Cancerous inhibitor of protein phosphatase 2A determines bortezomib-induced apoptosis in leukemia cells

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Online Supplementary Appendix

Design and Methods

Human leukemia cell lines, normal hematopoietic stem cells, primary leukemia cells, cell culture and western blot analysis

The HL-60, KG-1, K562 and MOLT-3 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). HL-60 is a human promyelocytic leukemia cell line and KG-1 is an AML cell line.[1] MOLT-3 is a human T-lymphoblastic leukemia cell line.[2] K562 is a human myelogenous leukemia cell line derived from the pleural effusion of a 53-year old female with CML in terminal blast crises.[3] Normal $CD34^+$ hematopoietic stem cells were obtained from 3 consenting autologous or allogeneic donors for hematopoietic stem cell transplantation and peripheral leukocytes were obtained from 4 consenting healthy volunteers. Primary leukemic cells from 13 consenting patients were also analyzed. Study protocols were approved by the institutional review board of Taipei Veterans General Hospital, and informed consent was obtained in accordance with the Declaration of Helsinki. Primary cells were collected from bone marrow (blasts >80%) from patients with newly diagnosed AML or acute lymphocytic leukemia. The WHO and FAB classifications of acute leukemia were used. Cells were frozen in medium containing 10% DMSO within 24 h of harvesting. All leukemia cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 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 leukemia cells treated with drugs at the indicated concentrations for various periods of time were prepared for immunoblotting of caspase-3, P-Akt, Akt, CIP2A, etc. Western blot analysis was performed as previously reported.[4]

Apoptosis analysis

Drug-induced apoptotic cell death was assessed by the following three methods: a) detection of DNA fragmentation with the Cell Death Detection ELISA kit (Roche Diagnostics Mannheim, Germany); b) Western blot analysis of caspase activation; and c) measurement of apoptotic cells by flow cytometry (sub-G1 analysis). ELISA was conducted according to the manufacturer's instructions.[5] For assessment of apoptosis (sub-G1) in primary cells, specific cell death was calculated with the formula: (xt-xm/100-xm)/100, where xt was the number of apoptotic cells in response to a given bortezomib concentration and xm were the apoptotic cells among the untreated cells.

Gene knockdown using siRNA

Smartpool siRNA reagents, including control (Scramble SiRNA, D-001810-01), and SiRNA for CIP2A (n. L-014135-01) were all purchased from Dharmacon (Chicago, IL, USA) as previously described.[4] Briefly, cells were transfected with siRNA (final concentration, 100 nM) in 6-well plates using the DharmaFECT4 transfection reagent (Dharmacon, Chicago, IL, USA) according to the manufacturer's instructions. After 48 h, the medium was replaced and the leukemia cells were incubated with bortezomib, harvested, and separated for Western blot analysis and for apoptosis analysis by flow cytometry.

HL-60 with constitutively active Akt (HL-60-Akt) and HL-60 with constitutively active CIP2A (HL-60-CIP2A cell)

HL-60 cells were transfected with myc-tagged Akt1 construct or myc-tagged CIP2A as previously described.[6] Briefly, following transfection, cells were incubated in the presence of G418 (0.78 mg/mL). After eight weeks of selection, surviving colonies, i.e. those arising from stably transfected cells, were selected and individually amplified. CIP2A cDNA (KIAA1524) was purchased from Origene (RC219918; Rockville, MD, USA) and constructed into pCMV6 vector. HL-60 cells with stable expression of CIP2A were selected as described above.

PP2A phosphatase activity

Protein phosphatase activity in the total cellular lysate was determined by measuring the generation of free phosphate from threonine phosphopeptide using the malachite green-phosphate complex assay according to the instructions provided by the manufacturer (Upstate Biotechnology, Lake Placid, NY, USA). 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 min 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.

Proteasome inhibitory activity

A 20S Proteasome Activity Assay kit (Chemicon International) was used to determine the proteasome inhibition in drug-treated cells. All procedures were conducted according to the manufacturer's instructions.[4] Briefly, cells were treated with or without bortezomib for the indicated length of time. Cells were then lysed and total protein was quantified. Equal amounts of total protein of each sample were used for incubation with the proteasome substrate (fluorophore-labeled substrate). Proteasome activity measurement was based on detection of the fluorophore after cleavage from the labeled substrate by a fluorometer with a 380/460 nm filter set.

NF-KB DNA-binding activity

The TransAM NF-κB p65 Transcription Factor Assay kit (Active Motif North America) was used to determine the DNA-binding activity of NF-κB in bortezomib-treated leukemia cells. The entire procedure was performed according to the manufacturer's manual as previously described.[4] Briefly, cells were exposed to bortezomib at the indicated doses for 6 h and cell lysates (nuclear extracts) were prepared. Nuclear NF-κB binding to related DNA fragments was determined by incubation with primary anti-p65 antibody, and quantified by a spectrometer at 450 nm after incubation with anti-IgG-horseradish peroxidase conjugate.

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 in an SPF-environment. All experimental procedures using these mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Taipei Veterans General Hospital. Each mouse was inoculated subcutaneously in the dorsal flank with 2×10^6 leukemia cells suspended in 0.1 mL serum-free medium containing 50% Matrigel (BD Biosciences, Bedford, MA, USA) under isoflurane anesthesia. Tumors were measured using calipers and their volumes calculated using a standard formula: width 2 × length × 0.52. When tumors reached 200-300 mm^3, mice were administered an intraperitoneal injection of bortezomib (0.5 mg/kg body weight) twice weekly for two weeks. Controls received vehicle. For each treatment subgroups (including controls), 6 tumor-bearing mice were used for data analysis.

DNA synthesis and cell proliferation of leukemia cells in vitro

DNA synthesis and cell proliferation were assessed by colorimetric assay using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and BrdU (colorimetric) kit (Roche Diagnostics, Mannheim, Germany). For MTT assay, cells were plated in a 96-well plate in 100 μl of 10% fetal bovine serum and RPMI per well and cultured up to 72 h. Cells were incubated for 4 h at 37°C with MTT; after incubation, medium was removed and cells were treated with DMSO for 5 min. Viability was evaluated by UV absorption spectrum at 550 nm with Microplate Reader Model 550 (Bio-Rad, Richmond, CA, USA). For BrdU assay, cells were plated in a 96-well plate in 100 μl of 10% fetal bovine serum and RPMI per well and cultured up to 72 h. Viability was evaluated by UV absorption spectrum at 450 nm with Microplate Reader Model 550 (Bio-Rad, Richmond, CA, USA).

Immunohistochemical staining

Paraffin-embedded tissue of bone marrow biopsy samples from 2 AML patients with over 80% blasts and one AML patient with approximately 25% blasts were immunohistochemically stained for CIP2A. Patients' sections (4 μm) on poly-1-lysine-coated slides were first de-waxed in xylene and rehydrated through graded alcohols. Antigen retrieval was performed by heating tissue slides in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 15 min. Slides were then rinsed using phosphate-buffered saline and treated with 3% hydrogen peroxide for 10 min, followed by incubation with blocking solution (2% fetal bovine serum and 1% bovine serum albumin) for 1 h at room temperature and then incubation with 1:100 dilution of rabbit polyclonal anti-p90 autoantigen (CIP2A) antibody (ab84547) (Abcam, UK) overnight at 4°C. On the second day, slides were thoroughly washed three times with phosphate-buffered saline and CIP2A signals were detected using the EnVisionTM system (Dako, USA). The slides were then counterstained with hematoxylin stain solution. Rabbit IgG was used as a control for antibody specificity. Bone marrow samples from 2 patients with idiopathic thrombocytopenic purpura were used for comparison with those from leukemia patients.

Human colony-forming unit granulocyte-macrophage (CFU-GM) assay in Petri dishes

$CD34^+$ normal hematopoietic cells were treated with bortezomib 0, 10, 50 and 100 nM for 6 h, each sample was washed and was then seeded in MethoCult-H4534 (StemCell Technologies, Vancouver, BC, Canada) medium in 35-mm dishes. This medium contains colony-stimulating factor (CSF), methylcellulose, fetal bovine serum, bovine serum albumin, 2-mercaptoethanol, glutamine, interleukin-3 (IL-3), GM-CSF and stem cell factor, without erythropoietin, as described by the manufacturer. Each culture sample contains $1x10^5$ low-density $CD34^+$ cells. Cultures were incubated at 37°C in 5% CO_2 for 14 days and colonies were scored.

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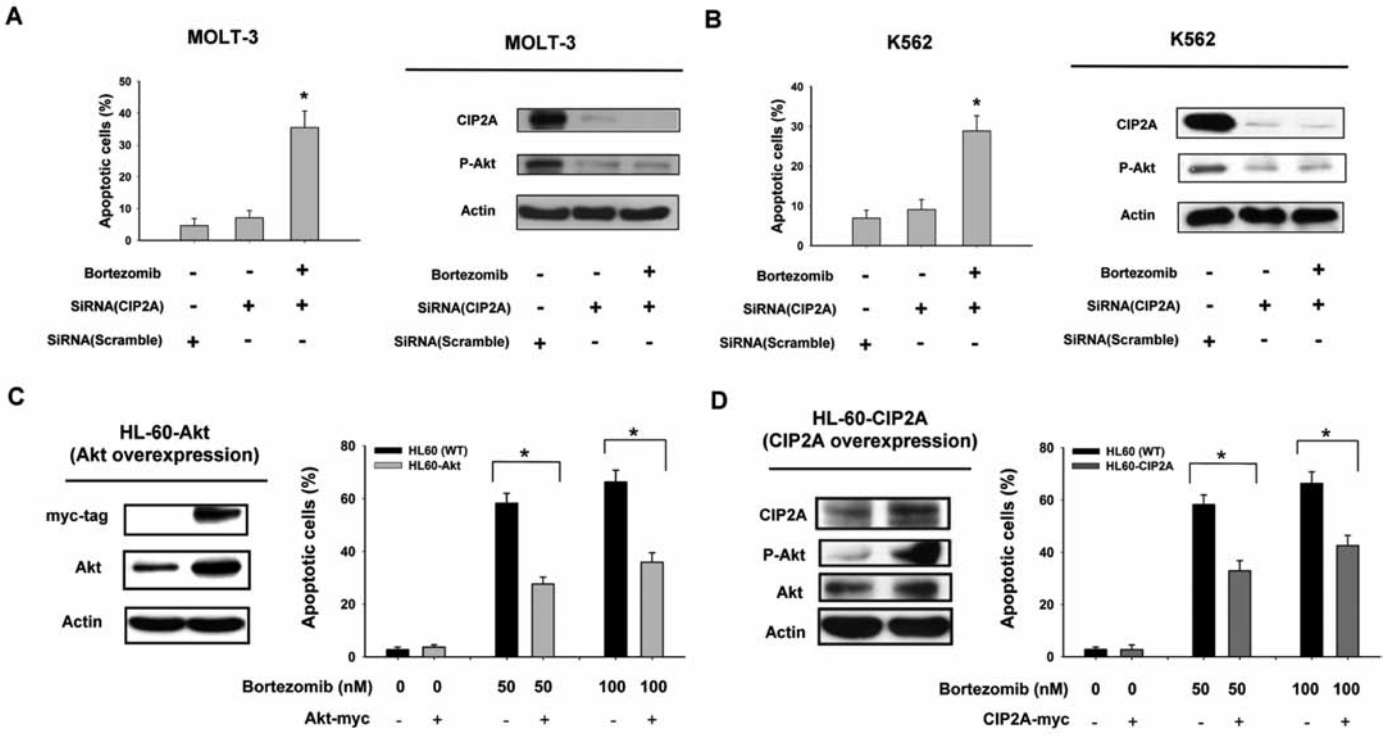
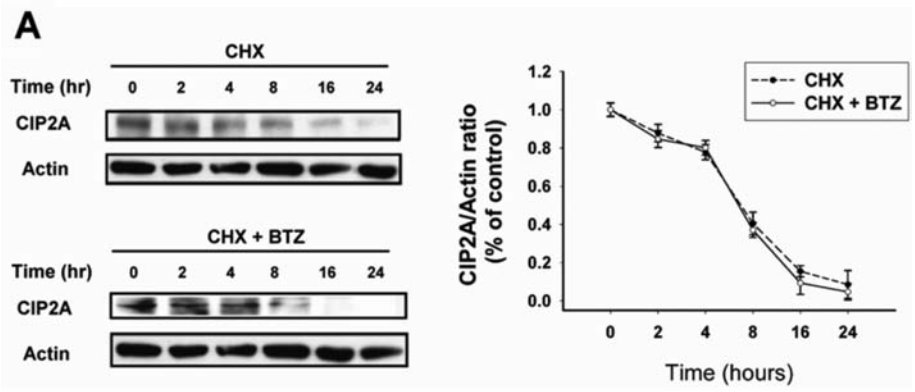
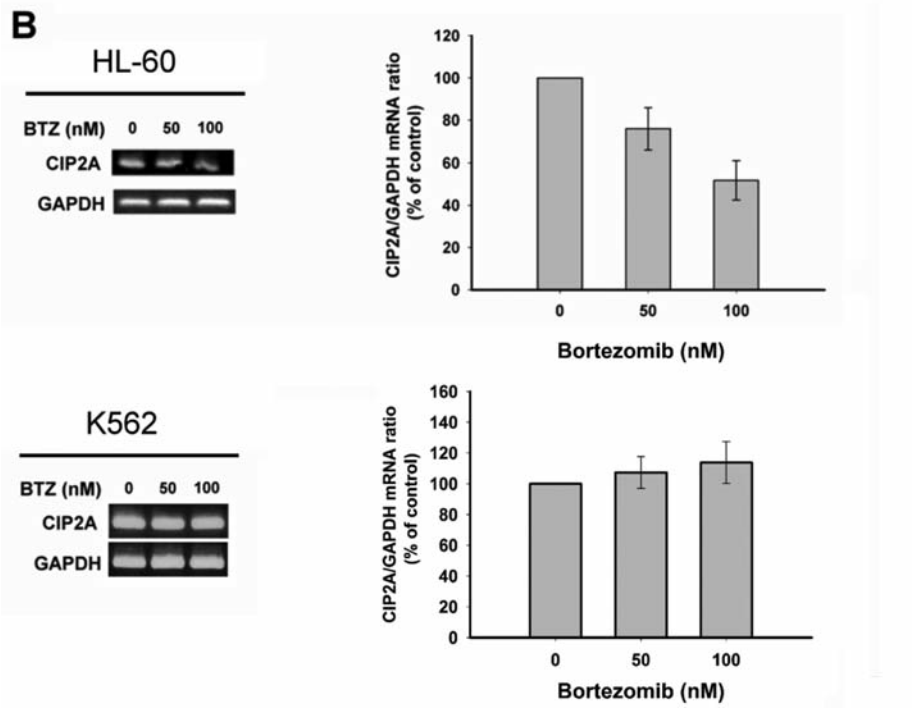
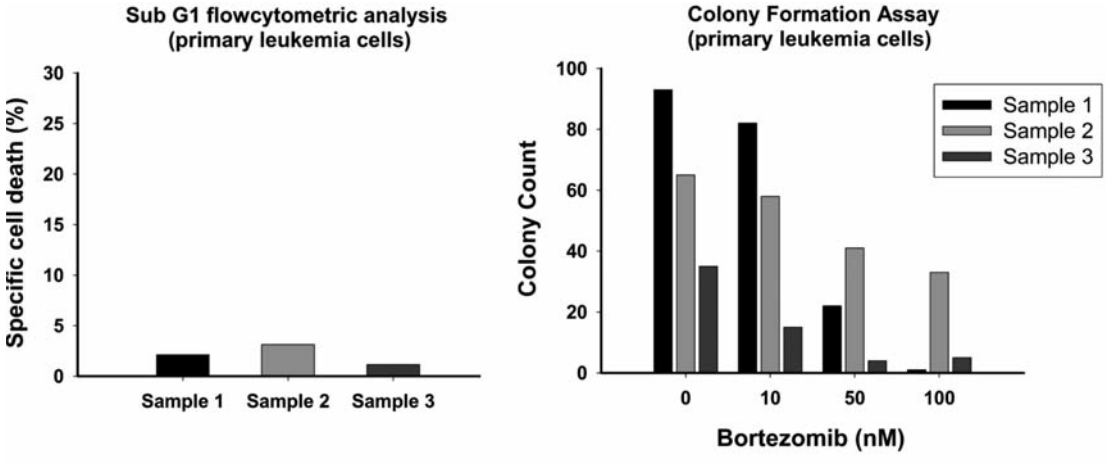


Online Supplementary Figure S1. (A) Downregulation of CIP2A by siRNA increases bortezomib-induced apoptosis in MOLT-3 cells (left) and is associated with downregulation of P-Akt (right). CIP2A siRNA treatment alone does not significantly induce apoptosis. Scramble SiRNA was used as control. Columns, mean (n=3); bars, SD; *P<0.05. Cells were transfected with either control or CIP2A siRNA for 48 h and then exposed to bortezomib at 100 nM for 12 h. Cell lysates were prepared and assayed for CIP2A and P-Akt by Western blotting. Representative of 3 independent experiments. (B) Similar effects by CIP2A siRNA and bortezomib treatments are seen in K562 cells (right and left). Cells were transfected and treated in same conditions as in (A). Columns, mean (n=3); bars, SD; *P<0.05. Representative of 3 independent experiments. (C) HL-60 cells with ectopic expression of myc-tagged Akt (Akt-myc) were generated (HL-60-Akt) (left) and treated with bortezomib. Ectopic expression of Akt-myc protected HL-60 cells from bortezomib's apoptotic effect (right). Columns, mean (n = 3); bars, SD; *P<0.05. HL-60 cells were transfected with Akt-myc and were selected for 8 weeks by G-418. Analysis of apoptotic cells was performed by flow cytometry after cells were sequentially exposed to DMSO or bortezomib 50 and 100 nM for 12 h. (D) HL-60 cells with ectopic expression of myc-tagged CIP2A were generated (HL-60-CIP2A). Note that HL-60-CIP2A cells also have constitutively high P-Akt (left). Ectopic expression of CIP2A also protects HL-60 cells from bortezomib's apoptotic effect (right). Columns, mean (n=3); bars, SD; *P<0.05. Cells were transfected with CIP2A-myc and were treated with DMSO or bortezomib in the same approach as (D).





Online Supplementary Figure S2. Bortezomib down-regulates transcription of CIP2A. (A) In bortezomib-sensitive HL-60 cells, the addition of bortezomib did not significantly affect CIP2A degradation, suggesting regulation of CIP2A by bortezomib occurs at pre-translation. After HL-60 cells were treated with 100 μg/mL translation inhibitor cyclohexamide (CHX) in the absence (top left) or presence of 50 nM bortezomib (bottom left) for the indicated length of time, the stability of CIP2A protein in whole-cell lysates were assessed by Western blot. (Right) immunoblots of CIP2A and actin from 3 independent experiments performed in 4A were scanned by a UVP BioSpectrum AC image system and quantitated using VisionWork LS software. (B) Bortezomib inhibits CIP2A mRNA in a dose-dependent manner in sensitive HL-60 cells (top). In resistant K562 cells, CIP2A mRNA was not inhibited by bortezomib (bottom). Cells were treated with bortezomib at 50 and 100 nM for 6 h, after which total RNA was isolated and CIP2A mRNA were semi-quantified using PCR as described in *Online Supplementary Design and Methods*. Columns, mean (n = 3); bars, SD.



Online Supplementary Figure S3. Effects of bortezomib on acute myeloid leukemia colony formation in primary leukemic cells unresponsive to short-term culture assay. (A) Short-term treatment (6 h) with bortezomib 100 nM did not significantly induce apoptosis in unresponsive primary leukemia cells. (B) Bortezomib reduced colony formation of primary leukemia cells from (A) by various degrees at the indicated doses (10, 50 and 100 nM). Colony formation assay for primary leukemia cells were performed by the same method for normal $CD34^+$ hematopoietic cells as described in in *Online Supplementary Design and Methods*.