Cheng et al., 2009). In addition, therapeutic biomarkers that may predict the response to sorafenib are not currently available. Therefore, to improve the treatment response in HCC, it is important to identify the molecular mechanism of sorafenib resistance.

PI3K/Akt signaling is an important survival/proliferative pathway involving various growth factors, cytokines, and activation of receptors (Liu et al., 2009). Akt is up-regulated in many human cancer types and links to oncogenesis to alter cellular functions (Liu et al., 2009). Akt-related molecules

**Fig. 1.** Establishment and verification of sorafenib-resistant Huh7 cell lines. A, the apoptotic effect of sorafenib in wide-type and resistant Huh7 HCC cells. Cells were exposed to sorafenib at the indicated concentrations for 48 h, and the apoptotic assay was analyzed by sub-G1 percentage. Columns, mean (n = 6); bars, mean ± S.D. *P < 0.05. B, dose-dependent apoptotic analysis of sorafenib in Hep3B and PLC5. Apoptotic cells were determined by flow cytometry. C, analysis of caspase activation and PARP cleavage. Wild-type and resistant Huh7 cells were exposed to sorafenib at the indicated doses (micromolar) in 5% FBS-containing medium for 48 or 72 h. Cell lysates were prepared and assayed for the activation of caspase-9, caspase-3, and PARP by Western blot. CF, cleaved form (activated form). D, the cytotoxic effects of sorafenib in Huh7 cells. Cells were exposed to sorafenib at the indicated concentrations for 24 h, and cell viability was analyzed by MTT assay. Columns, mean (n = 6); bars, mean ± S.D. *P < 0.05.
have also been noted for suppressing this vital oncogene in HCC treatment. PI3Ks are heterodimers that consist of a p110-catalytic and a p85-regulatory subunit. Under stimulation, the p85-regulatory subunit mediates the recruitment of Akt to the plasma membrane by interaction with phosphorylated motif on activated receptors. The majority of growth factors could be stimulators of the PI3K/Akt signaling cascade for oncogenesis. The conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate by p110-catalytic subunit is essential for the kinase activity of phosphatidylinositol 3-kinase-dependent 1, which phosphorylates and activates Akt at the plasma membrane. In contrast, phosphatase and tensin homolog (PTEN) inactivates PI3K by dephosphorylating PIP3 (Liu et al., 2009).

Several ATP-competitive inhibitors have been discovered for Akt, and some of them are phosphatidylinositol analogs, which according to previous reports might have problems with the specificity to other pleckstrin homology domain containing molecules and also have poor bioavailability (Lindsay et al., 2008; Liu et al., 2009). These concerns have led to investigations into allosteric inhibitors of Akt. Allosteric inhibitors are pleckstrin homology domain-dependent, show selectivity for the individual Akt isozymes, and inhibit the activity and the activation of Akt (Lindsay et al., 2008; Liu et al., 2009). 8-[4-(1-Aminocyclobutyl)phenyl]-9-phenyl-1,2,4-triazolo[3,4-f][1,6]naphthyridin-3(2H)-one dihydrochloride (MK-2206), an oral allosteric Akt inhibitor, has demonstrated positive phase I data (Liu et al., 2009; Hirai et al., 2010). The use of MK-2206 in several types of cancer is currently undergoing clinical investigation (Liu et al., 2009; Hirai et al., 2010).

To address the issue of sorafenib-induced resistance, we selected survival clones of Huh7 HCC cells through long-term exposure to sorafenib treatment. The sorafenib-resistant Huh7 clones showed resistance to sorafenib at the clinically relevant dose. In this study, we show that activation of the PI3K/Akt signaling pathway plays a key role in mediating resistance to sorafenib. The combination of MK-2206, an Akt inhibitor, and sorafenib overcomes such resistance.

Materials and Methods

Reagents and Antibodies. Sorafenib and MK-2206 were kindly provided by Bayer (West Haven, CT) and Merck & Co. (Whitehouse Station, NJ), respectively. For in vitro studies, sorafenib at various concentrations was dissolved in DMSO and then added to cells in 5% FBS-containing DMEM. Antibodies for immunoblotting, such as Akt1, Bad, Bax, Mcl-1, and poly(ADP-ribose) polymerase (PARP), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies, such as anti-pERK(1/2), extracellular signal-regulated kinase 2, cyclin D1, Bcl-2, Bcl-xl, Bid, caspase-3, caspase-8, caspase-9, and phospho-Akt (Ser473), were from Cell Signaling Technology (Danvers, MA). DR4 and DR5 were from BioLegend (San Diego, CA; flow cytometry) and Gen-Probe (San Diego, CA; Western blot).

Cell Culture. The Huh7 HCC cell line was obtained from the Health Science Research Resources Bank (JCRB0403; Osaka, Japan). Hep3B, PLC5, Sk-Hep-1, and HepG2 were obtained from American Type Culture Collection (Manassas, VA).

Cell Viability Analysis. The effect of individual test agents on cell viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The Huh7 cells were treated with sorafenib, MK-2206, and a combination of the two agents at various concentrations. Cell viability was quantified by measuring the absorbance at 570 nm. The IC50 values were calculated using GraphPad Prism software. Western blot analysis was performed to detect the expression levels of key proteins involved in the PI3K/Akt pathway and its downstream targets. The results showed that sorafenib-induced resistance is mediated by the activation of the PI3K/Akt signaling pathway, which can be overcome by the combination of MK-2206 and sorafenib.
2,5-diphenyl-2H-tetrazolium bromide (MTT) assay in six replicates. Cells (5000/well) were seeded and incubated in 96-well, flat-bottomed plates in DMEM with 10% FBS for 24 h and exposed to various concentrations of test agents in 5% FBS-supplemented DMEM for 24 h. Controls received DMSO vehicle at a concentration equal to that of drug-treated cells. The medium was removed and replaced by 200 μl of 0.5 mg/ml MTT in 10% FBS-containing DMEM, and cells were incubated in the CO2 incubator at 37°C for 2 h. Supernatants were removed from the wells, and the reduced MTT dye was dissolved in 200 μl/well DMSO. Absorbance at 570 nm was determined on a plate reader.

**Apoptosis Analysis.** The following three methods were used to assess drug-induced apoptotic cell death: the measurement of apoptotic cells by flow cytometry (sub-G1), Western blot analysis of caspase activation, and PARP cleavage.

**Raf-1 Activity.** The Raf-1 kinase assay kit was obtained from Millipore Corporation (Billerica, MA) and used according to the protocol provided by manufacturer. In brief, wide-type or resistant Huh7 cells were treated with 10 μM sorafenib for 24 h, and the Raf-1-containing extract was collected. The Huh7 lysate, including Raf-1 kinase protein, was incubated with recombinant MEK at 30°C for 30 min. Phospho-MEK1/2 antibody was used to evaluate the kinase activity of Raf-1.

**Statistical Analysis.** Comparisons of mean values were performed using the independent sample Student’s t test in SPSS for Windows 11.5 software (SPSS, Inc., Chicago, IL) (Chen et al., 2008).

**Results**

**Establishment and Verification of Sorafenib-Resistant Huh7 Cell Lines.** To investigate the molecular mechanism of the acquired resistance to sorafenib, we established several HCC cell lines by long-term exposure to sorafenib at low doses escalating to higher doses for a long period of time. We obtained two sorafenib-resistant cell lines. We examined the apoptotic effects of sorafenib by analyzing the sub-G1 percentage with flow cytometry. As shown in Fig. 1A, wild-type Huh7 cells showed apoptosis in the presence of sorafenib in a dose-dependent manner. In contrast, sorafenib-resistant Huh7 cells (Huh7-R1 and Huh7-R2) showed resistance to sorafenib-induced apoptosis, even at 10 μM (the highest clinical achievable concentration). We also examined the apoptotic effect of sorafenib in other HCC cell lines. Sorafenib exhibited apoptotic effects in Hep3B and PLC5 cells in a dose-dependent manner, giving results similar to those obtained with wild-type Huh7 (Fig. 1B). Furthermore, our data demonstrated that sorafenib induced the activation of caspase-9 and caspase-3 and the cleavage of PARP in a dose- and time-dependent manner in wild-type Huh7 cells, but not in Huh7-R1 or -R2 cells (Fig. 1C), indicating that our resistant cell lines did not respond to sorafenib treatment at the clinical relevant dose (10 μM). In addition, we examined the effect of sorafenib on cell viability in all Huh7

Fig. 3. Target validation of Akt as a resistant mechanism. A, left, overexpression of Akt protected HCC cells from sorafenib-induced apoptosis. Huh7 cells with ectopic expression of Akt1 were treated with sorafenib at the indicated doses for 48 h. The apoptotic cells were analyzed by sub-G1 percentage. Columns, mean (n = 3); bars, mean ± S.D. Right, apoptotic molecules were assayed in wild type or Akt-expressing Huh7 cells. The activation levels of caspase-9, caspase-3, and PARP were determined by immunoblots. CF, cleaved form. B, down-regulation of Akt by siRNA overcame the resistance to sorafenib in Huh7-R1 cells. Cells were transfected with either control or Akt siRNA for 24 h and then exposed to 10 μM sorafenib for 48 h. The activation of apoptosis was determined by caspase-9, caspase-3, and PARP through immunoblotting.
cells. Sorafenib had significant cytotoxic effects in wild-type Huh7 cells in a dose-dependent manner, whereas resistant Huh7 cells (Huh7-R1 and -R2) were less sensitive to sorafenib. These data indicate that Huh7-R1 and -R2 are resistant to the cytotoxic effect of sorafenib (Fig. 1D).

**Activation of PI3K/Akt Is Associated with Resistance to Sorafenib.** To identify the molecule mechanism of the resistance to sorafenib, we first examined the effect of Raf-1 activity on wild-type or resistant Huh7 cells in the presence of sorafenib treatment. We unexpectedly found that there was no significant different between Huh7 and Huh7-R1 cells in sorafenib-induced reduction of Raf-1 activity (Fig. 2A). The Raf-1 kinase activity on phospho-MEK1/2 was down-regulated significantly by sorafenib treatment in both Huh7 and Huh7-R cells, suggesting that Raf-1 may not play a role in mediating the drug resistance in resistant cells. It is noteworthy that we found that resistant cells showed up-regulation of Akt signaling in comparison with sensitive cells. As shown in Fig. 2B, phospho-Akt (Ser473 and Thr308) and Akt were overexpressed in resistant Huh7 cells. It is noteworthy that sorafenib induced apoptosis as shown by the activation of caspase-9 and PARP cleavage in wild-type Huh7, whereas no apoptosis was noted in resistant cells (Fig. 2B). In light of the significant role of the PI3K/Akt pathway in tumorigenesis, we examined the molecules related to the PI3K/Akt pathway to clarify its role in mediating the sorafenib resistance (Fig. 2C). PI3K is composed of a p85-regulatory subunit as well as a p110-catalytic subunit and mediates receptor binding, cellular localization, and enzyme activation by the cooperation of the two subunits. Our data showed that up-regulation of PI3K (p85) was found in Huh7-R1 and -R2. In contrast, PTEN, a PI3K suppressor, was down-regulated in resistant cells. We further assayed expression levels of downstream PI3K/Akt signaling proteins, which might also be affected in sorafenib-resistant cells. As shown in Fig. 2C, significant increases of mammalian target of rapamycin and S6 kinase were demonstrated in Huh7-R cells. In addition, we examined several known targets of sorafenib, including Erk, Mcl-1, and cyclin D1 in our HCC cells, and found that sorafenib down-regulated phospho-Erk, Mcl-1, and cyclin D1 in both sensitive and resistant cells, suggesting that these molecules may not play a role in mediating the resistance to sorafenib in our HCC cells (Fig. 2C).

**Target Validation of Akt in Sorafenib-Resistant Cells.** According to our previous findings, we hypothesized that the activation of Akt played a role in mediating the drug resistance; thus, next we validated the role of Akt in the effects of sorafenib. Our data showed that the percentage of apoptotic cells (Fig. 3A, left), along with the activation of caspases and the cleavage of PARP (Fig. 3A, right), increased in wild-type Huh7 cells under sorafenib treatment. However, Huh7 cells with ectopic expression of Akt were significantly protected from sorafenib-induced apoptosis (Fig. 4). MK-2206, an Akt inhibitor, sensitized resistant cells to sorafenib-induced apoptosis. A, resistant cells were exposed to sorafenib and/or MK-2206 at the indicated concentrations for 48 h, and apoptotic assay was analyzed by flow cytometry. Columns, mean (n = 6); bars, mean ± S.D. *, P < 0.05. B, the combination of sorafenib and MK-2206 induced significant cell death in resistant cells. Huh7-R1 and -R2 cells were treated with sorafenib at 10 μM and/or MK-2206 at 10 μM for 48 h. Cell lysates were assayed for phospho-Akt(Ser473), Akt, caspase-9, caspase-3, and PARP by Western blots. CF, cleaved form (activated form). C, the Akt-related proteins were assayed in the presence of 10 μM sorafenib and 10 μM MK-2206. Cell lysates were prepared for analysis of protein expression by Western blotting.
apoptosis, indicating that the activity of Akt could determine the drug sensitivity in HCC cells (Fig. 3A). We next employed small interference RNA (siRNA) to knock down Akt in HCC cells. As shown in Fig. 3B, down-regulation of Akt sensitized the Huh7-R1 cells to sorafenib-induced cell death, as shown by caspase activation and PARP cleavage (Fig. 3B). These data suggest that Akt, a vital survival factor, mediated resistance to sorafenib in HCC cell lines.

**MK-2206, an Akt Inhibitor, Sensitized Resistant Cells to Sorafenib-Induced Apoptosis.** We further explored the possibility of combining sorafenib with a novel drug that is potentially feasible for use in the clinic to overcome sorafenib-induced resistance. MK-2206, a highly selective non-ATP-competitive allosteric inhibitor of Akt, is currently undergoing clinical investigation for use with several types of a solid tumor. Our data showed that cotreatment with sorafenib and MK-2206 overcame the resistance to sorafenib in both Huh7-R1 and -R2 cells. The combination of these two drugs significantly increased the percentage of apoptotic cells in sorafenib-resistant Huh7 cells (Fig. 4A). In addition, down-regulation of phospho-Akt by MK-2206 sensitized resistant cells to sorafenib-induced cell death, as shown by the activation of caspases and PARP cleavage (Fig. 4B). It is noteworthy that MK-2206 alone did not show any significant effects on apoptosis in our resistant cells, suggesting that the inhibition of Akt is not sufficient to cause cell death in HCC cells. Moreover, we examined the molecules related to the PI3K/Akt signaling pathways and found that the 40-kDa proline-rich Akt substrate (PRAS40) was down-regulated in MK-2206-treated Huh7-R1 cells, indicating that MK-2206 inhibited Akt and its downstream signals in our cells. Other proteins, including p85, p110, PTEN, phosphatidylinositol 3-kinase-dependent 1, and mammalian target of rapamycin, were not altered significantly in the presence of combinational treatment for 48 h (Fig. 4C). Down-regulation of phospho-Erk was noted in sorafenib-treated cells, which is consistent with our previous finding (Fig. 2C). Therefore, our results indicated that the combination of sorafenib and Akt inhibitor may be a novel therapeutic strategy to overcome the sorafenib resistance in HCC.

**Sorafenib-Resistant HepG2 and Sk-Hep1 Cell Lines.** Besides Huh-7, we obtained two sorafenib-resistant cell lines from HepG2 and Sk-Hep1. We examined the cytotoxic effects of sorafenib in these cells. Sorafenib showed differential effects on cell viability in wild-type and resistant cells, indicating that HepG2-R and Sk-Hep1-R cells are resistant to the cytotoxic effect of sorafenib (Fig. 5, A and B). In addition, wild-type HepG2 and Sk-Hep1 cells showed apoptosis in the presence of sorafenib in a dose-dependent manner. However, sorafenib-resistant cells (HepG2-R and Sk-Hep1-R) showed resistance to sorafenib-induced apoptosis (Fig. 5, C and D). Moreover, resistant cells (HepG2-R and Sk-Hep1-R) had higher expressions of phospho-Akt (Ser473 and Thr308) and Akt. These data indicate that activation of Akt signaling pathway is present in resistant HepG2 and Sk-Hep1 cells (Fig. 5E).

**Discussion**

The recent clinical approval of sorafenib has marked the start of a new era in the pharmacological treatment of ad-
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References


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