**Bortezomib Sensitizes HCC Cells to CS-1008, an Antihuman Death Receptor 5 Antibody, through the Inhibition of CIP2A**

Kuen-Feng Chen\(^1,4\), Hui-Chuan Yu\(^1,4\), Chun-Yu Liu\(^5,6,7\), Hui-Ju Chen\(^1,4\), Yi-Ching Chen\(^1,4\), Duen-Ren Hou\(^8\), Pei-Jer Chen\(^1,4\), and Ann-Lii Cheng\(^2,3,4\)

**Abstract**

Previously, we have shown that bortezomib overcame TRAIL resistance in hepatocellular carcinoma (HCC) cells via the inhibition of Akt. Here, we report that bortezomib sensitizes these TRAIL-resistant cells, including Huh-7, Hep3B, and Sk-Hep1, to CS-1008, a humanized agonistic antihuman death receptor 5 antibody. Cancerous inhibitor of protein phosphatase 2A (CIP2A) mediated the sensitizing effect of bortezomib to CS-1008 through inhibiting protein phosphatase 2A (PP2A) activity. Combination treatment of bortezomib and CS-1008 downregulated CIP2A in a concentration- and time-dependent manner, and increased PP2A activity in HCC cells. Importantly, ectopic expression of CIP2A decreased Akt-related PP2A activity, indicating that CIP2A negatively regulates Akt-related PP2A activity in HCC cells. Moreover, silencing CIP2A by short interfering RNA enhanced CS-1008–induced apoptosis in HCC cells and ectopic expression of CIP2A in HCC cells abolished CS-1008–induced apoptosis, indicating that CIP2A plays an important role in the sensitizing effect of bortezomib to CS-1008. Finally, our in vivo data showed that CS-1008 and bortezomib combination treatment decreased tumor growth significantly. In conclusion, bortezomib sensitized HCC cells to CS-1008 through the inhibition of CIP2A.

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**Introduction**

Hepatocellular carcinoma (HCC) is the fifth most common solid tumor worldwide and remains a difficult malignancy to treat (1). Major obstacles hindering effective treatment for HCC include high recurrence after curative resection, underlying cirrhotic liver, and, importantly, the frequent resistance of advanced HCC to conventional chemotherapy and radiotherapy (2). The fact that most patients with advanced HCC do not respond well to current chemotherapeutic agents highlights the need for the development of novel targeted therapy for HCC (3). And, the promising trial result shown by the multikinase inhibitor sorafenib in patients with advanced HCC showed the potential of molecular-targeted therapy for advanced HCC (4).

Targeting apoptotic pathway is the foundation of many anticancer strategies. Among the possibilities, the pathway involving TNF-related apoptosis inducing ligand (TRAIL) is currently the most promising, as preclinical models suggest that apoptosis of tumor cells is achievable in vivo without lethal toxicities (5). TRAIL is a type II transmembrane protein that belongs to the TNF superfamily and functions through binding to death receptors (DR; ref. 6). Currently, 5 DRs that can be subdivided into 2 distinctive functional groups are known to bind to TRAIL (7, 8). DR4 (TRAIL-R1) and DR5 (TRAIL-R2) contain an effective cytoplasmic death domain that forms death-inducing signaling complex (DISC) on ligand binding and transduce proapoptotic signals (7, 8). The binding of TRAIL to DR4 or DR5 results in trimerization of the death domain-containing receptor, leading to the formation of a multiprotein complex designated the DISC, which involves participation of an adaptor molecule, Fas-associated protein with death domain (FADD) and activation of the initiator caspase-8.

CS-1008, a novel humanized agonistic antihuman DR5 antibody, was modified from a murine antihuman DR5 mAb, TRA-8 (9). CS-1008 has shown selective cytotoxicity toward tumor cells expressing DR5, such as colorectal adenocarcinoma cells, non–small-cell lung cancer cells, pancreatic carcinoma cells, and renal cell adenocarcinoma and has shown enhanced antitumor activity when in combination with gemcitabine or docetaxel (9). In a phase I trial, CS-1008 exerted no dose limiting toxicity at doses of up to 8 mg/kg weekly (10). CS-1008 is currently...
undergoing phase II trials in combination with sorafenib for the treatment of advanced HCC (NCI clinical trial: NCT01033240).

Despite reports of selective TRAIL-induced apoptosis in several types of tumor cells, many cancer cells are resistant to apoptosis induction by TRAIL and more and more evidence suggest that TRAIL alone may not be sufficient to efficiently induce apoptosis in many types of cancers, including HCC (11–14). Therefore, it is important both to understand the mechanisms underlying resistance and improve the potency of TRAIL-based therapeutic approaches, if TRAIL is going to be successfully used for cancer therapy. Proteasome inhibitors represent a highly promising class of anticancer agents to overcome TRAIL resistance or resensitize tumors to the apoptotic effect of TRAIL.

Bortezomib is the first proteasome inhibitor to be approved clinically for multiple myeloma andmantel cell lymphoma (15, 16). Bortezomib showed excellent antitumor activity against these 2 hematologic malignancies through blocking proteasome degradation of IkBα, an inhibitor of NF-κB (16). The fact that multiple cellular targets are affected by bortezomib suggests its potential advantage in enhancing multitumor activities in combination treatment with TRAIL in HCC. Indeed, in our previous study, exploring the antitumor activity of bortezomib against HCC cells (17), we showed that downregulation of p-Akt determines the sensitivity of bortezomib, and that proteasome inhibition is sufficient to efficiently induce apoptosis in many types of tumor cells, many cancer cells are resistant to apoptosis induction by TRAIL and more and more evidence suggest that TRAIL alone may not be sufficient to efficiently induce apoptosis in many types of cancers, including HCC (11–14). Therefore, it is important both to understand the mechanisms underlying resistance and improve the potency of TRAIL-based therapeutic approaches, if TRAIL is going to be successfully used for cancer therapy. Proteasome inhibitors represent a highly promising class of anticancer agents to overcome TRAIL resistance or resensitize tumors to the apoptotic effect of TRAIL.

Bortezomib sensitizes HCC cells to TRAIL through inhibition of p-Akt (14). Importantly, we found that protein phosphatase 2A (PP2A), a phosphatase which downregulates p-Akt, may mediate the effect of bortezomib on TRAIL sensitization (14). The mechanism by which bortezomib upregulates PP2A and, in turn, downregulates p-Akt was further delineated in our recent study on the synergistic interaction between bortezomib and sorafenib in HCC cells (18).

PP2A is a complex of serine/threonine protein phosphatase with broad substrate specificity and diverse cellular functions and consists of a dimeric core enzyme comprising of structural A and catalytic C subunits (the AC core enzyme), and heterogeneous regulatory B subunits (19, 20). It is known that there are several cellular inhibitors of PP2A, including SET (21) and cancerous inhibitor of PP2A (CIP2A; ref. 22). Notably, CIP2A (KIAA1524, P90), first cloned from patients with HCC (23), has been shown to promote anchorage-independent cell growth and in vivo tumor formation by inhibiting PP2A activity toward c-myc (22). Moreover, CIP2A is overexpressed in HCC and several other human malignancies (22, 24, 25) and is associated with clinical aggressiveness in human breast cancer (24), suggesting its role as an oncoprotein.

In this study, we found that bortezomib sensitizes TRAIL-resistant HCC cells to CS-1008 through the mediator CIP2A. We showed that CIP2A, through inhibition of PP2A-dependent p-Akt activity, mediates the sensitizing effect of bortezomib to CS-1008. The combination of bortezomib and CS-1008 downregulated CIP2A and, in turn, increased Akt-related PP2A activity in these HCC cells.

Materials and Methods

Reagents and antibodies

Bortezomib (Velcade) and CS-1008 were kindly provided by Millennium Pharmaceuticals and Daiichi Sankyo Pharmaceuticals, respectively. For in vitro studies, bortezomib or CS-1008 at various concentrations was dissolved in dimethyl sulfoxide (DMSO) and then added to cells in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 5% FBS. The final DMSO concentration was 0.1% after addition to the medium. Antibodies for immunoblotting such as anti-Akt1, anti-PARP, and anti–PP2A-C, were purchased from Santa Cruz Biotechnology. Other antibodies including anti–caspase-3, anti-CIP2A, and anti-p-Akt (Ser473) were from Cell Signaling.

Cell culture and Western blot analysis

The Sk-Hep1 and Hep3B cell lines were obtained from the American Type Culture Collection. The Huh-7 HCC cell line was obtained from the Health Science Research Resources Bank (Osaka, Japan; JCRB0403). All cells obtained from the American Type Culture Collection or the Health Science Research Resources Bank were immediately expanded and frozen down such that all cell lines could be restarted every 3 months from a frozen vial of the same batch of cells. No further authentication was done in our lab. Cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin G, 100 µg/mL streptomycin sulfate, and 25 µg/mL amphotericin B in a 37°C humidified incubator and an atmosphere of 5% CO2 in air. Lysates of HCC cells treated with drugs at the indicated concentrations for various periods of time were prepared for immunoblotting of caspase-3, PARP, p-Akt, and Akt. Western blot analysis was done as previously reported (17).

Apoptosis analysis

The following 3 methods were used to assess drug-induced apoptotic cell death: detection of DNA fragmentation with the Cell Death Detection ELISA kit (Roche Diagnostics), Western blot analysis of caspase activation and PARP cleavage, and measurement of apoptotic cells by flow cytometry (sub-G1). ELISA was conducted according to the manufacturer’s instructions (8).

Gene knockdown using short interfering RNA

Smartpool short-interfering RNA (siRNA) reagents, including a control (D-001810-10), and CIP2A (L-014135-01) were all purchased from Dharmacon and used according to the procedure described previously (17). Briefly, cells were transfected with siRNA (final concentration, 100 nmol/L) in 6-well plates using the DharmaFECT4 transfection reagent (Dharmacon), according to

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the manufacturer’s instructions. After 48 hours, the medium was replaced and the HCC cells were incubated with bortezomib, harvested, and separated for Western blot analysis and for apoptosis analysis by flow cytometry as described previously (17).

**Sk-Hep1 with constitutive active CIP2A**

CIP2A cDNA (KIAA1524) was purchased from OriGene (RC219918). Briefly, following transfection, cells were incubated in the presence of G418 (0.78 mg/mL). After 8 weeks of selection, surviving colonies, that is, those arising from stably transfected cells, were selected and individually amplified. Sk-Hep1 cells, with stable expression of CIP2A-myc, were then treated with bortezomib, harvested, and processed for Western blot analysis as described previously.

**Coimmunoprecipitation assay**

Cells were harvested and lysed on ice for 30 minutes in lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl, 0.5% Nonidet P-40, 1 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L Na₃VO₄, 5 mmol/L sodium pyrophosphate, and a protease inhibitor tablet). The cell lysates were centrifuged at 14,000 × g for 15 minutes, and the supernatants were recovered. Supernatants containing equal amounts of proteins were incubated with 2.5 mg of primary antibodies overnight at 4°C. The immunoprecipitates were harvested using protein G PLUS-agarose beads (Santa Cruz Biotechnology) that were washed once with regular washing buffer (50 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl, 1 mmol/L EDTA, and 0.5% Nonidet P-40), twice with high salt washing buffer (50 mmol/L Tris-HCl, 500 mmol/L NaCl, 1 mmol/L EDTA, and 0.5% Nonidet P-40), and once again with regular washing buffer. Immunoprecipitates were then eluted by boiling the beads for 5 minutes in SDS-PAGE sample buffer and characterized by Western blotting with appropriate antibodies.

**PP2A phosphatase activity**

The protein phosphatase activity in total cellular lysate was determined by measuring the generation of free phosphate from threonine phosphopeptide using the malachite green–phosphate complex assay as described by the manufacturer (Upstate Biotechnology). Cell lysates were prepared in a low-detergent lysis buffer (1% Nonidet P-40, 10 mmol/L HEPES, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L benzamidine, and 10 g/mL leupeptin). The phosphatase assay was done in a PP2A-specific reaction buffer (Upstate) containing 750 μmol/L phosphopeptide substrate. After 10 minutes of incubation at 30°C, the malachite dye was added, and free phosphate was measured by optical density at 650 nm. To avoid variability due to differences in the amounts of immunoprecipitated protein between samples, the phosphatase activities were normalized to the amount of PP2A immunoprecipitated, as detected and quantified by immunoblot analysis for each treatment group.

**Immunofluorescent staining**

Sk-Hep1 cells (wild-type or CIP2A-myc) were seeded on sterilized slides in a 10-cm dish overnight and then treated with DMSO or okadaic acid (wild-type) or forskolin (CIP2A-myc) for 24 hours. The cells were washed with PBS, fixed with 4% formaldehyde for 10 minutes at 37°C, and then washed with PBS twice. Cells were treated with 0.1% Triton X-100 and then blocked with 0.5% bovine serum albumin (BSA) in PBS at 37°C for 1 hour. Then cells were treated with anti-CIP2A or anti-p-Akt antibody (1:50 in PBS containing 0.5% BSA) at 4°C overnight. Fluorescein isothiocyanate–conjugated goat anti-mouse IgG (CIP2A; 1:1,000) or anti-rabbit IgG (p-Akt; 1:1,000) was applied for 1 hour at room temperature. The cells were observed under a fluorescence microscope (Leica DM2500).

**Xenograft tumor growth**

Male NCr athymic nude mice (5–7 weeks of age) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The mice were housed in groups and maintained under standard laboratory conditions on a 12-hour light-dark cycle. They were given access to sterilized food and water ad libitum. All experimental procedures using these mice were conducted in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of National Taiwan University. Each mouse was inoculated subcutaneously in the dorsal flank, with 1 × 10⁶ HCC cells suspended in 0.1 mL of serum-free medium containing 50% Matrigel (BD Biosciences). When tumors reached 150 to 200 mm³, mice were randomized divided into 4 groups (n = 8) and received an intraperitoneal injection of bortezomib (0.5 mg/kg body weight) twice weekly and/or intravenous injection of CS-1008 200 μg 3 times a week. Controls received vehicle consisting of 0.5% methylcellulose and 0.1% polysorbate 80 in sterile water. Data are representative of 3 independent experiments.

**Statistical analysis**

Tumor growth data points are reported as mean tumor volume ± SE. Comparisons of mean values were done using the independent samples t test in SPSS for Windows 11.5 software (SPSS Inc.).

**Results**

**Bortezomib enhances CS-1008–induced apoptosis in HCC cells**

To investigate the antitumor effect of CS-1008 on HCC cells, we first examined the apoptotic effect of CS-1008 in a panel of 3 human HCC cell lines, Huh-7, Sk-Hep1, and Hep3B at the clinical relevant concentrations. Apoptotic cells (sub-G₁) were determined by flow cytometry after 24 hours treatment. As shown in Fig. 1, HCC cells were quite resistant to CS-1008 and treatment with CS-1008 alone could not efficiently induce apoptosis in these HCC cells,
even up to a concentration of 800 ng/mL. However, combining bortezomib at 50 nmol/L with CS-1008 overcame the resistance and sensitized cells to apoptosis significantly in a concentration-dependent manner, starting from CS-1008 at a concentration of 200 ng/mL. We then examined the effect of a fixed concentration of CS-1008 on escalating concentrations of bortezomib and found that CS-1008 at a dose of 200 ng/mL increased
bortezomib-induced apoptosis in a concentration-dependent manner (Fig. 1B).

We further examined these apoptotic effects using an ELISA kit which detects DNA fragmentation. Our data showed that combination of CS-1008 and bortezomib increased DNA fragmentation in a dose- and time-dependent manner (Fig. 1C). These results indicate that a combination of CS-1008 and bortezomib overcame the resistance of HCC cells to the TRAIL receptor antibody, CS-1008.

Downregulation of CIP2A is associated with sensitizing effects of bortezomib in HCC cells

Our previous data suggested that the sensitizing effect of bortezomib on TRAIL resistance of HCC cells was mediated by the PP2A-Akt signaling pathway (14). Because CIP2A inhibits PP2A, we further hypothesized that inhibition of CIP2A may be associated with the sensitizing effect of bortezomib on CS-1008–induced apoptosis in HCC cells. As shown in Fig. 2A, combined treatment of bortezomib and CS-1008 reduced protein levels of CIP2A, in correlation with downregulation of p-Akt expression and induced apoptosis in all tested HCC cells. Evidence for the association between CIP2A inhibition and apoptosis induction was further strengthened by the time-dependent PARP cleavage in the combined bortezomib- and CS-1008–treated HCC cells (Fig. 2B). These results suggest that inhibition of CIP2A plays a key role in the mediating effects of the drug combination.

Target validation of CIP2A

Two different approaches were used to validate the role of CIP2A in the sensitizing effect of bortezomib on CS-1008–induced apoptosis in HCC cells. First, we knocked down protein expression of CIP2A by using siRNA. Sk-Hep1 cells were transfected with either scrambled siRNA as a control or CIP2A siRNA for 48 hours and were then exposed to DMSO or CS-1008 (200 ng/mL) for 24 hours. As shown in Fig. 3A, depletion of CIP2A by siRNA significantly reduced the resistance of CS-1008 in Sk-Hep1 cells in tandem with downregulation of p-Akt (Ser473) and enhanced apoptotic cell death. Notably, knockdown of CIP2A alone or in combination with CS-1008 showed similar effects on HCC cells.
downregulation of p-Akt, suggesting that adding CS-1008 does not enhance the effect of CIP2A on p-Akt. Next, we generated Sk-Hep1 (CIP2A-myc) cells with stably expressed CIP2A to investigate the sensitizing effect of bortezomib. Ectopic expression of CIP2A significantly abolished the sensitizing effect of bortezomib on CS-1008-induced apoptosis in comparison with wild-type Sk-Hep1 cells that underwent the same treatment \((P < 0.05; \text{Fig. 3B})\). Together, these results validated the importance of CIP2A inhibition in mediating the effect of bortezomib on CS-1008 sensitivity to HCC cells.

CIP2A inhibits Akt-associated PP2A activity

Our previous work showed that bortezomib increased PP2A activity in HCC cells \((14, 18)\). We, thus, next examined how bortezomib and CS-1008 combination treatment affected PP2A activity. As shown in Fig. 4A, bortezomib plus CS-1008 significantly upregulated PP2A activity in Sk-Hep1 and Huh-7 cells. Moreover, we found that treatment with either of the drugs alone or in combination did not significantly affect the protein level of PP2A complex including subunit A, B55, and C in Sk-Hep1 and HeP3B cells, except the drugs combination decreased PP2A-B55y in Huh-7 cells \((\text{Fig. 4B})\). These data suggested that the alterations of PP2A complex may not play a major role in mediating the sensitizing effect of bortezomib on CS-1008–treated HCC cells. Notably, CS-1008 and bortezomib combination treatment, compared with either drug alone, did not alter protein–protein interactions between Akt and PP2A significantly, suggesting that CIP2A does not affect their binding affinity directly \((\text{Fig. 4C})\). In addition, previous study has indicated that CIP2A inhibits c-myc–associated PP2A activity thereby stabilizing c-myc \((22)\). We, therefore, similarly examined Akt-related PP2A activity by coimmunoprecipitation of Akt in Sk-Hep1 cells. Our data indicated that ectopic expression of CIP2A reduced PP2A activity on Akt, suggesting that CIP2A plays a role in regulating Akt-related PP2A activity \((\text{Fig. 4D, left})\). In addition, the data of immunofluorescence staining also showed that the CIP2A-myc cells had higher p-Akt than the wild-type cells \((\text{Fig. 4D, right})\). The treatment of okadaic acid \((\text{a PP2A inhibitor})\) in wild-type cells enhanced the expression of p-Akt, whereas forskolin \((\text{a PP2A agonist})\) reduced the expression of p-Akt in CIP2A-myc cells \((\text{Fig. 4D, right})\). These data indicate that CIP2A reduces the PP2A activity on Akt in HCC cells.

Effect of bortezomib on HCC xenograft tumor

To confirm whether the sensitizing effect of bortezomib on CS-1008 has potentially relevant clinical implications, we assessed the in vivo effect of bortezomib and CS-1008 on Huh-7 xenograft tumors. Tumor-bearing mice were treated with vehicle or bortezomib at 0.5 mg/kg i.p. twice a week and/or CS-1008 200 \(\mu\)g 3 times a week for the duration of the in vivo experiment. Throughout the course of treatment, all mice tolerated the treatments well without observable signs of toxicity and had stable body weights. No gross pathologic abnormalities were noted at necropsy. As shown in Fig. 5A, tumor growth was significantly inhibited by treatment with bortezomib plus CS-1008 for 2 weeks \((\text{vs. control, } P < 0.05)\), and tumor size in the treatment group was only half that in the control group at the end of the experiment.
study. Treatment with CS-1008 alone had no significant effect on Huh-7 tumor growth (vs. control, $P > 0.05$) and bortezomib alone showed modest effect. Moreover, as shown in Fig. 5B, CIP2A protein levels decreased significantly in Huh-7 tumors treated with bortezomib in combination with CS-1008. Finally, an analysis of the effect of bortezomib on PP2A activity (Fig. 5C) showed a significant increase in PP2A phosphatase activity ($P < 0.05$) in Huh-7 tumors treated with bortezomib alone and bortezomib plus CS-1008. However, the treatment of CS-1008 alone did not show significant effects on CIP2A and the PP2A activity (vs. control, $P > 0.05$), which is consistent
with previous in vitro findings (Fig. 2A and Fig. 3A). These data indicate mediation of the effect via PP2A-dependent Akt inactivation in vivo. Further clinical investigation is warranted.

Discussion

In this study, we confirmed that CS-1008, a novel anti-human DR5 antibody with promising TRAIL-like anticancer activity, is not sufficient to kill HCC cells as a monotherapy. However, we found that bortezomib effectively sensitizes HCC cells to this novel anti-DR5 antibody. Importantly, we delineated the mechanism by which bortezomib sensitizes these resistant cells to CS-1008. Our results have several important implications. First, CIP2A may serve as a potential drug target in HCC. We identified CIP2A inhibition as a major determinant of sensitizing effects of bortezomib which is dissociated from proteasome inhibition. This suggests that other novel agents that are capable of downregulating CIP2A protein expression may also be good sensitizers of TRAIL-resistant cells. Indeed, knockdown of CIP2A by siRNA was able to restore CS-1008–induced apoptosis in resistant Sk-Hep1 cells (Fig. 3A). Moreover, Sk-Hep1 cells with ectopic overexpression of CIP2A were more resistant to the effect of bortezomib and CS-1008 combination treatment (Fig. 3B). As an oncoprotein, CIP2A may promote cancer cell aggressiveness, thereby facilitating resistance of cancer cells to apoptotic-inducing agents. Currently, there are no known specific CIP2A inhibitors and whether CIP2A is suitable as predictive markers for drug therapy remain to be determined. Future preclinical research exploring agents that inhibit CIP2A and clinicopathologic studies correlating CIP2A expression and drug sensitivity in HCC patients are warranted to further consolidate CIP2A as a good drug target. Notably, the data from a phase I study in human indicate that CS-1008 is well tolerated and the $c_{\text{min}}$ of CS-1008 is 14 mg/mL (1 mg/kg/wk; ref. 26).

Various mechanisms contribute to TRAIL resistance in cancers (12). Resistance to TRAIL can occur at any step in the apoptosis signaling cascade. For example, receptor level mutations or overexpression of DR4 or DR5 can lead to resistance (12). Defects in members participating in DISC assembly, such as FADD, caspase-8, and cellular FADD-like interleukin-1β-converting enzyme inhibitory protein can also result in resistance to TRAIL (27, 28). Furthermore, overexpression of antiapoptotic Bcl-2 family proteins (Bcl-2, Bcl-XL, Mcl-1, etc; refs. 29, 30), loss of proapoptotic protein function (Bax or Bak), and an inability to activate mitochondria during apoptosis, such as reduced release of mitochondria-derived activator of caspases (SMAC/DIABLO), have been shown to cause TRAIL resistance in mitochondria-dependent type II cancer cells (12, 31). Finally, aberrantly activated antiapoptotic pathways in various tumor cells, such as phosphoinositol-3-kinase (PI3K)/Akt signaling, mitogen-activated protein kinases pathway and NF-kB may contribute to development of TRAIL resistance (12, 32, 33). Interestingly, we discovered that through
CIP2A-PP2A-p-Akt regulatory mechanism, bortezomib overcomes CS-1008 resistance, supporting the hypothesis that constitutively active Akt signaling confers resistance of HCC cells to TRAIL and anti-DR5 CS-1008.

Previous literature, including our own work, has shown that bortezomib and other investigational proteasome inhibitors are capable of sensitizing cancer cells to TRAIL-induced apoptosis (14, 34–38). However, the molecular mechanisms underlying the sensitizing effect of bortezomib seem to be complicated and may be specific to cancer types. In certain cancer types, sensitization to TRAIL resistance has been reported to be through proteasome inhibition, including upregulation of TRAIL receptors (34, 38), activation of both extrinsic and intrinsic apoptosis pathways (39, 40), and probably, many other multiple molecular machineries. For example, Hetschko and colleagues (34) showed that the proteasome inhibitors MG132, via enhancement of transcription and surface expression of DR5, potentiates TRAIL sensitivity and reactivates apoptosis in TRAIL-resistant high-grade gliomas. In addition, our group showed that bortezomib is able to sensitize TRAIL resistance by its proteasome inhibition independent effects in TRAIL-resistant HCC cells. Importantly, despite the fact that TRAIL can activate NF-κB (41, 42) and that bortezomib effectively inhibits NF-κB signaling, it seems that in HCC cells, inhibition of NF-κB may not be the major determinant of TRAIL sensitization by bortezomib as shown by our previous work (14, 17).

Numerous studies have shown that a combination of chemotherapy and targeted agents can enhance the antitumor activity of TRAIL and its agonists through cross talk between the intrinsic and extrinsic apoptotic pathways (8). Among the many novel agents with potential to sensitize or overcome TRAIL resistance, the combination of bortezomib and CS-1008 represents a fascinating strategy for several reasons. Bortezomib is approved for clinical use and exerts particularly tolerable toxicity when combined with other cytotoxic chemotherapeutic agents (such as doxorubicin, melphalan, or vincristine), and even when used in combination with intensive salvage chemotherapy in patients with refractory hematologic malignancies (43, 44). CS-1008, for its part, although still under clinical investigation, has shown an excellent toxicity profile with low hepatic toxicity (9, 10, 45), which is particularly suitable for patients with HCC. In addition, as CS-1008 is DR5 specific, the potency of CS-1008 may not be affected by problems with DR4 mutations or dysfunction. DR5 mutations have been reported to be infrequent in HCC cells (46). However, the exact machinery by which bortezomib downregulates CIP2A is still unknown. It is possible that bortezomib affects the transcription or translation of CIP2A through an as yet unidentified mechanism or affects the degradation of CIP2A at post-translational level. Future work is needed in this area.

In summary, bortezomib sensitizes HCC cells to CS-1008–induced apoptosis through inhibiting a novel oncoprotein phosphatase interactive mechanism, the CIP2A-PP2A-p-Akt framework, and CIP2A may be a potential molecular target for HCC treatment. Moreover, combination of bortezomib, an agent with multiple cellular targets, and a specific anti-DR5 TRAIL agonist (CS-1008) is a promising anti-HCC–targeted therapy that warrants clinical trials. Future studies detailing the clinical role of CIP2A in HCC, and the machinery by which bortezomib affects CIP2A expression may lead to further progress in the development of molecular-targeted therapy for HCC.

Disclosure of Potential Conflicts of Interest
A-L. Cheng is a consultant for Daiichi-Sankyo.

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