PF-114, a novel selective inhibitor of BCR-ABL tyrosine kinase, is a potent inducer of apoptosis in chronic myelogenous leukemia cells

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Abstract. A t(9;22) chromosomal translocation which forms the chimeric tyrosine kinase breakpoint cluster region (BCR)-Abelson murine leukemia viral oncogene homolog 1 (ABL) is a key mechanism underlying the pathogenesis of chronic myelogenous leukemia (CML). Pharmacological inhibition of BCR-ABL with imatinib (Gleevec) has been reported as an effective targeted therapy; however, mutations (including the kinase domain of ABL) suppress the efficacy of inhibitors. PF-114, a derivative of the third generation BCR-ABL inhibitor ponatinib, demonstrated a high inhibitory activity against wild-type and mutant BCR-ABL forms, such as the clinically important T315I. Furthermore, PF-114 exhibited preferential kinase selectivity, safety, notable pharmacokinetic properties and therapeutic efficacy in a murine model. Investigation into the mechanisms of CML cell death revealed an exceptional potency of PF-114 (at low nanomolar concentrations) for the CML-derived K562 cell line, whereas leukemia cell lines that lack the chimeric tyrosine kinase were markedly more refractory. The molecular ordering of events mechanistically associated with K562 cell death included the dephosphorylation of CrkL adaptor protein followed by inhibition of ERK1/2 and Akt, G1 arrest, a decrease of phosphorylated Bcl-2-associated death promoter, Bcl-2-like protein 11, BH3 interacting-domain death agonist, Bcl-extra large and Bcl-2 family apoptosis regulator, and reduced mitochondrial transmembrane potential. Increased Annexin V reactivity, activation of caspases and poly(ADP-ribose) polymerase cleavage were proposed to lead to internucleosomal DNA fragmentation. Thus, PF-114 may be a potent inducer of apoptosis in CML cells. Nevertheless, activation of STAT3 phosphorylation in response to PF-114 may permit cell rescue; thus, a combination of BCR-ABL and STAT3 inhibitors should be considered for improved therapeutic outcome. Collectively, the targeted killing of BCR-ABL-positive cells, along with other beneficial properties, such as *in vivo* characteristics, suggests PF-114 as a potential candidate for analysis in clinical trials with CML patients.

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Abbreviations: BCR-ABL, breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1; CML, chronic myelogenous leukemia; CrkL, Crk-like protein; GSK-3β, glycogen synthase kinase-3β; JAK, Janus kinase; PARP, poly(ADP-ribose) polymerase; STAT3, signal transducer and activator of transcription 3

Key words: chronic myelogenous leukemia, breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1, protein kinase, adaptor proteins, signal transduction, cell cycle, apoptosis

Introduction

The breakpoint cluster region (BCR)-Abelson murine leukemia viral oncogene homolog 1 (ABL) chimeric tyrosine kinase has been mechanistically associated with in the pathogenesis of Philadelphia chromosome positive malignancies (1,2). Signaling initiated by BCR-ABL involves a number of pathways that regulate the survival and proliferation of chronic myelogenous leukemia (CML) cells (Fig. 1). Inhibition of BCR-ABL with imatinib mesylate, also known as Gleevec, has been reported to be notably efficient in patients with CML. Indeed, the use of imatinib markedly improved clinical outcome, with a 10 year survival of >80% of patients (3). Nevertheless, genetic and epigenetic mechanisms within imatinib-resistant cells can lead to relapse; thus, it is necessary to develop novel BCR-ABL inhibitors. A major criterion to

which these drugs must fit is the potency against both wild-type (native) and mutated BCR-ABL with minimal interaction with other protein kinases. Ponatinib, a Food and Drug Administration (FDA) approved, third generation BCR-ABL inhibitor for CML, is unique in its ability to downregulate native and all known clinical mutations (including the most frequent T315I) in the ABL kinase domain (4). However, side effects, particularly cardiovascular toxicity, have been associated with the limited target selectivity of ponatinib due to the attenuation of unrelated protein kinases (4-7).

Aiming to design an optimized compound that combines the potency of ponatinib (against the wild-type and mutant BCR-ABL CML cells) with an improved kinase selectivity profile, Chilov and Titov have developed 3-([1,2,4]triazolo[4,3-*a*] pyridin-3-ylethynyl)-4-methyl-N-(4-((4-methylpiperazin-1-yl) methyl)-3-(trifluoromethyl)phenyl)benzamide (PF-114) (8,9). PF-114 demonstrated excellent inhibitory potency against the wild-type and a number of clinically relevant ABL mutations, including T315I, E255K, Y253F and others in *in vitro* kinase assays; PF-114 exhibited notable selectivity in inhibiting ABL compared with other kinases. The nanomolar kinase inhibitory potency of PF-114 was paralleled by its efficacy against cultured leukemia cells and in murine xenografts (8). Importantly, at tolerable doses of orally administered PF-114, BCR-ABL positive human CML transplants in immunocompromised mice were completely inhibited for ≤240 days without tumor re-growth (8).

In the present study, we investigated the molecular mechanisms of CML cell death induced by PF-114. It was demonstrated that this compound is highly cytotoxic (at subnanomolar to low nanomolar concentrations) against the CML derived K562 cell line (native BCR-ABL), but not BCR-ABL negative leukemia cell lines, indicating notable target selectivity. Dephosphorylation of Crk-like protein (CrkL) adaptor protein (a BCR-ABL substrate) in K562 cells preceded the inhibition of pro-proliferative/pro-survival Akt-ERK1/2 signaling, and cell cycle arrest in G1 phase. Apoptotic signaling involved mitochondrial mechanisms, caspase activation, poly(ADP-ribose)polymerase (PARP) cleavage and DNA fragmentation. These results indicated that PF-114 is a potent, CML cell specific inhibitor of BCR-ABL signaling.

Materials and methods

Cell lines, culture conditions and drug treatment. Reagents were purchased from Sigma-Aldrich (Merck KGaA) unless specified otherwise. Human leukemia cell lines K562 (CML, BCR-ABL-positive), HL60 [FAB-M2 acute myeloblastic leukemia with maturation; (10)], U937 (monoblastic) and Jurkat (T-lymphocytic; all three harbor no BCR-ABL fusion protein) obtained from the American Type Culture Collection, were cultured in RPMI-1640 (PanEco) supplemented with 5% fetal calf serum (HyClone; GE Healthcare Life Sciences), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C, 5% CO_2 in a humidified atmosphere. Cells in the logarithmic phase of growth were used in the experiments. The synthesis of PF-114 was conducted as reported previously (8). This compound was dissolved in dimethyl sulfoxide as 10 mM stock solution and stored at -20˚C. Aqueous dilutions in water were made immediately before experiments.

CrkL phosphorylation assays. The total intracellular pool of CrkL protein [phosphorylated (p) and non-phosphorylated forms) was determined by flow cytometry (11). Cells were incubated for 2 h at 37˚C in the absence (control) or presence of 10, 30 and 100 nM PF-114, fixed with 2% buffered paraformaldehyde (10 min, 37˚C), then washed twice with saline and placed on ice. Cells were permeabilized with methanol for 30 min, washed with saline supplemented with 1% bovine serum albumin (BSA) and resuspended in 100 μl of 10% human serum (30 min, 4˚C) to prevent non-specific antibody binding. Then, cells were pelleted (2,700 x g, 5 min at room temperature), and the antibody to non-phosphorylated CrkL (Table S1; Santa Cruz Biotechnology, Inc.; 1:50) was added for 30 min at room temperature. Secondary antibody conjugated with AlexaFluor® 488 (Invitrogen; Thermo Fisher Scientific, Inc., cat. no. A27034; 1:1,000) was added, cells were incubated for 30 min at room temperature and washed with saline. For the detection of pCrkL, phycoerythrin (PE) conjugated antibody (Table S1; BD Biosciences, 1:5) was added followed by incubation for 30 min at room temperature and washing in saline with 1% BSA. Samples were analyzed by flow cytometry using FACSCanto II (BD Biosciences). Total CrkL was determined based on AlexaFluor 488 fluorescence in a fluorescein isothiocyanate-A channel whereas pCrkL positive cells were detected in a PE channel. A total of 20,000 fluorescent 'events' were collected per each sample. Data were analyzed with FACSDiva software 6.0 (BD Biosciences).

Cell death assays. The cytotoxicity of PF-114 was determined via an MTT assay (12). The assays were performed in 96-well microtiter plates (SPL Life Sciences). To each well, $5x10^3$ cells and PF-114 (up to 50 μM, 2-fold dilutions) were added. Cells were incubated for 72 h at 37˚C in a humidified CO_2-controlled atmosphere. The half-maximal inhibitory concentration (IC_{50}) was determined. Analysis of cell cycle distribution, as well as Annexin V-propidium iodide (PI; Thermo Fisher Scientific, Inc.) staining were conducted according to our previous study (12). The mitochondrial transmembrane electric potential was determined by flow cytometry using MitoTrackerRed (Thermo Fisher Scientific, Inc). Briefly, K562 cells ($5x10^4$ in 2 ml of culture medium) were treated with 10 nM PF-114 for 24 or 48 h at 37˚C, 5% CO_2) MitoTracker Red (1 μM) was added to the cells 2 h prior to the completion of drug exposure. Cells were washed with ice cold saline, resuspended in fresh saline and immediately analyzed by flow cytometry in PE-A channel. At least 30,000 fluorescent events were acquired per each sample. Data were processed using FACSDiva software.

For the detection of DNA fragmentation (13), K562 cells ($2x10^6$ in 10 ml of culture medium) were treated with PF-114 and lysed for 30 min on ice in buffer containing 0.35 M NaCl, 20 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 1 mM dithiothreitol and 0.5% NP-40. Buffered phenol (pH 8.0, 1:1 v/v) and chloroform (1:5 v/v) were added to the lysates. Samples were centrifuged for 10 min at 15,300 x g at 4˚C. Sodium acetate (3 M, pH 5.2; 0.1 v/v) and 1.5 v/v of ethanol were added to the aqueous phase. Samples were incubated overnight at -20˚C and centrifuged at 15,300 x g, 4˚C for 10 min. Pellets were dissolved in TAE buffer containing 10 μg/ml RNase A. DNA

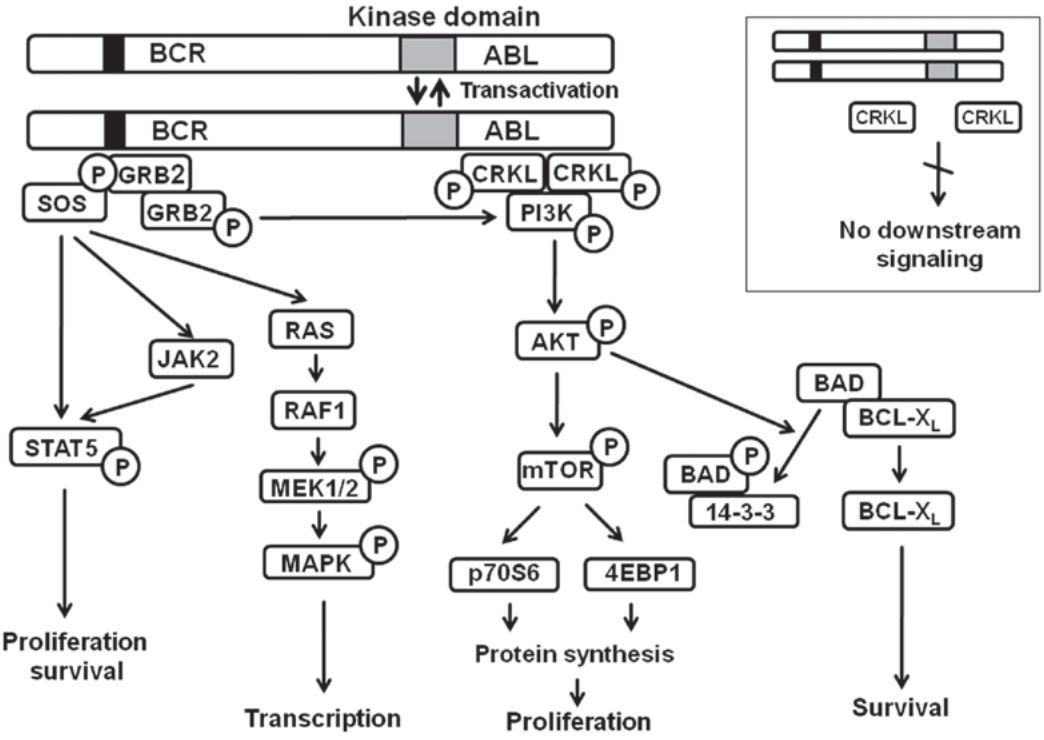

Figure 1. Signaling pathways regulated by BCR-ABL chimeric tyrosine kinase. Adapted from (10). AKT, protein kinase B; ABL, Abelson murine leukemia viral oncogene homolog 1; BCR, breakpoint cluster region; BCL-XL, B-cell lymphoma 2-extra large; BAD, BCL-2-associated death promoter; CRKL, Crk-like protein; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; STAT5, signal transducer and activator of transcription 5.

fragments were resolved by electrophoresis in 1% agarose gel, stained with ethidium bromide and visualized under UV light.

Immunoblotting. K562 cells ($1x10^5$ in 2 ml of culture medium) were treated with 0.1, 1, 10 and 100 nM PF-114 for 24 or 48 h, washed with ice cold saline and lysed for 30 min on ice in the buffer containing 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.1% sodium dodecylsulfate, 150 mM NaCl, 1 mM EDTA, protein inhibitor cocktail and 2 mM phenylmethylsulfonyl fluoride. Total protein concentration in lysates was determined by the Bradford method. Proteins were resolved in 10-15% SDS-PAGE (30 μg total protein per lane) and transferred onto a 0.2 μm nitrocellulose membrane (GE Healthcare). Non-specific protein-antibody interactions were blocked with 5% skimmed milk for 30 min. at room temperature. Primary antibodies (Table SI) were diltuted in Tris buffered saline with Tween-20 (TBST; 1:1,000) supplemented with 1% BSA. Membranes were incubated with primary antibodies overnight at 4˚C. Secondary antibodies conjugated with horseradish peroxidase were obtained from Cell Signaling Technology, Inc. (cat. nos. 7074 and 7076; Cell Signaling Technology, Inc.) and diluted in 5% skimmed milk in TBST (1:1,000). Membranes were incubated with secondary antibodies for 1 h at room temperature. Proteins were detected via enhanced chemiluinescence using the Image Quant LAS 4000 system (GE Healthcare).

Statistical analysis. Data are presented as the mean of four repeats ± standard deviation. Comparisons were performed using one-way ANOVA test with a Dunnett's post-hoc test (GraphPad Prism 6.0; GraphPad Software, Inc.) Each experimental group was compared to control cohort. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PF-114, a novel BCR-ABL inhibitor. A variety of chemotypes have been developed to inactivate BCR-ABL chimera. The tyrosine kinase ABL can be downregulated by targeting the ATP binding pocket and/or allosterically via interactions with the myristoil binding site [reviewed in (14)]. PF-114 is a fourth generation inhibitor of the BCR-ABL protein kinase and a close structural analog of the FDA approved drug ponatinib. The molecular design of PF-114 was based on the ponatinib scaffold to weaken the interactions of the inhibitor with off-target protein kinases, a major reason for the insufficient selectivity of ponatinib and a probable cause of its toxicity (7,15-17). The triazolopyridine moiety of PF-114 (Fig. 2A) that replaced the bioisosteric pyrazolopyrimidine moiety of ponatinib (Fig. 2B) has been designed to disfavor the hydrogen bonds between the inhibitor and protein kinases other than ABL. No hydrogen bonding with water molecules in the active site of B-Raf and other enzymes (but not ABL) was possible after replacing the nitrogen atom (an H bond acceptor) in ponatinib with the carbon atom in PF-114. Additionally, the formation of the CH...O=C hydrogen bond became unlikely due to the exchange of the CH moiety for the N atom in PF-114 that is repulsed from the main chain O=C. These modifications resulted in a more selective kinase inhibitory profile and an improved *in vivo* safety of PF-114 (8).

Preferential cytotoxicity of PF-114 to the BCR-ABL positive CML cell line. We compared the ability of PF-114 to induce

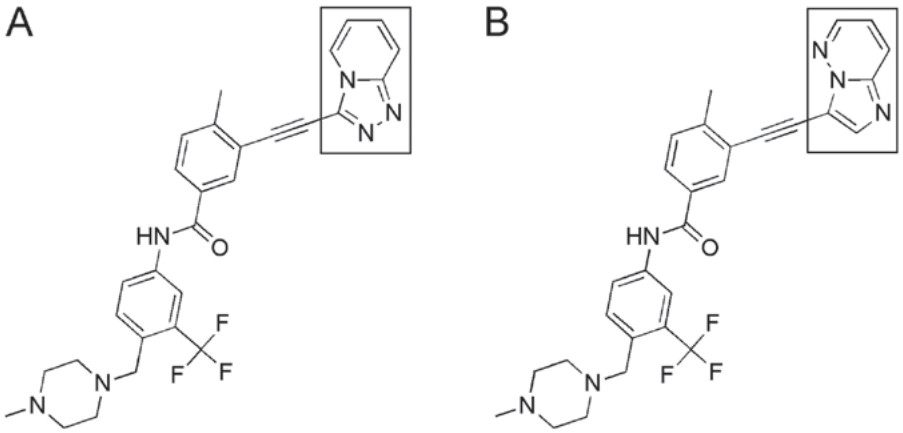

Figure 2. Chemical structures of breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1 inhibitors. (A) PF-114 and (B) ponatinib. Framed are the moieties in the ATP-binding pocket (triazolopyridine in PF-114 and pyrazolopyrimidine in ponatinib) that interact with the kinase hinge region.

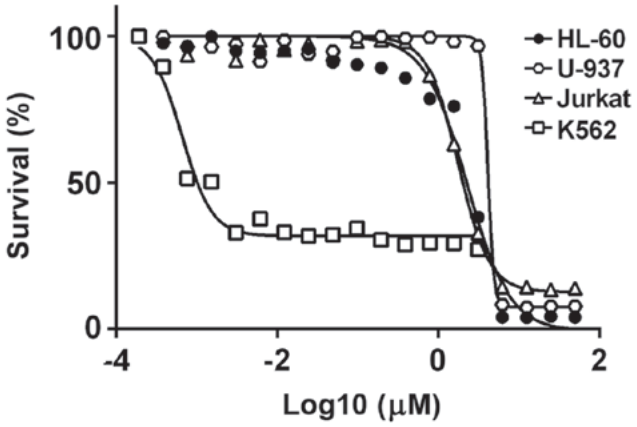

Figure 3. Differential cytotoxicity of PF-114 for breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1 positive and -negative leukemia cell lines. Human leukemia cell lines were treated with PF-114 for 72 h. Cell viability was analyzed via an MTT assay. Shown is one representative experiment of six with similar results. Each value is the mean of three independent measurements.

the death of leukemia cell lines depending on whether the cells carry the chimeric BCR-ABL protein. As presented in Fig. 3, PF-114 was notably potent (IC_{50}=1-5 nM after 72 h exposure) for the BCR-ABL-positive K562 cell line. The cytotoxic effect was time dependent: By 24 h, the percentage of viable cells (by MTT data conversion) remained high, whereas by 48 h the IC_{50} values peaked at their lowest levels in the low nanomolar range (Fig. S1). Conversely, the HL60, U-937 and Jurkat leukemia cell lines that lack BCR-ABL chimera were significantly less sensitive to PF-114, with IC_{50} values that were larger by three orders of magnitude. Furthermore, human donor lymphocytes were resistant to concentrations of PF-114 >1 μM (data not shown). These results indicated that PF-114 was preferentially cytotoxic against BCR-ABL positive cells.

PF-114 attenuates CrkL phosphorylation. In addition, whether the cytotoxicity of PF-114 arises due to the prevention of BCR-ABL mediated phosphorylation of its substrates was investigated. The CrkL adaptor protein is phosphorylated at Tyr207 by BCR-ABL (18); this event generates a scaffold for downstream proteins, thereby inducing BCR-ABL activated signaling. To determine the effects of PF-114 on CrkL phosphorylation, we treated K562 cells with this compound; the intracellular amounts of phosphorylated and non-phosphorylated CrkL pools were then measured. Intact K562 cells contained pCrkL whereas in the BCR-ABL negative leukemia cell lines (U937, HL-60 and Jurkat), only the non-phosphorylated protein was detected (Fig. 4A). Within 2 h following treatment with 30 nM PF-114 the amount of pCrkL in K562 cells decreased to ~25% of its initial level, indicating that PF-114 significantly attenuated BCR-ABL mediated CrkL phosphorylation compared with the control (Fig. 4B). Interestingly, this effect was reversible as the percentage of pCrkL was gradually restored following the removal of PF-114 and incubation of cells in drug free medium for ≥20 h. By this time pCrkL was detectable in ~80% of cells (data not shown).

PF-114 attenuates Akt-ERK1/2 and induces G1 arrest in BCR-ABL-positive CML cells. Non-phosphorylated CrkL fails to form dimers, which prevents the induction of signaling cascades. Accordingly, the phosphorylation of Akt and ERK1/2, the serine/threonine protein kinases generally known for their pro-survival and pro-proliferation activities (19-21), was markedly attenuated by exposure of K562 cells to nanomolar concentrations of PF-114 (Fig. 5A). Total amounts of Akt and ERK1/2 were markedly unchanged, indicating that the attenuation of Akt and ERK1/2 phosphorylation was a post-translational event. Importantly, the inhibition of Akt and ERK1/2 phosphorylation was paralleled by an increase in p27 expression (Fig. 5A) and cell arrest in G1 phase within 24 h exposure (Fig. 5B and C). These effects were observed at 1, 10 and 100 nM PF-114.

PF-114 induces apoptosis in K562 cells. Time course analysis of individual markers of programmed cell death in PF-114-treated K562 cells demonstrated the ordering of cell death-executing processes. By 24 h following treatment, Bad dephosphorylation was observed (Fig. 6A), an event that allows this protein to enter mitochondria, and interfere with the pro-survival Bcl-2 family proteins (22). In the same time period, changes in the mitochondrial transmembrane potential were measurable, with a notable portion of cells (>40%) exhibiting a decrease in this parameter (Fig. 6B).

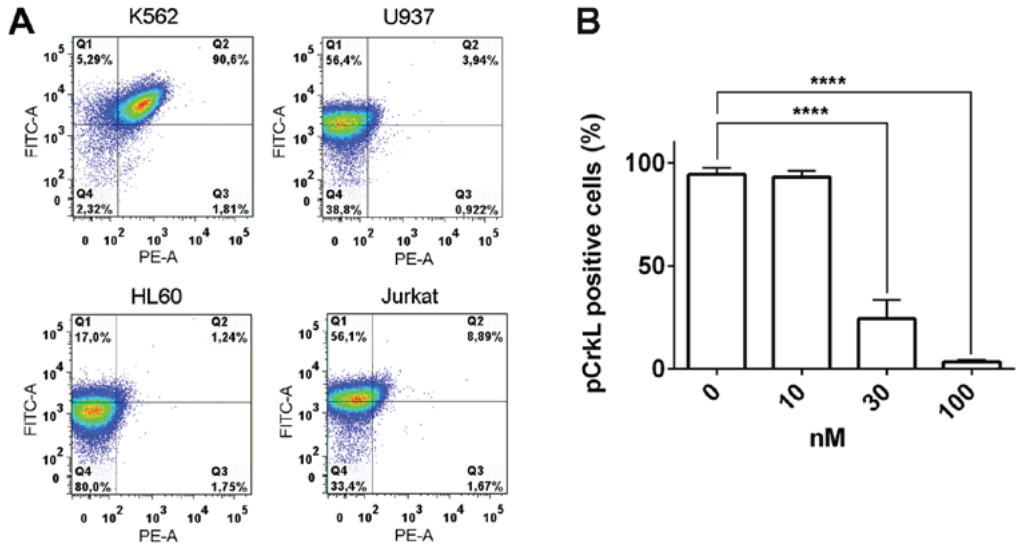

Figure 4. CrkL phosphorylation in K562 cells is sensitive to PF-114. (A) Cell line specific expression of pCrkL. The double colored flow cytometry-based assay indicated an increased fluorescence in the FITC channel (corresponds to staining of total CrkL) in all four cell lines, whereas PE fluorescence (corresponds to pCrkL staining) was detectable only in K562 cells. (B) Dose-dependent decrease of CrkL phosphorylation in K562 cells (2 h exposure). Data are presented as the mean ± standard deviation of four experiments. n=4. ****P<0.0001. FITC, fluorescein isothiocyanate; PE, phycoerythrin; pCRKL, phosphorylated Crk-like protein.

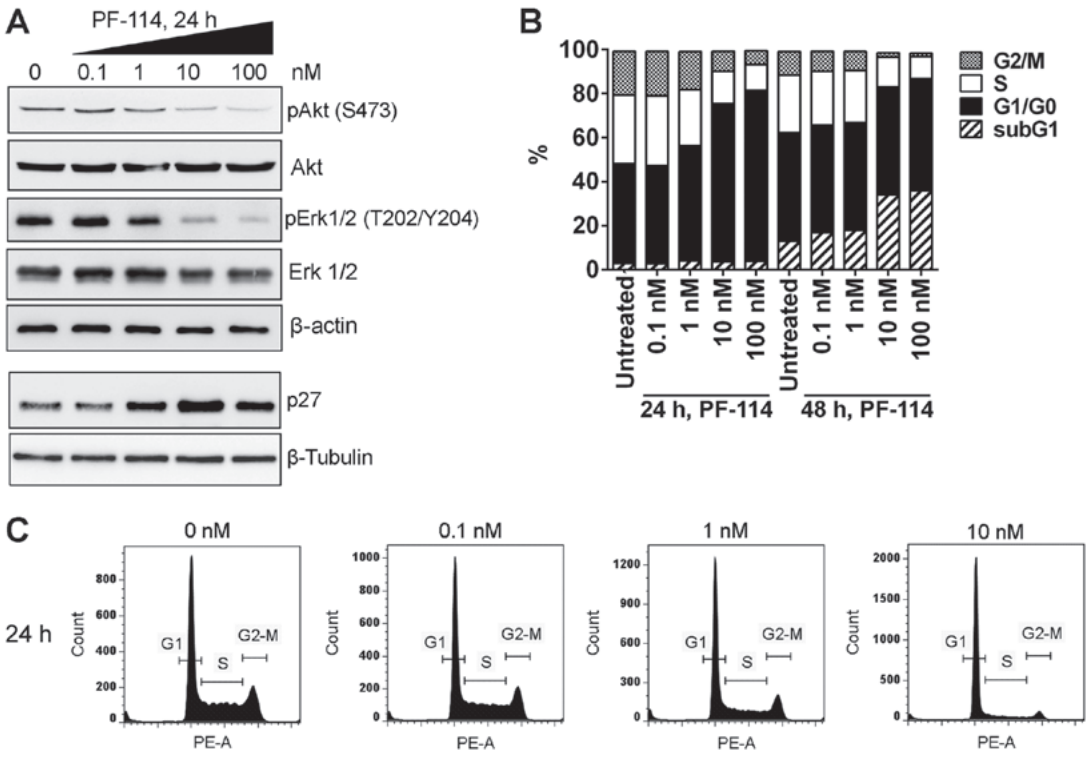

Figure 5. Effects of PF-114 on Akt and ERK1/2 phosphorylation, and the cell cycle. K562 cells were treated with various concentrations of PF-114 for 24 and 48 h. (A) A dose-dependent decrease in Akt and ERK1/2 phosphorylation, and the expression of p27 was observed. (B) Cell cycle distribution and (C) cell cycle arrest in G1 phase. Akt, protein kinase B; Erk, extracellular signal-regulated kinase.

By 48 h, the decreased mitochondrial transmembrane potential was detected in a predominant portion of cell population. Concomitant with this phenomenon was increased Annexin V staining (Fig. 6C), a marker of phosphatidylserine translocation across the plasma membrane of apoptotic cells (23). The processes of caspase-9 and PARP cleavage were revealed by 48 h; PARP cleavage generated a typical 89 kDa product and a 55 kDa band characteristic of later stages of death cascades (24). The proteolytic processing of caspase-3 was detectable by 24 h (data not shown). These events culminated to late apoptotic DNA degradation as indicated by the characteristic DNA fragments of 140-170 bp (Fig. 6E). The activation of caspase cascades led to a decrease in the expression of mitochondrial proteins BH3 interacting-domain death agonist (Bid), Bcl-extra large (XL) and Bcl-2 family apoptosis regulator (Mcl-1) achievable with notably high PF-114 concentrations (Fig. 7A). Bak expression

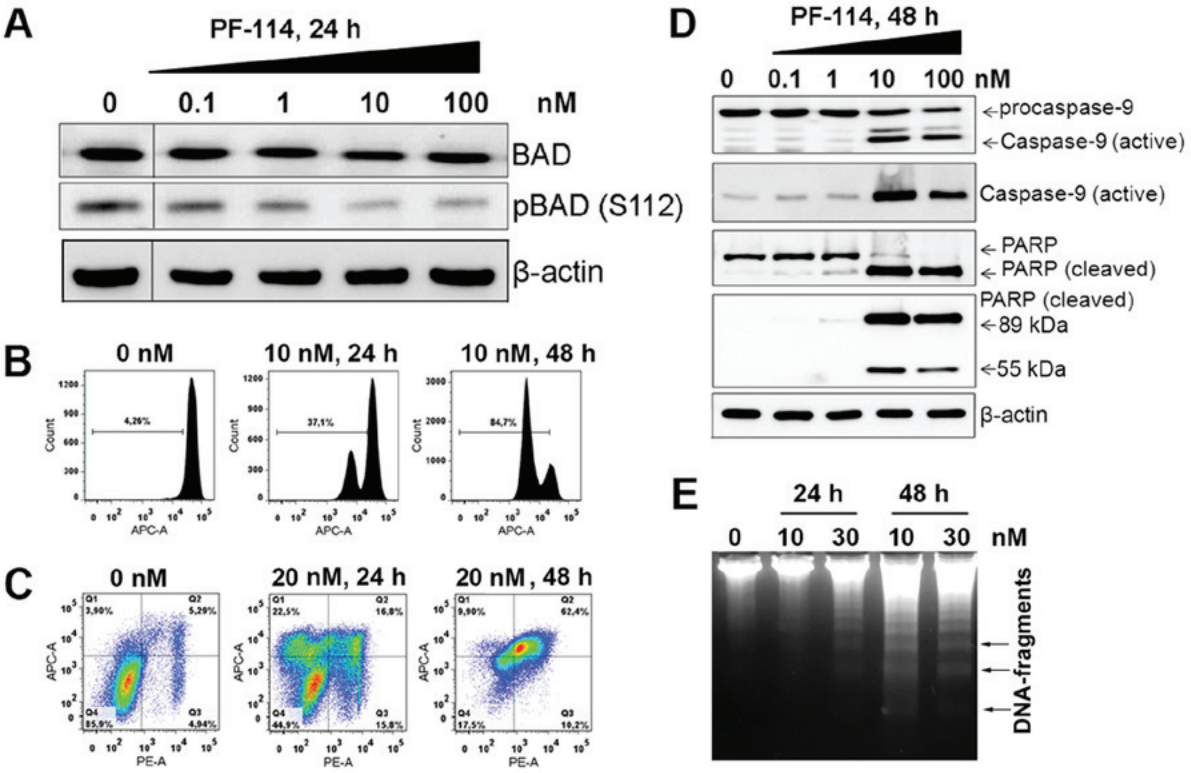

Figure 6. Apoptotic events are induced by PF-114 in K562 cells. (A) Decreased Bad phosphorylation; (B) reduced mitochondrial transmembrane electric potential; (C) increased Annexin V staining; (D) activation of caspase-9 and PARP cleavage, and (E) loss of DNA integrity (internucleosomal DNA fragmentation; arrows). APC, allophycocyanin; BAD, BCL-2-associated death promoter; p, phosphorylated; PARP, poly(ADP-ribose)polymerase; PE, phycoerythrin.

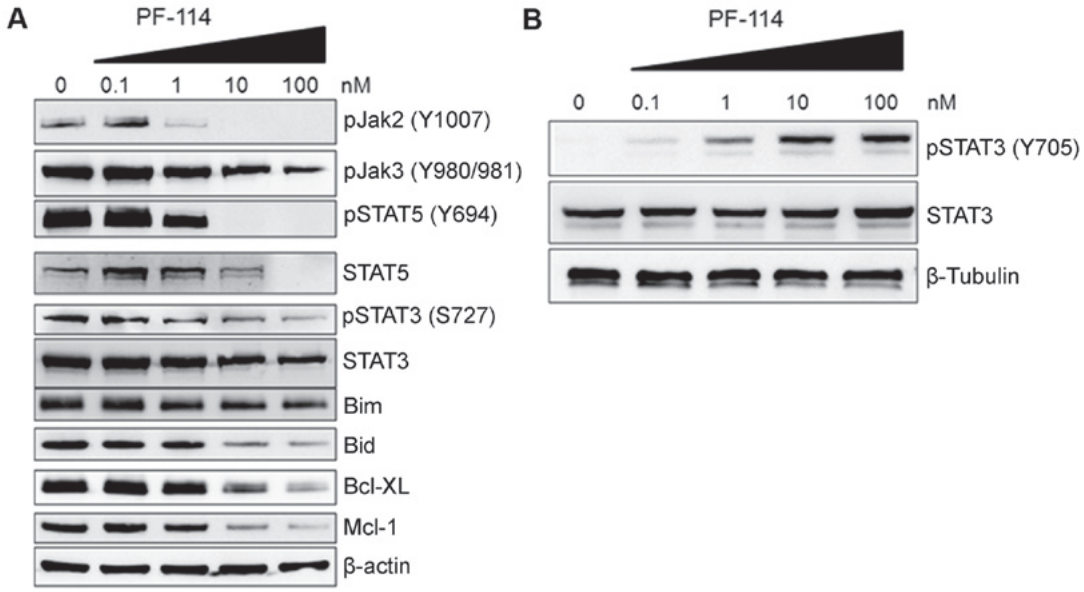

Figure 7. Effects of PF-114 on death/survival signaling. K562 cells were treated with various concentrations of PF-114 for 24 h. Proteins were analyzed by immunoblotting. (A) Mitochondrial and JAK/STAT proteins; (B) phosphorylation of STAT3 at Tyr705. Bcl-2, B-cell lymphoma-2; Bcl-XL, Bcl-2-extra large; Bid, BH3 interacting-domain death agonist; Bim, Bcl-2-like protein 11; Jak, Janus kinase-signal transducer; Mcl-1, Bcl-2 family apoptosis regulator; p, phosphorylated; STAT, signal transducer and activator of transcription.

was markedly unchanged, whereas that Bax levels markedly decreased at 100 nM PF-114 (Fig. S2). Collectively, typical apoptotic mechanisms may be implicated in PF-114-induced death of BCR-ABL positive, CML-derived K562 cells.

An imbalance between individual members of the superfamily of Janus kinase-signal transducer and activator of transcription proteins (JAK-STAT) has been reported to be associated with cell responses to various cytotoxic stimuli (25). As presented in Fig. 7A, pJAK2 at Tyr1007/1008 was markedly downregulated with 1 nM PF-114. Downregulation of pJAK3 was observed following treatment with higher concentrations of PF-114. Furthermore, individual amino acid residues within

the STAT3 phosphorylation sites were determined to be differentially sensitive to PF-114. We observed a dose-dependent increase in pSTAT3 Tyr705 with 1-100 nM PF-114 (Fig. 7B) whereas less pronounced Ser727 phosphorylation was detectable at 100 nM (Fig. 7A). Conversely, STAT5 phosphorylation appeared to be notably sensitive; low nanomolar concentrations of PF-114 abrogated STAT5 phosphorylation at Tyr694 (Fig. 7A). The expression of total STAT3 was markedly unchanged; total STAT5 protein expression decreased with higher PF-114 concentrations (10 and 100 nM).

In the present study, the effects of PF-114 and ponatinib were analyzed in K562 cells. The latter drug essentially induced similar responses at similar concentrations and time intervals as PF-114 (data not shown), which indicated the potential of the novel compound as a therapeutic agent for treating CML.

Discussion

The present study revealed that blocking BCR-ABL with the novel inhibitor PF-114 induced a tightly regulated ensemble of molecular events in K562 cells. Cell responses were manifested in a manner of cascades, suggesting a network involving protein kinases, and mitochondrial and transcriptional processes. Of note, dysregulation of signaling is not linear since several pathways are affected. This multiplicity of death mechanisms requires the inactivation of a key target downstream of BCR-ABL, that is, the CrkL adaptor protein; this can impair a number of kinase cascades. As a result, the divergent inactivation of pro-survival signaling was detectable in PF-114-treated CML cells. Downregulation of pAkt was concomitant with a decrease in pERK1/2, caspase activation, downregulation of mitochondrial proteins (Bid, Bcl-xL and Mcl-1) and reductions in the mitochondrial transmembrane potential. Collectively, these events may contribute to cell growth arrest and apoptosis. Therefore, targeting CrkL, an upstream (or proximal) coordinator of various pro-proliferative and pro-survival pathways, is therapeutically advantageous providing that certain mechanisms of cytotoxicity are non-functional as a result of a tumor genotype and/or preceding treatment.

Molecular ordering of signaling events in response to BCR-ABL inhibition revealed that cell cycle arrest preceded cell death. An elevation in p27 expression may be a candidate mechanism of G1 arrest in PF-114 treated K562 cells. This pivotal finding requires further investigation as p27 emerges as a therapeutic target in CML. Matrine, a plant alkaloid, significantly inhibited the proliferation of K562 cells, induced cell cycle arrest in G0/G1 and promoted apoptosis (26). These effects were regulated by a number of mechanisms, including p27 upregulation (27). Importantly, Liu *et al* (28) have demonstrated that this protein was critical for the maintenance of leukemia stem cells in CML-bearing mice. Furthermore, a combination of p27 silencing and a compound potent against imatinib-resistant K562 cells was synergistic (28). The complex regulation of p27 involves transcriptional and posttranscriptional events, including its translocation between the nucleus and the cytoplasm. The activity of Ca^{2+}-calmodulin dependent kinase IIγ reduces the amounts of nuclear p27, promoting the accumulation of this protein in the cytoplasm (29). Thus, p27-mediated G1 arrest and leukemia stem cell quiescence are alleviated, leading to blast crisis and disease progression (29). The mechanisms underlying the upregulation of p27 in response to PF-114 require further investigation. The results of the present study may provide suggests the necessity of drug combinations to increase the efficacy of targeted CML treatments.

Downregulation of the individual members of the JAK-STAT families by PF-114 requires further investigation. Lee *et al* (30) demonstrated that a variety of signaling cascades mediated by oncogenic protein kinases converge to STAT3. This transcription factor has been implicated in the protection of CML cells from apoptosis via several mechanisms (31-33). STAT3 undergoes a plethora of posttranslational modifications, in particular, phosphorylation, which regulate its genomic and non-genomic functions (34,35). STAT3 phosphorylation has been mechanistically associated with the emergence of resistance to imatinib (36). Eiring *et al* (37) reported a potent and selective SH2 domain inhibitor capable of reducing STAT3 phosphorylation and nuclear transactivation. This novel compound restored the sensitivity of CML cells to imatinib, including those from patients, otherwise resistant to tyrosine kinase inhibitors (37). Ruxolitinib, an inhibitor of JAK2, synergized with nilotinib in inducing CML cell death in a Phase I clinical trial (38). These findings favor for the combined use of BCR-ABL inhibitors with antagonists of various signaling cascades (39).

In pancreatic adenocarcinoma cells, imatinib and ponatinib exhibited no effect on pSTAT3 levels, but were elevated in response to an ERK blocker (40); however, as to which site in the STAT3 protein was phosphorylated remains unknown. In the present study, we observed opposing effects of PF-114 on two forms of pSTAT3; the levels of pSer727 decreased, whereas those of pTyr705 were elevated. The latter form was increased following treatment with >1 nM PF-114; however, pSer727 was attenuated only at 100 nM. In contrast, Ser727 but not Tyr705 phosphorylation was proposed as an important factor of glioblastoma radioresistance; pharmacological downregulation of pSTAT3 Ser727 markedly sensitized these cells to radiation (41). The phosphorylation at these two sites in STAT3 signifies the induction of differential epigenetic pathways in response to BCR-ABL inhibitors, including PF-114. Therefore, further investigation into the intracellular signaling mechanisms in PF-114-treated cells is required. Our future studies of the genome-wide transcriptional effects of PF-114 aim to identify genes that may influence the responses of CML cells to BCR-ABL inhibition.

Signaling events downstream of BCR-ABL inhibition may include mechanisms other than the ones described above. The present study reported that pAkt levels were decreased by PF-114; however, the levels of total glycogen synthase kinase-3 (GSK-3β) or its phosphorylated form were markedly unaltered in PF-114-treated cells (Fig. S3). In K562 cells, the role of GSK-3 as one of various substrates of Akt phosphorylation has been addressed by Zhou *et al* (42). The pharmacological inhibition of PI3K led to decreased pAkt levels, whereas the subsequent downregulation of pGSK-3β was less pronounced (42). This observation suggests, at least indirectly, that in K562 cells, GSK-3β could be phosphorylated by other kinases in addition to Akt. This hypothesis is in line with the synergistic effects of PI3K inhibitor and imatinib.

Furthermore, the PI3K-Akt-GSK-3β and the BCR-ABL-Akt pathways were proposed to act in concert, which suggests the role of signaling mechanisms that remain functional in cells treated with an BCR-ABL inhibitor. This further supports the need for combinations of the BCR-ABL inhibitor with other inhibitors of signal transduction.

In the present study, the effects of PF-114 on K562 cells that carry wild-type Abl in the context of BCR-ABL fusion protein were analyzed. To further evaluate the potential of PF-114 the cellular effects of the novel compound should be investigated against mutant forms of ABL kinase. As PF-114 potently inhibited different ABL mutants (including T315I) *in vitro*, in cell based assays and in animal models (8), the growth arrest/death mechanisms associated may be similar or identical in CML cells carrying wild-type or mutant Abl. However, the mutants may function beyond CrkL/BCR-ABL interactions, thereby circumventing its downstream cascades. Additionally, the T315I mutant is capable of restoring ERK1/2 signaling, STAT5 phosphorylation, factor-independent CML cell growth and leukemogenic potential in tetramerization-incompetent and other loss-of-function BCR-ABL mutants (43). These effects have been attributed to transphosphorylation of BCR protein by T315I mutants (44). These critical findings highlight the interplay between serine-threonine and tyrosine phosphorylation of BCR as an important mechanism of CML recurrence. Providing that T315I mutants confer growth advantages in the absence of the wild-type BCR-ABL variant that otherwise have notably increased transformation potential, T315I-driven phenomena may emerge after cells with wild-type BCR-ABL are eliminated by treatment (44). It is therefore critical to elucidate whether PF-114 can attenuate BCR transphosphorylation.

Our findings require further investigation. The present study aimed to identify the molecular mechanisms that comprise the response of one CML cell line to PF-114; however, which mechanism is closely associated with CML cell responses to PF-114 remains unknown. Thus, experiments involving the inactivation of individual pathways should be performed. Furthermore, our future efforts will be focused on mechanistic investigations using other BCR-ABL-positive cell lines and patient samples. Although Mian *et al* (8) demonstrated a high potency of PF-114 against CML cells possessing wild-type and different mutant forms of BCR-ABL, whether individual mechanisms of cell death are supported by our findings with K562 cells remains unknown. Finally, the mechanisms underlying cell death in cells with mutant BCR-ABL should be determined.

PF-114 is structurally similar to the FDA approved pan-BCR-ABL antagonist ponatinib. This compound is capable of inhibiting the wild-type as well as various clinically relevant mutant forms of chimeric BCR-ABL tyrosine kinase. We demonstrated that the CML-derived human K562 cell line that carries wild-type BCR-ABL was notably sensitive to PF-114. This agent at nanomolar concentrations rapidly blocked the phosphorylation of CrkL adaptor protein, thereby inhibiting a number of downstream signaling pathways, inducing cell cycle arrest and subsequent apoptosis. Together with favorable pharmacokinetics and good therapeutic efficacy in a murine model (8), this novel inhibitor may be considered as a potential therapeutic drug candidate for the treatment of CML (45).

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ESI, VVT, GGC, YVD and AAS made substantial contributions to the design of the study. AAZ, FNN and GGC synthesized PF-114. ESI, VVT, MAY, AIK, AVS and AAK performed the experiments and analyzed data. AAS wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interests.

References

1. Pasternak G, Hochhaus A, Schultheis B and Hehlmann R: Chronic myelogenous leukemia: Molecular and cellular aspects. J Cancer Res Clin Oncol 124: 643-660, 1998.
2. Lin X, Qureshi MZ, Attar R, Khalid S, Tahir F, Yaqub A, Aslam A, Yaylim I, De Carlos Back LK, Farooqi AA, *et al*: Targeting of BCR-ABL: Lessons learned from BCR-ABL inhibition. Cell Mol Biol (Noisy-le-grand) 62: 129-137, 2016.
3. Hochhaus A, Larson RA, Guilhot F, Radich JP, Branford S, Hughes TP, Baccarani M, Deininger MW, Cervantes F, Fujihara S, *et al*; IRIS Investigators: Long-term outcomes of imatinib treatment for chronic myeloid leukemia. N Engl J Med 376: 917-927, 2017.
4. Cortes JE, Kantarjian H, Shah NP, Bixby D, Mauro MJ, Flinn I, O'Hare T, Hu S, Narasimhan NI, Rivera VM, *et al*: Ponatinib in refractory Philadelphia chromosome-positive leukemias. N Engl J Med 367: 2075-2088, 2012.
5. Cortes JE, Kim DW, Pinilla-Ibarz J, le Coutre P, Paquette R, Chuah C, Nicolini FE, Apperley JF, Khoury HJ, Talpaz M, *et al*; PACE Investigators: A phase 2 trial of ponatinib in Philadelphia chromosome-positive leukemias. N Engl J Med 369: 1783-1796, 2013.
6. Hoy SM: Ponatinib: A review of its use in adults with chronic myeloid leukaemia or Philadelphia chromosome-positive acute lymphoblastic leukaemia. Drugs 74: 793-806, 2014.

7. Moslehi JJ and Deininger M: Tyrosine kinase inhibitor-associated cardiovascular toxicity in chronic myeloid leukemia. J Clin Oncol 33: 4210-4218, 2015.
8. Mian AA, Rafiei A, Haberbosch I, Zeifman A, Titov I, Stroylov V, Metodieva A, Stroganov O, Novikov F, Brill B, *et al*: PF-114, a potent and selective inhibitor of native and mutated BCR/ABL is active against Philadelphia chromosome-positive (Ph+) leukemias harboring the T315I mutation. Leukemia 29: 1104-1114, 2015.
9. Chilov GG and Titov IY: Protein kinase inhibitor (versions), use thereof for treating oncological diseases and based pharmaceutical composition. Patent RU2477723 (C2). Filed July 10, 2017; issued August 31, 2017.
10. Cellosaurus - a knowledge resource on cell lines. Version 29. https://web.expasy.org/cellosaurus/CVCL_0002.
11. Liu D: The adaptor protein Crk in immune response. Immunol Cell Biol 92: 80-89, 2014.
12. Shchekotikhin AE, Dezhenkova LG, Tsvetkov VB, Luzikov YN, Volodina YL, Tatarskiy VV Jr, Kalinina AA, Treshalin MI, Treshalina HM, Romanenko VI, *et al*: Discovery of antitumor anthra[2,3-b]furan-3-carboxamides: Optimization of synthesis and evaluation of antitumor properties. Eur J Med Chem 112: 114-129, 2016.
13. Rahbar Saadat Y, Saeidi N, Zununi Vahed S, Barzegari A and Barar J: An update to DNA ladder assay for apoptosis detection. Bioimpacts 5: 25-28, 2015.
14. Rossari F, Minutolo F and Orciuolo E: Past, present, and future of Bcr-Abl inhibitors: From chemical development to clinical efficacy. J Hematol Oncol 11: 84, 2018.
15. Haguet H, Douxfils J, Mullier F, Chatelain C, Graux C and Dogné JM: Risk of arterial and venous occlusive events in chronic myeloid leukemia patients treated with new generation BCR-ABL tyrosine kinase inhibitors: A systematic review and meta-analysis. Expert Opin Drug Saf 16: 5-12, 2017.
16. Wehrle J and von Bubnoff N: Ponatinib: A third-generation inhibitor for the treatment of CML. Recent Results Cancer Res 212: 109-118, 2018.
17. Zulbaran-Rojas A, Lin HK, Shi Q, Williams LA, George B, Garcia-Manero G, Jabbour E, O'Brien S, Ravandi F, Wierda W, *et al*: A prospective analysis of symptom burden for patients with chronic myeloid leukemia in chronic phase treated with frontline second- and third-generation tyrosine kinase inhibitors. Cancer Med 7: 5457-5469, 2018.
18. Aspinall-O'Dea M, Pierce A, Pellicano F, Williamson AJ, Scott MT, Walker MJ, Holyoake TL and Whetton AD: Antibody-based detection of protein phosphorylation status to track the efficacy of novel therapies using nanogram protein quantities from stem cells and cell lines. Nat Protoc 10: 149-168, 2015.
19. Lux MP, Fasching PA, Schrauder MG, Hein A, Jud SM, Rauh C and Beckmann MW: The PI3K pathway: Background and treatment approaches. Breast Care (Basel) 11: 398-404, 2016.
20. Dey N, De P and Leyland-Jones B: PI3K-AKT-mTOR inhibitors in breast cancers: From tumor cell signaling to clinical trials. Pharmacol Ther 175: 91-106, 2017.
21. Nussinov R, Tsai CJ, Jang H: A new view of pathway-driven drug resistance in tumor proliferation. Trends Pharmacol Sci 38: 427-437, 2017.
22. Correia C, Lee SH, Meng XW, Vincelette ND, Knorr KL, Ding H, Nowakowski GS, Dai H and Kaufmann SH: Emerging understanding of Bcl-2 biology: Implications for neoplastic progression and treatment. Biochim Biophys Acta 1853: 1658-1671, 2015.
23. Schutters K and Reutelingsperger C: Phosphatidylserine targeting for diagnosis and treatment of human diseases. Apoptosis 15: 1072-1082, 2010.
24. Chaitanya GV, Steven AJ and Babu PP: PARP-1 cleavage fragments: Signatures of cell-death proteases in neurodegeneration. Cell Commun Signal 8: 31, 2010.
25. Bousoik E and Montazeri Aliabadi H: 'Do We Know Jack' about JAK? A closer look at JAK/STAT signaling pathway. Front Oncol 8: 287, 2018.
26. Ma L, Zhu Z, Jiang L, Sun X, Lu X, Zhou M, Qian S and Jianyong L: Matrine suppresses cell growth of human chronic myeloid leukemia cells via its inhibition of the interleukin-6/Janus activated kinase/signal transducer and activator of transcription 3 signaling cohort. Leuk Lymphoma 56: 2923-2930, 2015.
27. Ma L, Xu Z, Wang J, Zhu Z, Lin G, Jiang L, Lu X and Zou C: Matrine inhibits BCR/ABL mediated ERK/MAPK pathway in human leukemia cells. Oncotarget 8: 108880-108889, 2017.
28. Liu C, Nie D, Li J, Du X, Lu Y, Li Y, Zhou J, Jin Y and Pan J: Antitumor effects of blocking protein neddylation in T315I-BCR-ABL leukemia cells and leukemia stem cells. Cancer Res 78: 1522-1536, 2018.
29. Gu Y, Zheng W, Zhang J, Gan X, Ma X, Meng Z, Chen T, Lu X, Wu Z, Huang W, *et al*: Aberrant activation of CaMKIIγ accelerates chronic myeloid leukemia blast crisis. Leukemia 30: 1282-1289, 2016.
30. Lee HJ, Zhuang G, Cao Y, Du P, Kim HJ and Settleman J: Drug resistance via feedback activation of Stat3 in oncogene-addicted cancer cells. Cancer Cell 26: 207-221, 2014.
31. Mencalha AL, Corrêa S, Salles D, Du Rocher B, Santiago MF and Abdelhay E: Inhibition of STAT3-interacting protein 1 (STATIP1) promotes STAT3 transcriptional up-regulation and imatinib mesylate resistance in the chronic myeloid leukemia. BMC Cancer 14: 866, 2014.
32. Wang H, Xie B, Kong Y, Tao Y, Yang G, Gao M, Xu H, Zhan F, Shi J, Zhang Y, *et al*: Overexpression of RPS27a contributes to enhanced chemoresistance of CML cells to imatinib by the transactivated STAT3. Oncotarget 7: 18638-18650, 2016.
33. Shi Y, Zhang Z, Qu X, Zhu X, Zhao L, Wei R, Guo Q, Sun L, Yin X, Zhang Y, *et al*: Roles of STAT3 in leukemia (Review). Int J Oncol 53: 7-20, 2018.
34. Guanizo AC, Fernando CD, Garama DJ and Gough DJ: STAT3: A multifaceted oncoprotein. Growth Factors 36: 1-14, 2018.
35. Sgrignani J, Garofalo M, Matkovic M, Merulla J, Catapano CV and Cavalli A: Structural biology of STAT3 and its implications for anticancer therapies development. Int J Mol Sci 19: E1591, 2018.
36. Al-Jamal HA, Jusoh SA, Yong AC, Asan JM, Hassan R and Johan MF: Silencing of suppressor of cytokine signaling-3 due to methylation results in phosphorylation of STAT3 in imatinib resistant BCR-ABL positive chronic myeloid leukemia cells. Asian Pac J Cancer Prev 15: 4555-4561, 2014.
37. Eiring AM, Page BDG, Kraft IL, Mason CC, Vellore NA, Resetca D, Zabriskie MS, Zhang TY, Khorashad JS, Engar AJ, *et al*: Combined STAT3 and BCR-ABL1 inhibition induces synthetic lethality in therapy-resistant chronic myeloid leukemia. Leukemia 31: 1253-1254, 2017.
38. Sweet K, Hazlehurst L, Sahakian E, Powers J, Nodzon L, Kayali F, Hyland K, Nelson A and Pinilla-Ibarz J: A phase I clinical trial of ruxolitinib in combination with nilotinib in chronic myeloid leukemia patients with molecular evidence of disease. Leuk Res 74: 89-96, 2018.
39. Singh H, Williams RT and Guy KR: Methods and compositions for the treatment of Bcr-Abl positive lymphoblastic leukemias. Patent US20150133462 A1. Filed May 23, 2013; issued May 14, 2015.
40. Sahu N, Chan E, Chu F, Pham T, Koeppen H, Forrest W, Merchant M and Settleman J: Cotargeting of MEK and PDGFR/STAT3 pathways to treat pancreatic ductal adenocarcinoma. Mol Cancer Ther 16: 1729-1738, 2017.
41. Ouédraogo ZG, Müller-Barthélémy M, Kemeny JL, Dedieu V, Biau J, Khalil T, Raoelfils LI, Granzotto A, Pereira B, Beaudoin C, *et al*: STAT3 serine 727 phosphorylation: A relevant target to radiosensitize human glioblastoma. Brain Pathol 26: 18-30, 2016.
42. Zhou Q, Chen Y, Chen X, Zhao W, Zhong Y, Wang R, Jin M, Qiu Y and Kong D: In vitro antileukemia activity of ZSTK474 on K562 and multidrug resistant K562/A02 cells. Int J Biol Sci 12: 631-638, 2016.
43. Haberbosch I, Rafiei A, Oancea C, Ottmann GO, Ruthardt M and Mian AA: BCR: A new target in resistance mediated by BCR/ABL-315I? Genes Cancer 7: 36-46, 2016.
44. Mian AA, Schüll M, Zhao Z, Oancea C, Hundertmark A, Beissert T, Ottmann OG and Ruthardt M: The gatekeeper mutation T315I confers resistance against small molecules by increasing or restoring the ABL-kinase activity accompanied by aberrant transphosphorylation of endogenous BCR, even in loss-of-function mutants of BCR/ABL. Leukemia 23: 1614-1621, 2009.
45. NIH: U.S. National Library of Medicine. ClinicalTrials. Study to evaluate tolerability, safety, pharmacokinetics and preliminary efficacy of PF-114 for oral administration in adults with Ph+ chronic myeloid leukemia, which is resistant to the 2-nd generation Bcr-Abl inhibitors or Has T315I mutation in the BCR-ABL gene. NCT02885766. https://clinicaltrials.gov/ct2/show/NCT02885766.