Bortezomib sensitizes HCC cells to CS-1008, an anti-human death receptor 5 antibody, through the inhibition of CIP2A

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Abbreviations: HCC, hepatocellular carcinoma; PP2A, protein phosphatase 2A; CIP2A, cancerous inhibitor of PP2A; PI3K, phosphatidylinositol-3-kinase; PDK1, phosphatidylinositol-3-kinase dependent 1; PARP, poly (ADP-ribose) polymerase; DMEM, Dulbecco’s modified Eagle’s medium, FBS, fetal bovine serum; i.p., intraperitoneal; s.c., subcutaneous.
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Dr. Ann-Lii Cheng is a consultant for Daiichi-Sankyo. Other authors have nothing relevant to this manuscript to disclose.
Abstract:

Previously, we have demonstrated 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 anti-human 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 down-regulated 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 small interference 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.
**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 demonstrated by the multikinase inhibitor sorafenib in patients with advanced HCC demonstrated the potential of molecular targeted therapy for advanced HCC (4).

Targeting apoptotic pathway is the foundation of many anti-cancer strategies. Among the possibilities, the pathway involving tumor necrosis factor (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 (DRs) (6). Currently five DRs that can be subdivided into two 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) upon 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 multi-protein complex designated the death-inducing signaling complex (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 anti-human DR5 antibody, was modified from a murine anti-human DR5 mAb, TRA-8 (9). CS-1008 has shown selective cytotoxicity towards tumor cells expressing DR5, such as colorectal adenocarcinoma cells, non-small-cell lung cancer cells, pancreatic carcinoma cells, and renal cell adenocarcinoma and has demonstrated 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 (PIs) represent a highly promising class of anticancer agents to overcome TRAIL resistance or re-sensitize tumors to the apoptotic effect of TRAIL.

Bortezomib is the first PI to be approved clinically for multiple myeloma and mantel cell lymphoma (15, 16). Bortezomib demonstrated excellent anti-tumor activity against these two hematologic malignancies through blocking proteasome degradation of IkB, an inhibitor of NF-κB (16). The fact that multiple cellular targets are affected by bortezomib suggests its potential advantage in enhancing antitumor 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 down-regulation of p-Akt determines bortezomib’s sensitivity, and that bortezomib-induced apoptosis may not be associated with proteasome inhibition in HCC cells. Moreover, we confirmed that bortezomib sensitized HCC cells to TRAIL through inhibition of p-Akt (14). Importantly, we found that protein phosphatase 2A (PP2A), a phosphatase which down-regulates p-Akt, may mediate the effect of bortezomib on TRAIL sensitization (14). The mechanism by which bortezomib upregulates PP2A and in turn down-regulates 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) (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 over-expressed 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 demonstrated 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 down-regulated 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 (Cambridge, MA), and Daiichi Sankyo Pharmaceuticals (Tokyo, Japan), respectively. For *in vitro* studies, bortezomib or CS-1008 at various concentrations was dissolved in DMSO and then added to cells in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal bovine serum (FBS). The final DMSO concentration was 0.1% after addition to the medium. Antibodies for immunoblotting such as anti-Akt1, -PARP and -PP2A-C, were purchased from Santa Cruz Biotechnology (San Diego, CA). Other antibodies including anti-caspase-3, -CIP2A and -P-Akt (Ser473) were from Cell Signaling (Danvers, MA).

*Cell culture and western blot analysis.* The Sk-Hep1, and Hep3B cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The Huh-7 HCC cell line was obtained from the Health Science Research Resources Bank (HSRRB; Osaka, Japan; JCRB0403). All cells obtained from ATCC or HSRRB 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, Akt, etc. Western blot analysis was performed as previously reported (17).

**Apoptosis analysis.** The following three 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 siRNA.** Smartpool siRNA reagents, including a control (D-001810-10), and CIP2A (L-014135-01) were all purchased from Dharmacon (Chicago, IL) and used according to the procedure described previously (17). Briefly, cells were transfected with siRNA (final concentration, 100 nM) in 6-well plates using the Dharma-FECT4 transfection reagent (Dharmacon, Chicago, IL) according to 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; Rockville, MD). Briefly, following transfection, cells were incubated in the presence of G418 (0.78 mg/mL). After 8 weeks of selection, surviving colonies, i.e., 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.
**Co-immunoprecipitation assay.** Cells were harvested and lysed on ice for 30 minutes in lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM Na$_3$VO$_4$, 5 mM 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 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40), twice with high salt washing buffer (50 mM Tris-HCl, 500 mM NaCl, 1 mM 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, Lake Placid, NY). Cell lysates were prepared in a low-detergent lysis buffer (1% Nonidet P-40, 10 mM HEPES, 150 mM NaCl, 10% glycerol, 1 mM PMSF, 5 mM benzamidine, and 10 g/mL leupeptin). The phosphatase assay was performed in a PP2A-specific reaction buffer (Upstate) containing 750 μM 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 h. The cells were washed with PBS, fixed with 4% formaldehyde for 10 min 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 in PBS at 37°C for 1 h. Then cells were treated with anti-CIP2A or anti-P-Akt antibody (1:50 in PBS containing 0.5% bovine serum albumin) at 4°C overnight. FITC-conjugated goat anti-mouse IgG (CIP2A; 1:1000) or anti-rabbit IgG (P-Akt; 1:1000) was applied for 1 h 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 performed in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of National Taiwan University. Each mouse was inoculated s.c. in the dorsal flank with $1 \times 10^6$ HCC cells suspended in 0.1 ml of serum-free medium containing 50% Matrigel (BD Biosciences, Bedford, MA). When tumors reached 150-200 mm³, mice were randomized divided into four groups (N = 8), and received an intra-peritoneal injection of bortezomib (0.5 mg/kg body weight) twice weekly and/or intra-venous injection of CS-1008 200 μg three times a week. Controls
received vehicle consisting of 0.5% methylcellulose and 0.1% polysorbate 80 in sterile water. Data are representative of three independent experiments.

**Statistical analysis.** Tumor growth data points are reported as mean tumor volume ± SE. Comparisons of mean values were performed using the independent samples $t$ test in SPSS for Windows 11.5 software (SPSS, Inc., Chicago, IL).

**Results:**

**Bortezomib enhances CS-1008-induced apoptosis in HCC cells.** To investigate the anti-tumor effect of CS-1008 on HCC cells, we first examined the apoptotic effect of CS-1008 in a panel of three 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 Figure 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 nM 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.

**Down-regulation of CIP2A is associated with bortezomib and CS-1008 combination treatment 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). Since 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 down-regulation 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 small interference RNA (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 another 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 down-regulation of P-Akt (Ser473) and enhanced apoptotic cell death. Notably, knockdown of CIP2A alone or in combination with CS-1008 showed similar effects on down-regulation 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) (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 demonstrated 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 Figure 4A, bortezomib plus CS-1008 significantly up-regulated 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, B56γ and C in Sk-Hep1 and Hep3B cells, except the drugs combination decreased PP2A-B55γ in Huh-7 cells (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 (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 co-immunoprecipitation 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 (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 (Fig. 4D, right). The treatment of okadaic acid (a PP2A inhibitor) in wild type cells enhanced the expression of P-Akt whereas forskolin (a PP2A agonist) reduced the expression of P-Akt in CIP2A-myc cells (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 μg three 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 two weeks (*versus* control, *P* < 0.05) and tumor size in the treatment group was only half that in the control group at the end of the study. Treatment with CS-1008 alone had no significant effect on Huh-7 tumor growth (*versus* 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 the 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 (*versus* control, *P* > 0.05), which is consistent with previous *in vitro* findings (Fig. 2A & 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 anti-cancer 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 bortezomib’s sensitizing effects which is dissociated from proteasome inhibition. This suggests that other novel agents that are capable of down-regulating 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 (Figure 3A). Moreover, Sk-Hep1 cells with ectopic over-expression of CIP2A were more resistant to the effect of bortezomib and CS-1008 combination treatment (Figure 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 pre-clinical research exploring agents that inhibit CIP2A and clinicopathological 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 $14 \mu g/ml$ (1 mg/kg/week) (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-1b-converting enzyme inhibitory protein (cFLIP) can also result in resistance to TRAIL (27, 28). Furthermore, overexpression of anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-XL, Mcl-1, etc) (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 nuclear factor-kappa B (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 demonstrated that bortezomib as well as 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 up-regulation 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 et al. (34) demonstrated 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 demonstrated 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-kB (41, 42) and that bortezomib effectively inhibits NF-kB signaling, it seems that in HCC cells, inhibition of NF-kB may not be the major determinant of TRAIL sensitization by bortezomib, as demonstrated 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 hematological 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 down-regulates 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, as well as the machinery by which bortezomib affects CIP2A expression may lead to further progress in the development of molecular-targeted therapy for HCC.

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**Figure legends**

Fig. 1 Bortezomib enhances CS-1008-induced apoptosis in HCC cells. A, Concentration-escalation effects of CS-1008 with 50 nM bortezomib on apoptosis in 3 TRAIL-resistant HCC cells. B, Concentration-escalation effects of bortezomib with 200 ng/ml CS-1008. HCC cells were exposed to bortezomib and/or CS-1008 at the indicated concentrations in DMEM with 5% FBS in 6-well plates for 24 hr and apoptotic cells were assessed by flow cytometry. Points, mean; bars, SD (n = 3). C, Effects of bortezomib on DNA fragmentation in HCC. Cells were exposed to CS-1008 (200 ng/ml) and/or bortezomib at the indicated concentrations (nM) in DMEM with 5% FBS for 24 hr. Cell lysates were prepare and analyzed for cell death ELISA. Data are representative of three independent experiments. D, The chemical structure of bortezomib.

Fig. 2 Down-regulation of CIP2A is associated with sensitizing effects of bortezomib in HCC cells. A, Effects of bortezomib on protein levels of CIP2A, P-Akt, Akt and Bcl-2 family in HCC cells. HCC cells treated with bortezomib (100 nM) and/or CS-1008 (200 ng/ml) for 24 hours and cell lysates were prepared for western blot. Data are representative of three independent experiments. B, Time-dependent analysis of CIP2A level and apoptotic death with bortezomib and CS-1008 combination treatment.
Sk-Hep1 cells were exposed to bortezomib and/or CS-1008 for the indicated period of time. Cell lysates were prepared and assayed for CIP2A, P-Akt, Akt, and PARP. CF, cleaved form (activated form).

Fig. 3  In vitro target validation of CIP2A. A, Down-regulation of CIP2A by siRNA overcomes the resistance to CS-1008 in Sk-Hep1 cells. Bottom panel, protein levels of CIP2A. Top panel, analysis of apoptotic cells. Cells were transfected with either control SiRNA or CIP2A SiRNA for 48 hours and then exposed to CS-1008 (200 ng/ml) for 24 hours. For analysis of apoptotic cells (subG1), cells were analyzed by flow cytometry. Columns, mean; bars, SD (n = 3). B, Protective effects of ectopic expression of CIP2A on apoptosis induced by the combination of CS-1008 and bortezomib in Sk-Hep1 cells. Right, protein levels of Akt in both wild type and Huh7-Akt cells. WT, wild type. Left, analysis of apoptotic cells. Columns, mean; bars, SD (n = 3). Sk-Hep1 cells were transfected with CIP2A with myc tag and were selected for 8 weeks by G-418. Analysis of apoptotic cells was performed by flow cytometry after cells were exposed to the combination of bortezomib 100 nM and CS-1008 200 ng/ml for 24 hours.

Fig. 4  CIP2A inhibits Akt-associated PP2A activity. A, Analysis of PP2A activity in HCC cells. Columns, mean; bars, SD (n = 3). Cells (Sk-Hep1 or Huh-7) were treated with DMSO or bortezomib (100 nM) and CS-1008 (200 ng/ml) for 24 hours. Cell lysates were prepared for detecting PP2A activity. B, Effects of bortezomib and/or CS-1008 on protein levels of PP2A complex. Cells were exposed to bortezomib (100 nM) and/or CS-1008 (200 ng/ml) for 24 hours. C, Effects of bortezomib on the dynamic PP2A/Akt interactions in
Sk-Hep1 cells. Cells were treated with bortezomib (100 nM) and/or CS-1008 (200 ng/ml) for 24 hours, and cell lysates were immunoprecipitated with anti-PP2A-C antibodies. The immunoprecipitates were probed with Akt1 and PP2A-C by western blot. D, Left, Over-expression of CIP2A decreased Akt-associated PP2A activity. Cell lysates from Sk-Hep1 cells (wild type, or CIP2A-myc) were immunoprecipitated by anti-Akt1 antibodies. Cell lysates were prepared for detecting PP2A activity. Columns, mean; bars, SD (n = 3). Right, CIP2A and P-Akt staining. Top, Sk-Hep1 wild type cells; bottom, CIP2A-overexpression cells. OA, okadaic acid (a PP2A inhibitor). Forskolin (a PP2A agonist). Cells were treated with DMSO or OA (wild type) or FK (CIP2A-myc) for 24 hours then stained with CIP2A (red) or P-Akt (green) and examined under a fluorescence microscope.

Fig. 5 In vivo effect of bortezomib on Huh-7 xenograft nude mice. A, effects of bortezomib and/or CS-1008 on tumor growth. Points, mean (n = 6); bars, SE. Each mouse was inoculated s.c. in the dorsal flank with 1 million Huh-7 cells suspended in Matrigel. When the tumor reached a volume of 200 mm³, mice received treatments of vehicle or 0.5 mg/kg bortezomib i.p. twice weekly and/or CS-1008 200 µg i.v. three times a week for the duration of the study. B, Western blot analysis of CIP2A in Huh-7 and PLC5 tumors. C, Analysis of PP2A activity in tumors. Values are means ± SD (n = 3). The figure is a representative of three independent experiments.
Fig. 1

A

Sk-Hep1

Hep3B

Huh-7

B

Sk-Hep1

Hep3B

Huh-7

C

Sk-Hep1

Hep3B

Huh-7

D

Bortezomib
Fig. 5

A

![Graph showing tumor size over days of treatment with different treatments: Vehicle, Bortezomib, CS1008, and Bortezomib + CS1008. The graph includes error bars and significance levels (P-values) for the comparison between groups.]

B

![Western blot analysis showing protein levels of CIP2A, P-Akt, Akt, and Actin for different treatments: Vehicle, Bortezomib, CS1008, and Bortezomib + CS1008.]

C

![Bar graph showing PP2A activity as a percentage of control for different treatments: Vehicle, Bortezomib (Bz), CS1008 (CS), and Bortezomib + CS1008 (Bz + CS). The graph includes error bars and significance levels (P-values) for the comparison between groups.]
Molecular Cancer Therapeutics

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