Bortezomib sensitizes HCC cells to CS-1008, an anti-human death receptor 5 antibody, through the inhibition of cancerous inhibitor of protein phosphatase 2A

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

Departments of [1] Medical Research, [2] Oncology, [3] Internal Medicine, and [4] National Center of Excellence for Clinical Trial and Research, National Taiwan University Hospital and National Taiwan University College of Medicine; [5] Division of Hematology and Oncology, Department of Medicine, Taipei Veterans General Hospital; [6]School of Medicine, and [7]Institute of Biopharmaceutical Sciences**,** National Yang-Ming University; [8] Department of Chemistry, National Central University, Taiwan

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Contact Information:

For reprint requests, correspondence, and proofs:

Ann-Lii Cheng, Department of Internal Medicine and Department of Oncology, National Taiwan University Hospital, No 7, Chung-Shan S Rd, Taipei, Taiwan. Tel: 886-2-23123456 ext: 67251; Fax: 886-2-23711174; Email: alcheng@ntu.edu.tw

Author's email addresses:

Kuen-Feng Chen, kfchen1970@ntu.edu.tw; Hui-Chuan Yu, gane-mai@yahoo.com.tw; Chun-Yu Liu, liuchunyu_tw@yahoo.com.tw; Yi-Ching Chen, cf0817@yahoo.com.tw; Duen-Ren Hou, drhou@cc.ncu.edu.tw; Pei-Jer Chen, peijerchen@ntu.edu.tw; Ann-Lii Cheng, alcheng@ntu.edu.tw

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.

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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 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 dose- 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 other three DRs, decoy receptor 1 (DcR1), decoy receptor 2 (DcR2), and the soluble osteoprotegerin (OPG), lack a functional death domain required to initiate apoptosis and act as inhibitors of TRAIL's apoptotic activity (6-8). 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. In so-called type I cells, the initiator caspase-8 activates (cleaves) the downstream effector caspase-3, -6, and -7 to execute the extrinsic apoptotic pathway (9). In type II cells, caspase-8 activates the BH3-interacting domain death agonist (Bid) from the intrinsic (mitochondrial) apoptotic pathway (9). TRAIL, thus, induces apoptosis by triggering both extrinsic and intrinsic apoptotic pathways. Using an agonistic ligand or monoclonal antibody to activate DR4 or DR5, therefore, represents an attractive therapeutic approach.

CS-1008, a novel humanized agonistic anti-human DR5 antibody, was modified from a murine anti-human DR5 mAb, TRA-8 (10). 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 (10). In a phase I trial, CS-1008 exerted no dose limiting toxicity at doses of up to 8 mg/kg weekly (11). 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 (12-15). 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 (16, 17). Bortezomib demonstrated excellent anti-tumor activity against these two hematologic malignancies through blocking proteasome degradation of IκB, an inhibitor of NF-κB (17). 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 (18), 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 (15). Importantly, we found that protein phosphatase 2A (PP2A), a phosphatase which down-regulates p-Akt, may mediate the effect of bortezomib on TRAIL sensitization.(15). 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 (19).

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 (20, 21). The regulatory B subunits are believed to play key roles in controlling the localization and specific activity of different PP2A

holoenzymes (22). In particular, PP2A plays a vital role in regulating cell proliferation through dephosphorylation of various oncogenic kinases, such as Raf, Akt and ERK (22-24). It is known that there are several cellular inhibitors of PP2A, including SET (25) and cancerous inhibitor of PP2A (CIP2A) (26) . Notably, CIP2A (KIAA1524, P90), first cloned from patients with HCC (27), has been shown to promote anchorage-independent cell growth and *in vivo* tumor formation by inhibiting PP2A activity toward c-Myc (26). Moreover, CIP2A is over-expressed in HCC and several other human malignancies (26, 28, 29) and is associated with clinical aggressiveness in human breast cancer (28), 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 downregulated CIP2A and in turn increased Akt-related PP2A activity in these HCC cells. This sensitizing effect was confirmed by *in vivo* nude mice model showing that CS-1008 in combination with bortezomib decreased tumor growth in Huh-7 tumors. Through the CIP2A-PP2A-pAkt mechanism described, bortezomib in combination with CS-1008 may constitute a promising strategy for the treatment of HCC.

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, 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 (Manassas, VA). The Huh-7 HCC cell line was obtained from the Health Science Research Resources Bank (Osaka, Japan; JCRB0403). 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% CO_2 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 (18).

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 (18). 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 (18).

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_3VO_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.

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 1×10^6 HCC cells suspended in 0.1 ml of serum-free medium containing 50% Matrigel (BD Biosciences, Bedford, MA). When tumors reached 150-200 mm^3, mice received an intra-peritoneal injection of bortezomib (0.5 mg/kg

body weight) twice weekly and/or CS-1008 200 μg three times a week. Controls received vehicle.

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 resistant 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 dose-dependent manner starting from CS-1008 at a concentration of 200 ng/ml. We then examined the effect of a fixed-dosed of CS-1008 on escalating doses of bortezomib and found that CS-1008 at a dose of 200 ng/ml increased bortezomib-induced apoptosis in a dose-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 (15). 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-10080 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. 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 (15, 19). 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. Moreover, we found that treatment with either of the drugs alone or in combination did not affect the protein level of PP2A complex including subunit A, B56γ and C (Fig. 4B). 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 (26). 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).

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 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 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 plus CS-1008, indicating 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.

Various mechanisms contribute to TRAIL resistance in cancers (13). 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 (13) . 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 (30, 31). Furthermore, overexpression of anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-XL, Mcl-1, etc) (32, 33), loss of proaoptotic 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 (13, 34). 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 (13, 35, 36). Interestingly, we discovered that through CIP2A-PP2A-p-Akt regulatory mechanism, bortezomib overcomes CS-1008 resistance, supporting the hypotheis 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 (15, 37-41). 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 (37, 41), activation of both extrinsic and intrinsic apoptosis pathways (42, 43), and probably many other multiple molecular machineries. For example, Hetschko et al. (37) 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 we 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 (44, 45) 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 (15, 18).

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. First, the combination is safe. 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 (46, 47). CS-1008, for its part, although still under clinical investigation, has shown an excellent toxicity profile with low hepatic toxicity (10, 11, 48), 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 (49). 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.

Reference:

1. Verslype C, Van Cutsem E, Dicato M, et al. The management of hepatocellular carcinoma. Current expert opinion and recommendations derived from the 10th World Congress on Gastrointestinal Cancer, Barcelona, 2008. Ann Oncol 2009; 20 Suppl 7: vii1-vii6.

2. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. Nat Rev Cancer 2006; 6: 674-87.

3. Tanaka S, Arii S. Molecularly targeted therapy for hepatocellular carcinoma. Cancer Sci 2009; 100: 1-8.

4. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008; 359: 378-90.

5. Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. Nat Rev Cancer 2008; 8: 782-98.

6. Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. Oncogene 2003; 22: 8628-33.

7. Rowinsky EK. Targeted induction of apoptosis in cancer management: the emerging role of tumor necrosis factor-related apoptosis-inducing ligand receptor activating agents. J Clin Oncol 2005; 23: 9394-407.

8. Wiezorek J, Holland P, Graves J. Death receptor agonists as a targeted therapy for cancer. Clin Cancer Res 2010; 16: 1701-8.

9. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998; 94: 491-501.

10. Yada A, Yazawa M, Ishida S, et al. A novel humanized anti-human death receptor 5 antibody CS-1008 induces apoptosis in tumor cells without toxicity in hepatocytes. Ann Oncol 2008; 19: 1060-7.

11. M. N. Saleh IP, T. E. Wood, J. Posey, III, J. Shah, R. Carlisle, S. Wojtowicz-Praga and A. Forero-Torres. A phase I study of CS-1008 (humanized monoclonal antibody targeting death receptor 5 or DR5), administered weekly to patients with advanced solid tumors or lymphomas. J Clin Oncol 2008; 26:3537.

12. Shin EC, Seong YR, Kim CH, et al. Human hepatocellular carcinoma cells resist to TRAIL-induced apoptosis, and the resistance is abolished by cisplatin. Exp Mol Med 2002; 34: 114-22.

13. Zhang L, Fang B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer Gene Ther 2005; 12: 228-37.

14. Pathil A, Armeanu S, Venturelli S, et al. HDAC inhibitor treatment of hepatoma cells induces both TRAIL-independent apoptosis and restoration of sensitivity to TRAIL. Hepatology 2006; 43: 425-34.

15. Chen KF, Yeh PY, Hsu C, et al. Bortezomib overcomes tumor necrosis

factor-related apoptosis-inducing ligand resistance in hepatocellular carcinoma cells in part through the inhibition of the phosphatidylinositol 3-kinase/Akt pathway. J Biol Chem 2009; 284: 11121-33.

16. Orlowski RZ, Kuhn DJ. Proteasome inhibitors in cancer therapy: lessons from the first decade. Clin Cancer Res 2008; 14: 1649-57.

17. Voorhees PM, Orlowski RZ. The proteasome and proteasome inhibitors in cancer therapy. Annu Rev Pharmacol Toxicol 2006; 46: 189-213.

18. Chen KF, Yeh PY, Yeh KH, Lu YS, Huang SY, Cheng AL. Down-regulation of phospho-Akt is a major molecular determinant of bortezomib-induced apoptosis in hepatocellular carcinoma cells. Cancer Res 2008; 68: 6698-707.

19. Chen KF, Yu HC, Liu TH, Lee SS, Chen PJ, Cheng AL. Synergistic interactions between sorafenib and bortezomib in hepatocellular carcinoma involve PP2A-dependent Akt inactivation. J Hepatol 2009; 52: 88-95.

20. Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem J 2001; 353: 417-39.

21. Xu Y, Xing Y, Chen Y, et al. Structure of the protein phosphatase 2A holoenzyme. Cell 2006; 127: 1239-51.

22. Mumby M. PP2A: unveiling a reluctant tumor suppressor. Cell 2007; 130: 21-4.

23. Adams DG, Coffee RL, Jr., Zhang H, Pelech S, Strack S, Wadzinski BE. Positive regulation of Raf1-MEK1/2-ERK1/2 signaling by protein serine/threonine phosphatase 2A holoenzymes. J Biol Chem 2005; 280: 42644-54.

24. Bielinski VA, Mumby MC. Functional analysis of the PP2A subfamily of protein phosphatases in regulating Drosophila S6 kinase. Exp Cell Res 2007; 313: 3117-26.

25. Neviani P, Santhanam R, Trotta R, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. Cancer Cell 2005; 8: 355-68.

26. Junttila MR, Puustinen P, Niemela M, et al. CIP2A inhibits PP2A in human malignancies. Cell 2007; 130: 51-62.

27. Soo Hoo L, Zhang JY, Chan EK. Cloning and characterization of a novel 90 kDa 'companion' auto-antigen of p62 overexpressed in cancer. Oncogene 2002; 21: 5006-15.

28. Come C, Laine A, Chanrion M, et al. CIP2A is associated with human breast cancer aggressivity. Clin Cancer Res 2009; 15: 5092-100.

29. Li W, Ge Z, Liu C, et al. CIP2A is overexpressed in gastric cancer and its depletion leads to impaired clonogenicity, senescence, or differentiation of tumor cells. Clin Cancer Res 2008; 14: 3722-8.

30. Okano H, Shiraki K, Inoue H, et al. Cellular FLICE/caspase-8-inhibitory protein

as a principal regulator of cell death and survival in human hepatocellular carcinoma. Lab Invest 2003; 83: 1033-43.

31. Eggert A, Grotzer MA, Zuzak TJ, et al. Resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. Cancer Res 2001; 61: 1314-9.

32. Kim SH, Ricci MS, El-Deiry WS. Mcl-1: a gateway to TRAIL sensitization. Cancer Res 2008; 68: 2062-4.

33. Fulda S, Meyer E, Debatin KM. Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression. Oncogene 2002; 21: 2283-94.

34. Zhang XD, Zhang XY, Gray CP, Nguyen T, Hersey P. Tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of human melanoma is regulated by smac/DIABLO release from mitochondria. Cancer Res 2001; 61: 7339-48.

35. Bortul R, Tazzari PL, Cappellini A, et al. Constitutively active Akt1 protects HL60 leukemia cells from TRAIL-induced apoptosis through a mechanism involving NF-kappaB activation and cFLIP(L) up-regulation. Leukemia 2003; 17: 379-89.

36. Ehrhardt H, Fulda S, Schmid I, Hiscott J, Debatin KM, Jeremias I. TRAIL induced survival and proliferation in cancer cells resistant towards TRAIL-induced apoptosis mediated by NF-kappaB. Oncogene 2003; 22: 3842-52.

37. Hetschko H, Voss V, Seifert V, Prehn JH, Kogel D. Upregulation of DR5 by proteasome inhibitors potently sensitizes glioma cells to TRAIL-induced apoptosis. FEBS J 2008; 275: 1925-36.

38. Sayers TJ, Brooks AD, Koh CY, et al. The proteasome inhibitor PS-341 sensitizes neoplastic cells to TRAIL-mediated apoptosis by reducing levels of c-FLIP. Blood 2003; 102: 303-10.

39. Feng X, Yan J, Wang Y, et al. The proteasome inhibitor bortezomib disrupts tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression and natural killer (NK) cell killing of TRAIL receptor-positive multiple myeloma cells. Mol Immunol; 47: 2388-96.

40. Liu J, Qu XJ, Xu L, et al. Bortezomib Synergizes TRAIL-Induced Apoptosis in Gastric Cancer Cells. Dig Dis Sci 2010.

41. Liu X, Yue P, Chen S, et al. The proteasome inhibitor PS-341 (bortezomib) up-regulates DR5 expression leading to induction of apoptosis and enhancement of TRAIL-induced apoptosis despite up-regulation of c-FLIP and survivin expression in human NSCLC cells. Cancer Res 2007; 67: 4981-8.

42. Seki N, Toh U, Sayers TJ, et al. Bortezomib sensitizes human esophageal squamous cell carcinoma cells to TRAIL-mediated apoptosis via activation of both extrinsic and intrinsic apoptosis pathways. Mol Cancer Ther 2010; 9: 1842-51.

43. Brooks AD, Jacobsen KM, Li W, Shanker A, Sayers TJ. Bortezomib sensitizes human renal cell carcinomas to TRAIL apoptosis through increased activation of caspase-8 in the death-inducing signaling complex. Mol Cancer Res 2010; 8: 729-38.
44. Franco AV, Zhang XD, Van Berkel E, et al. The role of NF-kappa B in TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of melanoma cells. J Immunol 2001; 166: 5337-45.
45. Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA, Goodwin RG. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity 1997; 7: 813-20.
46. Dunleavy K, Pittaluga S, Czuczman MS, et al. Differential efficacy of bortezomib plus chemotherapy within molecular subtypes of diffuse large B-cell lymphoma. Blood 2009; 113: 6069-76.
47. Messinger Y, Gaynon P, Raetz E, et al. Phase I study of bortezomib combined with chemotherapy in children with relapsed childhood acute lymphoblastic leukemia (ALL): a report from the therapeutic advances in childhood leukemia (TACL) consortium. Pediatr Blood Cancer 2010; 55: 254-9.
48. Sung ES, Park KJ, Lee SH, et al. A novel agonistic antibody to human death receptor 4 induces apoptotic cell death in various tumor cells without cytotoxicity in hepatocytes. Mol Cancer Ther 2009; 8: 2276-85.
49. Jeng YM, Hsu HC. Mutation of the DR5/TRAIL receptor 2 gene is infrequent in hepatocellular carcinoma. Cancer Lett 2002; 181: 205-8.

Figure legends

Fig. 1 Bortezomib enhances CS-1008-induced apoptosis in TRAIL-resistant HCC cells. A, Dose-escalation effects of CS-1008 with 50 nM bortezomib on apoptosis in 3 TRAIL-resistant HCC cells. B, Dose-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 hours 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 doses (nM) in DMEM with 5%FBS for 24hr. Cell lysates were prepare and analyzed for cell death ELISA. Data are representative of three independent experiments.

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). *P < 0.05. 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). *P < 0.05. 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). *$P < 0.05$. Sk-Hep1 sells 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 24hours, and cell lysates were immunoprecipitated with anti-PP2A-C antibodies. The immunoprecipitates were probed with Akt1 and PP2A-C by western blot. D, Over-expression of CIP2A decreased Akt-associated PP2A activity. *Columns*, mean; *bars*, SD (n = 3). *P < 0.05. 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). *P < 0.05.

Fig. 5 *In vivo* effect of bortezomib on Huh-7 xeonograft nude mice. A, effects of bortezomib and/or CS-1008 on tumor growth. Points, mean (n = 6); bars, SE. *, P < 0.05. Each mouse was inoculated s.c. in the dorsal flank with 1 million Huh-7 or PLC5 cells suspended in Matrigel. When the tumor reached a volume of 200 mm^3, mice received treatments of vehicle or 1.0 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; $*P < 0.05$). The figure is a representative of three independent experiments.

Fig. 1

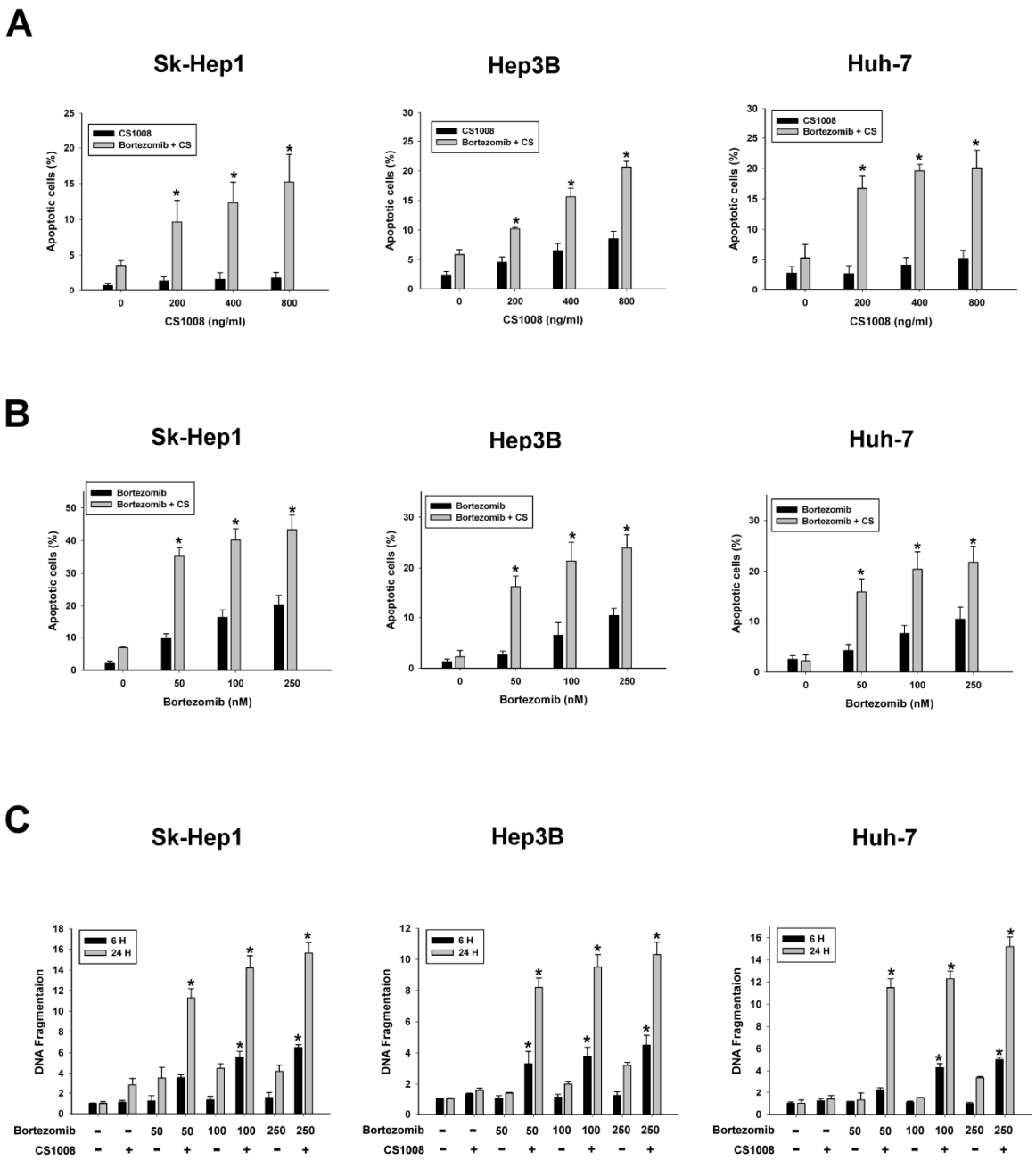

A
Sk-Hep1
Hep3B
Huh-7
CS1008
Bortezomib + CS
Apoptotic cells (%)
CS1008 (ng/ml)
B
Bortezomib
Bortezomib + CS
Apoptotic cells (%)
Bortezomib (nM)
C
6 H
24 H
DNA Fragmentaion
Bortezomib
CS1008

Fig. 2

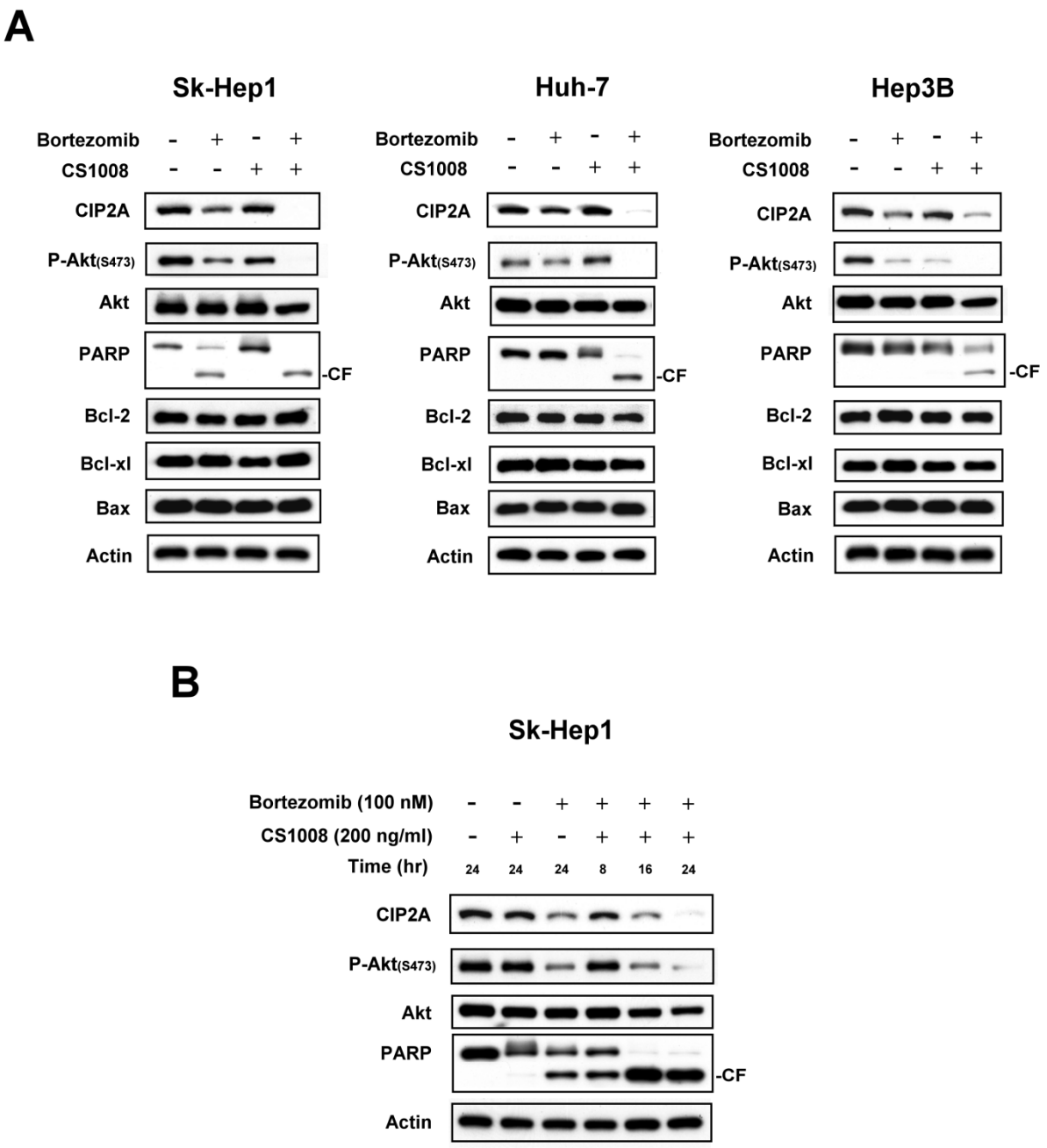

A
Sk-Hep1
Huh-7
Hep3B
Bortezomib - + - +
CS1008 - - + +
CIP2A
P-Akt(S473)
Akt
PARP
-CF
Bcl-2
Bcl-xl
Bax
Actin
B
Sk-Hep1
Bortezomib (100 nM) - - + + + +
CS1008 (200 ng/ml) - + - + + +
Time (hr) 24 24 24 8 16 24
CIP2A
P-Akt(S473)
Akt
PARP
-CF
Actin

Fig. 3

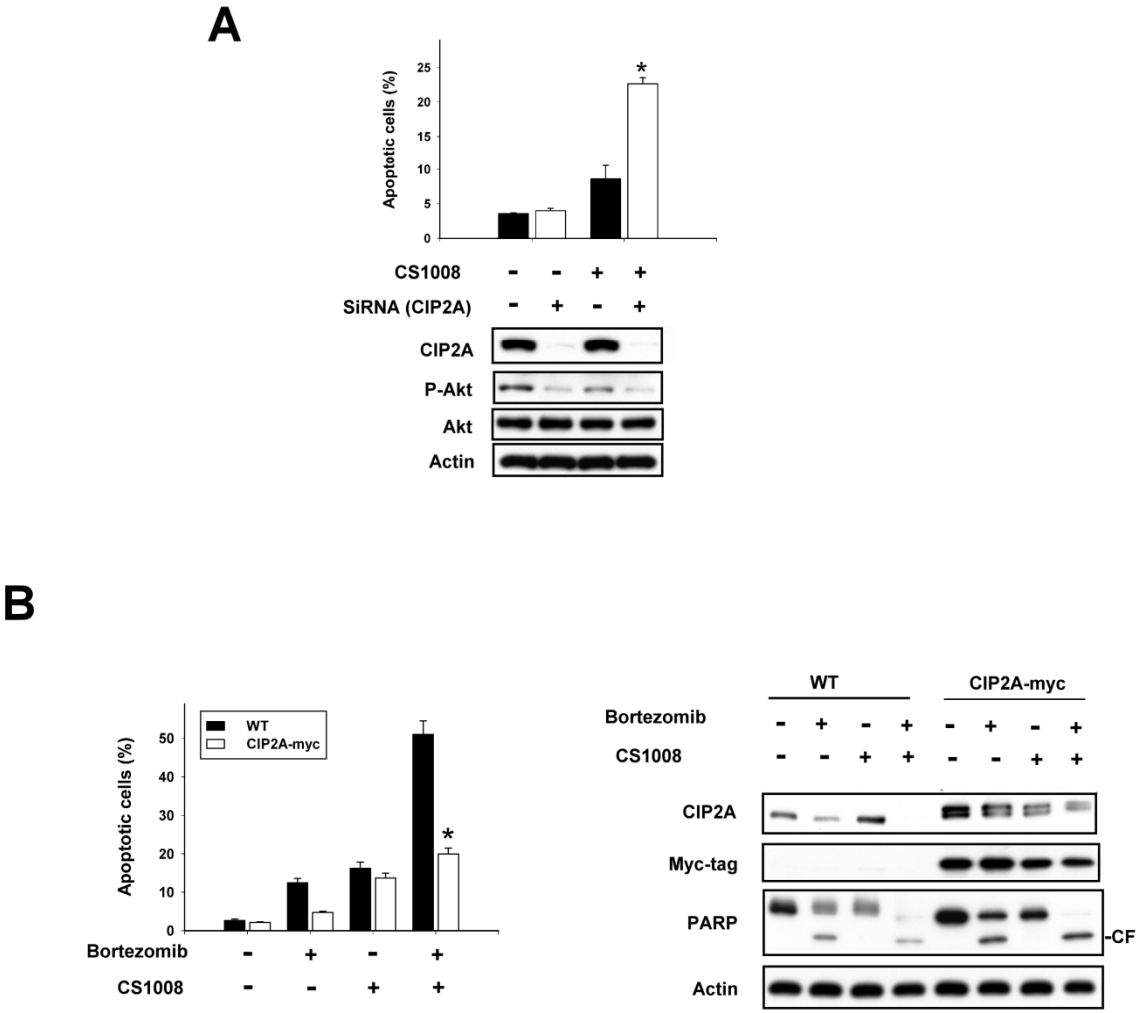

A
Apoptotic cells (%)
25
20
15
10
5
0
*
CS1008 - - + +
SiRNA (CIP2A) - + - +
CIP2A
P-Akt
Akt
Actin
B
WT
CIP2A-myc
Apoptotic cells (%)
50
40
30
20
10
0
*
Bortezomib - + - +
CS1008 - - + +
WT
CIP2A-myc
Bortezomib - + - + - + - +
CS1008 - - + + - - + +
CIP2A
Myc-tag
PARP
-CF
Actin

Fig. 4

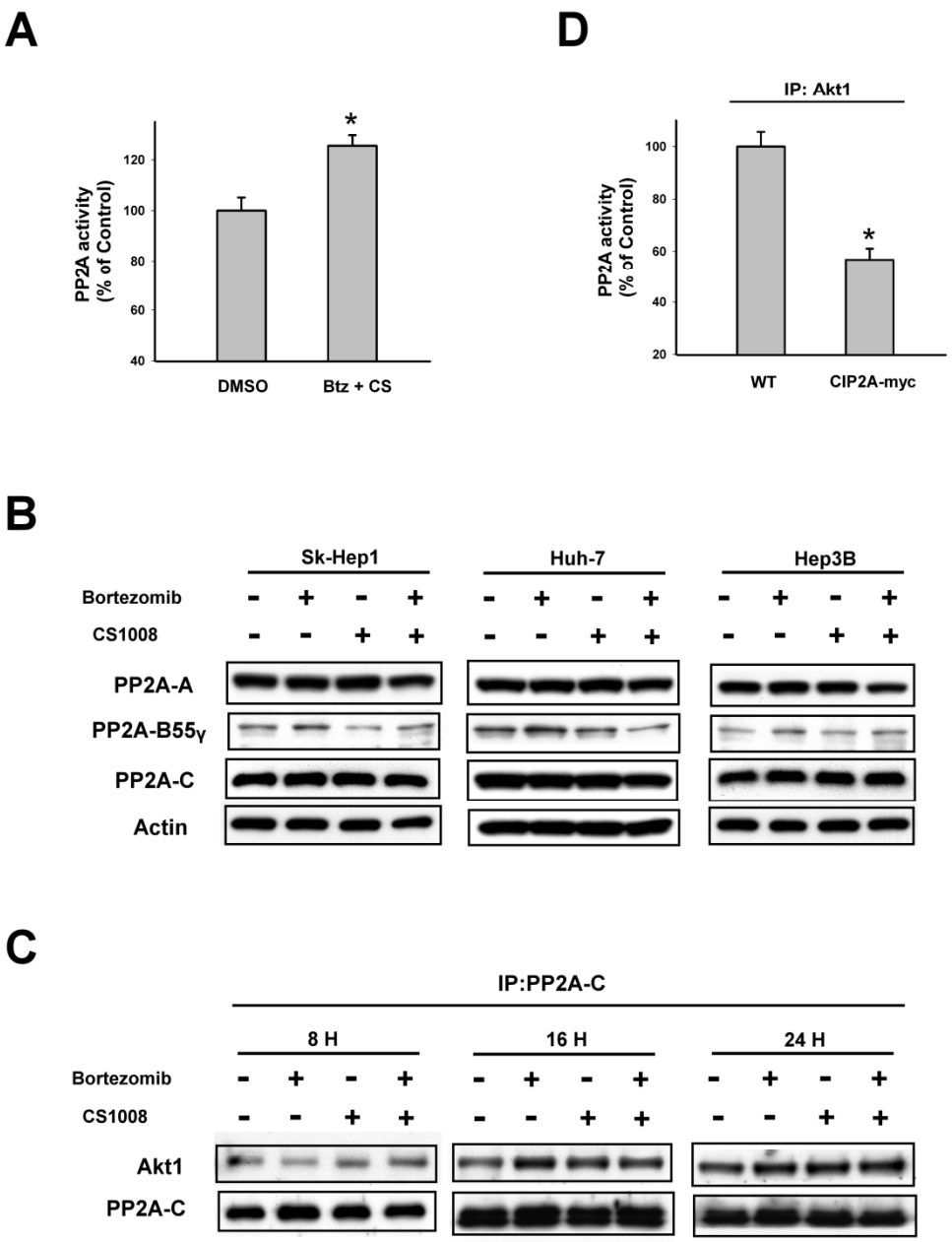
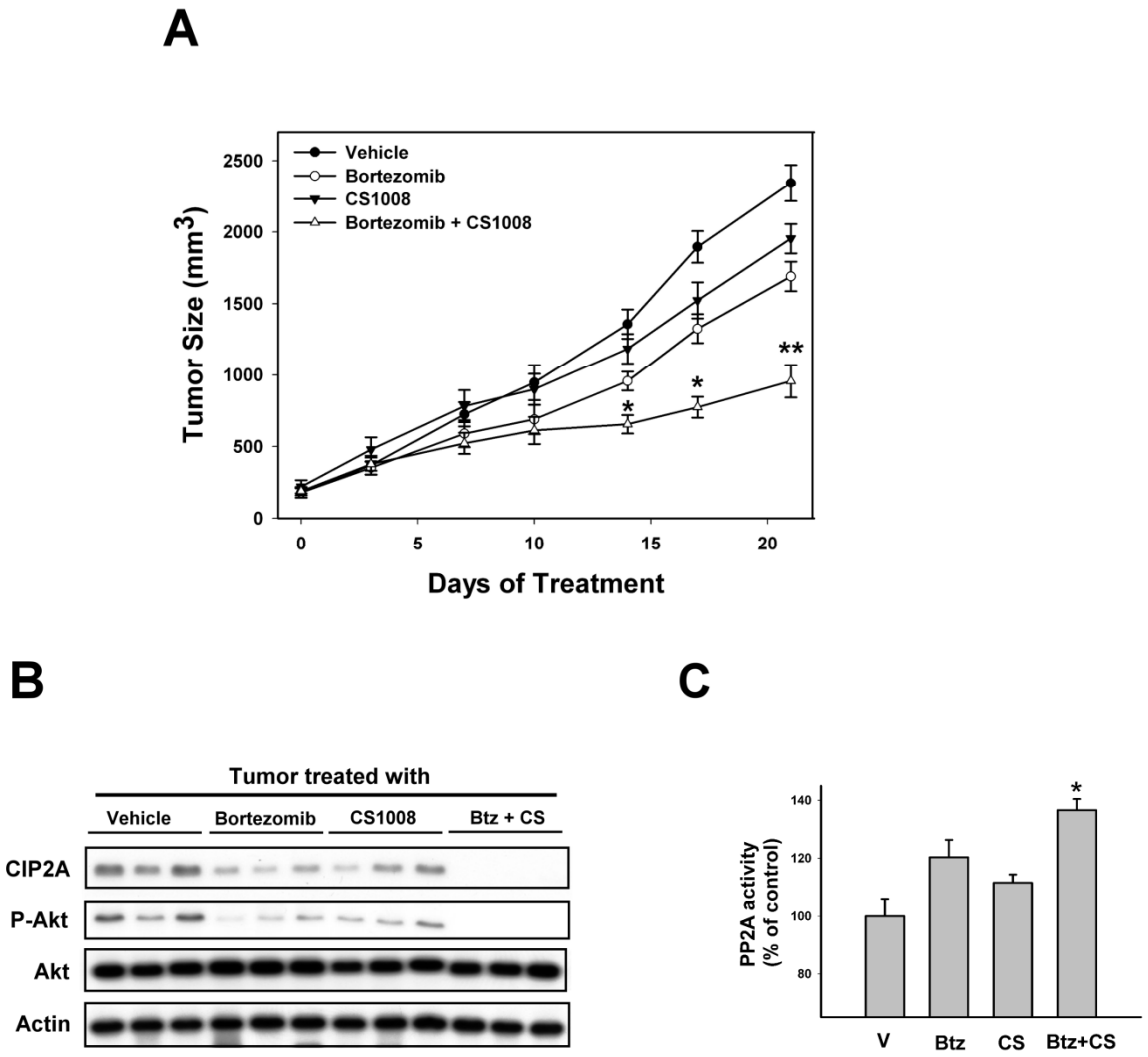

A
PP2A activity (% of Control)
120
100
80
60
40
*
DMSO
Btz + CS
D
IP: Akt1
PP2A activity (% of Control)
100
80
60
40
20
*
WT
CIP2A-myc
B
Sk-Hep1
Huh-7
Hep3B
Bortezomib
CS1008
PP2A-A
PP2A-B55γ
PP2A-C
Actin
C
IP:PP2A-C
8 H
16 H
24 H
Bortezomib
CS1008
Akt1
PP2A-C

Fig. 5


A
Vehicle
Bortezomib
CS1008
Bortezomib + CS1008
Tumor Size (mm3)
Days of Treatment
B
Tumor treated with
Vehicle
Bortezomib
CS1008
Btz + CS
CIP2A
P-Akt
Akt
Actin
C
PP2A activity (% of control)
V
Btz
CS
Btz+CS