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Efficacy of ponatinib against ABL tyrosine kinase inhibitor-resistant leukemia cells

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ABSTRACT

Because a substantial number of patients with chronic myeloid leukemia acquire resistance to ABL tyrosine kinase inhibitors (TKIs), their management remains a challenge. Ponatinib, also known as AP24534, is an oral multi-targeted TKI. Ponatinib is currently being investigated in a pivotal phase 2 clinical trial. In the present study, we analyzed the molecular and functional consequences of ponatinib against imatinib- or nilotinib-resistant (R) K562 and Ba/F3 cells. The proliferation of imatinib- or nilotinib-resistant K562 cells did not decrease after treatment with imatinib or nilotinib. Src family kinase Lyn was activated. Point mutation Ba/F3 cells (E334V) were also highly resistant to imatinib and nilotinib. Treatment with ponatinib for 72 h inhibited the growth of imatinib- and nilotinib-resistant cells. The phosphorylation of BCR-ABL, Lyn, and Crk-L was reduced. This study demonstrates that ponatinib has an anti-leukemia effect by reducing ABL and Lyn kinase activity and this information may be of therapeutic relevance.

1. Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by the Philadelphia chromosome (Ph) [1]. This translocation results in the expression of the BCR-ABL fusion protein, which is a constitutively active tyrosine kinase that activates downstream molecules [2]. The ABL tyrosine kinase inhibitor (TKI) imatinib was the first TKI against CML, and was effective for the treatment of patients in the chronic phase (CP) of CML [3]. However, CML patients develop resistance to imatinib; BCR-ABL-dependent imatinib resistance can be caused by a BCR-ABL point mutation and BCR-ABL amplification [4]. A point mutation in BCR-ABL is the most common mechanism of imatinib resistance in BCR-ABL-positive leukemia patients, and more than 50 point mutations have been identified. BCR-ABL-independent imatinib resistance is caused by the activation of Src kinase families [4]. Recently, other ABL TKIs, e.g., dasatinib and nilotinib, have also been used clinically. Dasatinib and nilotinib was approved as a first-line treatment in newly diagnosed CML-CP patients. However, treatment with dasatinib or nilotinib was discontinued in a number of CML patients because of adverse events, intolerance, or other reasons [5]. Thus, alternative strategies are required to improve the outcome of CML patients.

Ponatinib, formally AP24534, is an oral multi-targeted TKI [6]. Ponatinib is the product of a structure-based approach for the design of small molecule TKIs and inhibits the native and mutant forms of BCR-ABL, including T315I. Ponatinib suppressed the emergence of mutations at a concentration of 40 nM [6]. Ponatinib was enrolled in the phase 2 PACE (ponatinib Ph+ acute lymphoblastic leukemia (ALL) and CML evaluation) trial in patients with resistant or intolerant CML and Ph+ ALL [7].

In the present study, we investigated the mechanism of imatinib and nilotinib resistance by using the representative BCR-ABL-positive cell line K562 and Ba/F3 BCR-ABL random mutagenesis. Our data indicate that Lyn is activated in imatinib- and nilotinib-resistant cells. We also established and analyzed imatinib- or nilotinib-resistant BCR-ABL mutant cells. We report here that ponatinib can inhibit the proliferation of ABL TKI-resistant Ph-positive leukemia cells. Collectively, these results demonstrate a novel role for resistance to TKIs and present a potential target for resistance to TKIs in BCR-ABL-positive cells.

2. Materials and methods

2.1. Reagents and antibodies

Ponatinib was purchased from Shanghai Biochempartner Co., Ltd. (Shanghai, China). Imatinib and nilotinib were kindly provided by Novartis (Basel, Switzerland). Stock solutions of nilotinib and ponatinib were dissolved in dimethyl sulfoxide and subsequently diluted to the desired concentration in growth medium. Imatinib was dissolved in distilled water. RPMI 1640 medium and fetal calf serum was purchased from Life Technologies (Carlsbad, CA, USA). Anti-phospho Abl, anti-phospho Crk-L, anti-cleaved caspase 3, and poly (ADP-ribose) polymerase (PARP) antibodies (Abs) were

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purchased from Cell Signaling (Beverly, MA, USA). All other reagents were obtained from Sigma (St. Louis, MI, USA).

2.2. Cell culture and mutagenesis

The human CML cell line K562 was obtained from the American Type Culture Collection (Manassas, VA, USA). The BCR-ABL-positive cell line Ba/F3 BCR-ABL with wild-type (wt) and mutant Ba/F3 cells (T315I) was established previously [8]. To establish the imatinib- or nilotinib-resistant sublines, K562 cells were cultured in RPMI1640 medium in the presence of gradually increasing concentrations of imatinib or nilotinib and the medium was changed every week [9]. These cells were maintained in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum with 1% penicillin/streptomycin in a humidified incubator at 37 °C. ABL tyrosine kinase-resistant K562 cells were maintained with the indicated concentrations of imatinib or nilotinib. In some experiments, Ba/F3 BCR-ABL mutagenesis cells were cultured in RPMI1640 medium with imatinib (5 μM) or nilotinib (2 μM) for a month to establish nilotinib-resistant cells.

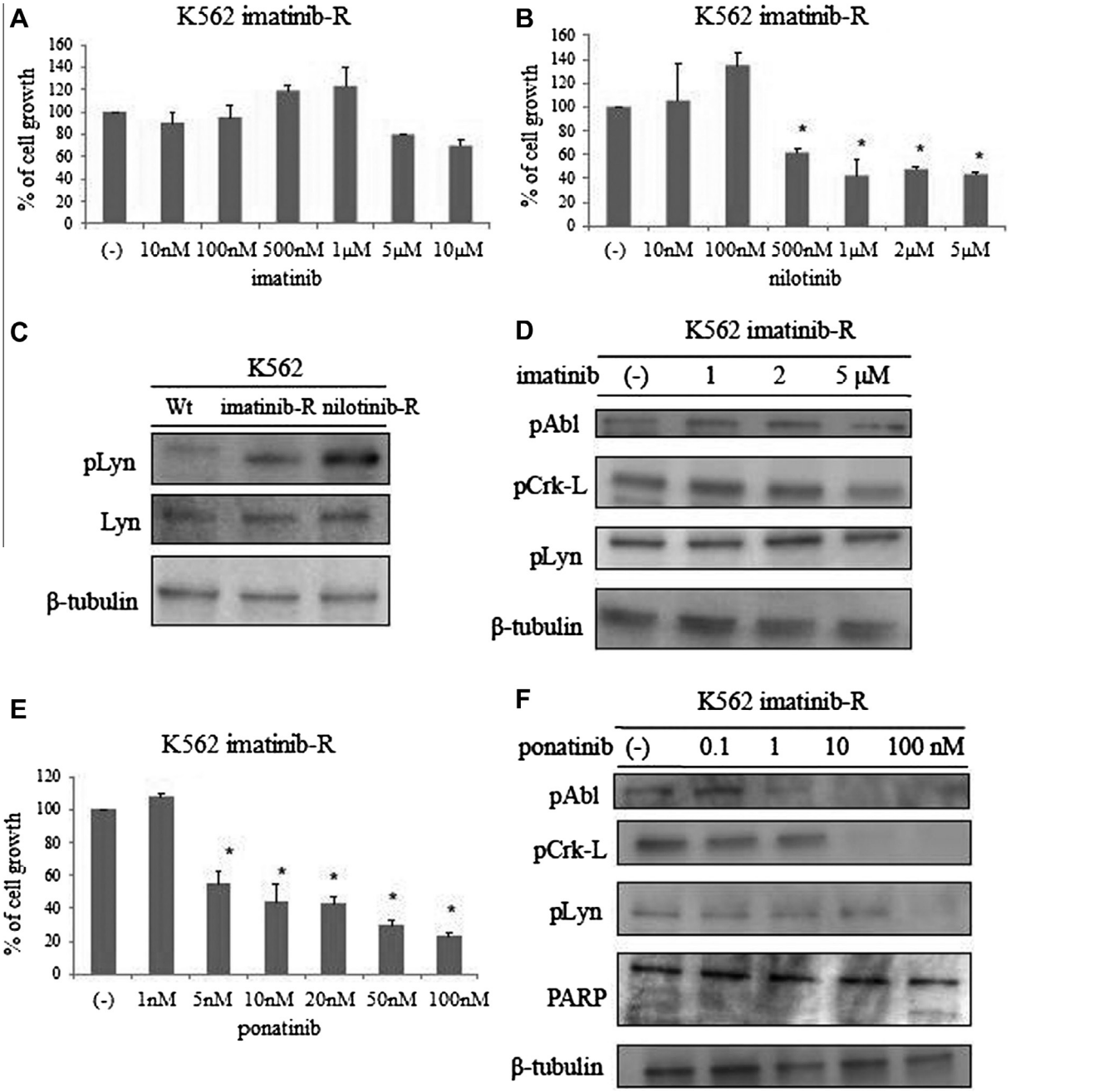

Fig. 1. Analysis of imatinib-resistant K562 cells. (A and B) Imatinib-resistant K562 cells were treated with various doses of imatinib or nilotinib, and growth inhibition was determined by a cell proliferation assay. These experiments were performed in triplicate. $P < 0.05$ vs. untreated control. (C) Whole cell lysates obtained from imatinib- or nilotinib-resistant cells and the K562 parental cell line were subjected to immunoblotting analysis using phospho-Lyn, Lyn, and β-tubulin Abs. (D) Imatinib-resistant-K562 cells were treated with imatinib for 24 h. Whole cell lysates were analyzed by immunoblotting with phospho-specific Abl, Crk-L, and Lyn Abs. β-tubulin was used as the loading control. (E) Imatinib-resistant K562 cells were treated with various doses of ponatinib for 72 h. Viable cell numbers were calculated. These experiments were performed in triplicate. $P < 0.05$ vs. untreated control. (F) Imatinib-resistant K562 cells were treated with the indicated concentrations of ponatinib for 24 h. Total cell extracts were analyzed by immunoblotting with phospho-specific Abl, Crk-L, Lyn, and cleaved PARP Abs. β-tubulin was used as the loading control. Results in A–F are representative of at least three reproducible experiments.

2.3. BCR-ABL mutation analysis

Direct sequencing was carried out according to standard methods and performed by SRL (Tokyo, Japan).

2.4. Cell proliferation assay

Cell proliferation was assessed as described previously [10]. Each assay was performed in quadruplicate.

2.5. Immunoblot analysis

Western blot analysis was performed as described previously [10,11]. In brief, after stimulation, the cells were washed with ice-cold phosphate-buffered saline and lysed in RIPA lysis buffer. The protein content of the lysates was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The lysates were loaded onto polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated with the primary Abs of interest at

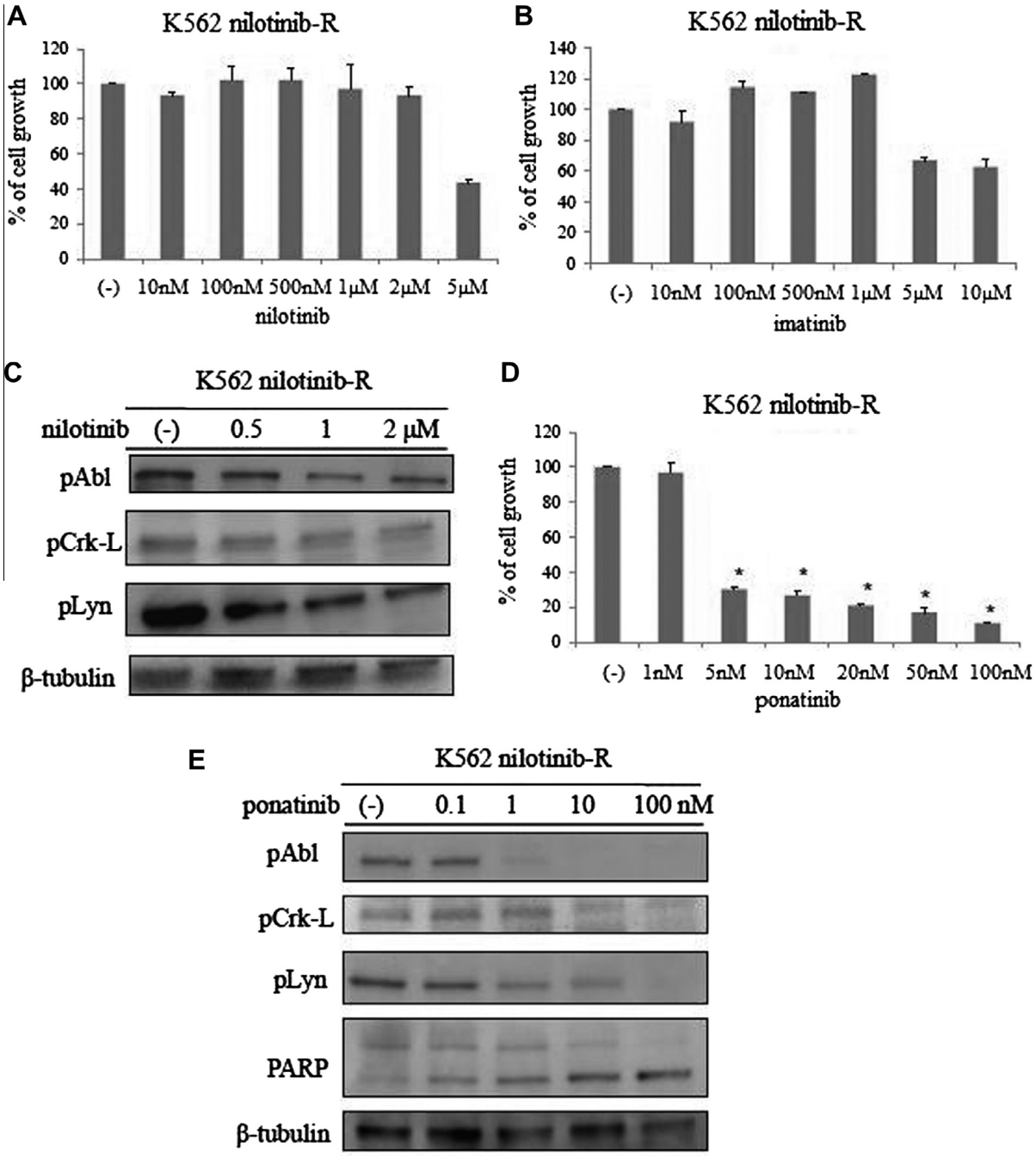

Fig. 2. Analysis of nilotinib-resistant K562 cells. (A and B) Nilotinib-resistant K562 cells were treated with various doses of nilotinib or imatinib, and growth inhibition was determined by a cell proliferation assay. $P < 0.05$ vs. untreated control. (C) Nilotinib-resistant-K562 cells were treated with nilotinib for 24 h. Whole cell lysates were analyzed by immunoblotting with phospho-specific Abl, Crk-L, and Lyn Abs. β-tubulin was used as the loading control. (D) Nilotinib-resistant K562 cells were treated with various doses of ponatinib for 72 h. Viable cell numbers were calculated. $P < 0.05$ vs. untreated control. (E) Nilotinib-resistant K562 cells were treated with the indicated concentrations of ponatinib for 24 h. Total cell extracts were analyzed by immunoblotting with phospho-specific Abl, Crk-L, Lyn, and cleaved PARP Abs. β-Tubulin was used as the loading control. Results in A–E are representative of at least three reproducible experiments.

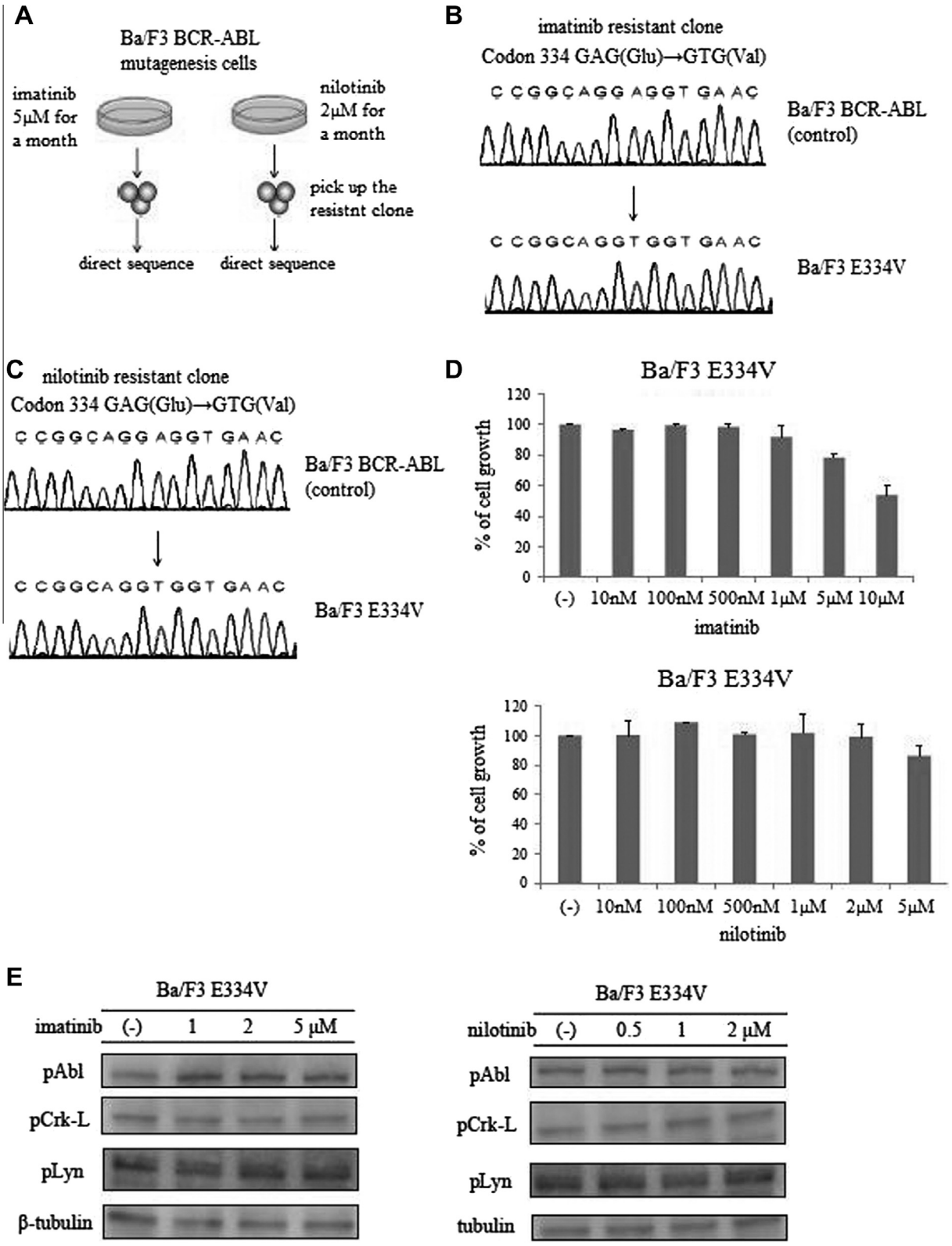

Fig. 3. Establishment of imatinib- or nilotinib-resistant BCR-ABL-positive Ba/F3 cells (Ba/F3 E334V). (A) Scheme of the experimental strategy for the imatinib- or nilotinib-resistant cells. (B and C) Direct sequencing of the BCR-ABL kinase domain was performed by using imatinib- or nilotinib-resistant Ba/F3 BCR-ABL cells. Nucleotide sequence analyses of the ABL kinase domain of imatinib- or nilotinib-resistant Ba/F3 BCR-ABL are shown. (D) Ba/F3 E334V cells were treated with various doses of imatinib or nilotinib, and cellular growth was analyzed. These experiments were performed in triplicate. (E) Ba/F3 E334V cells were treated with imatinib or nilotinib for 24 h. Whole cell lysates were analyzed by immunoblotting with phospho-specific Abl, Crk-L, and Lyn Abs. β-tubulin was used as the loading control. Results in A–E are representative of at least three reproducible experiments.

the appropriate dilution for 1 h. The blots were then probed with the secondary Abs and developed using an enhanced chemiluminescence system (ECL; Amersham Phamacia Biotech, Buckinghamshire, UK).

2.6. Statistical analysis

Differences between the treatment groups, in terms of dose response and apoptosis, were determined using Student's *t* test. *P* values <0.05 were considered significant.

3. Results and discussion

3.1. Analysis of imatinib-resistant K562 cells

First, the expression levels of BCR-ABL were determined in K562 and imatinib-resistant K562 cells by using BCR-ABL fluorescence *in situ* hybridization. We found that BCR-ABL amplification was not increased in imatinib-resistant K562 cells compared with the parental cell line (data not shown). In order to determine whether mutations in the ABL kinase domain affect the binding efficacy of imatinib, direct sequence analyses of the ABL kinase region were examined in K562 and imatinib-resistant K562 cells; however, no point mutation was identified (data not shown). Next, these cell lines were evaluated using a cell proliferation assay. The parental cell line was sensitive to imatinib and nilotinib (data not shown); however, imatinib-resistant K562 cells were highly resistant to imatinib (Fig. 1A). In contrast, imatinib-resistant K562 cells were resistant to nilotinib, but cellular growth was inhibited by a high concentration of nilotinib (Fig. 1B). We next examined intracellular signaling in these cell lines. In the imatinib- or nilotinib-resistant cells, we found that the Src family kinase Lyn was activated compared with the parental cell line (Fig. 1C). Phosphorylation of BCR-ABL and Crk-L was not reduced after imatinib treatment in the resistant cells (Fig. 1D). Ponatinib reportedly represents a promising molecule for patients with a wild type and BCR-ABL point mutation [6]; therefore, we next investigated the effects of ponatinib in the imatinib-resistant cell lines. Imatinib-resistant K562 cells were cultured with the indicated concentrations of ponatinib and cell proliferation was analyzed. Imatinib-resistant K562 cells were sensitive to low concentrations of ponatinib (Fig. 1E). In immunoblot analysis, phosphorylation of BCR-ABL and Crk-L was reduced by ponatinib. We also found that phosphorylation of Lyn was reduced after ponatinib treatment in imatinib-resistant K562 cells (Fig. 1F).

3.2. Analysis of nilotinib-resistant K562 cells

First, the expression levels of BCR-ABL were determined. BCR-ABL amplification was not increased in nilotinib-resistant K562 cells compared with the parental cell line (data not shown). Direct sequence analyses of the ABL kinase region were also examined, but no point mutation was identified (data not shown). Next, these cell lines were evaluated using a cell proliferation assay. Nilotinib-resistant K562 cells were highly resistant to nilotinib (Fig. 2A). We found nilotinib-resistant K562 cells were also resistant to imatinib (Fig. 2B). In immunoblot analysis, phosphorylation of BCR-ABL and Crk-L was not reduced after nilotinib treatment (Fig. 2C). Nilotinib-resistant K562 cells were cultured with the indicated concentrations of ponatinib. Nilotinib-resistant K562 cells were sensitive to low concentrations of ponatinib (Fig. 2D). Phosphorylation of BCR-ABL and Crk-L was reduced by ponatinib. We also found that phosphorylation of Lyn was reduced after ponatinib treatment in nilotinib-resistant K562 cells (Fig. 2E). Resistance to ABL TKI is commonly associated with the reactivation of BCR-ABL signaling. In the present study, we demonstrated that a BCR-ABL point mutation and BCR-ABL amplification were not detected in the imatinib- or nilotinib-resistant K562 cells; however, the Src family kinase Lyn was activated.

3.3. Analysis of imatinib- or nilotinib-resistant BCR-ABL point mutation Ba/F3 cells

Several studies have analyzed the response rates of patients with a BCR-ABL mutation to second-generation ABL TKIs. Nilotinib is a potent and selective ABL TKI and effective in imatinib-resistant BCR-ABL point mutation cells, excluding those with the T315I mutation. Patients with the E255K/V, Y253F/H, or F359C/V mutations reportedly have a less favorable outcome with nilotinib therapy [12]. In order to generate resistance to imatinib or nilotinib, we established new ABL TKI resistant cell line by using a Ba/F3 BCR-ABL random mutagenesis screen. These cell lines were cultured with the indicated concentrations of imatinib 5 μM or nilotinib 2 μM and resistant clones were selected (Fig. 3A). We first examined the sequence of the ABL kinase region in the imatinib- or nilotinib-resistant Ba/F3 cells. To date, more than 50 individual mutations, conferring variable resistance to imatinib, have been described in patients with CML. We identified a novel mutation, codon 334 GAG (Glu) to GTG (Val) (E334V), in this region of BCR-ABL in Ba/F3 BCR-ABL imatinib resistant cells (Fig. 3B). In nilotinib resistant Ba/F3 cells, we also identified the same point mutant, E334V (Fig. 3C). The Ba/F3 E334V cells were evaluated using a cell proliferation assay. We found that Ba/F3 E334V cells were highly resistant to imatinib and nilotinib (Fig. 3D). We also evaluated intracellular signaling. Phosphorylation of BCR-ABL and the downstream molecule Crk-L was not inhibited by imatinib or

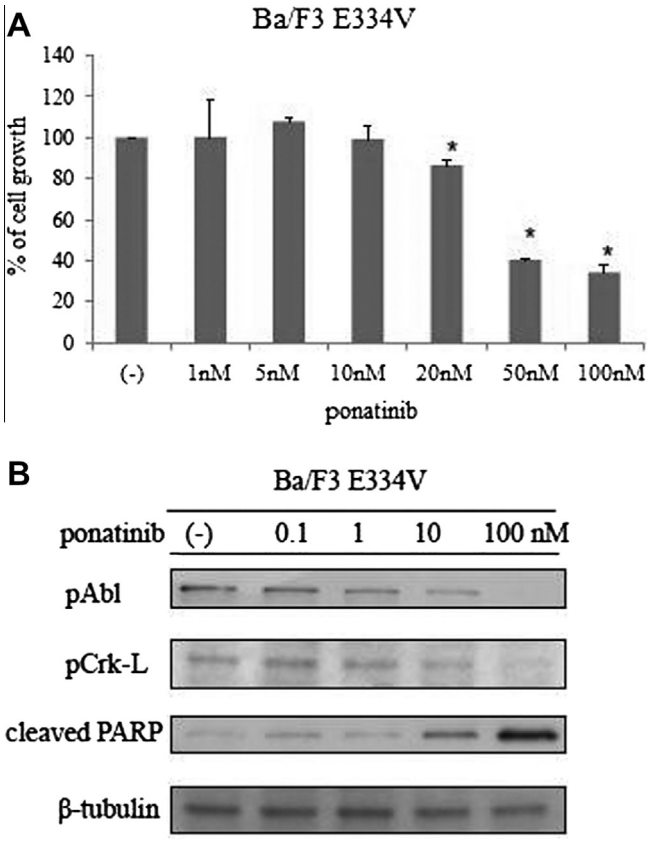

Fig. 4. Effect of ponatinib on imatinib- or nilotinib-resistant Ba/F3 E334V cells. (A) Ba/F3 E334V cells were treated with various doses of ponatinib for 72 h. Viable cell numbers were calculated. These experiments were performed in triplicate. $P < 0.05$ vs. untreated control. (B) Ba/F3 E334V cells were treated with the indicated concentrations of ponatinib for 24 h. Total cell extracts were analyzed by immunoblotting with phospho-specific Abl, Crk-L, and cleaved PARP Abs. β-tubulin was used as the loading control. Results in A and B are representative of at least three reproducible experiments.

nilotinib (Fig. 3E). In this study, we established a novel BCR-ABL point mutation cell line and these results indicated that the E334V point mutant provided resistance not only to imatinib but also to nilotinib.

3.4. Efficacy of ponatinib against imatinib- or nilotinib-resistant Ba/F3 E334V cells

O'Hare et al., which indicated that ponatinib is effective in BCR-ABL point mutation cells [6]. We investigated whether ponatinib inhibited the BCR-ABL mutant using cellular proliferation and immunoblot assays. We found Ba/F3 E334V cells were sensitive to ponatinib, even though a high concentration of ponatinib was needed to achieve an equivalent loss of cell viability (Fig. 4A). In immunoblot analysis, phosphorylation of BCR-ABL and Crk-L was reduced by ponatinib. In contrast, PARP activation was found after ponatinib treatment in a dose-dependent manner, suggesting that apoptosis was induced by ponatinib (Fig. 4B). Thus, our work demonstrated that ponatinib is effective in E334V mutant cells.

4. Conclusion

We show here, for the first time, that ponatinib is induces apoptosis in imatinib- or nilotinib-resistant BCR-ABL positive cell lines. Ponatinib was evaluated in a phase II clinical trial of patients with refractory CML and other hematological malignancies. Ponatinib is approved for the treatment of CML and Ph-positive ALL patients who are resistant or intolerant to prior TKI therapy. The imatinib- or nilotinib-resistant cell lines were sensitive to ponatinib by the inhibition of BCR-ABL and the Src family kinase Lyn. Our results suggest that this information may be of therapeutic relevance for patients resistant to ABL TKIs.

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