The Bcl-xL Inhibitor, ABT-737, Efficiently Induces Apoptosis and Suppresses Growth of Hepatoma Cells in Combination with Sorafenib

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Tumor cells are characterized by uncontrolled proliferation, often driven by activation of oncogenes, and apoptosis resistance. The oncogenic kinase inhibitor sorafenib can significantly prolong median survival of patients with advanced hepatocellular carcinoma (HCC), although the response is disease-stabilizing and cytostatic rather than one of tumor regression. Bcl-xL (B cell lymphoma extra large), an antiapoptotic member of the B cell lymphoma-2 (Bcl-2) family, is frequently overexpressed in HCC. Here, we present *in vivo* evidence that Bcl-xL overexpression is directly linked to the rapid growth of solid tumors. We also examined whether ABT-737, a small molecule that specifically inhibits Bcl-xL but not myeloid cell leukemia-1 (Mcl-1), could control HCC progression, especially when used with sorafenib. Administration of ABT-737, even at an *in vivo* effective dose, failed to suppress Huh7 xenograft tumors in mice. ABT-737 caused the levels of Mcl-1 expression to rapidly increase by protein stabilization. This appeared to be related to resistance to ABT-737, because decreasing Mcl-1 expression levels to the baseline by a small interfering RNA–mediated strategy made hepatoma cells sensitive to this agent. Importantly, administration of ABT-737 to Mcl-1 knockout mice induced severe liver apoptosis, suggesting that tumor-specific inhibition of Mcl-1 is required for therapeutic purposes. Sorafenib transcriptionally down-regulated Mcl-1 expression specifically in tumor cells and abolished Mcl-1 up-regulation induced by ABT-737. Sorafenib, not alone but in combination with ABT-737, efficiently induced apoptosis in hepatoma cells. This combination also led to stronger suppression of xenograft tumors than sorafenib alone. *Conclusion:* Bcl-xL inactivation by ABT-737 in combination with sorafenib was found to be safe and effective for anti-HCC therapy in preclinical models. Direct activation of the apoptosis machinery seems to unlock the antitumor potential of oncogenic kinase inhibitors and may produce durable clinical responses against HCC. (HEPATOLOGY 2010;52:1310-1321)

The B cell lymphoma-2 (Bcl-2) family proteins regulate the mitochondrial pathway of apoptosis, a major form of cell death.[1] They include five antiapoptotic proteins, Bcl-2, B cell lymphoma extra large (Bcl-xL), myeloid cell leukemia-1 (Mcl-1), Bcl-2–related protien A1 (Bfl-1), and Bcl-2-like 2 (Bcl-w), and two structurally related proapoptotic proteins, Bcl-2–antagonist/killer (Bak) and Bcl-2–

Abbreviations: ALT, alanine aminotransferase; Bad, Bcl-2–associated agonist of cell death; Bak, Bcl-2–antagonist/killer; Bax, Bcl-2–associated X protein; Bcl-2, B cell lymphoma-2; BH3, Bcl-2 homology domain-3; Bid, BH3-interacting domain death agonist; cDNA, complementary DNA; HA, hemagglutinin; HCC, hepatocellular carcinoma; Mcl-1, myeloid cell leukemia-1; mRNA, messenger RNA; RT-PCR, reverse-transcription polymerase chain reaction; siRNA, small interfering RNA; USP9X, ubiquitin-specific peptidase 9 X-linked; WST, water-soluble tetrazolium.

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associated X protein (Bax), as well as several structurally diverse proapoptotic Bcl-2 homology domain-3 (BH3)-only proteins like Bcl-2–associated agonist of cell death (Bad), BH3-interacting domain death agonist (Bid), and Bcl-2-like 11 (Bim). Bak and Bax, effector molecules in this family, homo-oligomerize into proteolipid pores within the mitochondrial outer membrane, leading to release of cytochrome c followed by activation of downstream caspases, such as caspase-3/7, which dismantle a variety of cellular substrates, leading to cell death. Antiapoptotic Bcl-2 proteins function as regulators of apoptosis by directly or indirectly antagonizing Bak and Bax activity to maintain cellular integrity. BH3-only proteins, sensors of apoptosis, are activated by a variety of cellular stresses and either directly activate Bak and Bax or neutralize antiapoptotic Bcl-2 proteins, inducing cell death. Because tumor cells encounter a variety of cellular stresses, such as genotoxic and environmental factors, overexpression of antiapoptotic Bcl-2 family proteins is commonly observed and leads to survival of tumor cells.[2] We and others have reported that Bcl-xL is frequently overexpressed in human hepatocellular carcinomas (HCCs).[3–6] These reports point to the resistance of hepatoma cells to a wide variety of stress-inducing conditions. For example, Bcl-xL blocks p53-induced apoptosis in hepatoma cells, implying that Bcl-xL overexpression may be one of the mechanisms by which HCC survives under genotoxic conditions.[3] In addition, Bcl-xL overexpression was found to be associated with poor overall survival and disease-free survival after surgical resection for HCC.[7] These findings suggest that Bcl-xL may be a therapeutic target for HCC, although this possibility has not yet been addressed. Bcl-xL is also expressed in normal hepatocytes and plays a critical role in maintaining their integrity.[8] Thus, special caution is necessary when Bcl-xL inactivation is applied to therapeutic purposes.

Despite advances in understanding the mechanisms of cell death and the biology of Bcl-2 family proteins, therapeutic strategies for HCC targeting apoptotic molecules have been hampered due to a lack of specific inhibitors. ABT-737 is one of the first small-molecule inhibitors of Bcl-2 family proteins and opens the field for cancer treatment targeting the apoptosis machinery. ABT-737, designed as a Bad mimetic, binds and neutralizes Bcl-2, Bcl-xL, and Bcl-w, but not Mcl-1 or Bfl-1.[9-11] It has single-agent activity in a number of hematopoietic cancers and some solid tumors.[12,13] Its orally available derivative, ABT-263, is in early clinical trials against lymphoid malignancies, small-cell lung cancer, and chronic lymphocytic leukemia, with some promising results.[14] In this study, we investigated the impact of ABT-737 in treating human hepatoma in culture and using a xenograft model. We found that hepatoma cells are relatively resistant to ABT-737, presumably due to reciprocal up-regulation of Mcl-1 upon ABT-737 exposure. Although concomitant Mcl-1 inhibition appears to be effective for inducing apoptosis by ABT-737, it should be done in a tumor-specific manner, because administration of ABT-737 leads to liver deterioration in Mcl-1 knockout mice. Finally, sorafenib, an anti-HCC agent recently approved by the U.S. Food and Drug Administration, down-regulates Mcl-1 expression in a tumor-specific manner and induces apoptosis and tumor growth suppression in cooperation with ABT-737. Combination therapy with sorafenib and a Bcl-xL inhibitor seems to be an attractive strategy for controlling tumor progression in HCC.

Materials and Methods

Cell Lines and Reagents. Primary human hepatocytes were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured with the provided medium. Human hepatoma cell lines were cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO). Cycloheximide was purchased from Nacalai Tesque (Kyoto, Japan), sorafenib tablets were purchased from Bayer HealthCare (Osaka, Japan), and ABT-737 was kindly provided by Abbott Laboratories (Abbott Park, IL). They were dissolved with dimethyl sulfoxide for *in vitro* use.

Hela Cells Expressed Bcl-xL with the Tet-on System. pcDNA3HABcl-xL, an expression vector coding human Bcl-xL tagged with hemagglutinin (HA), was provided by Dr. G. Nunez (University of Michigan Medical School, Ann Arbor, MI). The pcDNA4/TOHABcl-xL was constructed by inserting the complementary DNA (cDNA) for Bcl-xL gene with HA-tag from pcDNA3HABcl-xL into the EcoRI site of pcDNA4/TO (Invitrogen, Carlsbad, CA). TREx-Hela cells (Invitrogen) were transfected with pcDNA4/

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TOHABcl-xL using Lipofectin (Invitrogen). The cells were cultured with DMEM containing 1.1 μg/mL zeocin, and zeocin-resistant clones were isolated. After examination of HA–Bcl-xL induction by doxycycline, two clones (Hela–Bcl-xL$^{Tet\text{-}on}$ clone A, clone B) were established and used for further experiments.

Mice. Conditional Bcl-xL knockout mice (*bcl-x$^{flox/flox}$ Alb-Cre* [albumin/cre recombinase]) and Mcl-1 knockout mice (*mcl-1$^{flox/flox}$ Alb-Cre*) were previously described.[15] Balb/c nude mice (CAnN.Cg-Foxn1^{nu}/CrlCrlj) were purchased from Charles River Laboratories (Yokohama, Japan). They were maintained in a specific pathogen–free facility and treated with humane care with approval from the Animal Care and Use Committee of Osaka University Medical School.

Apoptosis Assay. The *in vitro* apoptosis assay, measurement of caspase-3/7 activity, and the water-soluble tetrazolium salt (WST) assay, were described previously.[16] The *in vivo* apoptosis assay, measurement of serum alanine aminotransferase (ALT) level, and caspase-3/7 activity and histological analyses were also previously described.[15]

Western Blot Analysis. Whole-cell extracts from cultured cells or tissues were prepared and subjected to western blot. For immunodetection, the following antibodies were used: anti–Bcl-xL antibody and anti-human Mcl-1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA); anti-mouse Mcl-1 antibody from Rockland (Gilbertsville, PA); anti-Bid antibody, anti-Bax antibody, and anti-cleaved caspase-3 antibody from Cell Signaling Technology (Beverly, MA); anti-Bak antibody from Millipore (Billerica, MA); anti-Bim antibody from Assay Design (Ann Arbor, MI); anti-ubiquitin-specific peptidase 9, X-linked (USP9X) antibody from Abnova (Taipei, Taiwan); and anti–beta actin antibody from Sigma-Aldrich (St. Louis, MO) or Cell Signaling Technology.

Xenograft Tumor. To produce a xenograft tumor, 3×10^6 to 5×10^6 Hela–Bcl-xL$^{Tet\text{-}on}$ clone A or Huh7 cells were subcutaneously injected to Balb/c nude mice. For induction of HA–Bcl-xL, the mice that were injected with Hela–Bcl-xL$^{Tet\text{-}on}$ clone A cells were fed with water containing 100 μg/mL doxycycline. For anticancer therapy, ABT-737 was administered as described.[17] Sorafenib tablets were crushed and orally administered with water containing 12.5% Cremophor EL (Sigma-Aldrich) and 12.5% ethanol. We estimated the volume of the xenograft tumor using the following formula: tumor volume = $\pi/6 \times (\text{major axis}) \times (\text{minor axis})^2$.

Small RNA Interference. Hepatoma cell lines were transfected with Stealth select RNAi (set of three oligonucleotides, Invitrogen) RNA interference (RNAi) directed against Mcl-1 or USP9X. A Stealth RNAi negative control kit (set of three oligonucleotides, Invitrogen) was used as a control for sequence-independent effects following Stealth RNAi delivery. The transfections were carried out using Lipofectamine RNAiMAX (Invitrogen) according to the reverse transfection protocol.

Real-Time Reverse-Transcription Polymerase Chain Reaction. Real-time reverse-transcription PCR (RT-PCR) was performed as previously described.[15] Mcl-1 messenger RNA (mRNA) expressions were measured using TaqMan Gene Expression Assays (Assay ID: Hs03043899_m1) and were corrected with the quantified expression level of beta actin mRNA measured using TaqMan Gene Expression Assays (Assay ID: Hs99999903_m1).

Statistical Analysis. Data are presented as mean ± standard deviation. Differences between two groups were determined using the Student *t* test for unpaired observations unless otherwise noted. Multiple comparisons were performed by analysis of variance followed by Scheffe post hoc correction. $P < 0.05$ was considered statistically significant.

Results

Bcl-xL Overexpression Is a Molecular Mechanism of Rapid* In Vivo *Tumor Growth. Research has shown that Bcl-xL overexpression confers resistance to apoptosis in a variety of tumor cells. To examine its impact on tumor growth *in vivo*, we generated the Hela–Bcl-xL$^{Tet\text{-}on}$ cell line which expresses the modified tetracycline repressor molecule (rtTA) and Bcl-xL under control of tetracycline-responsive cis-elements. We chose Hela cells as a model because they expressed a relatively small amount of Bcl-xL in comparison with human hepatoma cells including Huh7, Hep3B, and HepG2 (Fig. 1A). Tetracycline analogue doxycycline treatment efficiently induced Bcl-xL in Hela–Bcl-xL$^{Tet\text{-}on}$ cells as expected (Fig. 1B) and conferred resistance to apoptosis as evidenced by significantly lower levels of caspase-3/7 activity in culture (Fig. 1C), although it did not have a significant effect on cell growth assay (Fig. 1D). Next, we subcutaneously injected Hela–Bcl-xL$^{Tet\text{-}on}$ cells into nude mice. When subcutaneous tumors grew to approximately 1 cm, the mice were randomly assigned to two groups: a doxycycline-drinking group and a water-drinking group. Subcutaneous tumors grew rapidly in the doxycycline-drinking group compared with the water-drinking group (Fig. 1E). As expected, xenograft tumors displayed higher levels of Bcl-xL expression than those in the water drinking group (Fig.

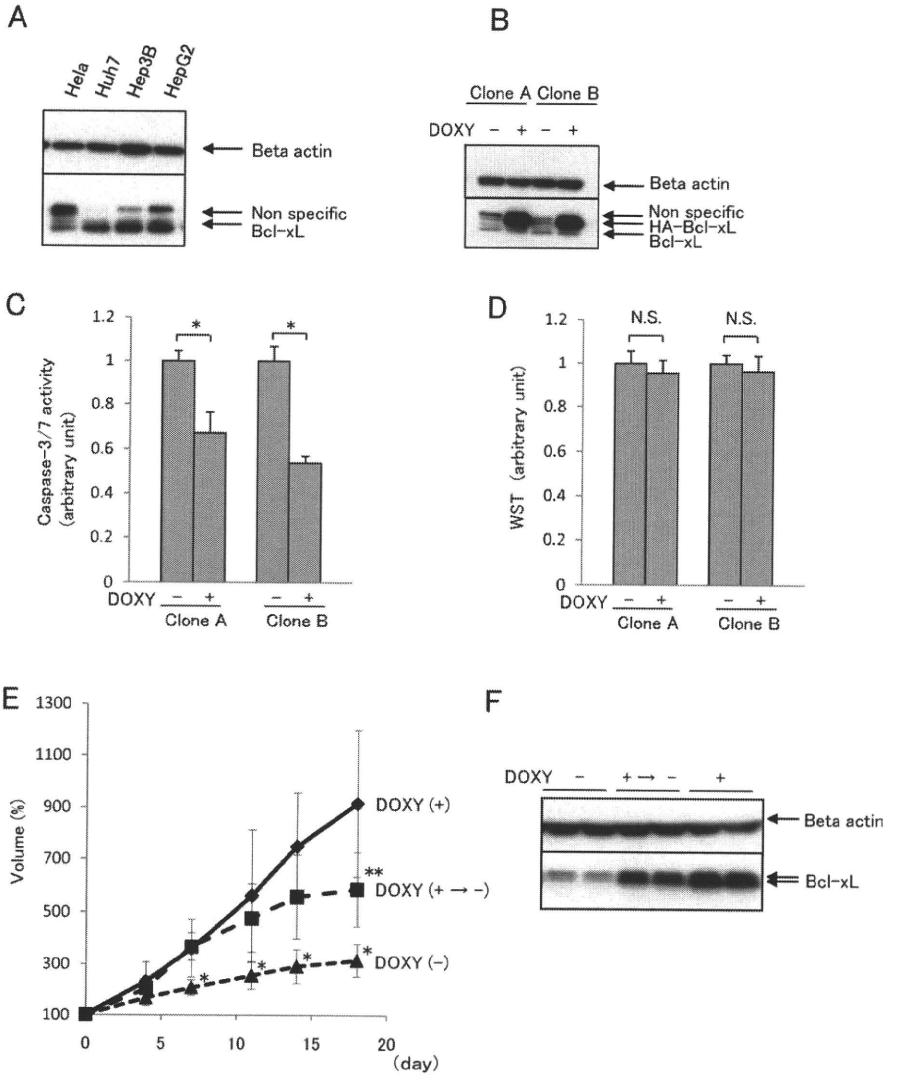

Fig. 1. Bcl-xL overexpression in vitro and in vivo by the Tet-on system. (A) Western blot analysis of Bcl-xL in human hepatoma cells and Hela cells. (B,C,D) Bcl-xL overexpression in vitro. Two independent clones of Hela-Bcl-xLTet-on cells were cultured with or without 1 μM doxycycline (DOXY) for 24 hours. (B) Western blot analysis of Bcl-xL. (C,D) Caspase-3/7 activity in culture supernatant and cell viability by the WST assay (N = 4). *P < 0.05. N.S., not significant. (E,F) Bcl-xL overexpression in vivo. Nude mice carrying xenograft tumors of Hela-Bcl-xL$^{Tet\text{-}on}$ clone A were randomly assigned to water given with or without 100 mg/mL doxycycline for 7 days. After 7 days, the mice of the doxycycline-drinking group were randomly assigned to two groups: one in which doxycycline drinking was continued and the other in which water was given instead (N = 5 or 6 per group). (E) The percentage of xenograft tumor volume. (F) Western blot analysis of xenograft tumor for the expression of Bcl-xL. *P < 0.05 versus the other two groups. **P < 0.05 versus the DOXY (+) group.

1F). In addition, switching the mice to water drinking at 7 days after doxycycline drinking decreased Bcl-xL expression and retarded tumor growth compared with continuing doxycycline drinking (DOXY + → − versus DOXY +, respectively; Fig. 1F). These results indicate that Bcl-xL overexpression was directly linked to rapid growth of tumors *in vivo* and suggest that Bcl-xL may be a therapeutic target for inhibiting tumor progression, especially for Bcl-xL–overexpressing tumors.

Bcl-xL Inhibitor ABT-737 Dose-Dependently Induces Apoptosis of Hepatoma Cells but Fails to Suppress Tumor Growth in a Xenograft Model. To examine the impact of pharmaceutical inactivation of Bcl-xL overexpressed in hepatoma cells, Huh7 and Hep3B hepatoma cells were cultured with escalating doses of ABT-737. ABT-737 dose-dependently activated caspase-3/7 in hepatoma cells and suppressed tumor growth at high dosages (Fig. 2A,B). To examine the *in vivo* effect of ABT-737, nude mice were subcutaneously injected with Huh7 cells to generate xenograft tumors and were randomly assigned into two groups when the diameter of the subcutaneous tumors reached approximately 1 cm: ABT-737 injection group and vehicle injection group. Administration of ABT-737 at 50 mg/kg body weight/day for 7 days failed to suppress tumor growth (Fig. 2C). In contrast, mild ALT elevation and thrombocytopenia were observed in ABT-737–injected mice (Fig. 2D). Previous research has demonstrated that both are observed in mice after ABT-737 administration,[17,18] confirming that the dose injected in the present experiment is sufficient for inducing a biological effect of ABT-737 *in vivo*.

ABT-737 Posttranscriptionally Increases Expression of Mcl-1. To examine the mechanisms underlying relative resistance of hepatoma cells to ABT-737, we examined the expression profile of the Bcl-2 family

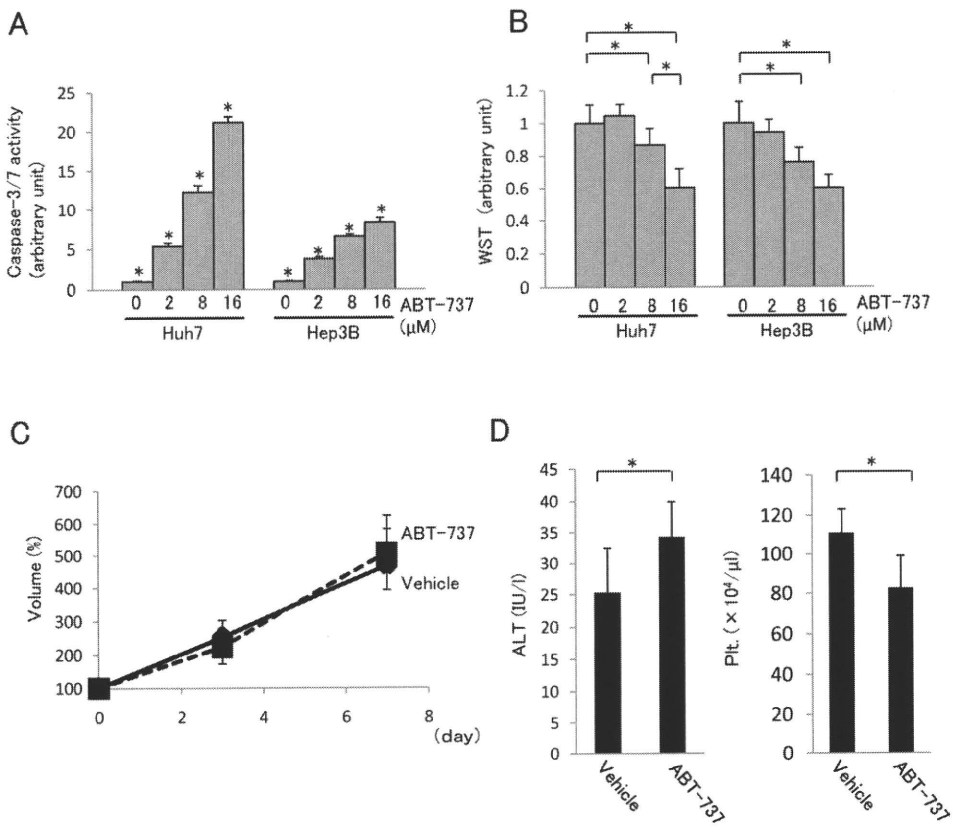

Fig. 2. Apoptosis and growth of hepatoma cells treated with ABT-737 *in vitro* and *in vivo*. (A,B) Huh7 and Hep3B cells were treated with indicated doses of ABT-737 for 24 hours (N = 4). (A) Caspase-3/7 activity of culture supernatant. $*P < 0.05$ versus all other groups. (B) Cell viability by the WST assay. $*P < 0.05$. (C,D) Nude mice carrying xenograft tumors of Huh7 cells were intraperitoneally administered 50 mg/kg ABT-737 or vehicle daily for 7 days. (N = 9 for each group.) (C) The percentage of xenograft tumor volume. (D) Serum ALT levels and circulating platelet count. $*P < 0.05$.

proteins. Administration of ABT-737 did not affect expression of proapoptotic multidomain members Bak and Bax or BH3-only proteins Bid and Bim in cultured hepatoma cell lines Huh7 and Hep3B (Fig. 3A). Although the slower migrating species of Bim at 4 hours was increased, this change disappeared at 24 hours. In agreement with previous research,[19,20] Mcl-1 was constitutively expressed in hepatoma cells. Of importance is the finding that the levels of Mcl-1 expression were rapidly increased as early as 4 hours after ABT-737 exposure. Expression of mcl-1 mRNA did not differ between ABT-737–treated cells and vehicle-treated cells (Fig. 3B), suggesting the involvement of a posttranscriptional mechanism. Because Mcl-1 is a rapid-turnover protein, the levels of Mcl-1 may be regulated by protein degradation.[21] To examine this, we treated hepatoma cells with cycloheximide, a well-established protein synthesis inhibitor, in the presence or absence of ABT-737. Cycloheximide-induced rapid decline in Mcl-1 expression was substantially blocked in the presence of ABT-737, suggesting that ABT-737 significantly delays degradation and prolongs the stability of Mcl-1 (Fig. 3C). Recently, it was reported that the deubiquitinase USP9X is involved in stabilization of Mcl-1.[22] In this study, western blot analysis revealed that the levels of USP9X expression were not changed in Huh7 and Hep3B with ABT-737 (Supporting Fig. 1A). Furthermore, USP9X down-regulation by small interfering RNA (siRNA) could not block the Mcl-1 up-regulation induced by ABT-737 (Supporting Fig. 1B).These results suggest that USP9X was not involved in Mcl-1 up-regulation induced by ABT-737. Of importance is the finding that Mcl-1 expression was also up-regulated after administration of ABT-737 in our xenograft model (Fig. 3D). Because Mcl-1 is not a target of ABT-737, relative resistance to ABT-737 of hepatoma cells may be due, at least in part, to posttranscriptional induction of Mcl-1.

Mcl-1 Knockdown Sensitizes Hepatoma Cells to ABT-737. To examine the impact of Mcl-1 induction in hepatoma cell resistance to ABT-737, we silenced Mcl-1 expression through use of three different siRNAs. Western blot analysis revealed that Mcl-1 siRNA2 and siRNA3 completely knocked down Mcl-1 expression in Hep3B cells, whereas Mcl-1 siRNA1 did so only partially (Fig. 4A). Mcl-1 knockdown or a medium dose of ABT-737 (4 μM) modestly activated caspase-3/7 in Hep3B cells, whereas both substantially activated caspase-3/7 (Fig. 4B). In addition, Mcl-1 knockdown or ABT-737 alone failed to suppress the growth of tumor cells but caused significant suppression when used together (Fig. 4C). Caspase-3 activation was also confirmed by western blots (Fig. 4A). It should be noted that Mcl-1 siRNA1 reduced Mcl-1

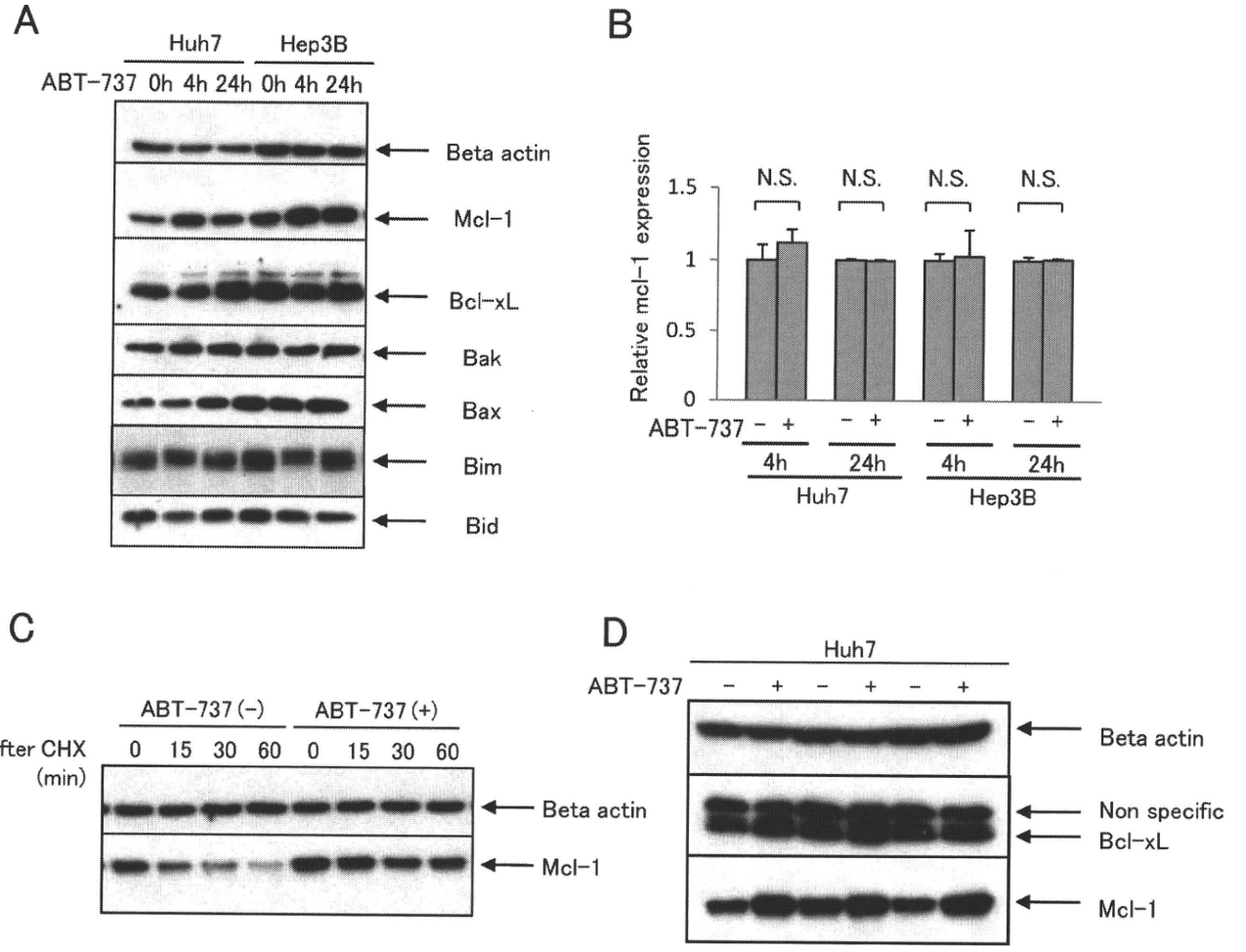

Fig. 3. Up-regulation of Mcl-1 in human hepatoma cells by ABT-737 in vitro and in vivo. (A,B) Huh7 and Hep3B cells were cultured with 4 μM ABT-737 for the indicated times. (A) Western blot analysis for the expression of Bcl-2 family proteins. (B) Real-time RT-PCR analysis for mcl-1 mRNA expression (N = 6). The levels were normalized to each group without ABT-737. N.S., not significant. (C) Huh7 cells were cultured with or without 4 μM ABT-737 for 4 hours and then further treated with 1 mM cycloheximide (CHX) for the indicated times. Western blot analysis for Mcl-1 expression. (D) Nude mice carrying xenograft tumors of Huh7 cells were intraperitoneally administered 50 mg/kg ABT-737 or vehicle daily for 7 days. Western blot analysis of xenograft tumor after 7-day treatment for the expression of Bcl-2 family proteins.

expression in ABT-737-treated cells to levels similar to those of untreated cells (Fig. 4A). Even in this case, mcl-1 knockdown enhanced caspase activation and growth suppression of Hep3B cells induced by ABT-737. Similar data were obtained with another hepatoma cell line, Huh7 (Fig. 4A and Supporting Fig. 2). These results indicate that Mcl-1 up-regulation induced by ABT-737 is involved in the resistance of hepatoma cells to ABT-737 and suggest that combination therapy with ABT-737 and Mcl-1 inhibitor may be predictably effective *in vivo*.

We previously reported that, similar to Bcl-xL, Mcl-1 plays an important role in apoptosis resistance of normal hepatocytes. In addition, knockdown of both Mcl-1 and Bcl-xL led to impaired liver development during embryogenesis.[15] Thus, the concern arises that simultaneous inactivation of both Bcl-xL and Mcl-1 may cause severe liver injury in adults. To examine this possibility, we injected ABT-737 into hepatocyte-specific Mcl-1 knockout mice or wild-type littermates. ABT-737 injection into wild-type mice led to mild liver apoptosis, which is consistent with our previous finding,[17] whereas injection into Mcl-1 knockout mice induced massive liver apoptosis leading to severe liver injury, and most animals died within 1 day (Fig. 4D,E). This result indicates that inactivation of both Bcl-xL and Mcl-1 may be hazardous and that Mcl-1 inactivation should be done in a tumor-specific manner.

Sorafenib Down-Regulates Mcl-1 Expression in Hepatoma Cells Much More Strongly than in Normal Liver Cells. Previous research has shown that sorafenib down-regulates Mcl-1 expression in hepatoma cells in a mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK)-independent manner.[16,23] In the present study, to examine whether Mcl-1

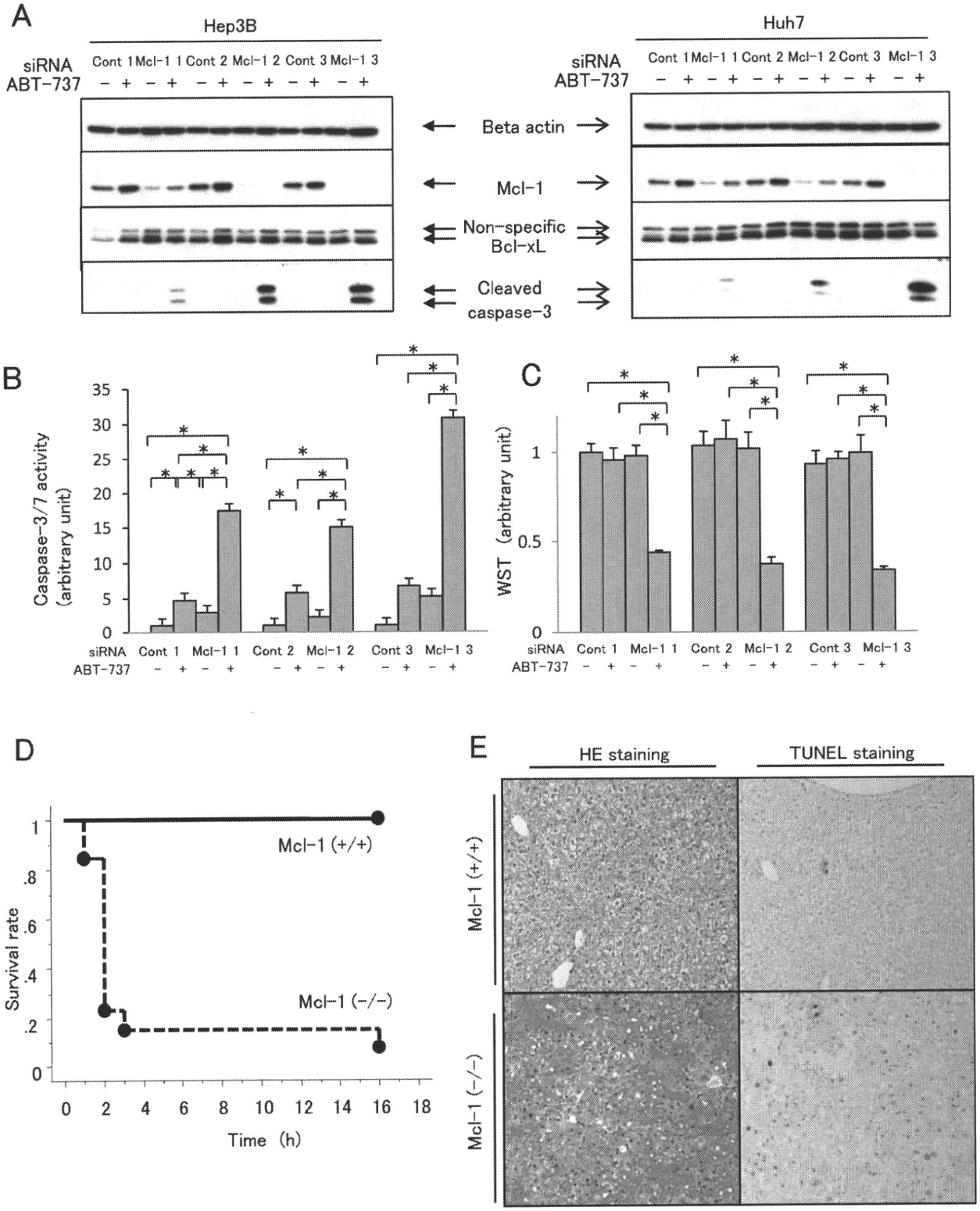

Fig. 4. Effects of ABT-737 under inhibition of Mcl-1 in vitro and in vivo. (A-C) Hep3B and Huh7 cells were transfected with Mcl-1 siRNAs (Mcl-1 1, Mcl-1 2, and Mcl-1 3) or control siRNAs (Cont 1, Cont 2, Cont 3). Forty-eight hours after transfection, they were treated with or without 4 μM ABT-737 for 24 hours (N = 4). (A) Western blot analysis for the expression of Mcl-1, Bcl-xL, and cleaved caspase-3. (B) Caspase-3/7 activities of supernatant in Hep3B culture dishes. *P < 0.05. (C) Cell viability of Hep3B cells by the WST assay. *P < 0.05. (D,E) Wild-type mice (Mcl-1 +/+) and hepatocyte-specific Mcl-1 knockout mice (Mcl-1 −/−) were intraperitoneally administered 50 mg/kg of ABT-737. (D) Survival curve of the mice (N = 13 or 15). (E) Hematoxylin and eosin (HE) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining of the liver sections 16 hours after administration of ABT-737 with wild-type mice and immediately after death with Mcl-1 knockout mice. Representative photographs are shown.

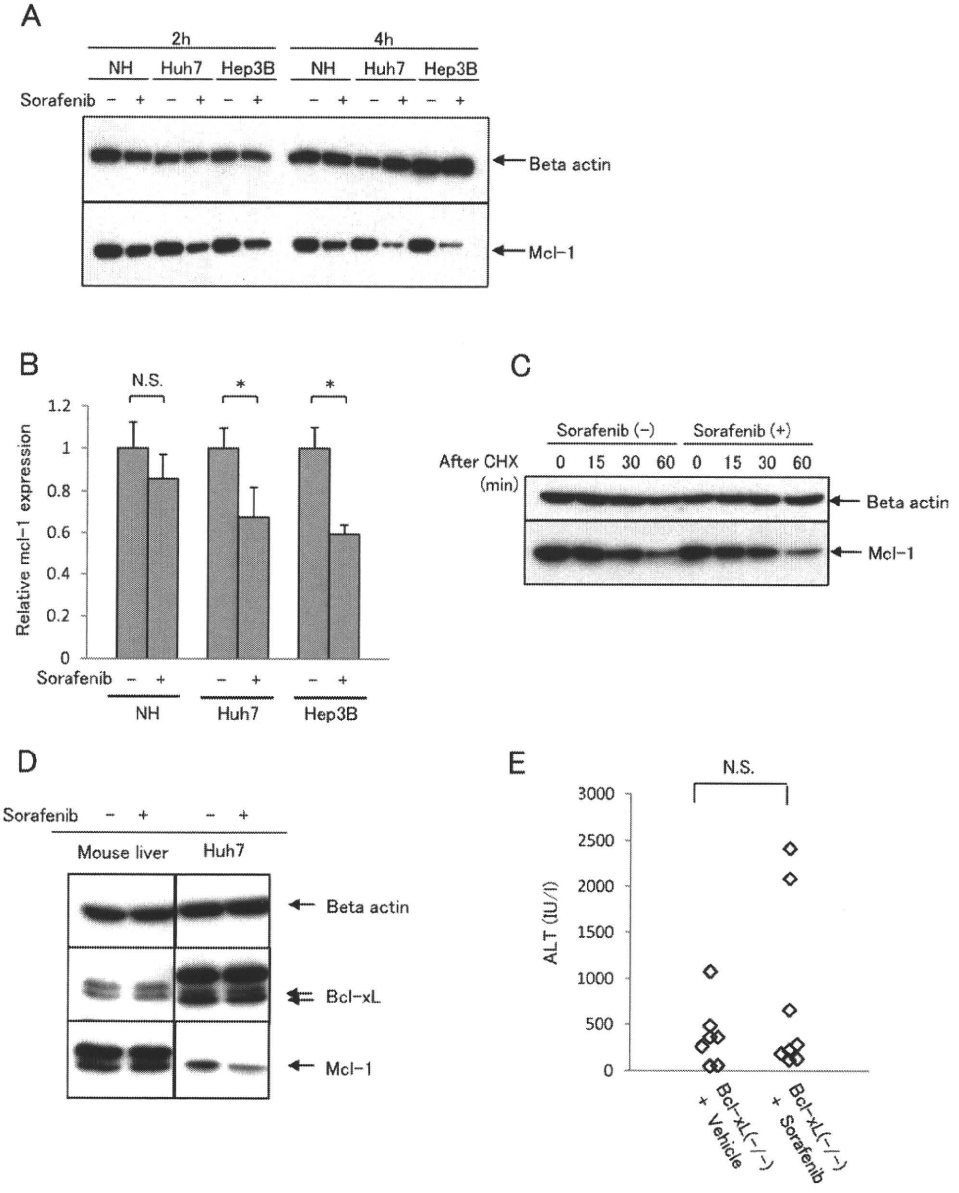

Fig. 5. Effects of sorafenib on the Mcl-1 expression in hepatoma cells and nontransformed cells in vitro and in vivo. (A,B) Normal hepatocytes (NH) and Huh7 and Hep3B cells were treated with 5 μM sorafenib. (A) Western blot analysis for the expression of Mcl-1 after treatment for 2 or 4 hours. (B) Real-time RT-PCR analysis for mcl-1 expression after treatment for 4 hours (N = 6). The levels were normalized to each group without sorafenib. N.S., not significant. *P < 0.05. (C) Huh7 cells were cultured with 1 mM cycloheximide (CHX) with or without 5 μM sorafenib for the indicated times. Western blot analysis for Mcl-1 expression. (D) Nude mice carrying xenograft tumors of Huh7 cells were orally administered 30 mg/kg sorafenib or vehicle daily for 3 days. Western blot analysis for the expression of Bcl-xL and Mcl-1 in the xenograft tumor of Huh7 cells and the liver of mice. (E) Hepatocyte-specific Bcl-xL knockout mice (Bcl-xL −/−) were orally administered daily 30 mg/kg sorafenib or vehicle daily for 3 days. The serum ALT levels are shown (N = 7 or 8). The difference between two groups was determined using Mann-Whitney's U test. N.S., not significant.

suppression of sorafenib is tumor-specific, nontransformed human hepatocytes and hepatoma cell lines were treated with sorafenib. Sorafenib down-regulated Mcl-1 expression in all hepatoma cell lines tested, but had a lesser effect on nontransformed human hepatocytes (Fig. 5A). Sorafenib down-regulated mcl-1 mRNA expression in Huh7 and Hep3B hepatoma cells but not in nontransformed hepatocytes (Fig. 5B). To examine the posttranscriptional effect of sorafenib for Mcl-1 expression, we treated Huh7 cells with cycloheximide in the presence or absence of sorafenib. Cycloheximide-induced decline in Mcl-1 expression was not accelerated by sorafenib (Fig. 5C). This result indicated that, in contrast to the case of ABT-737, sorafenib does not affect the degradation process of Mcl-1.

We also examined Mcl-1 expression in the liver as well as xenograft tumors. Administration of sorafenib significantly suppressed Mcl-1 expression in Huh7 xenograft tumors but not in the liver (Fig. 5D). To examine the safety of sorafenib in the absence of Bcl-xL *in vivo*, we administered sorafenib to hepatocyte-specific Bcl-xL knockout mice. These mice had elevated levels of serum ALT at baseline, as we reported previously,[8] but displayed neither further ALT elevation nor lethal liver failure upon sorafenib administration (Fig. 5E). Taken together, these results indicate that sorafenib did not affect Mcl-1 expression in the liver and therefore did not cause further liver injury even if Bcl-xL was inactivated.

ABT-737 Induced Apoptosis of Hepatoma Cells and Suppressed Growth of Xenograft Tumor with Sorafenib Coadministration. To examine the impact of coadministration of sorafenib and ABT-737 on

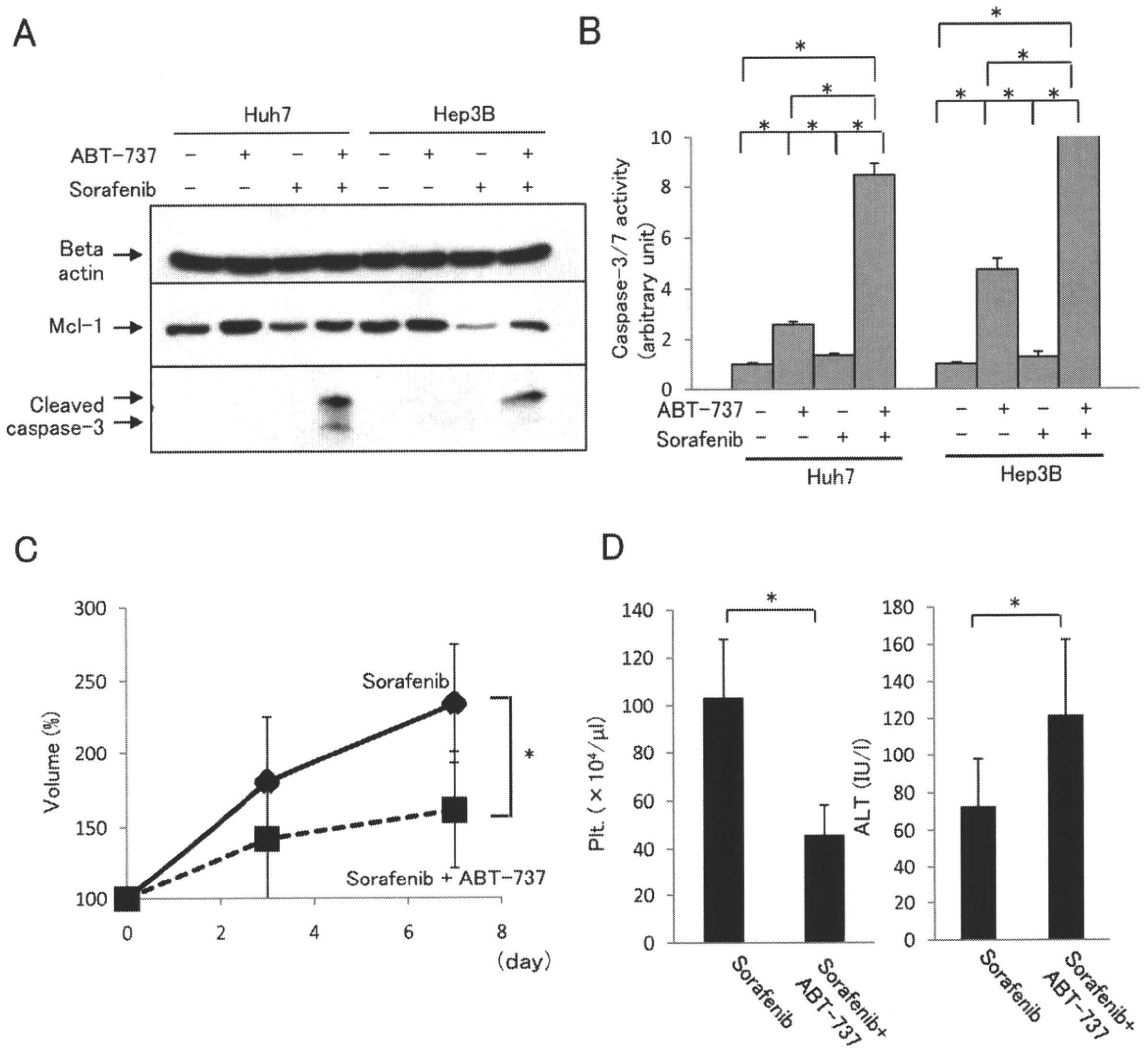

Fig. 6. Effects of ABT-737 with sorafenib treatment in vitro and in vivo. (A,B) Huh7 and Hep3B cells were treated with or without 4 μM ABT-737 together with or without 5 μM sorafenib. (A) Western blot analysis for the expression of Mcl-1 and cleaved caspase-3. (B) Caspase-3/7 activity of culture supernatants. *$P < 0.05$. (C,D) Nude mice carrying xenograft tumors of Huh7 cells were intraperitoneally administered daily 50 mg/kg ABT-737 or vehicle with daily oral administration of 30 mg/kg sorafenib for 7 days ($N = 8$ or 11). (C) The percentage of xenograft tumor volume. (D) Circulating platelet (Plt.) count and serum ALT levels. *$P < 0.05$.

inducing apoptosis, we treated Huh7 and Hep3B hepatoma cells with ABT-737 and/or sorafenib. Although ABT-737 up-regulated Mcl-1 expression in Huh7 and Hep3B cells, sorafenib abolished the Mcl-1 up-regulation induced by ABT-737; the levels of Mcl-1 expression of cells treated with both were similar to those of nontreated cells (Fig. 6A). Sorafenib failed to activate caspase-3/7 in hepatoma cells by itself, but efficiently activated it in the presence of ABT-737 (Fig. 6B). It was also confirmed by efficient cleavage of caspase-3 on western blot analysis (Fig. 6A).

To examine whether ABT-737 has an antitumor effect in the presence of sorafenib, we administered ABT-737 and sorafenib together to nude mice bearing Huh7 xenograft tumors. Although even sorafenib alone significantly suppressed tumor growth compared with the vehicle alone (Supporting Fig. 3), coadministration of ABT-737 and sorafenib led to significant further suppression of tumor growth compared to administration of sorafenib alone (Fig. 6C). Similar to administration of ABT-737 as a single agent, coadministration of sorafenib and ABT-737 also induced mild thrombocytopenia and ALT elevation (Fig. 6D). However, coadministration did not induce further morbidity or mortality in mice, suggesting that this regimen is safe and effective for controlling HCC progression.

Discussion

Tumor cells have two characteristic features: uncontrolled proliferation and apoptosis resistance. Uncontrolled proliferation, driven by activation of a variety

of oncogenes, is directly linked to tumor growth. Apoptosis resistance is believed to be required for the oncogene-induced aberrant proliferation, because without it, tumor cells tend to undergo apoptosis.[24] However, the direct link between apoptosis resistance and growth of solid tumors *in vivo* has not been well studied. Clarifying this point is very important, especially because a very recent study by Weber et al.[25] produced the contradictory finding that aged hepatocyte-specific Mcl-1 knockout mice develop HCC-like lesions, suggesting a link between hepatocarcinogenesis and increased proliferation resulting from increased apoptosis. In the present study, we used conditional expression of Bcl-xL in tumor cells to show that Bcl-xL overexpression, which is frequently found in human HCC, can be directly linked to tumor growth *in vivo*, although it did not promote significant cell growth *in vitro*. Our results suggest that tumor cells encounter a variety of cellular stresses and require antiapoptosis to survive *in vivo* rather than *in vitro*. Thus, we consider antiapoptosis to be an important mechanism for progression of a solid tumor, even if it may not be the case for tumor development as suggested by Weber et al.[25] Our finding also provides proof of the concept that Bcl-xL may be a target for therapy against HCC progression.

In the present study, we showed that, unlike hematopoietic malignancy, hepatoma cells are relatively resistant to ABT-737. Although ABT-737 dose-dependently induced apoptosis in hepatoma cells, a relatively high dose of ABT-737 (more than 8 μM) was required to suppress tumor growth *in vitro*. Importantly, administration of an *in vivo* effective dose of ABT-737 (50 mg/kg) failed to suppress xenograft tumors. We found increased expression of Mcl-1 in cultured hepatoma cells as well as xenograft tumors upon ABT-737 treatment. This may be part of the mechanism of their relative resistance to ABT-737 because hepatoma cells were highly sensitive to this agent if Mcl-1 expression levels were kept constant by an siRNA strategy. Previous articles have reported that Mcl-1 knockdown makes some tumor cells sensitive to ABT-737.[26,27] The present study showed that ABT-737 up-regulation of Mcl-1 rather than Mcl-1 expression itself may be a mechanism of tumor cell resistance to this agent.

A recent study demonstrated that long-term exposure to ABT-737 made initially sensitive lymphoma cell lines resistant to this agent via up-regulation of Mcl-1.[28] In this study, Mcl-1 up-regulation in the ABT-737–resistant lymphoma cells were reported to be mediated by transcriptional up-regulation. In the present study, hepatoma cells showed immediate, posttranscriptional up-regulation of Mcl-1. This rapid response may contribute to the difficulty of treating hepatoma cells with ABT-737 compared with lymphoma cells in which ABT-737 is reported to be effective not only *in vitro*[29] but also *in vivo*.[30] The mechanism by which hepatoma cells posttranscriptionally up-regulate Mcl-1 upon ABT-737 exposure is not clear at present. However, our study has shown that Mcl-1 up-regulation was mediated by delayed degradation of Mcl-1 protein in ABT-737–treated cells without involving the USP9X deubiquitinase. ABT-737 is a Bad mimetic small molecule and preferentially binds with the BH3-binding groove of Bcl-xL. This binding may release endogenous BH3-only proteins such as Bim and Bid and presumably Bak and Bax from Bcl-xL and these unleashed Bcl-2 proteins may then bind Mcl-1. The interaction between Mcl-1 and the unleashed Bcl-2 proteins may cause increased Mcl-1 stability. Because Bak/Bax and Bid/Bim function as effectors and activators for the mitochondrial pathway of apoptosis, respectively, their binding with Mcl-1 may also cause apoptosis resistance to ABT-737.

Not only efficacy but also safety is an important point when considering a therapeutic strategy for cancer. Tumor cells sometimes share similar mechanisms for survival with normal cells. Indeed, HCCs overexpress Bcl-xL, but this molecule also plays an important role in maintaining the integrity of normal hepatocytes.[8] In the present study, we administered ABT-737 to Mcl-1 knockout mice and demonstrated that inactivation of both Bcl-xL and Mcl-1 could induce lethal hepatitis. We previously reported that Bcl-xL and Mcl-1 are required for liver development during embryogenesis,[15] and the present study also revealed the critical importance of both molecules in the adult liver. Recently, the possibility of combination therapy for down-regulation of Bcl-xL and Mcl-1 has been reported *in vitro*.[26,27,31] The present study, for the first time, focused on the *in vivo* safety of this strategy.

Regarding safety concerns about the inactivation of both Mcl-1 and Bcl-xL, sorafenib is an attractive agent because as we have revealed in this study, it down-regulates Mcl-1 expression in a relatively specific manner in tumor cells. Experiments with sorafenib administration into Bcl-xL knockout mice confirmed the safety of coadministration of sorafenib and ABT-737. The underlying mechanisms by which sorafenib down-regulates Mcl-1 in a tumor-specific manner are not clear. Some reports have shown that the down-regulation of Mcl-1 by sorafenib is independent of MEK/ERK,[16,23,32] but is dependent on Raf, AKT (protein kinase B), and Tyr705 phosphorylation of signal transducer and activator of transcription 3 (STAT3).[33,34]

Together with the report that activation of Ras/Raf and STAT3 pathways was found in HCC,[35] these pathways in tumor cells may be more activated than in healthy cells and result in the specificity of Mcl-1 down-regulation in tumor cells by sorafenib. Further experiments are needed to clarify this point.

Sorafenib belongs to a recently approved new class of targeted therapeutics that inhibit the oncogenic kinase pathway for HCC. It has been found to significantly prolong survival of patients with advanced HCC, although its effect appeared to be one of maintaining a stable disease state rather than inducing tumor regression.[36,37] It is speculated that sorafenib produces anticancer effects through a variety of ways such as suppression of angiogenesis and cell cycle arrest of tumor cells. Although it down-regulates Mcl-1,[16,23,32-34] its effect on apoptosis has not been clearly understood. In the present study, we found that sorafenib could not efficiently induce apoptosis in hepatoma cells by itself. This might explain why this agent elicits predominantly disease-stabilizing, cytostatic responses rather than tumor regression. Adding ABT-737 efficiently induced apoptosis of hepatoma cells, clearly indicating that the target of ABT-737, presumably Bcl-xL, blocks the apoptosis-inducing potency of sorafenib. Furthermore, coadministration of ABT-737 and sorafenib led to stronger suppression of xenograft tumor growth than did administration of sorafenib alone. These results suggest that combining sorafenib with ABT-737 may be an attractive strategy for producing durable clinical responses to combat HCC.

In conclusion, we have demonstrated that the inhibition of Bcl-xL by ABT-737 under sorafenib administration was safe and effective for anti-HCC therapy in preclinical models. ABT-737, a direct activator of apoptosis machinery, may unlock the potent antitumor potential of oncogenic kinase inhibitors such as sorafenib.

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■ Poster Discussion Session #4026

TACEとソラフェニブの併用は，中等度進行期肝細胞癌のアジア人患者で安全かつ有効

— Phase Ⅱ Trial of Study in Asia of the Combination of TACE (Transcatheter arterial chemoembolization) with Sorafenib in Patients with Hepatocellular Carcinoma Trial (START) : 2nd Interim Safety and Efficacy Analysis —

Internal Medicine, Asan Medical Center, University of Ulsan College of Medicine　Chung YH, et al.

切除不能な中等度進行期肝細胞癌のアジア人患者において，肝動脈化学塞栓療法（TACE）にソラフェニブを追加する治療法は安全かつ有効性が示唆されることが，第Ⅱ相多施設試験STARTの中間解析の結果，明らかになった。韓国Asan Medical CenterのChung氏らの研究グループが，4日のポスターディスカッションのセッションで報告した。

肝細胞癌（HCC）は全世界の患者のうち約50%がアジア-太平洋地域で発生している。アジアでは特に中等度進行期患者におけるファーストラインの非根治治療として，TACEがHCC管理の要となっている。

一方でソラフェニブは進行性HCC患者における全生存の向上が確認された，初の全身治療薬であり，有効的な治療オプションとされている。

START試験は，BCLC病期分類がBでTACE未治療の被験者を対象に，ドキソルビシンによるTACEにソラフェニブ（800mg/日）を追加する治療法を検討したもの。ECOG PS 0～1，Child-Pugh A/B（スコアが7以下），腫瘍径10cm以下といった参加基準が設けられ，63例が対象となった。

TACEではリピオドール®ウルトラフルイド（5～20mL）とドキソルビシン（30～60mg）のエマルジョン注入後，吸収性粒子で閉塞。最初のTACEを試験1日目とし，4日目からソラフェニブ400mgを1日2回内服，次のTACEが始まる4日前に一旦中断した後，TACEから4日目に再開するという治療サイクルを6～8週間ごとに，最高6サイクルまで繰り返した。

毎回TACEから4週間後に腹部のCTスキャンとαフェトプロテインの測定を行い，TACEを継続する必要があるかどうかを評価。CTスキャンで腫瘍組織が確認されなくなった場合はTACEを中止し，忍容性がある場合はソラフェニブによる治療だけを継続し，3カ月間隔でCTスキャンとαフェトプロテインの測定を行った。新しい病変が認められた場合，TACE再開が可能かどうかを評価した。

安全性の中間評価は，ソラフェニブによる治療を最低1回受けた，最初の被験者63例を対象とし，有効性分析は，これらのうち腫瘍評価を最低2回実施した50例の被験者を対象とした。

被験者の年齢中央値は58.7歳，94.0%がChild-Pugh Aで，77.8%がB型肝炎であった。追跡期間中央値は137日であった。

薬剤関連の有害事象は133件発生し，グレード3/4は39例。このうち手足皮膚反応が最も多く10例で，ALT上昇と好中球減少がそれぞれ8例であった（**表1**）。多くが軽度から中等度であり，有害事象により治療を中止した被験者はいなかった。

有効性分析では，18例が完全奏効し，30例が部分奏効または安定，2例が増悪していた（**図1**）。

今回の中間解析で，TACEとソラフェニブの併用は安全性と忍容性があり，良好な奏効率が示唆されたことから，研究グループは今後，本治療の有効性を検証するため，さらに臨床試験が必要で

表1　Sorafenib＋TACEによるAE

	sorafenib＋TACE (n＝63)	
	all grades	grade 3/4
all categories	133	39
hand-foot skin reaction	62	10
ALT elevation	17	8
neutropenia	8	8
fatigue/rash/others	46	25

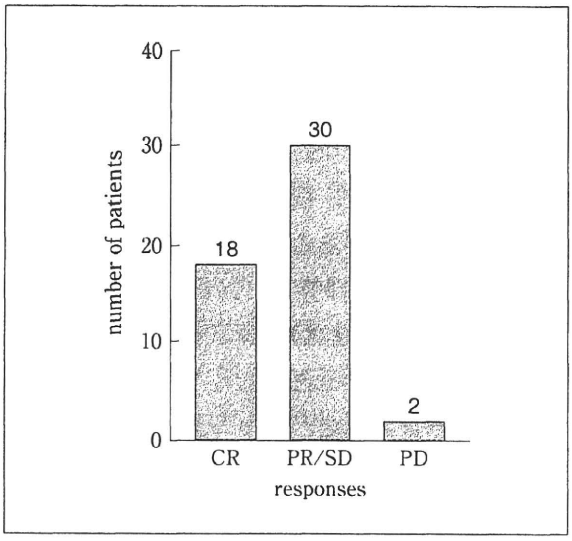

図1　奏効率

ある，と結論した。

監修・コメント

杏林大学医学部付属病院　腫瘍内科　　古瀬　純司 先生

ソラフェニブは切除不能進行性肝細胞癌で生存期間の延長に関する有用性が確認された唯一の薬剤として広く用いられている。肝細胞癌ではTACEが多く行われており，ソラフェニブを併用することで，TACEの効果の増強が期待されている。日本と韓国でTACE後ソラフェニブを内服するプラセボコントロール試験が行われたが，有用性が示唆されたもののnegativeな結果に終わった。今回のSTART試験はTACEとほぼ同時にソラフェニブを投与する方法を採っており，中間解析の結果であるが，安全性は十分であることが示された。また有効性も示唆されており，今後の展開が期待される。

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CORRESPONDENCE Open Access

Eastern asian expert panel opinion: designing clinical trials of molecular targeted therapy for hepatocellular carcinoma

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Abstract

The largest burden of hepatocellular carcinoma (HCC) lies in Asia, secondary to hepatitis B virus (HBV) infection. Improved survival with sorafenib has fostered new research but many challenges remain in designing clinical trials. The disease, its management, and populations affected by it are heterogeneous worldwide and within Asia. An expert conference of Eastern Asian oncologists and hepatologists was convened to foster consensus in clinical trial design. The panel identified key areas that need to be addressed to facilitate clinical trials in Asia. Stratification by viral etiology is desirable within Asia and by region in global trials. Antiviral therapy should also be considered as a stratification factor and incorporated into HCC management in trials. The panel agreed that histological diagnosis is not required for trial entry and that Barcelona-Clinic Liver Cancer (BCLC) staging is acceptable for trials as long as portal hypertension can be better defined with standardized methodology. Consensus in treatment must be sought to allow multi-national trials and it must be recognized that first-line sorafenib is not largely feasible in Asia. Finally, Asian nations must be urged to participate in clinical trials, many of which are ongoing, to advance new treatment options in this challenging disease.

Background

Over 600,000 cases of hepatocellular carcinoma (HCC) are diagnosed annually worldwide and the mortality-to-incidence rate ratio is second only to pancreatic cancer [1,2]. The incidence of HCC varies widely by geographical region. Asia carries the largest burden with 55% of all cases occurring in China [1]. Age-standardized incidence rates per 100,000 persons for men are 45.0 in Korea (1999-2001) [3], 37.9 in China (2002) [1], and 23.1 in Japan (2002) [1]. Corresponding rates for women are 12.0, 14.2, and 7.6. Globally, the predominant cause of HCC is viral infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) [4].

Hepatocellular carcinoma is refractory to cytotoxic chemotherapy [5] and the failure of cytotoxic regimens has led to a bleak outlook. However, the recent development of molecular targeted therapies is changing the landscape and offering hope. Researchers have found new optimism for initiating clinical trials after sorafenib showed efficacy in advanced disease [6]. Currently, trials are planned or ongoing in all stages of HCC; however, many issues remain [7]. Most salient is the variability in management practices both between Asia and the West and within Asia. Key differences are apparent in the etiology, diagnosis, staging, and treatment of HCC among countries. These differences complicate the conduct of international clinical trials that will foster approval and availability of new therapeutic entities.

In order to forge a better understanding of how HCC clinical practices in the Eastern Asian region compare to current global clinical trial requirements, an expert conference was held. Participants of the panel (the authors) are oncologists and hepatologists representing China, Hong Kong, Japan, Korea, and Taiwan who have an expertise in treating HCC. Each panelist offered insight, reviewed herein, about how HCC is managed across Eastern Asia and how management practices and clinical trial requirements can be unified to advance new treatments, particularly targeted agents, for HCC.

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Etiology

Viral etiology varies by region with HBV predominating in non-Japanese Asians and accounting for approximately 70-80% of cases. In Japan, most of Europe, and in the United States, HCV is more common than HBV among viral etiologies [3,8-11]. However, in the United States, 67% of HCC cases are seronegative for both viruses [10].

The increased incidence of HBV-HCC in Eastern Asia compared to Japan and Western nations leads to different management issues and prognosis that affect clinical trial design. Hepatitis C virus-HCC is more likely to develop in the background of cirrhosis than HBV-HCC [12]. Therefore, the underlying liver disease may differ in HCC patients by region, a factor that weighs heavily in treatment decisions.

Survival differences have been observed according to geographic region and viral etiology, though the reasons for these observations remain unclear. In clinical trials of systemic therapy for advanced HCC, trials done in Asian countries reported inferior survival compared with trials done in non-Asian countries [13]. Possible reasons include variation in genetic and/or epigenetic aberrations between different viral etiologies and the propensity for Asian physicians to use local therapy more aggressively and in later stages, resulting in enrollment of a more advanced patient population to trials of systemic therapy. Survival between HBV-HCC and HCV-HCC appears similar in early-stage, resectable HCC, if staging and other clinical parameters are considered [14]. However, two retrospective studies have found poorer survival in HBV-HCC among patients with unresectable, advanced disease [15,16]. Attributing the survival difference to viral etiology alone is difficult but demonstrates the need for considering the potential differences in clinical trials.

Additionally, in contrast to HCV, HBV reactivates with immune suppression, complicating treatment with immunosuppressive regimens [17,18]. The predominance of HBV-HCC in Asia is associated with increased use of antiviral agents to prevent viral reactivation during HCC treatment. Antiviral therapy with lamivudine has reduced the incidences of HBV reactivation and hepatitis, reduced the severity of hepatitis episodes, led to fewer disruptions in chemotherapy, and reduced mortality related to HBV reactivation in clinical trials of patients with HCC or other cancers who are receiving chemotherapy [19-22]. Anti-viral therapy following curative resection, radiofrequency ablation, or other local, non-chemotherapeutic treatments for HBV-HCC, has been shown to increase residual liver volume and/or function and may prolong survival [23-25]. Furthermore, interferon, given after curative therapy, may increase recurrence-free survival rates [26,27]. These benefits indicate that use of antiviral therapy is an important confounding factor in HCC clinical trials.

A separate international expert panel has recommended stratification according to region for global trials but discouraged further stratification according to etiology [7]. However, in light of the confounding factors described herein, the current panel agreed that trials within Eastern Asia should include stratification by HBV or HCV etiology. Further, antiviral therapy should be both considered as a stratification factor and incorporated into the overall management of patients in international HCC clinical trials.

Screening

Stage at diagnosis differs both within Eastern Asia and between Eastern Asia and Western nations. Using TNM-based staging systems, China and Japan have relatively high proportion of patients diagnosed at Stage I or II compared to Hong Kong and Korea. In the United States, a higher percentage of patients are diagnosed with distant metastasis compared to Asian countries [28,29].

The differences may reflect variable screening practices. The proportion of patients who receive screening in the United States appears to vary according to the individual's healthcare. Only 25% of family practice physicians report routinely screening appropriate patients for HCC compared to 84% of physicians who are members of the Association for the Study of Liver Diseases (AASLD) [30,31]. In a study of 157 patients diagnosed with HCC at three US Veteran Affairs (VA) medical centers, 39% of patients with a known risk factor for HCC received screening [32]. With the exception of Hong Kong, where screening has been conducted in the context of study, screening high-risk populations is the standard of care in Asia. With diagnosis occurring at earlier stages, Eastern Asian countries are better able to utilize curative therapies, significantly affecting treatment paradigms and clinical trial populations.

Diagnosis

Both pathological and clinical diagnostic procedures vary according to country. The majority of pathological diagnoses are made by core biopsy in Korea, China, and Hong Kong, with fine needle aspiration (FNA) used infrequently. In contrast, 30% or fewer of pathological diagnoses are made by core biopsy in Japan, and Taiwan. Taiwan employs FNA in approximately 10% of cases but utilizes surgery for pathologic diagnosis in approximately 38% of cases. Protocols designating biopsy-proven HCC as an enrollment requirement would conflict with current practices in Japan and

Taiwan. The panel agreed that for trials conducted in the advanced/metastatic setting, histological confirmation of HCC is not necessary. Further, pre-treatment biopsy may result in tumor seeding which would complicate neoadjuvant trials.

Staging

A variety of staging systems are employed worldwide [33-36]. Several of these systems are based on the tumor-node-metastasis (TNM) paradigm or incorporate TNM groupings as a variable [33-35,37]. Other systems, such as the Barcelona-Clinic Liver Cancer (BCLC) staging system, incorporate measures of liver function and underlying disease. Complicating international clinical trial design is the variable use of these systems both within Asia and globally. Each region of Asia represented by the panel currently utilizes a different system. In China, the revised Staging Criteria of Primary Liver Cancer is used. This system was developed by the Chinese Society of Liver Cancer. The system uses criteria based on size, number and location of tumors, lymph node spread, extrahepatic metastasis, portal vein thrombosis, and liver function (Child-Pugh scores) [38]. In Japan, both the staging system and treatment algorithm apply liver function as the first category of evaluation rather than tumor size. Hong Kong does not have a unified staging system. Although BCLC is considered a valuable tool for a treatment algorithm in Hong Kong, the system is considered less useful for prognostication. The Chinese University Prognostic Index (CUPI) [37] has been found useful for prognostication at one center due to the more advanced population [39]. Korea employs a modified International Union Against Cancer (UICC) system and Taiwan uses BCLC.

The TNM-based staging systems have an important drawback: these systems do not account for underlying liver disease [40]. In HCC, the presence of liver disease is a common and important prognostic factor that is integral in determining treatment [40,41]. For these reasons, TNM-based systems have limited value in the comprehensive management of HCC. The Child-Pugh (CP) score is a widely-accepted system to evaluate liver function. Despite empirical selection of variables, this tool represents a simple, bedside tool that predicts mortality in cirrhotic patients with a degree of accuracy not substantially less than the more statistically sound model for end-stage liver disease (MELD) [42]. The BCLC staging system incorporates measures of liver function (portal hypertension, bilirubin, and CP scores at higher stages) and has emerged as the standard for clinical trial design [6,43]. However, this system is not generally used in Eastern Asia with the exception of Taiwan. China, specifically, has failed to adopt this system due to the omission of portal vein thrombosis as a factor, which has been shown to independently predict mortality [41]. Additionally, BCLC includes portal venous hypertension which requires an invasive procedure to measure that is not standard practice in Asia. However, the panel indicated that, if required for clinical trials seeking United States Food and Drug Administration approval, BCLC would be acceptable if the protocols also incorporated portal vein hypertension - measured and defined with non-invasive standardized methodology - and further evaluation of liver function.

Treatment Practices

Treatment practices vary somewhat throughout Eastern Asia and no unified treatment algorithm exists. Japan, China, Hong Kong, Korea, and Taiwan each use separate treatment algorithms, all of which differ from the BCLC treatment algorithm [7,44,45]. Such variations in treatment practices cause challenges in defining treatment protocols for international clinical trials.

Potentially Curative Treatment Options

Resection is utilized more often in Eastern Asia versus Western nations, which may reflect diagnosis at earlier stages and less cirrhosis in Asia [46]. In some centres in China, Taiwan, and Japan, between 34-40% of patients undergo resection, while the proportion is approximately 10-20% in others. In parts of East Asia [47,48], patients with recurrence undergo re-resection. Local ablation is performed in approximately 15% of patients in China, Hong Kong, and Taiwan and approximately 30% of patients in Japan. Liver transplant is the only treatment modality that offers a cure both for HCC and the underlying liver disease, but its application is limited both in Eastern Asia and the West.

Nonsurgical Local Treatments

Although TACE and transarterial embolization (TAE) are standards of care, significant heterogeneity exists among countries and institutions with respect to the types of embolizing materials and techniques utilized. Embolizing materials used typically include a mixture of iodized oil (lipiodol) and an anthracycline (epirubicin or doxorubicin) or cisplatin followed by gelatin sponge particles (Japan, Taiwan, Hong Kong). Nonetheless, other agents are used, particularly in China where 5-fluorouracil (5-FU) and mitomycin-C may be employed. Japan uses HAI with cisplatin alone, 5-FU and cisplatin (FP), or 5-FU and interferon. Currently, no consensus has been reached regarding the interval between procedures or endpoints. Other local therapies are variably utilized and include intratumoral injection, laser therapy, cryotherapy, microwave coagulation therapy, hepatic arterial infusion (HAI), intraarterial radiotherapy with yttrium-90 and conformal external radiotherapy.

Systemic Therapy With Sorafenib

Targeted therapy has been employed only for advanced disease [7,44,45]. A multitude of targeted therapies have been investigated for use in HCC; however, only sorafenib is approved for use in Asian and Western countries. These approvals were based on improved survival in the SHARP trial and the parallel Asian phase III trial [6,49]. Although sorafenib has been approved in Asia, the agent is not widely used largely due to cost [50]. Cost-sharing programs have been started in some countries to manage this issue. Such programs have been successful in that they expand usage; however, lack of long-term coverage renders the practice unsustainable.

In addition to cost, emerging evidence suggests that sorafenib may be less well tolerated by Asian patients compared to Western patients. Hand-foot skin reaction (HFSR) appears to be more frequent in Asians, particularly lower-grade reactions. Hand-foot skin reaction (all grades) occurred in 21% of patients in the US SHARP study; the rate was 45% in the Asian phase III sorafenib trial [6,46]. Grade 3 event rates were 8% in SHARP compared with 11% in the Asian trial. Korean and Japanese studies have reported rates of 56%-57% (all grades) [51,52]. In the Korean population, HFSR was the most common reason for treatment interruption. Indeed, dose reductions for HFSR were more frequent in the Asian phase III trial (11%) than in SHARP (5%) [6,46] The panelists noted that in practice, dose reduction or use of a reduced starting dose of sorafenib is common in Asia. Lower dosing is being investigated in small Asian trials. In a Japanese phase I study, sorafenib 200 mg twice daily led to a 38% incidence of HFSR [52].

Though HFSR is most common, some differences between Westerners and Asians may be present with respect to the drug's effect on the liver. The Korean population experienced a 4% rate of grade 3 or 4 hyperbilirubinemia associated with marked ALT elevations [51]. Individual differences in drug metabolism may be present. Increased bilirubin was reported separately in a patient with UGT1A1 polymorphism; the authors proposed that sorafenib inhibition of UGT1A1 in this patient may have contributed to the hyperbilirubinemia [53].

Other Systemic Therapies

Systemic cytotoxic chemotherapy has failed to prolong survival in advanced HCC [5]. Small studies of cytotoxic chemotherapy plus biochemical modulation may achieve tumor control in patients with good performance status and liver function reserves and no hypersplenism [54-56]. In Korea, chemotherapy is used as part of concurrent chemoradiotherapy protocols at some centers. In Hong Kong, systemic cytotoxic chemotherapy is considered when a patient fails or is ineligible for anti-VEGF therapy. Chemotherapy was not recommended in Japanese treatment guidelines.

In China, use of traditional Chinese medicine (TCM) is common and unique compared to Western nations. These medicines can be categorized according to two main purposes: 1) promoting liver health and delaying cirrhosis and 2) countering the side effects of chemotherapy. Panelists indicated that the first type of TCM must be allowed in clinical trials; excluding these treatments would severely restrict enrollment. However, the second type of TCM could potentially be excluded if required.

Investigational Targeted Therapy

Targeted agents are at the forefront of HCC clinical research. Promoting clinical trial participation in Asia is important to foster development of new drugs appropriate for this population. Recently completed phase II trials of new treatments are described below and ongoing phase II and III trials of targeted therapies in HCC are reviewed in Table 1.

The combination of sorafenib and chemotherapy has been investigated in phase II trials. A randomized phase II trial found superior outcomes with the combination of sorafenib plus doxorubicin compared to placebo plus doxorubicin [57]. Median progression-free and overall survival times were 6.9 months and 13.8 months in the sorafenib arm compared to 2.8 months and 6.5 months in the placebo arm, respectively. The combination was associated with a 21% incidence of left ventricular dysfunction, though mostly of grade 1 or 2 severity. The SECOX trial evaluated sorafenib plus capecitabine and oxaliplatin [58]. Response was observed in 14% with stable disease in 61%. Median time to progression (TTP) was 7.1 months and median survival was 10.2 months. Toxicities included HFSR, diarrhea, and neutropenia. When sorafenib was paired with metronomic tegafur/uracil (UFT; 125 mg/m^2 twice daily), the combination led to overall response and stable disease rates of 6% and 51%, respectively [59]. Median progression-free survival was 3.7 months and median survival was 7.4 months. The most common grade 3 or 4 adverse events were fatigue (15%), HFSR (9%), and bleeding (8%).

Sunitinib has been evaluated at various doses and schedules. The SAKK 77/06 trial utilized sunitinib 37.5 mg/day continuously in 45 Swiss patients [60]. Median progression-free survival (PFS) was 2.8 months and median survival was 9.3 months. The most frequent grade 3/4 toxicities were fatigue in 24% and thrombocytopenia in 18%. Two US studies evaluated sunitinib 37.5 mg daily for 4 weeks every 6 weeks [61,62]. Response rates were 3%-6% and stable disease rates were 35%-47%. One study reported PFS and survival; median PFS was 4.0 months and median survival was 9.9 months.

Table 1 Ongoing Phase II/III Trials in Advanced HCC

Study Name Clinicaltrials.gov Identifier	Phase	Intervention	Setting	Location
Advanced Disease				
Targeted Agents With Cytotoxic Therapy				
NCT00832637	II	Erlotinib + gemcitabine + oxaliplatin	Prior systemic therapy allowed	US
HOG GI06-101 NCT00532441	II	Erlotinib + docetaxel	Third-line or less	US
NCT00384800	II	Thalidomide + tegafur/uracil	No prior chemotherapy	Taiwan
NCT00519688	II	Thalidomide + tegafur/uracil	No prior chemotherapy	Taiwan
NCT00862082	I/II	Sorafenib + PR104 Sorafenib	First-line	US, Asia
Anti-VEGF Agents as Monotherapy				
BRISK NCT00858871	III	Brivanib + placebo Sorafenib + placebo	First-line	International
NCT00825955	III	Brivanib + placebo BSC + placebo	Sorafenib failure	International
NCT00699374	III	Sunitinib Sorafenib	First-line	International
NCT00247676	II	Sunitinib	First-line	France, Korea, Taiwan
Other Targeted Agents as Monotherapy				
NCT00225290	III	Thalidomide Placebo	Any line Poor liver reserve	Taiwan
NCT00033462	II	Erlotinib	First- or second-line	US
NCT00077441	II	Bortezomib	First-line	US, Australia, Korea, HK
NCT00390195	I/II	Everolimus (weekly or daily)	Any line	Taiwan
NCT00920192	I/II	Foretinib	Any line	Taiwan, HK
Combination Targeted Therapy				
SEARCH NCT00901901	III	Sorafenib + erlotinib Sorafenib	First-line	International
NCT00881751	II	Erlotinib + bevacizumab Sorafenib	First-line	US
NCT00365391	II	Erlotinib + bevacizumab	First- or second-line	US
TCOGP-1209 NCT00971126	I/II	Thalidomide + sorafenib	First-line	Taiwan
NCT00828594	I/II	Everolimus + sorafenib Placebo + sorafenib	First-line	International
NCT00791544	I/II	AVE1642* +/- sorafenib or erlotinib	Any line	France
Earlier-stage Disease				
STORM NCT00692770	III	Sorafenib Placebo	Adjuvant (post-resection or -local ablation)	International
BRISK-TA NCT00908752	III	Brivanib + TACE Placebo + TACE	BCLC B	International
NCT00921531	III	Thalidomide + TACE TACE	BCLC A-B	China
NCT00728078	II/III	Thalidomide, low dose	Adjuvant (post-RFA)	China
START NCT00990860	II	Sorafenib + TACE	BCLC B	Taiwan
NCT00855218	II	Sorafenib + TACE Placebo + TACE	BCLC B	International
COTSUN NCT00919009	II	Sorafenib + TACE	TNM III/IVa	Korea
NCT00576199	II	Bevacizumab	Pre- and Post-TACE	HK

Table 1 Ongoing Phase II/III Trials in Advanced HCC *(Continued)*

JLOG 0901 NCT00933816	I/II	Sorafenib + fluorouracil/platinum HAI	Not suitable for resection, ablation, TACE	Japan
NCT00293436	I/II	Erlotinib + celecoxib	Adjuvant (post-resection, -TACE, or -RFA), high-risk	US

BSC, best supportive care; ECOG, Eastern Cooperative Oncology Group; HAI, hepatic arterial infusion; HK, Hong Kong; HOG, Hoosier Oncology Group; JLOG, Japan Liver Oncology Group; RFA, radiofrequency ablation; TACE, transarterial chemoembolization; US, United States; VEGF, vascular endothelial growth factor
*Anti-insulin-like growth factor receptor-1 monoclonal antibody

The most common grade 3/4 toxicities were fatigue and elevated liver function tests. A study in Europe and Asia that evaluated high-dose sunitinib (50 mg daily for 4 weeks every 6 weeks) found similar response and stable disease rates but higher toxicity with four grade 5 events [63].

Other multiple receptor tyrosine kinase inhibitors that target VEGF under investigation include brivanib, linifanib (formerly ABT-869), vandetanib, and pazopanib. Brivanib inhibits VEGF and fibroblast growth factor; a phase II trial showed median survival of 10 months in treatment-naive patients [64] and a 58% stable disease rate in patients who failed one prior antiangiogenic therapy [65]. The most frequent grade 3/4 toxicities were hyponatremia (41%), fatigue (16%), and AST elevation (19%) [64]. Linifanib inhibits VEGF and PDGF receptor tyrosine kinases. A phase II study (n = 44; 84% treatment-naïve) showed a response rate of 7%, median PFS of 3.7 months and median survival of 9.3 months [66]. Toxicities are consistent with anti-VEGF agents. A phase II, placebo-controlled study of vandetanib, which targets VEGFR, EGFR, and RET signaling, showed activity in HCC but failed to meet its primary endpoint of tumor stabilization in a Taiwanese trial [67]. A phase I dose-ranging study of pazopanib, which inhibits VEGF, PDGF, and c-kit, showed evidence of activity [68].

Phase II trials of erlotinib plus bevacizumab are promising. In 16 previously untreated patients, the combination led to a median TTP of 2.3 months and median survival of 13.7 months [69]. In 40 patients, 73% of whom were previously untreated, the response rate was 25%, median PFS was 9.0 months, and median survival was 15.7 months [70]. In 58 patients, 76% of whom were previously untreated, median PFS times were 8.8 months in patients with no prior therapy, 7.9 months in patients previously treated with sorafenib, and 6.6 months in those previously treated with therapy other than sorafenib [71]. Corresponding median survival times were 15.6 months, 13.3 months, and 14.4 months. In all studies, adverse events were consistent with the individual drug profiles.

Asian Panel Opinions on Clinical Trial Design

In 2008, the American Association for the Study of Liver Diseases (AASLD) published a framework for clinical trial design in HCC [7]. During the current expert panel meeting, participants provided their views about clinical trial design from an Asian perspective. These views are outlined in Table 2.

The Asian panel also provided additional insights into clinical trial issues specific to disease stage. The panel noted a great need for trials in resectable disease. The panel felt that testing compounds in the adjuvant setting before establishing efficacy in the metastatic setting is possible, citing positive phase II adjuvant results with muparfostat (formerly PI-88) [72] and noting the need for effective therapies in this setting. The panel also expressed interest in chemoprevention with sorafenib and other agents after resection or local ablation. In unresectable disease, especially where locoregional therapy is indicated, placebo-controlled trials remain feasible, though the panel acknowledged opportunities are limited. In this setting, it may be beneficial to limit enrollment to patients who experience a maximal response after TACE based on modified EASL criteria [73]. Such a requirement would facilitate identification of subsequent disease progression across patients. However, additional research is necessary to identify the best clinical endpoints in this setting. Because it remains difficult to differentiate recurrent disease from a second primary cancer, time to development of a new lesion may be an appropriate outcome in this setting. Finally, in the advanced/metastatic setting, the panel felt that developing new agents in the second-line setting is warranted.

Summary

Hepatocellular carcinoma is a disease of variable incidence and etiology that is managed differently worldwide. This expert panel has identified key areas that need to be addressed to facilitate clinical trials in Asia. Stratification by viral etiology is desirable within Asia and by region in global trials. Antiviral therapy should also be considered as a stratification factor and incorporated into HCC management in trials. The panel agreed with AASLD that histological diagnosis is not required for trial entry. Staging and treatment plans vary significantly. The panel felt BCLC staging is acceptable for trials as long as portal vein hypertension can be measured and defined with non-invasive standardized