

Blockade of the ERK or PI3K-Akt signaling pathway enhances the cytotoxicity of histone deacetylase inhibitors in tumor cells resistant to gefitinib or imatinib

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Abstract

Deregulated activation of protein tyrosine kinases, such as the epidermal growth factor receptor (EGFR) and Abl, is associated with human cancers including non-small cell lung cancer (NSCLC) and chronic myeloid leukemia (CML). Although inhibitors of such activated kinases have proved to be of therapeutic benefit in individuals with NSCLC or CML, some patients manifest intrinsic or acquired resistance to these drugs. We now show that, whereas blockade of either the extracellular signal-regulated kinase (ERK) pathway or the phosphatidylinositol 3-kinase (PI3K)–Akt pathway alone induced only a low level of cell death, it markedly sensitized NSCLC or CML cells to the induction of apoptosis by histone deacetylase (HDAC) inhibitors. Such enhanced cell death induced by the respective drug combinations was apparent even in NSCLC or CML cells exhibiting resistance to EGFR or Abl tyrosine kinase inhibitors, respectively. Co-administration of a cytostatic signaling pathway inhibitor may contribute to the development of safer anticancer strategies by lowering the required dose of cytotoxic HDAC inhibitors for a variety of cancers.

Key words: non-small cell lung cancer, chronic myeloid leukemia, MEK inhibitor, PI3K inhibitor, HDAC inhibitor, drug resistance.

Introduction

Protein tyrosine kinases play key roles in the regulation of cell proliferation, migration, and survival [1]. Aberrant activity of these enzymes can result from genetic alterations acquired early in tumorigenesis and remains an essential aspect of tumor cell physiology throughout subsequent disease progression [2]. For example, abnormal activation of EGFR¹ that results from overexpression or activating mutation of this receptor tyrosine kinase has been associated with many types of human cancer including those of the lung, breast, colon, ovary, and bladder [3]. In addition, constitutive activation of the tyrosine kinase Abl that results from the formation of a fusion protein with Bcr is associated with CML [4]. Targeted inhibition of these protein tyrosine kinases has therefore emerged as an attractive strategy for cancer treatment [5]. Small-molecule inhibitors of EGFR or Abl, including gefitinib [6] and imatinib [7], respectively, have been developed.

Aberrant activation of the EGFR tyrosine kinase is most frequently associated with NSCLC [3]. Specific inhibition of the kinase activity by gefitinib or erlotinib has proved to be of therapeutic benefit in individuals with NSCLC [8]. Whereas most NSCLCs with activating mutations of EGFR, such as the deletion of five amino acids (Δ E746-A750) or the missense mutation L858R, are sensitive to these EGFR TKIs [9], those that overexpress the wild-type receptor are refractory to such treatment [10]. Furthermore, a second mutation (T790M) of EGFR that confers acquired resistance to gefitinib has been found to develop in some NSCLC patients treated with this drug [11]. Similarly, although imatinib is effective in most CML patients expressing the Bcr-Abl

fusion protein, point mutations such as T315I develop in some individuals during continued imatinib therapy, resulting in acquired resistance to the inhibitor [12]. Development of effective strategies to treat NSCLC or CML patients who develop resistance to these TKIs is thus required.

The ERK and PI3K-Akt signaling pathways function downstream of EGFR and Bcr-Abl and are constitutively activated in many NSCLC and CML cells. We recently showed that specific blockade of the ERK pathway by an inhibitor of ERK kinase (MEK) sensitizes tumor cells with constitutive activation of this pathway to apoptotic cell death induced by an HDAC inhibitor [13]. We here show that blockade of either the ERK pathway or the PI3K-Akt pathway markedly enhances the cytotoxicity of HDAC inhibitors in NSCLC and CML cells in a manner independent of their respective sensitivity to gefitinib or imatinib.

Materials and Methods

Agents and antibodies. Gefitinib and imatinib (methanesulfonate salt) were obtained from LC Laboratories, and PX-866 and HC-toxin were from Sigma. PD184352 was synthesized as described previously [14]. Antibodies to ERK isoforms 1 and 2 (ERK1/2), to EGFR, and to c-Abl were from Santa Cruz Biotechnology; those to diphosphorylated ERK1/2, to Tyr¹¹⁷³-phosphorylated EGFR, and to β -actin were from Sigma; and those to Akt, to Ser⁴⁷³-phosphorylated Akt, to Tyr²⁴⁵-phosphorylated c-Abl,

and to Asp¹⁷⁵-cleaved caspase-3 were from Cell Signaling Technology.

Cell culture. Human lung adenocarcinoma PC-9, H1650, and H1975 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum [15]. The human CML cell line K562 as well as immortalized murine bone marrow-derived pro-B cells stably expressing either native human Bcr-Abl (BaF3[Bcr-Abl]) or the T315I mutant of Bcr-Abl (BaF3[Bcr-Abl^{T315I}]) [16] were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Flow cytometry. Cells exposed to various agents were harvested by treatment with trypsin, fixed with 70% ethanol, incubated with DNase-free RNase A (100 µg/ml, Sigma), stained with propidium iodide (20 µg/ml), and analyzed for DNA content with the use of a FACSCalibur flow cytometer and Cell Quest Pro software (Becton Dickinson) [17]. For detection of ROS accumulation, cells were incubated for 30 min at 37°C with 10 µM CM-H₂DCFDA and then monitored for fluorescence as previously described [13, 18].

Immunoblot analysis. Cell lysates were prepared as described [19, 20] and fractionated by SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride membrane and probed with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Promega). Immune complexes were visualized with enhanced chemiluminescence reagents (GE Healthcare

Bio-sciences).

Results and Discussion

EGFR mutations are not the only determinant of gefitinib sensitivity in NSCLC cells.

We examined the gefitinib sensitivity of several NSCLC cell lines expressing activated mutant forms of EGFR, including PC-9 (Δ E746-A750 deletion), H1650 (Δ E746-A750), and II-18 (L858R mutation) cells. Although gefitinib inhibited EGFR tyrosine kinase activity (as reflected by EGFR autophosphorylation) in all these NSCLC cells in a concentration-dependent manner, it inhibited the proliferation of PC-9 and II-18 cells but not that of H1650 cells (Fig. 1A). Furthermore, gefitinib induced a concentration-dependent increase in the proportion of PC-9 cells with a fractional DNA content (cells in sub-G₁ phase), a characteristic feature of apoptosis [17], without eliciting a similar effect in H1650 or II-18 cells.

At concentrations of $\geq 0.1 \mu\text{M}$, gefitinib inhibited the activation of Akt and ERK1/2, major signaling molecules that function downstream of EGFR, as well as induced the activation of caspase-3 in PC-9 cells (Fig. 1B). In contrast, gefitinib inhibited the activation of ERK1/2 but not that of Akt in II-18 cells, whereas it had no effect on the activation state of ERK1/2 or Akt in H1650 cells. The PI3K-Akt pathway is a major determinant of cell survival [21], whereas the ERK pathway is a major regulator of cell

proliferation [22]. Our results suggest that blockade of both the ERK and PI3K-Akt pathways is required for the induction of apoptosis in the NSCLC cell lines examined. Gefitinib-induced inhibition of the ERK pathway but not of the PI3K-Akt pathway appeared to result only in suppression of proliferation in H1975 cells. Consistent with this notion, treatment with the combination of the MEK inhibitor PD184352 (10 μ M) and the PI3K inhibitor PX-866 (5 μ M) to suppress the ERK and PI3K-Akt pathways, respectively, induced apoptosis in all three NSCLC cell lines (Fig. 2A, B). Furthermore, the combination of gefitinib and PX-866 induced apoptosis in H1975 cells as effectively as did the combination of PD184352 and PX-866 (data not shown). In this context, combined inhibition of the ERK and PI3K-Akt pathways in vivo was recently shown to result in marked antitumor activity in basal-like or triple-negative breast cancers, which are poorly responsive to traditional therapies and have a poor prognosis [23]. Although the molecular mechanisms by which inhibition of EGFR tyrosine kinase activity by gefitinib results in such diverse responses among tumor cells remain to be elucidated, our results indicate that activating mutations of EGFR are not the sole determinant of gefitinib sensitivity in NSCLC cells.

Blockade of the ERK or PI3K-Akt pathway enhances the cytotoxicity of HDAC inhibitors in NSCLC cells irrespective of gefitinib sensitivity.

HC-toxin, an HDAC inhibitor, induced apoptotic cell death in PC-9, H1650, and H1975 cells, with the proportion of cells in sub-G₁ phase increasing up to 80 to 90% in

the presence of HC-toxin at concentrations increasing up to 5 μM (Fig. 2A). Blockade of the ERK pathway by PD184352 (10 μM) or blockade of the PI3K-Akt pathway by PX-866 (5 μM) alone had only a slight or moderate effect on the induction of apoptosis in these tumor cells. However, blockade of either pathway enhanced the pro-apoptotic effect of HC-toxin in each NSCLC cell line, with this enhancement being most prominent at a low concentration (0.1 μM) of HC-toxin that alone showed only limited apoptosis-inducing activity. Blockade of the ERK pathway by the MEK inhibitor U0126 (20 μM) or blockade of the PI3K-Akt pathway by the PI3K inhibitor LY294002 (10 μM) also enhanced the cytotoxicity of other structurally distinct HDAC inhibitors such as valproic acid and trichostatin A in these cell lines (data not shown). Gefitinib also enhanced the pro-apoptotic activity of HC-toxin in PC-9 and H1650 cells, but not in H1650 cells, consistent with the ability of gefitinib to suppress the ERK or PI3K-Akt pathway in these cell lines (Fig. 1B). Together, these results indicate that specific blockade of the ERK or PI3K-Akt pathway sensitizes NSCLC cells expressing activated mutants of EGFR to the induction of cell death by HDAC inhibitors, apparently in a manner independent of the sensitivity of these tumor cells to gefitinib.

The cytotoxicity of HDAC inhibitors is associated with the intracellular accumulation of ROS [13, 18]. The ERK [24] and PI3K-Akt [25] pathways have been implicated in protection of cells from oxidative stress. We therefore next examined whether blockade of these pathways might enhance the ability of HDAC inhibitors to induce the accumulation of ROS in NSCLC cells. Although treatment of PC-9, H1650, or H1650 cells with 0.1 μM HC-toxin, 0.1 or 1 μM gefitinib, 10 μM PD184352, or 5 μM

PX-866 alone did not induce substantial accumulation of ROS, the combination of 0.1 μ M HC-toxin and either 10 μ M PD184352 or 5 μ M PX-866 resulted in ROS accumulation to the same marked extent as that induced by a high concentration (1 μ M) of HC-toxin alone (Fig. 2C). Furthermore, the combination of HC-toxin and gefitinib induced marked or moderate accumulation of ROS in PC-9 and H1650 cells, respectively, but had no such effect in H1650 cells. The free radical scavenger NAC suppressed not only the accumulation of ROS (Fig. 2C) but also the increase in the proportion of dead cells with a fractional DNA content (Fig. 2A) as well as the activation of caspase-3 (data not shown) induced by the combination of HC-toxin and either gefitinib, PD184352, or PX-866. These results thus indicate that blockade of the ERK or PI3K-Akt pathway markedly sensitizes all three NSCLC cell lines to HDAC inhibitor-induced ROS accumulation, and that the accumulated ROS mediate the induction of apoptosis by the respective drug combinations.

Although the combination of PD184352 and PX-866 induced apoptotic cell death efficiently in these NSCLC cells, it did not induce substantial accumulation of ROS (Fig. 2C). Furthermore, cell death induced by the combination of PD184352 and PX-866 were affected only slightly by the presence of NAC (data not shown). Although the molecular mechanisms by which combined blockade of the ERK and PI3K-Akt pathways induces apoptosis in tumor cells remain to be elucidated, such death induction appears to be largely independent of ROS accumulation.

Combination of either an ERK pathway or PI3K-Akt pathway inhibitor and an HDAC

inhibitor kills cells harboring imatinib-resistant Bcr-Abl^{T315I}

We next examined whether the combination of an ERK or PI3K-Akt pathway inhibitor and an HDAC inhibitor might kill imatinib-resistant CML cells. For these experiments, we used the human CML cell line K562 expressing native Bcr-Abl as well as an immortalized murine pro-B cell line stably expressing either native human Bcr-Abl (BaF3[Bcr-Abl] cells) or the T315I mutant of Bcr-Abl (BaF3[Bcr-Abl^{T315I}] cells); the latter cells are a widely studied model for CML cells resistant to even second-generation Abl TKIs including dasatinib [16].

Imatinib inhibited in a concentration-dependent manner the tyrosine kinase activity of Bcr-Abl (as reflected by Bcr-Abl autophosphorylation), the activation of Akt and ERK1/2, and cell proliferation in K562 and BaF3[Bcr-Abl] cells (Fig. 3). It also induced a prominent increase in the proportion of dead cells with a fractional DNA content as well as the activation of caspase-3 in these cells (Fig. 3). In contrast, imatinib failed to inhibit Bcr-Abl tyrosine kinase activity, the activation of Akt or ERK1/2, or cell proliferation as well as to induce apoptotic cell death or caspase-3 activation in BaF3[Bcr-Abl^{T315I}] cells.

HC-toxin induced cell death in a concentration-dependent manner not only in K562 and BaF3[Bcr-Abl] cells but also in BaF3[Bcr-Abl^{T315I}] cells (Fig. 4A). Although blockade of the ERK pathway or the PI3K-Akt pathway alone showed little effect on the induction of cell death, combined suppression of both pathways effectively induced apoptosis in all three cell lines. Furthermore, blockade of either pathway markedly

sensitized BaF3[Bcr-Abl^{T315I}] as well as K562 and BaF3[Bcr-Abl] cells to HC-toxin-induced cell death; blockade of the ERK pathway appeared more effective in this regard than did that of the PI3K-Akt pathway. Thus, whereas 0.25 μ M HC-toxin or 10 μ M PD184352 alone induced only a small increase in the proportion of apoptotic cells in the BaF3[Bcr-Abl^{T315I}] cell line, the combination of these two agents markedly increased the extent of cell death to a level similar to that apparent with 5 μ M HC-toxin. The combination of a low concentration (0.1 or 0.25 μ M) of HC-toxin and either 10 μ M PD184352 or 5 μ M PX-866 also induced marked accumulation of ROS, and both this effect and that on cell death induced by the respective drug combinations were inhibited by NAC in all three cell lines. These results indicate that the combination of a low concentration of an HDAC inhibitor and either an ERK or PI3K-Akt pathway inhibitor effectively kills CML cells, irrespective of their sensitivity to Abl TKIs and via a mechanism that involves the enhanced accumulation of ROS.

Conclusion

We have shown that the combination of either an ERK pathway inhibitor or a PI3K-Akt pathway inhibitor and a low concentration of an HDAC inhibitor, each of which alone exhibits only marginal apoptosis-inducing activity, efficiently kills NSCLC and CML cells in a manner independent of their sensitivity to EGFR or Abl TKIs, respectively. The efficacy of these drug combinations was greater than that of the combination of ERK pathway and PI3K-Akt pathway inhibitors. We have previously

shown that blockade of the ERK pathway [26] or the PI3K-Akt pathway [27] enhances the induction of apoptosis by microtubule-destabilizing agents in tumor cells in which the respective signaling pathway is constitutively activated. Co-administration of a cytostatic signaling pathway inhibitor, such as a MEK inhibitor or a PI3K inhibitor, might thus represent a means to achieve safer anticancer strategies through lowering the required dose of cytotoxic anticancer drugs such as HDAC inhibitors or microtubule-destabilizing agents in a wide variety of cancer cells, including those resistant to EGFR or Abl TKIs. Consistent with this notion, optimal use of molecularly targeted therapies has recently been proposed to lie in combination treatment, either with classic cytotoxic drugs or with other targeted therapies [22, 28].

Acknowledgments

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Footnote

¹Abbreviations: EGFR, epidermal growth factor receptor; CML, chronic myeloid leukemia; NSCLC, non-small cell lung cancer; TKI, tyrosine kinase inhibitor; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; HDAC, histone deacetylase; ROS, reactive oxygen species; CM-H₂DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; NAC; *N*-acetyl-L-cysteine.

Figure Legends

Fig. 1. Sensitivity of NSCLC cells expressing active mutant EGFRs to gefitinib. **(A)** PC-9, H1650, or II-18 cells were incubated for the indicated times with the indicated concentrations of gefitinib, after which the cells were harvested by exposure to trypsin. Viable cells were counted with a hemocytometer after staining with trypan blue (upper panels). Alternatively, the cells were incubated for 48 h with the indicated concentrations of gefitinib, after which the proportion of cells in sub-G₁ phase was determined by flow cytometry (lower panels). **(B)** Cells were incubated for 24 h with the indicated concentrations of gefitinib, after which cell lysates (30 µg of protein) were subjected to immunoblot analysis with antibodies to phosphorylated (p-) or total forms of EGFR, Akt, or ERK1/2, to activated caspase-3, or to β-actin (loading control). Data in (A, upper panels) and (B) are representative of three separate experiments; those in (A, lower panels) are means ± SD from three separate experiments.

Fig. 2. Effects of blockade of the ERK or PI3K-Akt pathway on the cytotoxicity of HC-toxin in NSCLC cells. **(A)** Cells were incubated for 48 h with 0.1 µM (PC-9 cells) or 1 µM (H1650 and II-18 cells) gefitinib, 10 µM PD184352 (PD), 5 µM PX-866 (PX), or both 10 µM PD184352 and 5 µM PX-866 (PD + PX), in the absence or presence of the indicated concentrations of HC-toxin (HC) or of both 0.1 µM HC-toxin and 10 mM NAC, after which the proportion of cells in sub-G₁ phase was determined by flow cytometry. **(B)** Cells were incubated for 24 h with the indicated additions as described in

(A), after which cell lysates (30 μ g of protein) were subjected to immunoblot analysis with antibodies to the indicated proteins. (C) Cells were incubated for 24 h with the indicated additions as described in (A), labeled with CM-H₂DCFDA, and analyzed by flow cytometry to determine the percentage of cells manifesting ROS accumulation. Data in (A) and (C) are means \pm SD from three separate experiments; those in (B) are representative of three separate experiments.

Fig. 3. Sensitivity of cells expressing Bcr-Abl fusion proteins to imatinib. (A) K562, BaF3[Bcr-Abl], or BaF3[Bcr-Abl^{T315I}] cells were incubated for the indicated times with the indicated concentrations of imatinib. Viable cells were then counted with a hemocytometer after staining with trypan blue (upper panels). Alternatively, cells were incubated for 48 h with the indicated concentrations of imatinib and were then analyzed for the proportion of cells with a fractional DNA content by flow cytometry (lower panels). (B) Cells were incubated for 24 h with the indicated concentrations of imatinib, after which cell lysates (30 μ g of protein) were subjected to immunoblot analysis with antibodies to the indicated proteins. Data in (A, upper panels) and (B) are representative of three separate experiments; those in (A, lower panels) are means \pm SD from three separate experiments.

Fig. 4. Effects of blockade of the ERK or PI3K-Akt pathway on the cytotoxicity of HC-toxin in cells harboring Bcr-Abl fusion proteins. (A) Cells were incubated for 48 h with 1 μ M (K562 cells) or 5 μ M (BaF3[Bcr-Abl] and BaF3[Bcr-Abl^{T315I}] cells) imatinib,

10 μ M PD184352, 5 μ M PX-866, or the combination of 10 μ M PD184352 and 5 μ M PX-866, in the absence or presence of the indicated concentrations of HC-toxin or of both 0.1 or 0.25 μ M HC-toxin and 10 mM NAC, after which the proportion of cells in sub-G₁ phase was determined by flow cytometry. **(B)** Cells were incubated for 24 h with the indicated additions as described in (A), after which cell lysates (30 μ g of protein) were subjected to immunoblot analysis with antibodies to the indicated proteins. **(C)** Cells were incubated for 24 h with the indicated additions as described in (A) and were then stained with CM-H₂DCFDA for determination of the percentage of cells manifesting ROS accumulation in five randomly selected microscopic fields (total of >300 cells). Data in (A) and (C) are means \pm SD from three separate experiments; those in (B) are representative of three separate experiments.

Fig.1. K.Ozaki *et al.*

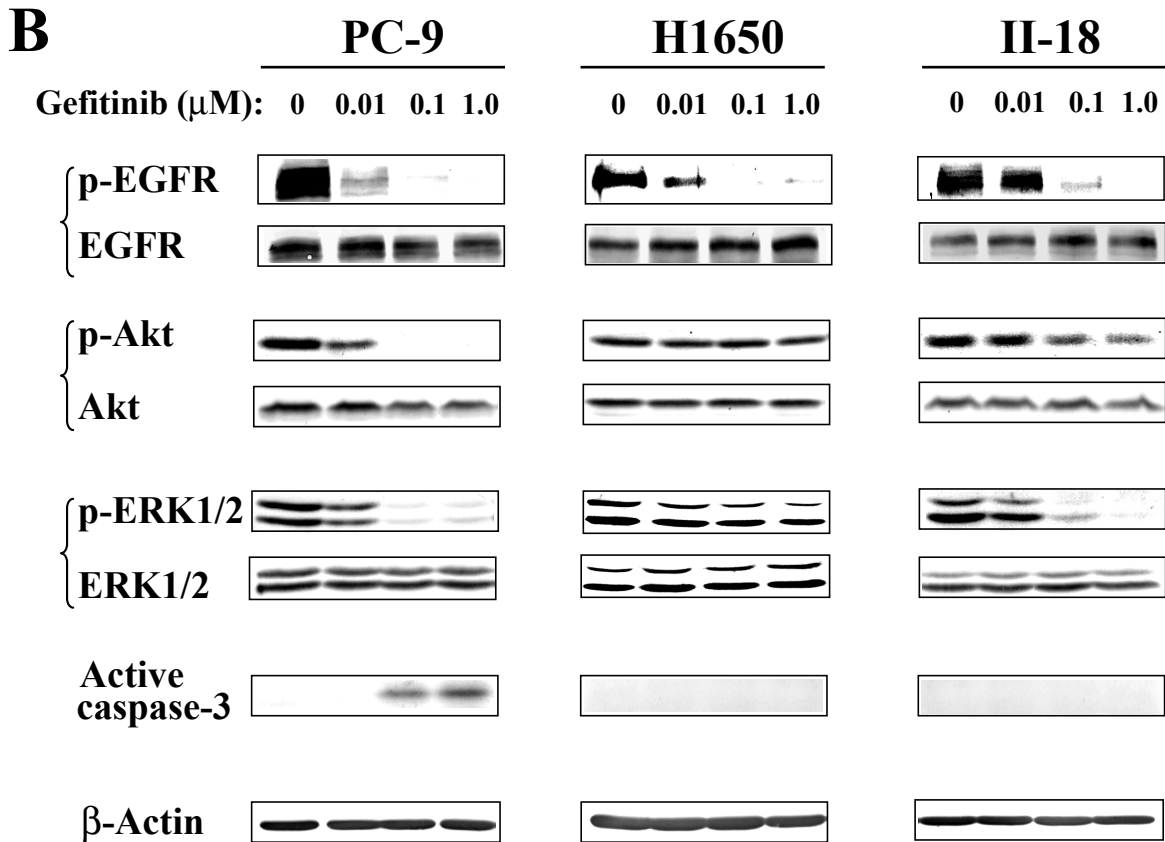
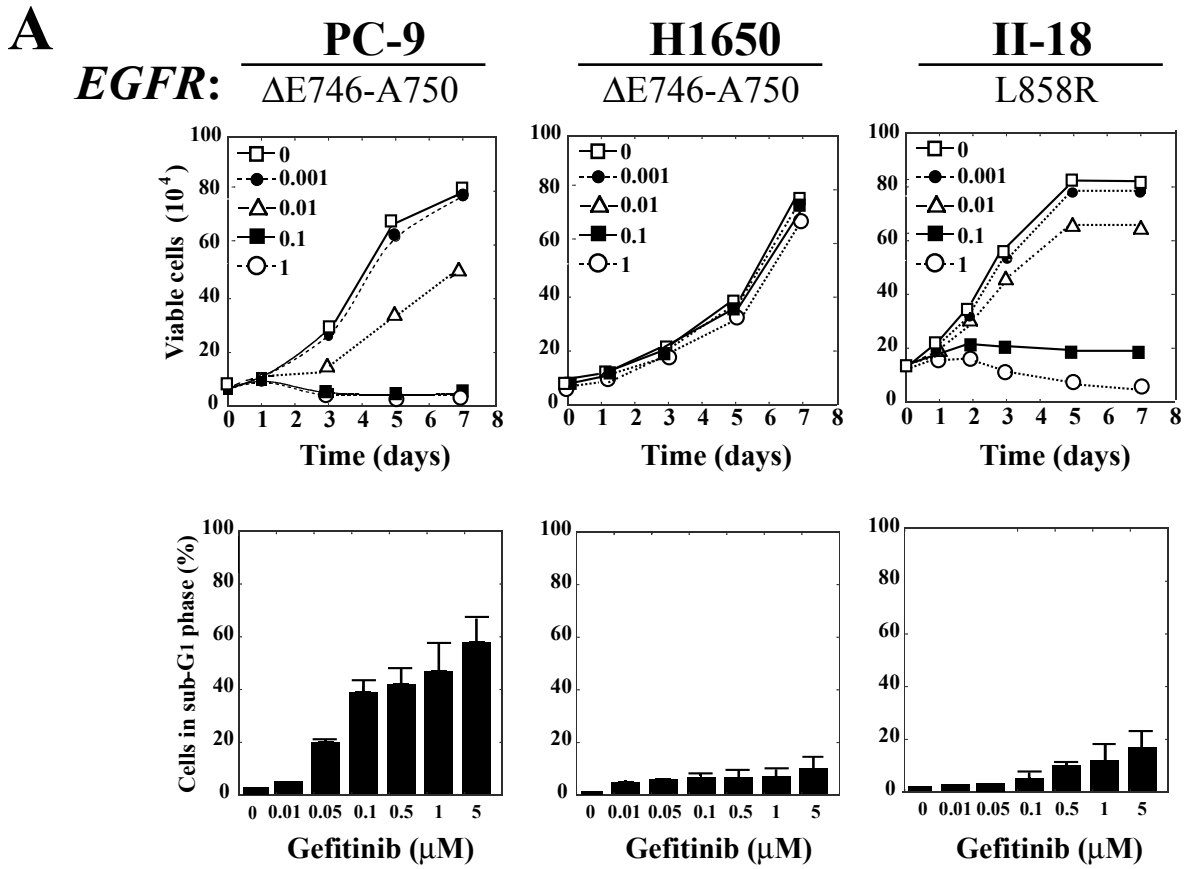


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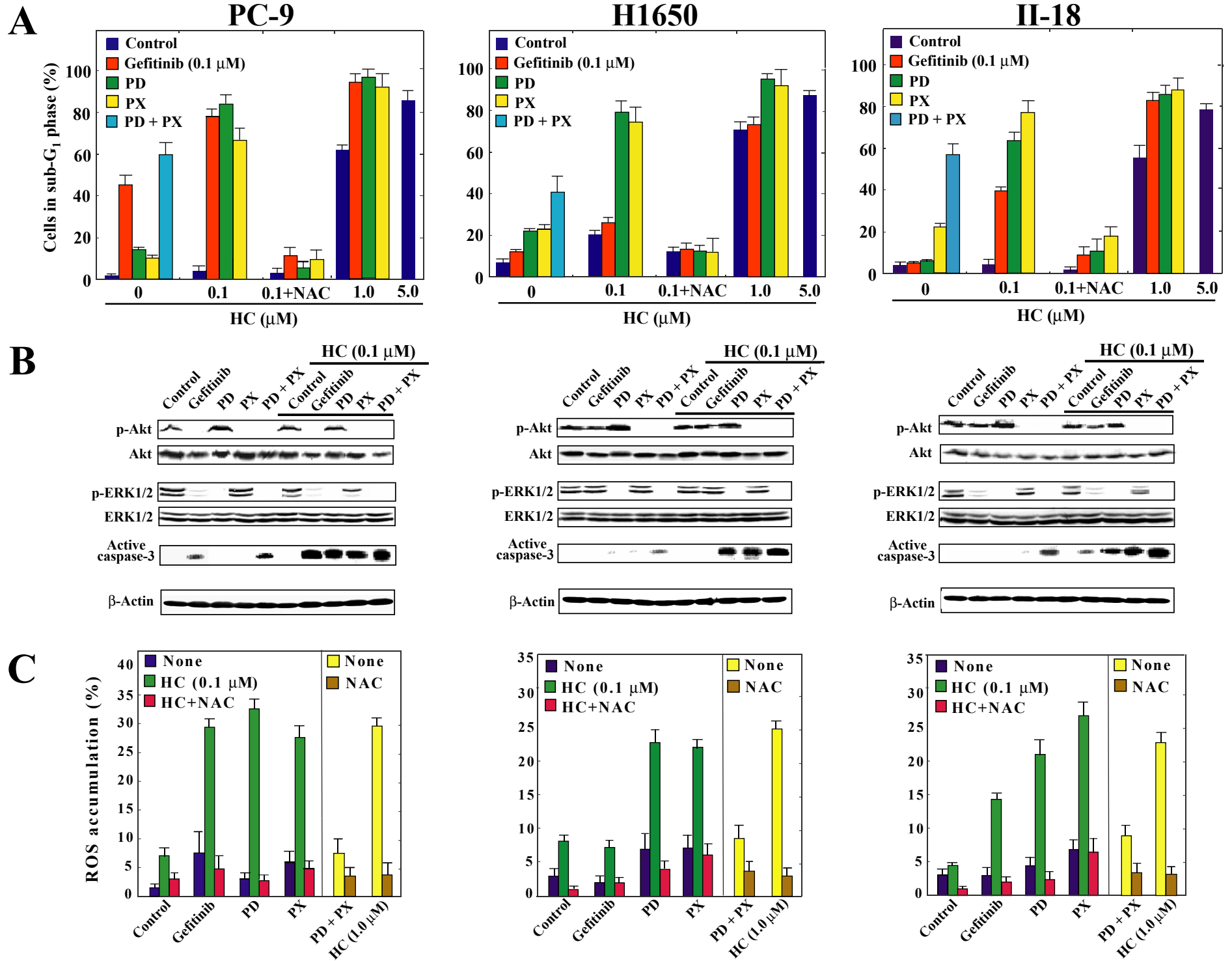


Fig.3. K.Ozaki *et al.*

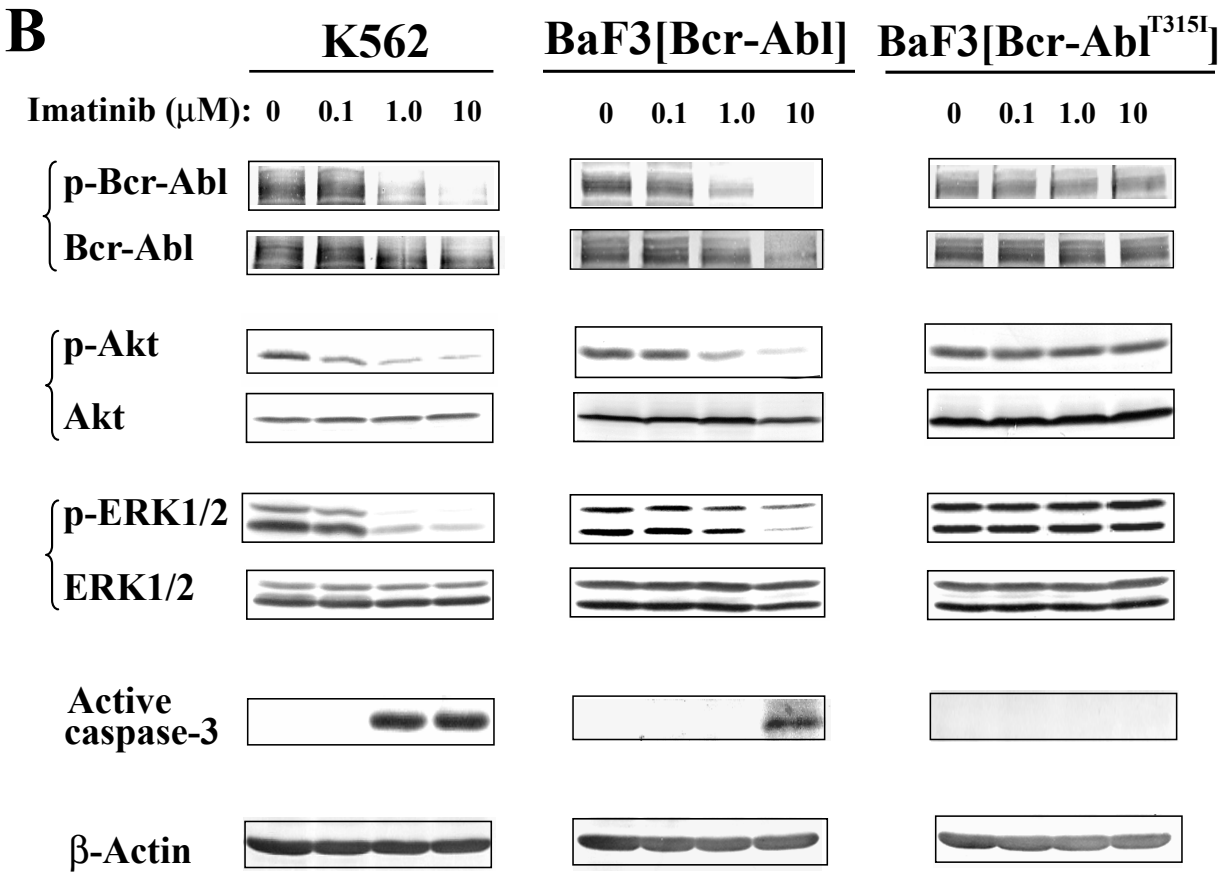
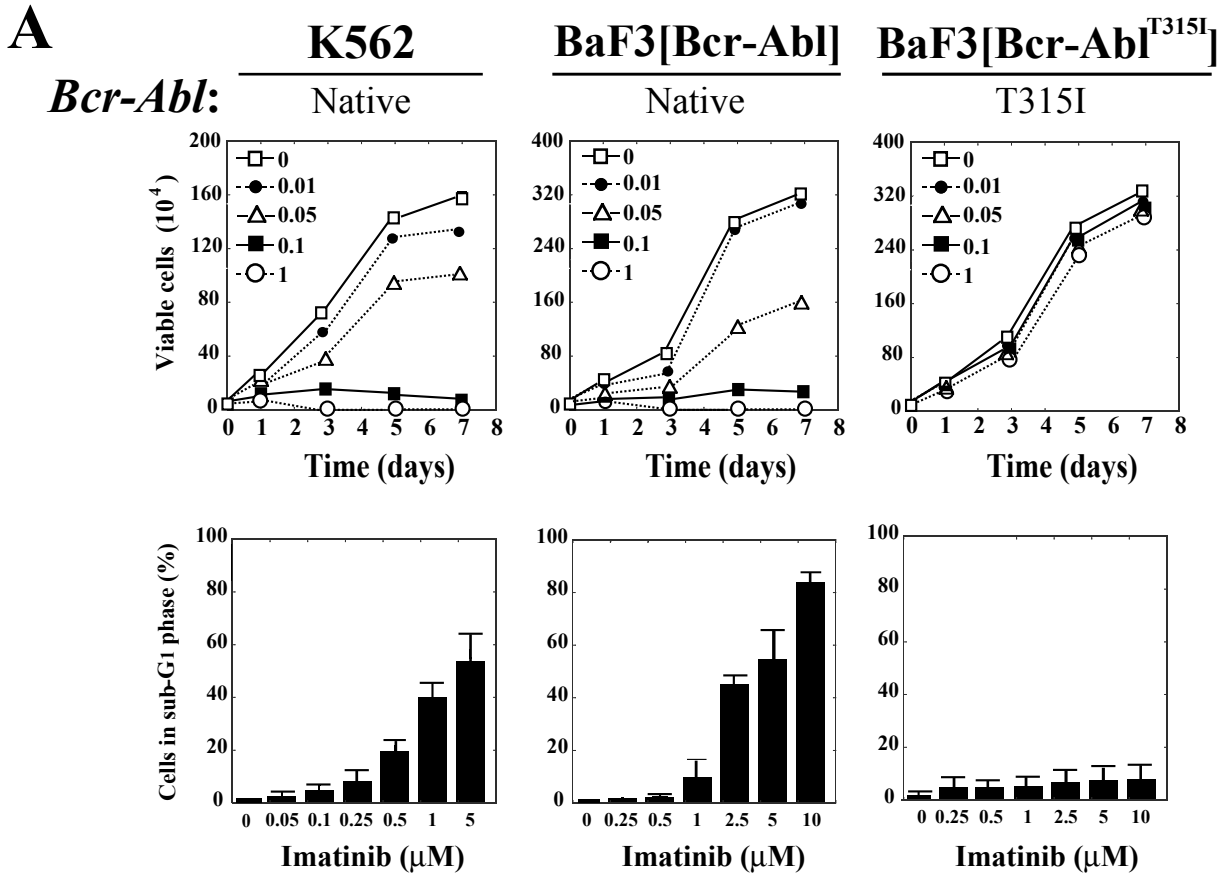
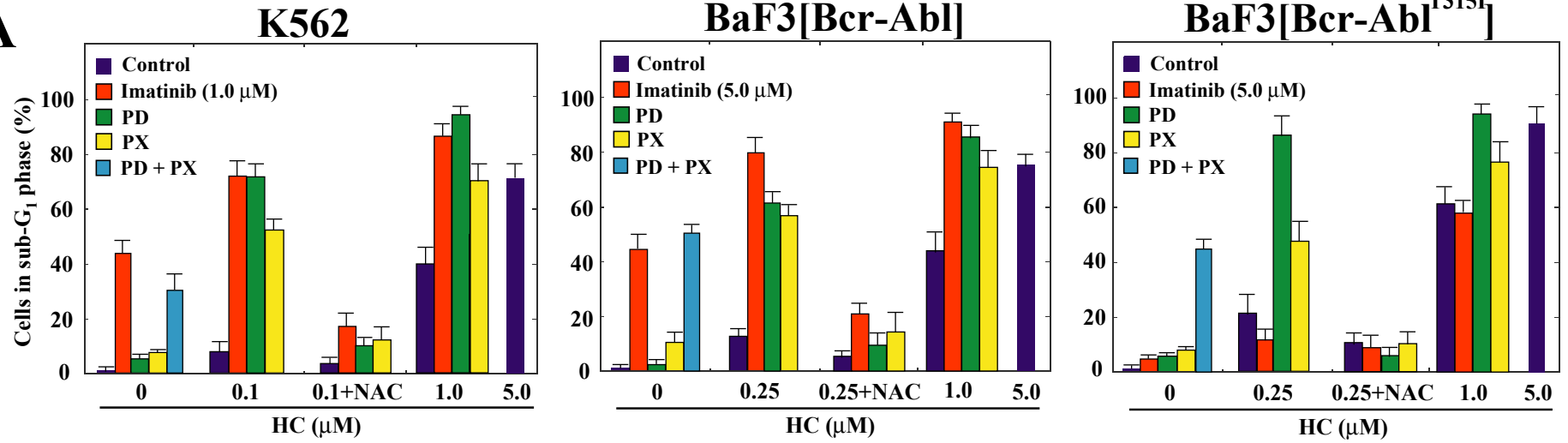
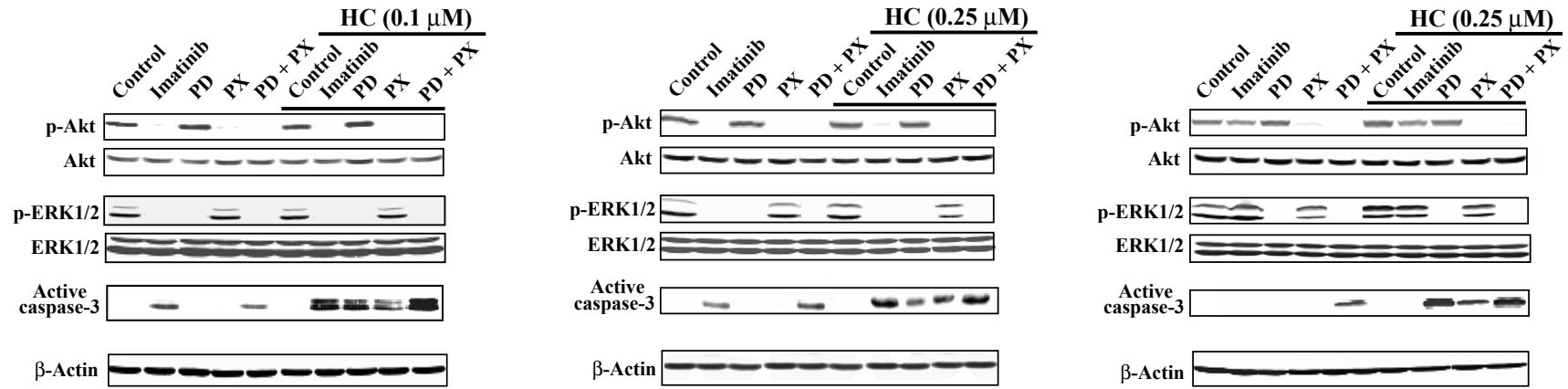


Fig.4. K. Ozaki *et al.*

A



B



C

