

Combination of panobinostat with ponatinib synergistically overcomes imatinib-resistant CML cells

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The major mechanism of imatinib (IM) resistance of CML is the reactivation of ABL kinase either through *BCR-ABL* gene amplification or mutation. We investigated the cytotoxicity of a pan-ABL tyrosine kinase inhibitor, ponatinib, and a pan-histone deacetylase inhibitor, panobinostat, against IM-resistant CML cells *in vitro*. Two different IM-resistant cell lines, K562/IM-R1 and Ba/F3/T3151 were evaluated in comparison with their respective, parental cell lines, K562 and Ba/F3. K562/IM-R1 overexpressed *BCR-ABL* due to gene amplification. Ba/F3/T3151 was transfected with a *BCR-ABL* gene encoding T3151-mutated *BCR-ABL*. Ponatinib inhibited the growth of both K562/IM-R1 and Ba/F3/T3151 as potently as it inhibited their parental cells with an IC_{50} of 2–30 nM. Panobinostat also similarly inhibited the growth of all of the cell lines with an IC_{50} of 40–51 nM. This was accompanied by reduced histone deacetylase activity, induced histone H3 acetylation, and an increased protein level of heat shock protein 70, which suggested disruption of heat shock protein 90 chaperone function for *BCR-ABL* and its degradation. Importantly, the combination of ponatinib with panobinostat showed synergistic growth inhibition and induced a higher level of apoptosis than the sum of the apoptosis induced by each agent alone in all of the cell lines. Ponatinib inhibited phosphorylation not only of *BCR-ABL* but also of downstream signal transducer and activator of transcription 5, protein kinase B, and ERK1/2 in both K562/IM-R1 and Ba/F3/T3151, and the addition of panobinostat to ponatinib further inhibited these phosphorylations. In conclusion, panobinostat enhanced the cytotoxicity of ponatinib towards IM-resistant CML cells including those with T3151-mutated *BCR-ABL*.

Chronic myelogenous leukemia is a malignant transformation of hematopoietic stem cells. The clinical stages of CML consist of three different phases: a chronic phase that is characterized by excessive proliferation, but retains the capacity for differentiation; an accelerated phase that shows a rapid progression after 4–6 years of the chronic phase; and a blast crisis that results in a fatal acute leukemia.⁽¹⁾ The genetic abnormality of CML is a reciprocal t(9,22)(q34;q11) chromosomal translocation, the so-called Philadelphia chromosome. This abnormality generates a *BCR-ABL* fusion gene, resulting in the expression of a leukemia-specific oncoprotein, *BCR-ABL*, which is a potent tyrosine kinase that plays a central role in CML pathogenesis.^(2–5)

Current first-line treatment options for CML include the TKI IM, and the second-generation agents, nilotinib and dasatinib. These TKIs all inhibit the *BCR-ABL* tyrosine kinase and have dramatically improved the prognosis of CML patients.^(6–9) Nevertheless, a small percentage of CML patients are primarily refractory or secondarily resistant to these TKIs.^(10,11) Moreover, the prognosis of patients in blast crisis is still poor despite the use of these agents because of drug resistance. The major mechanism of drug resistance of CML is reactivation of the ABL kinase either through *BCR-ABL* gene mutation or through

gene amplification. Approximately 40% and 20% of the observed drug resistance is due to *BCR-ABL* gene mutation and gene amplification, respectively.^(12,13) Therefore, new agents that can overcome the reactivation of ABL kinase are needed.

Histone deacetylase inhibitors are emerging anticancer therapeutics. Histone deacetylase inhibitors promote the acetylation of histones in treated cells, which results in chromatin in an opened and transcriptionally permissive state, leading to apoptosis or the inhibition of proliferation. Recently, a pan-HDAC inhibitor, panobinostat (formerly LBH589), has been reported to have promising anticancer activity.⁽¹⁴⁾ Panobinostat is a hydroxamate analog and clinical studies of this agent are currently underway for various hematological malignancies including Hodgkin's lymphoma, cutaneous T-cell lymphoma, AML, myelodysplastic syndrome, and multiple myeloma.^(14,15) Histone deacetylase inhibitors also induce the acetylation of non-histone proteins such as HSP90, thereby inhibiting its chaperone function. If panobinostat has such a function, then panobinostat may suppress the association between HSP90 and its client protein, *BCR-ABL*, leading to *BCR-ABL* polyubiquitination and proteasomal degradation.^(13,14,16) Thus, HDAC inhibitors may overcome the cellular resistance of CML cells to TKIs.

The T315I mutation arises in the BCR-ABL kinase domain from the beginning or during treatment with TKIs including IM, nilotinib, and dasatinib and this mutation has been identified in up to 20% of patients with TKI-resistant CML.^(13,17) This mutation confers CML resistance not only to IM but also to the second-generation TKIs such as nilotinib and dasatinib.^(18,19) The T315I residue is located in the gatekeeper region of the ATP-binding site of BCR-ABL, resulting in structural inhibition of the binding of IM, nilotinib, and dasatinib to this region.^(19,20) A new pan-ABL tyrosine kinase inhibitor, ponatinib, is structurally designed to accommodate T315I mutation through its carbon-carbon triple bond linkage.⁽¹⁹⁾ Ponatinib has been investigated in a phase II PACE clinical trial in patients who had CML or Philadelphia chromosome-positive acute lymphoblastic leukemia with resistance or intolerance to nilotinib or dasatinib or with BCR-ABL T315I mutation. By 12 months of treatment, 56% of 267 patients with chronic phase CML had achieved a major cytogenetic response.⁽¹⁷⁾ Thus, ponatinib is a promising therapeutic option in patients with all kinds of BCR-ABL mutation, including T315I.

We hypothesized that the combination of panobinostat and ponatinib may overcome drug resistance and result in high therapeutic efficacy in CML through the combination of the different mechanisms of action of each agent. To test this hypothesis, the K562/IM-R1 cell line and the Ba/F3/T315I cell line were used to evaluate the cytotoxicity of panobinostat and ponatinib. The K562/IM-R1 cell line that was established in our previous study overexpresses BCR-ABL due to *BCR-ABL* gene amplification.⁽²¹⁾ The Ba/F3/T315I cell line is transfected with a *BCR-ABL* gene encoding T315I-mutated BCR-ABL.⁽²²⁾

Materials and Methods

Cell lines and reagents. Imatinib mesylate and panobinostat were kindly supplied by Novartis-Pharma (Basel, Switzerland). Ponatinib was purchased from ARIAD Pharmaceuticals (Cambridge, MA, USA). Imatinib mesylate was dissolved in sterile water; panobinostat and ponatinib were dissolved in DMSO. The human CML cell line K562 and its IM-resistant variant K562/IM-R1, and the BCR-ABL-transfected cell line Ba/F3 and its IM-resistant variant Ba/F3/T315I, were cultured in RPMI-1640 media with 10% FBS and maintained in a 5% CO₂-humidified atmosphere at 37°C.

Proliferation assay. To evaluate the proliferative activity of each cell line, the XTT assay was carried out according to the manufacturer's instructions (Roche, Indianapolis, IN, USA) with slight modifications.⁽²¹⁾ Briefly, 1 mL cells (5×10^4 /mL) was incubated for 24 h in a 24-well plate, followed by the addition of a 10- μ L aliquot of different concentrations of IM, panobinostat, or ponatinib, alone or in combination. The cells were incubated for a further 72 h, and then a 100- μ L aliquot was transferred to a 96-well microplate. The cells were mixed with 50 μ L XTT and incubated for another 4 h at 37°C. Absorbance at 480 nm was analyzed using spectrophotometry with a fluorescent microplate reader (SpectraMax 250; Molecular Device Japan, Ashiya, Japan). The IC₅₀ values were calculated from the growth inhibition curves generated for each treatment.

Calculation of CI. Combination index analysis provides quantitative information on drug interactions. The CI method was based on that described by Chou and Talalay,⁽²³⁾ and the values were determined by using the IC₅₀ values of their cell lines and the computer software CalcuSyn (version 2.0)

(Biosoft, Great Shelford, UK). Combination index values less than, equal to, and more than 1 indicate synergism, additivity, and antagonism, respectively.

Quantitation of apoptotic cell death. To evaluate cytotoxicity, apoptotic cell death was determined as phosphatidylserine externalization by using the annexin V-FLUOS Staining kit (Roche). At 48 h after treatment, the cells were collected by centrifugation (253 g for 5 min) and washed in PBS and centrifuged again. The pellets were resuspended in 50 μ L FITC-conjugated annexin V mixed with 50 μ L propidium iodide. Samples were added to 500 μ L HEPES buffer and analyzed by FACS analysis using FACSCanto II (BD Bioscience, Franklin Lakes, NJ, USA). Annexin V-positive cells were considered to be apoptotic.

Western blot analysis. Protein lysates were extracted from the cells (1×10^7 cells) after treatment for 24 h using the Qproteome Mammalian Protein Prep Kit (Qiagen, Hilden, Germany). The lysates were applied to 7.5% or 12% Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA, USA) and were electrophoresed and transferred onto Immobilon-P membranes (Millipore, Billerica, MA, USA). The membranes were probed using standard techniques with the primary antibodies, and subsequently with the secondary antibodies. Amersham ECL Prime Western Blotting Detection Reagent and ImageQuant LAS4000mini (GE Healthcare Life Sciences, Little Chalfont, UK) were used to visualize and quantify protein signals.

Rabbit polyclonal anti-BCR, anti-phospho-BCR (Tyr177), anti-AKT, anti-phospho AKT (Ser473), anti-ERK1/2, anti-phospho-ERK1/2, anti-histone H3 and anti-HSP70, rabbit monoclonal anti-phospho-STAT5 (Tyr694) (all from Cell Signaling Technology, Beverly, MA, USA), mouse monoclonal anti-STAT5 (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-acetylated histone H3 (Millipore), mouse monoclonal anti-HSP90 (Enzo Life Science, Farmingdale, NY, USA), and anti-actin (Sigma, St. Louis, MO, USA) antibodies were used as the primary antibodies. Blocking One and Blocking One-P (Nacalai Tesque, Kyoto, Japan) were used for the antibody dilutions. Anti-rabbit IgG-HRP-linked antibody (Cell Signaling Technology) and anti-mouse IgG-HRP-linked antibody (GE Healthcare Life Sciences) were used as the secondary antibodies.

Activity of HDAC. Activity of HDAC was determined using an HDAC assay kit (Active Motif, Carlsbad, CA, USA). Briefly, 30 μ L nuclear extracts, which were extracted from cell samples treated for 12 h with the Qproteome Nuclear Protein kit (Qiagen), were incubated with 1 mM HDAC substrate in the assay buffer in a total volume of 50 μ L for 1 h at 37°C, followed by termination of the reaction by addition of 50 μ L HDAC developing solution. The fluorescence intensity was measured at a wavelength of 405 nm.

Statistical analyses. All statistical analyses were carried out using Microsoft Excel 2007 software (Microsoft, Redmond, WA, USA). All graphs were generated using GraphPad Prism software (version 5.0) (GraphPad Software, San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Synergistic cytotoxicity of panobinostat and ponatinib towards CML cell lines. Growth inhibitory effects of IM, or of panobinostat or ponatinib alone or in combination, on K562 cells, K562/IM-R1 cells, Ba/F3 cells, and Ba/F3/T315I cells were

determined using the XTT assay (Tables 1,2 and Figs 1,2). This assay indicated that K562/IM-R1 cells and Ba/F3/T3151 cells were 12-fold and 13-fold more IM-resistant, respectively, than their parental counterpart (Table 1, Fig. 1). In contrast, panobinostat equally inhibited the growth of K562 cells, K562/IM-R1 cells, Ba/F3 cells, and Ba/F3/T3151 cells with IC_{50} values of between 40–51 nM (Table 2, Fig. 2). Ponatinib also inhibited the growth of both IM-resistant K562/IM-R1 cells and Ba/F3/T3151 cells as potently as it inhibited the growth of their respective parental K562 and Ba/F3 cells (Table 2, Fig. 2). Importantly, the combination of panobinostat and ponatinib showed enhanced growth inhibitory effects on all of the cell lines compared to either agent alone (Table 2, Fig. 2). The CI that was calculated based on their IC_{50} values clearly showed the synergy between these agents in all of the cell lines (Fig. 3).

We further evaluated drug-induced apoptosis of these cells using flow cytometry. When the cells were treated for 48 h with panobinostat or ponatinib either alone or in combination,

Table 1. Sensitivity of CML cell lines to imatinib

	IC_{50} (μ M)			
	K562	K562/IM-R1	Ba/F3	Ba/F3/T3151
Imatinib	0.6	7.6 (12.6)	0.6	7.8 (13.0)

Cells were incubated with imatinib for 72 h, followed by XTT assay. IC_{50} values, which indicate the 50% growth inhibitory concentration, are shown. Numbers in parentheses indicate fold resistance relative to parental cells.

Table 2. Sensitivity of CML cell lines to panobinostat and/or ponatinib

	IC_{50} (μ M)				
	K562	K562/IM-R1	Ba/F3	Ba/F3/T3151	HL60
Panobinostat	50.0	51.0	40.0	47.0	29.9
Ponatinib	2.0	3.8	5.0	30.0	796
Combination	0.7	1.3	3.7	10.0	32.1

Cells were incubated with the same concentration of panobinostat, ponatinib, or their combination for 72 h, followed by XTT assay. IC_{50} values, which indicate the 50% growth inhibitory concentration, are shown. HL60 cells that do not express the BCR-ABL protein were used as a negative control.

the combination of the two agents induced greater apoptotic cell death than the sum of apoptotic cell death induced by each agent alone in all of the cell lines (Table 3, Fig. 4a–d). Moreover, 30 nM ponatinib combined with 30 nM panobinostat was as cytotoxic as 50 nM ponatinib alone against Ba/F3/T3151 cells (Fig. 4e).

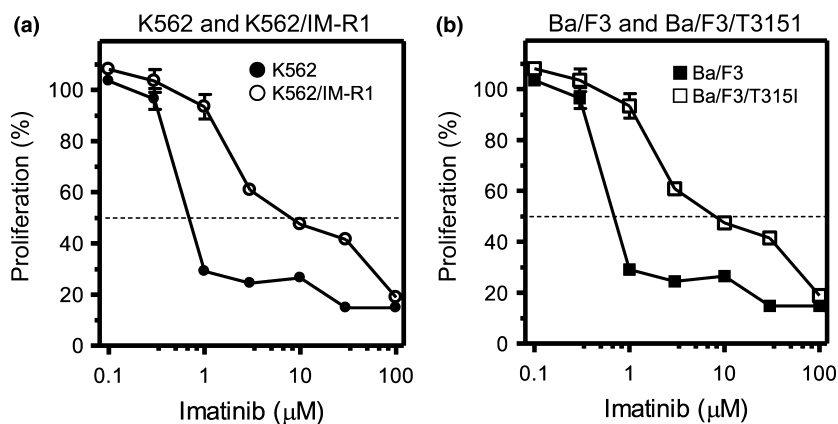
Thus, panobinostat and ponatinib showed synergistic cytotoxicity towards IM-resistant cell lines, whose resistance was caused by either *BCR-ABL* gene amplification or by BCR-ABL T3151 mutation. The cytotoxicity was as potent as that shown towards IM-sensitive cell lines.

Inhibition of BCR-ABL autophosphorylation in CML cell lines by panobinostat and/or ponatinib. The protein expression levels of BCR-ABL and autophosphorylated BCR-ABL were determined by Western blotting of cell lines after treatment with panobinostat or ponatinib, alone or in combination. The constitutive expression level of BCR-ABL was higher in K562/IM-R1 cells than in K562 cells, indicating the overexpression of the ABL kinase by *BCR-ABL* gene amplification in this cell line (Fig. 5a). This result was consistent with our previous findings.⁽²¹⁾

Treatment with ponatinib inhibited the autophosphorylation of BCR-ABL in all cell lines, indicating inhibition of ABL kinase activity. The expression level of BCR-ABL was unchanged by this treatment. Treatment with panobinostat reduced the protein expression of BCR-ABL in all cell lines, suggesting that it induced degradation of the BCR-ABL protein (Fig. 5b–e). The detected autophosphorylation of BCR-ABL was also reduced by panobinostat, which reflected the decreased expression of the BCR-ABL protein in the presence of panobinostat. This degradation of BCR-ABL might be attributable to the disruption of the chaperone function of HSP90 by panobinostat. It was noted that the combination of ponatinib with panobinostat augmented the inhibition of BCR-ABL autophosphorylation in all of the cell lines (Fig. 5b–e).

Inhibition of BCR-ABL downstream signal pathways in CML cell lines by panobinostat and/or ponatinib. BCR-ABL-dependent signal transduction is critical to the vigorous proliferation of CML cells. Signal transducer and activator of transcription 5, AKT, and ERK1/2 are signaling molecules that are located downstream in BCR-ABL signal pathways.⁽¹²⁾ Ponatinib inhibited the phosphorylation of STAT5, AKT, and ERK1/2 in all of the cell lines including in the cell line with T3151 mutated BCR-ABL (Fig. 5b–e). Panobinostat also reduced the phosphorylation of these downstream signals in all of the cell lines in a concentration-dependent manner (Fig. 5b–e). Moreover, the phosphorylation of downstream signals was more potently

Fig. 1. Cellular sensitivity to imatinib mesylate (IM). The CML cell lines and their respective IM-resistant variants, K562 and K562/IM-R1 cells (a), Ba/F3 and Ba/F3/T3151 cells (b) were incubated for 72 h with IM at various concentrations, followed by the XTT assay for evaluation of cell proliferation. Proliferation was evaluated as a percentage of that in cells not treated with IM. The dotted line indicates 50% proliferation that was used to calculate the IC_{50} values.



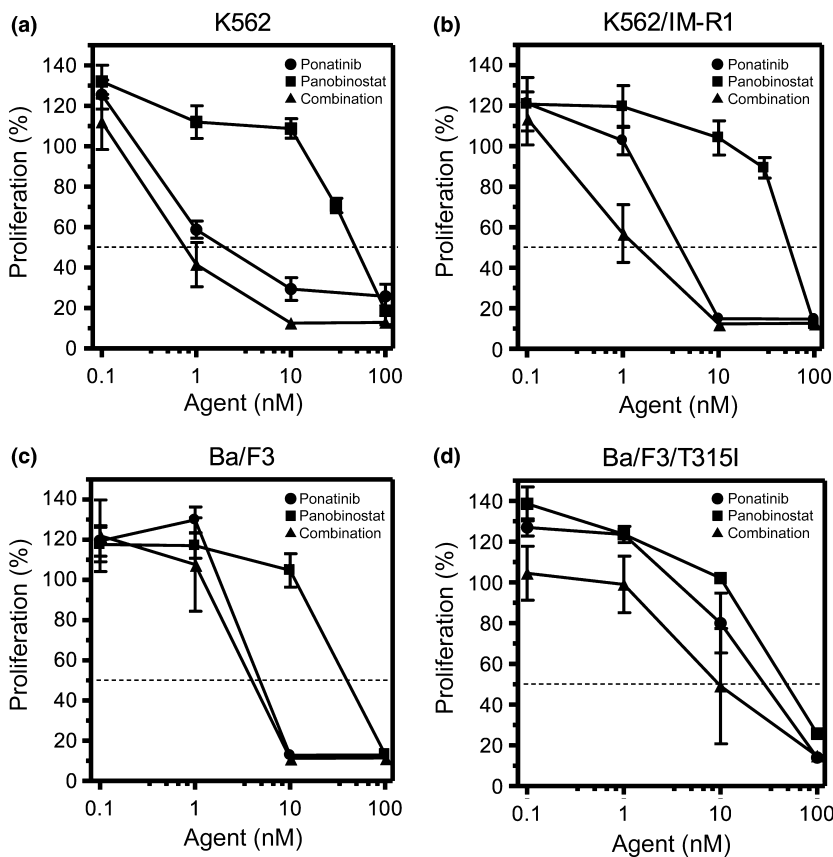


Fig. 2. Cellular sensitivity to panobinostat and/or ponatinib. The CML cell lines and their respective imatinib mesylate (IM)-resistant variants, K562 cells (a) K562/IM-R1 cells (b), Ba/F3 cells (c), and Ba/F3/T3151 cells (d) were incubated with ponatinib or panobinostat, alone or in combination, for 72 h at the indicated concentrations within the range of 0.1–100 nM, followed by XTT assays for evaluation of cell proliferation. Proliferation was evaluated as a percentage of that in cells not treated with ponatinib and/or panobinostat. The dotted line indicates 50% proliferation that was used to calculate the IC₅₀ values.

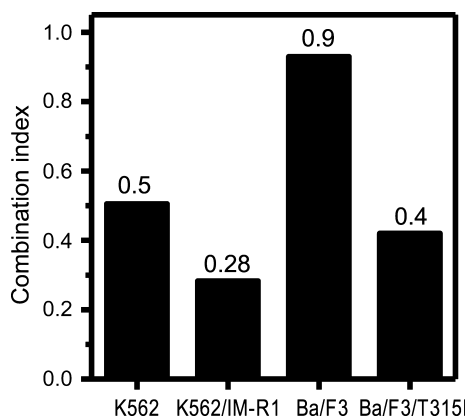


Fig. 3. Combination index (CI) values evaluated for panobinostat and ponatinib. The CI values evaluated for panobinostat and ponatinib for the indicated cell lines were calculated using the computer software CalcuSyn (version 2.0). CI < 1, synergistic effect; CI = 1, additive effect; CI > 1, antagonistic effect.

inhibited by the combination of ponatinib and panobinostat than by each single agent (Fig. 5b–e). The strong inhibition of phosphorylation of STAT5 in K562 and Ba/F3 cells, of AKT in K562/IM-R1 and Ba/F3 cells, and of ERK1/2 in K562 and K562/IM-R1 cells by 5 nM ponatinib alone, may have masked any additional effect of panobinostat. Interestingly, the expression of the AKT protein was reduced by panobinostat, suggesting the possibility that AKT is a client protein of HSP90. Thus, the inhibition of ABL-kinase activity by ponatinib combined with the induced degradation of BCR-ABL by

Table 3. Induction of apoptosis in CML cell lines by panobinostat and/or ponatinib

	Mean values (%)			
	K562	K562/IM-R1	Ba/F3	Ba/F3/T3151
Panobinostat	20.4	17.8	5.4	12.4
Ponatinib	34.1	23.3	24.7	36.7
Combination	70.3	65.4	44.9	71.9

Cells were incubated with panobinostat, ponatinib, or their combination at a concentration of 10 nM (K562 cells, K562/IM-R1 cells, Ba/F3 cells) or 30 nM (Ba/F3/T3151 cells) per agent for 48 h, followed by flow cytometric analysis of apoptosis. Apoptotic cell death was evaluated by annexin V positivity. Mean values are shown.

panobinostat resulted in a synergistic decrease in the phosphorylation of BCR-ABL and its downstream signaling pathways.

Inhibition of HDAC activity, induction of histone H3 acetylation, and augmentation of HSP70 protein expression in CML cell lines by panobinostat or panobinostat plus ponatinib. The major mechanism of action of panobinostat is inhibition of HDAC activity. The activity of HDAC was therefore measured in all of the cells after treatment with panobinostat or with panobinostat plus ponatinib. The activity of HDAC was significantly inhibited by panobinostat regardless of the presence of ponatinib in all of the cell lines (Fig. 6). Activity of HDAC was unchanged in these cell lines after treatment with IM or ponatinib alone (data not shown).

Western blot analysis showed the acetylation of histone H3 in cells after treatment that included panobinostat (Fig. 7a). The level of the non-histone protein, HSP90 was unchanged

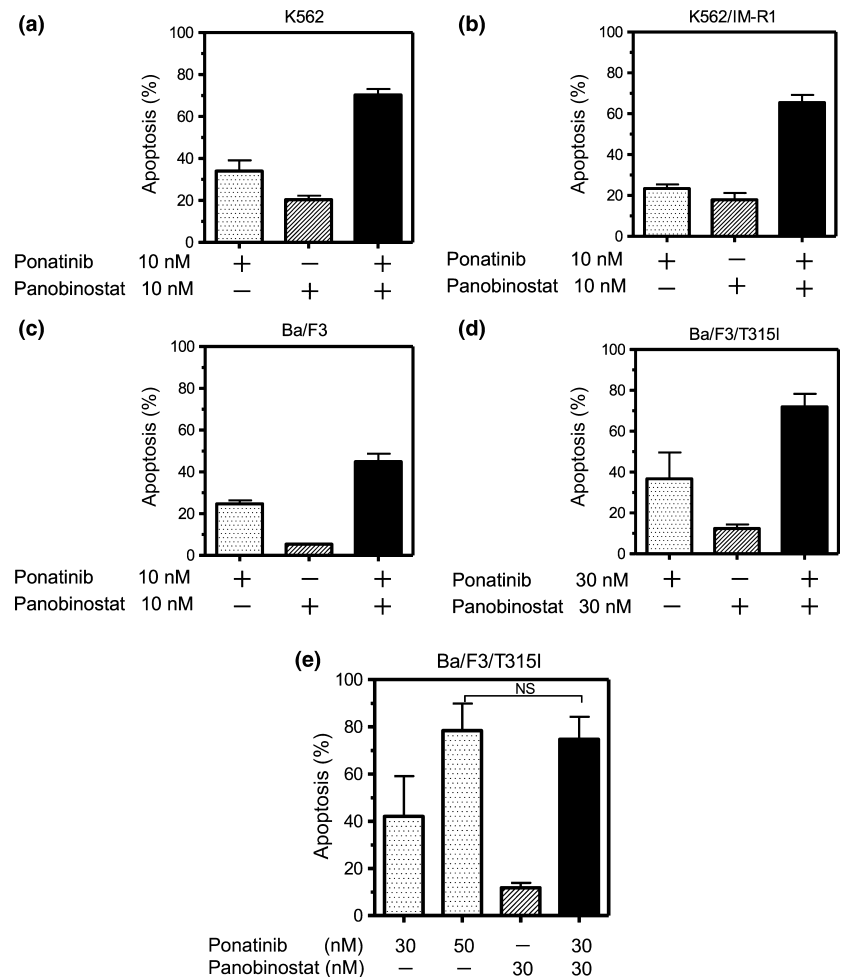


Fig. 4. Effect of panobinostat and/or ponatinib on apoptosis of CML cell lines. The CML cell lines were treated for 48 h with the indicated concentrations of panobinostat or ponatinib, alone or in combination. Apoptotic cell death was evaluated by FACS analysis of annexin V positivity (a–d). (e) The pro-apoptotic effect of treatment of Ba/F3/T3151 cells with 50 nM ponatinib alone, with a combination of 30 nM ponatinib and 30 nM panobinostat, or with 30 nM panobinostat alone was also evaluated. N.S., not significant ($P > 0.05$).

by treatment of K562 cells with panobinostat or with panobinostat plus ponatinib. Nevertheless, panobinostat increased the protein level of HSP70 in K562 cells (Fig. 7b), which is suggestive of inhibition of the chaperone function of HSP90.⁽²⁴⁾

Discussion

Imatinib resistance in CML is attributed to a variety of mechanisms including reactivation of the BCR-ABL kinase, increased efflux of the drug through P-glycoprotein, and compensation for the loss of BCR-ABL signaling by other kinase pathways such as Src-family kinases.^(12,25–32) Therefore, to overcome IM resistance the use of new agents with anti-CML activity that is mediated through different mechanisms of action is necessary. As the major reason for IM resistance is reactivation of the BCR-ABL kinase, we investigated the cytotoxic effects of the new BCR-ABL TKI ponatinib combined with the HDAC inhibitor panobinostat against IM-resistant cell lines, including a cell line that expresses a BCR-ABL kinase that is active due to T315I mutation.

The present study clearly shows that both ponatinib and panobinostat showed antiproliferative effects and cytotoxicity against the IM-resistant variant K562/IM-R1 cells with BCR-ABL amplification and Ba/F3/T315I cells with BCR-ABL with a T315I kinase mutation (Figs 2–4d). Moreover, the combination of panobinostat and ponatinib showed synergistic antiproliferative and cytotoxic effects on these cell lines (Fig. 3). Thus,

ponatinib inhibited the constitutive ABL kinase activity in cells expressing gene-amplified active ABL or in cells expressing ABL with a T315I mutation (Fig. 5b–e). This inhibition of ABL kinase activity was accompanied by inhibition of the phosphorylation of downstream signaling molecules. Panobinostat inhibited HDAC activity, resulting in acetylated histone H3, which might result in inhibition of the proliferation of CML cells.⁽¹⁴⁾ Moreover, HSP70 expression was augmented by panobinostat, suggesting that the chaperone function of HSP90 for BCR-ABL was disrupted (Fig. 7b).^(24,33) Therefore, synergistic cytotoxicity exerted by the combination of panobinostat and ponatinib was attributed to their different mechanisms of action: inhibition of the ABL kinase and inhibition of HDAC activity, respectively.

Fiskus *et al.*⁽³⁴⁾ reported that panobinostat combined with nilotinib induced a greater loss of cell viability and apoptosis of IM-resistant CML cells, including cells expressing BCR-ABL with the T315I mutation, than panobinostat or nilotinib alone. In that study, it was not determined how panobinostat enhanced the activity of nilotinib, which structurally cannot contact the BCR-ABL protein with the T315I mutation. For the present study, we therefore chose to study ponatinib, which can structurally contact the BCR-ABL protein with the T315I mutation. Okabe *et al.*⁽³⁵⁾ reported that the combination of vorinostat and ponatinib had synergistic effects against CML cells including cells expressing BCR-ABL with the T315I mutation. In their study, co-treatment with vorinostat and ponatinib was cytotoxic towards Ba/F3/T315I cells and

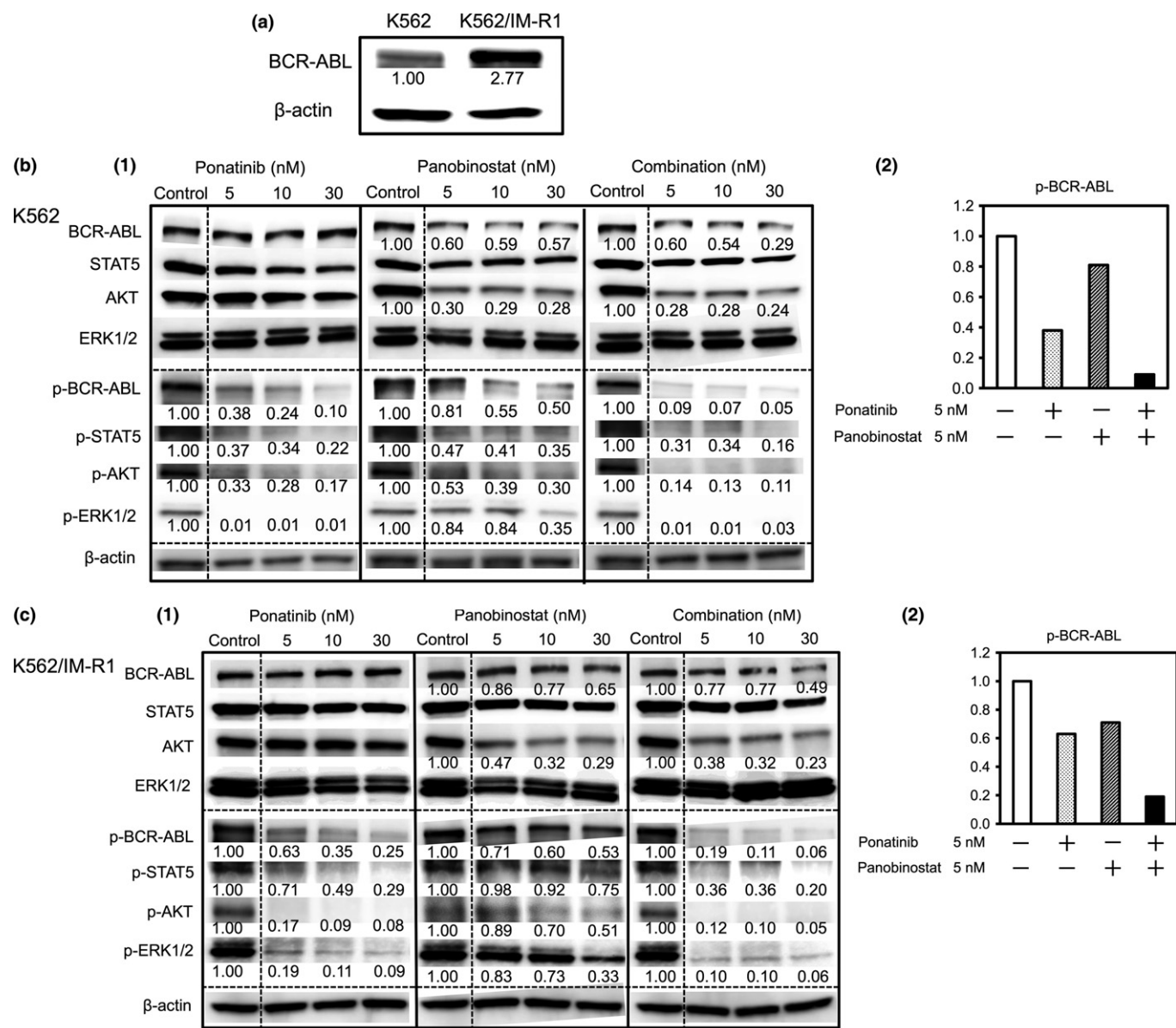


Fig. 5. Effect of panobinostat and/or ponatinib on the expression of BCR-ABL, phospho-BCR-ABL, and downstream signaling molecules in CML cell lines. All cell lines were treated for 24 h with panobinostat, ponatinib, or their combination at the indicated concentrations followed by Western blotting of BCR-ABL expression (a). (b–e) (1) Western blotting of BCR-ABL, STAT5, AKT, and ERK1/2 proteins (top) or their phosphorylated (p-) forms (bottom). β-Actin was assayed as a loading control. Band intensity was determined using densitometry and is indicated below each band. (b–e) (2) The combination of ponatinib with panobinostat at the indicated concentrations inhibited p-BCR-ABL expression more strongly than the sum of each agent alone.

ponatinib-resistant Ba/F3 cells through reduction in the phosphorylation of CRKL, a signaling molecule downstream of BCR-ABL, and through enhancement of caspase 3 and poly (ADP-ribose) polymerase activity. Our present study showed that the combination of panobinostat and ponatinib also displayed synergistic antiproliferative and cytotoxic effects towards not only cells expressing BCR-ABL with the T315I mutation but also towards cells with *BCR-ABL* gene amplification (Figs 2–4d). Moreover, we showed that panobinostat and the combination of panobinostat with ponatinib inhibited HDAC activity (Fig. 6), induced histone H3 acetylation (Fig. 7a), and augmented HSP70 expression (Fig. 7b). These data suggest that the combination of panobinostat and ponatinib may hold promise for the treatment of IM-resistant cells.

The BCR-ABL protein activates several molecular mechanisms to inhibit apoptosis.⁽³³⁾ Thus, BCR-ABL increases the phosphorylation and the activity of STAT5, resulting in increased expression of the anti-apoptotic BCL-XL protein. BCR-ABL also induces activation of the phosphoinositide 3-kinase pathway, resulting in activated AKT kinase that leads to the phosphorylation and inactivation of downstream signaling proteins such as Bcl-2-associated death promoter and caspase 9 that regulate apoptosis. Moreover, BCR-ABL activates Ras/Raf/ERK1/2 and nuclear factor-κB activities, which together inhibit apoptosis through multiple mechanisms.^(33,36) In the present study, ponatinib inhibited both the autophosphorylation of BCR-ABL and phosphorylation of downstream signaling molecules, resulting in the induction of apoptosis. It

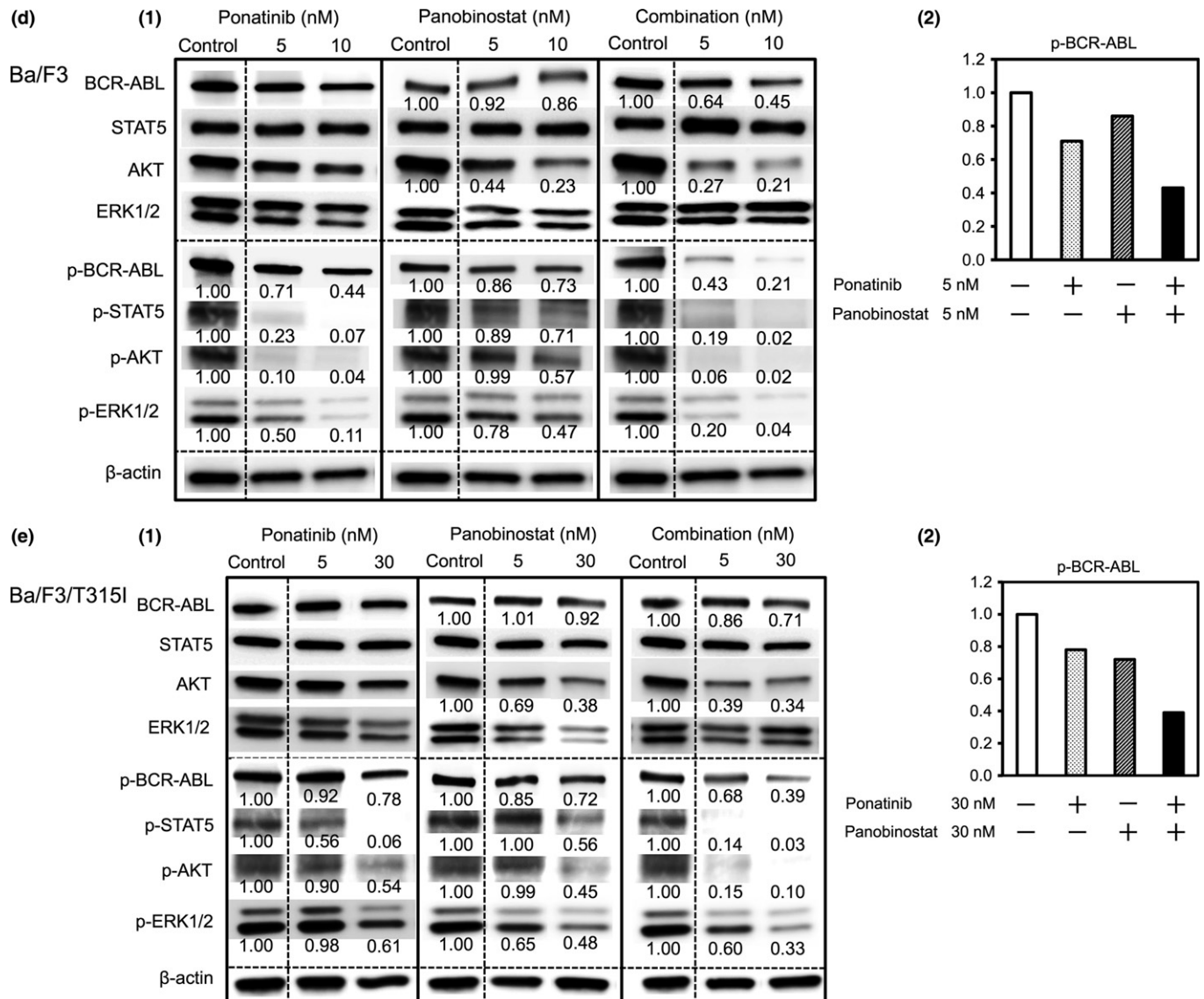


Fig. 5. continued.

has been reported that panobinostat also decreases the levels of generic oncogenic targets, including not only BCR-ABL but also Fms-related tyrosine kinase 3, c-Raf, and AKT through attenuation of the chaperone function of HSP90.^(14,34,37) Heat shock protein 90 is a non-histone protein that is associated with HDAC6 and that functions as a chaperone of the BCR-ABL protein, protecting it from degradation. This protection of BCR-ABL supports activation of the aforementioned downstream pathways, cell cycle proliferation, and apoptosis.⁽¹⁴⁾ Accordingly, HSP90 has also been previously suggested to be an important therapeutic target of CML.^(34,35)

In the present study, panobinostat reduced BCR-ABL and AKT protein expression (Fig. 5b–e), and increased HSP70 levels (Fig. 7b). These data are suggestive of a decreased chaperone function of HSP90 that would lead to the degradation of BCR-ABL in both wild-type and IM-resistant cells.^(14,33,34,38) Moreover, the induction of histone H3 acetylation by panobinostat (Fig. 7a) was consistent with a previous report that panobinostat did not exert antiproliferative effects

without induction of acetylated histone H3.⁽¹⁴⁾ Importantly it was observed that the autophosphorylation of BCR-ABL following treatment with the combination of ponatinib and panobinostat was lower than that following treatment with either agent alone. These results suggested that inhibition of the phosphorylation of BCR-ABL by ponatinib combined with the degradation of BCR-ABL and AKT induced by panobinostat would exert synergistic inhibitory effects on BCR-ABL signaling.

In the HDAC assay, panobinostat alone or the combination of panobinostat with ponatinib reduced HDAC activity (Fig. 6). The reduction in HDAC activity tended to be greater when the cells were treated with ponatinib together with panobinostat compared to treatment with panobinostat alone ($P = 0.33$ for K562 cells, $P = 0.16$ for K562/IM-R1 cells, $P = 0.16$ for Ba/F3 cells, and $P = 0.18$ for Ba/F3/T315I cells; panobinostat versus panobinostat plus ponatinib). Gonzalez-Zuñiga *et al.*⁽³⁹⁾ reported that, in Alzheimer's disease, c-abl stabilized HDAC2, resulting in the repression of neuronal gene expression. In that study, a transgenic mouse model of

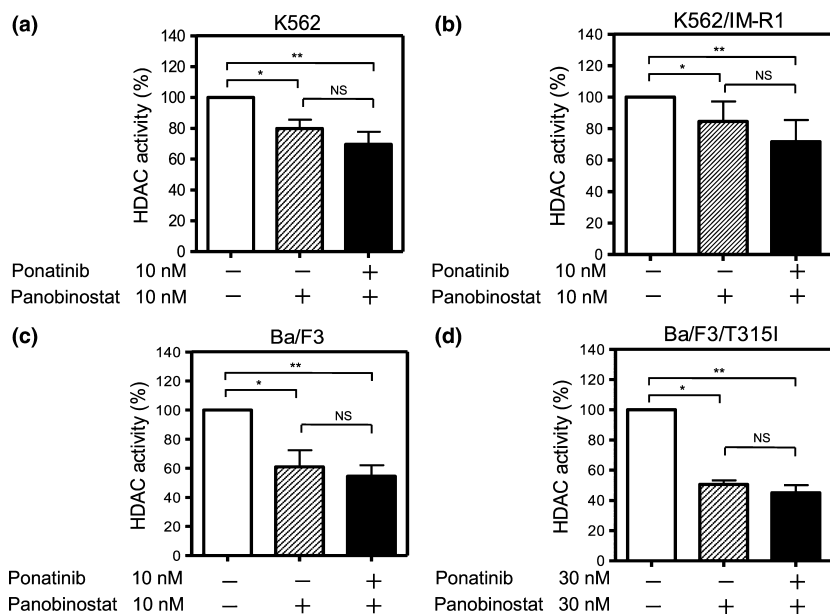


Fig. 6. Effect of panobinostat or panobinostat plus ponatinib on histone deacetylase (HDAC) activity in CML cell lines. All cell lines were treated for 12 h with the indicated concentration of panobinostat or panobinostat plus ponatinib. HDAC activity was then determined using an HDAC assay kit. Activity is indicated relative to that in the absence of treatment (100%). K562, * $P = 0.026$, ** $P = 0.023$; K562/IM-R1, * $P = 0.047$, ** $P = 0.013$; Ba/F3, * $P = 0.014$, ** $P = 0.037$; Ba/F3/T315I, * $P = 0.025$, ** $P = 0.041$. N.S., not significant ($P > 0.05$).

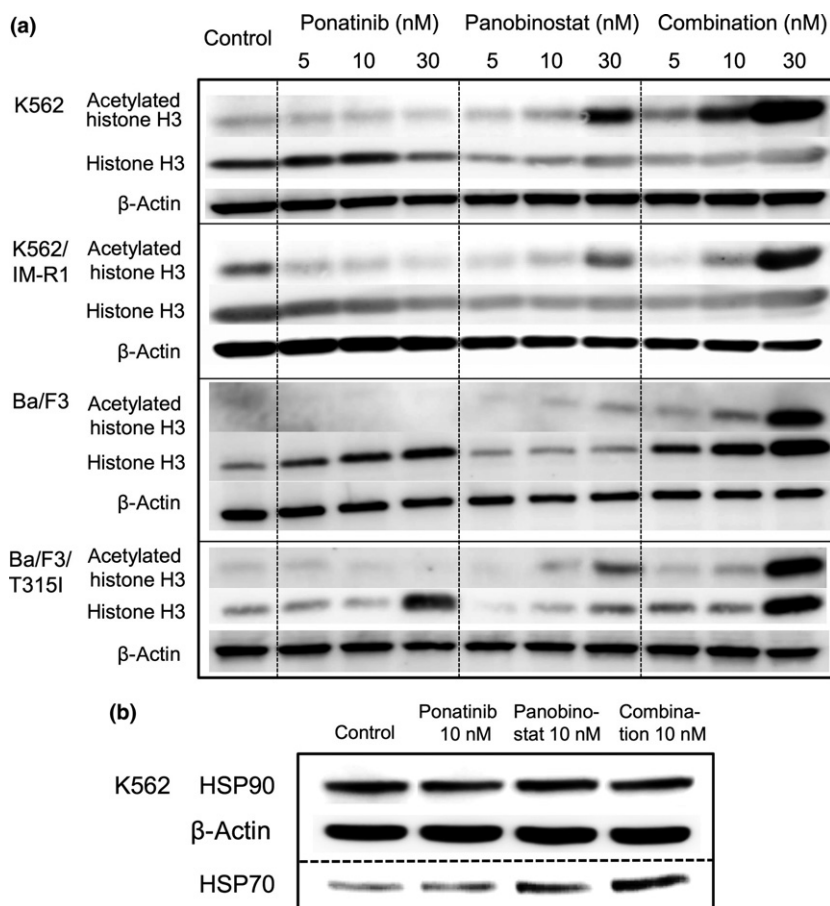


Fig. 7. (a) Effect of panobinostat and/or ponatinib on the acetylation status of histone H3 and on the expression level of heat shock protein (HSP)70 in CML cell lines. All cell lines were treated for 24 h with panobinostat, ponatinib, or their combination at the indicated concentrations. Western blotting was then carried out to evaluate the protein expression levels of histone H3 and acetylated histone H3. β -Actin was assayed as a loading control. (b) Western blotting was also used to evaluate the protein expression levels of HSP90 and HSP70 in K562 cells at 24 and 18 h, respectively, after treatment with 10 nM panobinostat, ponatinib, or their combination.

Alzheimer's disease was treated with IM, which resulted in decreased HDAC2 levels, suggesting that the BCR-ABL protein was also involved in stabilizing HDAC activity. Therefore, ponatinib might also be associated with the regulation of HDAC through unknown epigenetic mechanisms.

Adverse clinical effects of ponatinib include serious vascular occlusion events such as myocardial infarction or stroke.⁽⁴⁰⁾ In

the PACE trial, patients usually take ponatinib at a dose of 45 mg/day (as a starting dose).⁽¹⁷⁾ It was reported that the trough level of ponatinib reaches 40 nM when patients take ponatinib at a dose of 30 mg/day.^(41,42) In our study, even in IM-resistant cells that display active BCR-ABL, including BCR-ABL with the T315I mutation, combination therapy of panobinostat and ponatinib at a dosage of 30 nM per agent

exerted synergistic cytotoxic effects (Fig. 4d). Flow cytometric analysis of apoptosis indicated that the combination of these two agents at a concentration of 30 nM each agent had similar cytotoxic effects towards Ba/F3/T315I cells as 50 nM ponatinib alone (Fig. 4e). O'Hare *et al.*⁽¹⁹⁾ reported that ponatinib suppressed the emergence of any single mutation of BCR-ABL at a concentration of 40 nM. Our study suggested that, when panobinostat is combined with ponatinib, low concentrations of these agents might be sufficient to exert strong cytotoxic effects towards CML cells, including towards IM-resistant cells. Therefore, combining a low concentration of ponatinib with a low concentration of panobinostat might be a possible solution to the problem of adverse effects of higher concentrations of ponatinib.

In conclusion, this is the first report on the combination of ponatinib and panobinostat. We have clearly shown that treatment with a combination of panobinostat and ponatinib synergistically overcame IM resistance that was mediated either through *BCR-ABL* gene amplification or through BCR-ABL T315I mutation. This synergistic effect might be attributable to inhibition of ABL kinase activity combined with degradation of the BCR-ABL protein. The combination of these two agents

might offer a new therapeutic strategy with excellent anti-CML activity and reduced toxicity.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

AKT	protein kinase B
CI	combination index
HDAC	histone deacetylase
HSP	heat shock protein
IM	imatinib mesylate
PACE	Ponatinib Ph-positive acute lymphoblastic leukemia and CML Evaluation
STAT5	signal transducer and activator of transcription 5
TKI	tyrosine kinase inhibitor

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