Janus kinase 2 regulates Bcr–Abl signaling in chronic myeloid leukemia

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Despite the success of imatinib mesylate (IM) in the early chronic phase of chronic myeloid leukemia (CML), patients are resistant to IM and other kinase inhibitors in the later stages of CML. Our findings indicate that inhibition of Janus kinase 2 (Jak2) in Bcr–Abl+ cells overcomes IM resistance although the precise mechanism of Jak2 action is unknown. Knocking down Jak2 in Bcr–Abl+ cells reduced levels of the Bcr–Abl protein and also the phosphorylation of Tyr177 of Bcr–Abl, and Jak2 overexpression rescued these knockdown effects. Treatment of Bcr–Abl+ cells with Jak2 inhibitors for 4–6 h but not with IM also reduced Bcr–Abl protein and pTyr177 levels. In vitro kinase experiments performed with recombinant Jak2 showed that Jak2 readily phosphorylated Tyr177 of Bcr–Abl (a Jak2 consensus site, YvnV) whereas c-Abl did not. Importantly, Jak2 inhibition decreased pTyr177 Bcr–Abl in immune complexes but did not reduce levels of Bcr–Abl, suggesting that the reduction of Bcr–Abl by Jak2 inhibition is a separate event from phosphorylation of Tyr177. Jak2 inhibition by chemical inhibitors (TG101209/WP1193) and Jak2 knockdown diminished the activation of Ras, PI-3 kinase pathways and reduced levels of pTyrSTAT5. These findings suggest that Bcr–Abl stability and oncogenic signaling in CML cells are under the control of Jak2.

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Introduction

Our previous experiments suggest that Janus kinase 2 (Jak2) has an important role in Bcr–Abl+ cells, and that Bcr–Abl expression leads to activation of Jak2.1–3 Bcr–Abl is known to drive the Grb2-Ras-Raf-Mek1/2 Erk pathway and the PI-3 kinase pathway involving Gab2,4,4 the Jak2-STAT3 pathway7,8 and the Bcr–Abl-STAT5 pathway.9,10 Phosphorylation of Tyr177 Bcr–Abl is a critical event required for development of chronic myeloid leukemia (CML), as the Y177F mutant of Bcr–Abl expresses resistance to IM and other kinase inhibitors in the later stages of CML.20,21

Residual CML disease appears to involve primitive progenitor cells, which have been shown to be present in niches in the bone marrow.23,24 These cells are considered quiescent and not Bcr–Abl dependent.25–27 These findings require a search for new therapeutic targets and compounds to eradicate these tyrosine kinase inhibitors-resistant cells from the bone marrow niche. Jak2 is an important signaling component in hematopoietic cells, as it transmits signals generated by interaction of cytokines such as IL-3 with the IL-3 receptor. The α- and β-chains of the IL-3 receptor are part of a large dodecamer structure in which two Jak2 molecules are bound to the β-chain in close proximity to each other.28 Jak2, as a result of interaction of IL-3 with the IL-3 receptor, becomes activated by autophosphorylation at Tyr1007. Jak2, as a result of interaction of IL-3 with the IL-3 receptor, becomes activated by autophosphorylation at Tyr1007.28,29 Studies by Huang et al.30 suggest that IL-3 signaling driven by activated Jak2 is enhanced by Jak1 interaction with Jak2. Our new findings indicate that Jak2 controls Bcr–Abl signaling in CML cells, as either Jak2 knockdown or Jak2 inhibition drastically reduced the levels of Bcr–Abl and the phosphorylation of Tyr177 within Bcr–Abl causing reduction of oncogenic signaling.

Materials and methods

See Supplementary Material and Methods.

Results

Jak2 knockdown reduced levels of Bcr–Abl protein and Tyr 177 phosphorylated form of Bcr–Abl in mouse hematopoietic and CML cell lines

Our findings with Bcr–Abl+ cell lines including cells expressing imatinib mesylate (IM)-resistant forms of Bcr–Abl indicate that Jak2 is a critical player in CML.20,22 We began exploring the mechanisms behind this critical role of Jak2 in CML. We found that Jak2 controls Bcr–Abl protein levels, as Jak2 knockdown causes a rapid disappearance of Bcr–Abl (Figure 1). We used a specific mouse form of Jak2 small interfering RNA to knockdown Jak2 in Bcr–Abl+ 32D mouse myeloid cells (Figure 1a).
Jak2 knockdown dramatically reduced levels of the pTyr Bcr–Abl protein. To offset the possible nonspecific effects of Jak2 knockdown, we rescued Jak2 knockdown by transducing Jak2 cDNA into cells at the time of Jak2 knockdown. The rescued cells had restored levels of pTyr Bcr–Abl in cells corresponding to the restored expression of Jak2, suggesting that Jak2 controls the expression of Bcr–Abl.

We also used an inducible form of specific human Jak2 short hairpin RNA to knockdown Jak2 in three CML cell lines, namely BV173, KBM-7 and K562-R. Levels of Jak2 were reduced and also Bcr–Abl protein levels were drastically reduced following Jak2 knockdown in K562-R cells (Figures 1b and c). Reversal of Jak2 knockdown (removal of the inducer doxycycline) restored the expression of the Bcr–Abl protein (Figure 1d).

In Jak2 knockdown experiments, levels of Tyr177 phosphorylation within Bcr–Abl were also strongly inhibited as expected as Bcr–Abl had also disappeared. Importantly, rescue experiments partially restored levels of pTyr177 (Figures 1a–d). Similar results were observed by Jak2 knockdown in CML cell lines BV173 and KBM7 (Supplementary Figure 1a–c).

Jak2 phosphorylated the tyrosine 177 Bcr sequence found in Bcr–Abl
An analysis of the Bcr–Abl sequence revealed various tyrosine residues that are Jak2 consensus phosphorylating sites (YxxV/L/I) (Supplementary Table 1). We observed that tyrosine 177 within Bcr–Abl (YxxV) fits the consensus Jak2 phosphorylation motif. To test whether Jak2 would phosphorylate Tyr177, we made a Bcr peptide that contains sequences surrounding the tyrosine 177 sequence of Bcr–Abl, and used that peptide as a target for Jak2 (Figures 2a–c). Purified recombinant Jak2 (pH1 domain) readily phosphorylated this peptide and this phosphorylation was strongly inhibited by the selective Jak2 inhibitor TG101209 (TG) but not by IM (Figures 2a and b). We note that TG is a potent inhibitor of Jak2’s ability to phosphorylate Tyr 177 in kinase assays with an IC50 of less than 0.01 μM (reported to be 6 nM) (Supplementary Figure 1d).

Jak2 immune complexes isolated from Bcr–Abl + 32D cells also phosphorylated the Tyr 177 Bcr peptide and phosphorylation was inhibited by TG but not by IM (Figure 2a). These Jak2 immune complexes contain Bcr–Abl, Jak2 and HSP90 and other signaling members such as Akt and STAT3. Thus, although Bcr–Abl is present in the Jak2 immune complex (see Supplementary Figure 1a, b), it does not phosphorylate Tyr 177 as IM does not inhibit tyrosine phosphorylation of the peptide (Figure 2a). We note that purified near full-length recombinant c-AbI kinase only poorly phosphorylated the Tyr 177 site in the Bcr peptide (Figure 2c).

Jak2 inhibition of phosphorylation of Tyr177 is a separate event from disappearance of Bcr–Abl
To determine whether the disappearance of Bcr–Abl could be separated from the inhibition of Tyr 177 phosphorylation, we performed kinase assays with immune complexes harvested from Bcr–Abl + 32D cells with anti-Jak2 antibodies, and determined whether Jak2 inhibition would decrease levels of pTyr177 and whether levels of Bcr–Abl in the immune complexes would also be reduced by Jak2 inhibition. The Bcr–Abl protein was not decreased in these immune complexes by Jak2 inhibition but importantly levels of pTyr177 were strongly decreased (Figures 2d, e). Similarly, levels of pTyr Bcr–Abl were not reduced by treatment of the kinase reaction mixture with 5 and 10 μM TG (Figure 2d). Importantly, Jak2 and Bcr–Abl coprecipitated in immune complexes (Supplementary Figure 1e and f). These results indicate that the events leading to the decrease of Bcr–Abl occurred within intact cells but not in immune complexes from these same cells and, more importantly, the inhibition of phosphorylation of Tyr177 by TG can readily occur in these subcellular fractions under conditions wherein Bcr–Abl levels were stable. Importantly, we found that Jak2 inhibition caused only background levels of apoptosis during the first 4 h of treatment wherein Bcr–Abl levels were strongly reduced (Supplementary Figure 5c).

Jak2 inhibitor TG101209 rapidly decreased the levels of pTyr177 Bcr–Abl
We tested the effects of Jak2 inhibition in various Bcr–Abl + cell lines and CML cell lines. Jak2 inhibition reduced levels of active Jak2 in a dose-dependent manner. The 50% point of Jak2 inhibition as measured by pTyr1007 phosphorylation was estimated to be 5 μM as estimated by quantitation of the intensity values (Figure 3a). Similarly, the level of Bcr–Abl pTyr177 was inhibited in a dose-dependent manner and the 50% inhibitory point was estimated to be 5 μM. The 50% inhibitory point for Bcr–Abl reduction was estimated to be ~7.5 μM. Interestingly, the selective Jak2 inhibitor TG rapidly decreased levels of phosphorylation of Tyr177 of Bcr–Abl but also decreased levels of Bcr–Abl during this time period (Figure 3b). The CML cell line K562-R showed rapid loss of pTyr177 Bcr–Abl and the

Figure 1  Knockdown of Jak2 strongly reduced levels of the Bcr–Abl protein and pTyr 177 of Bcr–Abl in Bcr–Abl + cells and CML cell lines. (a) Knockdown of Jak2 and rescue of Jak2 expression in Bcr–Abl + 32D cells (32Dp210) using Jak2-specific small interfering RNA. 32Dp210 contains a b3a2 form of Bcr–Abl expressed in mouse myeloid 32D cells. (b) Knockdown of Jak2 in K562-R CML cell line by an inducible Jak2-specific short hairpin RNA. Jak2 knockdown was initiated by addition of doxycycline (2 μg/ml) for 3 days. (c) Non-targeted short hairpin RNA has no effects on Jak2 expression and Bcr–Abl expression. (d) Reversal of Jak2 knockdown restores levels of pTyr177 and the Bcr–Abl protein. After a 3-day induction, doxycycline was withdrawn from the culture for 6 days. Western blotting with the appropriate antibodies was performed in these studies.

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Bcr–Abl protein after 3 h (Figure 3c), as did KBM7 CML cells (Supplementary Figure 1g). Reductions of pTyr177 Bcr–Abl and Bcr–Abl were seen in CML cell line K562 (Supplementary Figure 1h). Cells from a blast crisis CML patient treated with TG101209 also had a reduction of pTyr177 Bcr–Abl and Bcr–Abl protein (Figure 3d). We also showed that Jak2 inhibition rapidly reduced the levels of total pTyr proteins (Figure 3e).

The Jak2 consensus sites include YxxV/L/I and there are a number of these Jak2 consensus sites in Bcr–Abl (Supplementary Table 1). We wondered whether YxxF would also be a site that phosphorylated Jak2, as F-like V/L/I is a hydrophobic amino acid. There are three such sites in Bcr–Abl including Tyr 360. We had made a mouse monoclonal antibody against the pTyr360 sequence of Bcr. This sequence-specific pTyr monoclonal antibody detected a signal in Bcr–Abl, and Jak2 inhibition by a new Jak2 inhibitor WP1193 (see below) dramatically reduced the level of pTyr360 Bcr–Abl within 60 min (Supplementary Figure 2a), suggesting that Jak2 also phosphorylates Tyr360 of Bcr–Abl.

pTyr177 within P160 BCR is also rapidly reduced by Jak2 inhibition

Similar to Bcr–Abl, phosphorylation of Tyr177 within P160 BCR was also rapidly reduced by treatment with 10 μM TG (Supplementary Figure 1i). As Bcr is believed to form heterotetramers with Bcr–Abl, these results suggest that Jak2 would phosphorylate Tyr177 within Bcr–Abl and Bcr in these heterotetramers. It is unknown whether phosphorylation of Bcr on Tyr177 would contribute to activation of Ras and PI-3 kinase pathways in CML cells.

CML 34+ cells respond to Jak2 inhibition by reduction of pTyr177 Bcr–Abl and Bcr–Abl

Cells were harvested from the peripheral blood of a CML blast crisis patient, having 98% blasts. After CD34 selection, sufficient cells were available for western blotting (3 million cells). Flow cytometry results indicated that 87% of the cells bound to the CD34 beads were CD34+ (results not shown). Western blotting revealed that Jak2 inhibition by TG reduced levels of pTyr177 and Bcr–Abl in CD34+ cells (Figure 3f). We note that CD34+ cells from this patient were resistant to IM but sensitive to TG (Supplementary Figure 4a).

Cord blood CD34+ cells showed loss of Bcr–Abl and pTyr177 Bcr–Abl caused by Jak2 inhibition

We examined the effects of Jak2 inhibition in CD34+ cord blood cells transduced with BCR–ABL.12 The cells were treated for 6 h with 2.5 and 10 μM TG, pTyr177 Bcr–Abl and Bcr–Abl were drastically reduced as was Jak2 and activated Jak2 (pTyr 1007) following treatment of CD34+ cells with TG (Figure 3g). These results with CD34+ cord blood cells, which were recently transduced with Bcr–Abl, would be consistent with Jak2 inhibiting early progenitor cells in CML patients.
Inhibition of the Bcr–Abl kinase by IM had little effect on the phosphorylation of Tyr177 of Bcr–Abl in short-term experiments

Importantly, IM, a well-known Abl kinase inhibitor, did not inhibit tyrosine phosphorylation of Tyr177 of Bcr–Abl for up to 3 h within Bcr–Abl+ cells nor was the level of the Bcr–Abl protein affected during 3-h treatment with IM (Figure 3h). These results provide further evidence that Tyr177 of Bcr–Abl is a site phosphorylated by Jak2 and does not result from a Bcr–Abl autophosphorylation reaction. However, treatment of Bcr–Abl+ cells with IM for longer times, as expected, drastically decreased Bcr–Abl protein levels in a dose-dependent manner (1–10 μM, 16 h, not shown).

Jak2 inhibition reduced activation of the Ras, PI-3 kinase and STAT5 pathways

We determined whether binding of Grb2 to Bcr–Abl was similarly decreased by Jak2 inhibition. It is known that Grb2 binds to pTyr177 of Bcr–Abl and pTyr Shc; Shc also contains the
Grb2-binding sequence YvnV sequence. Shc associates with Bcr–Abl and like pTyr177, drives the Ras and PI-3 kinase pathways. Grb2 levels in the Bcr–Abl immunocomplex was reduced by 60% within 120 min of TG treatment compared with the control (Figure 4a). Jak2 inhibition by TG reduced levels of Ras GTP within 60 min in 32Dp210 cells (Figures 4b and c), which is consistent with the observed decrease in Grb2 binding to Bcr–Abl. We also determined whether Jak2 inhibition decreased PI-3 kinase activation. It is known that pTyr177 binds Grb2 which in turn binds Gab2.6 Our previous studies indicate that Jak2 activation leads to phosphorylation of Tyr 452 of Gab2, which is involved in binding to the regulatory subunit of the PI-3 kinase leading to its activation.6 In our current studies, pTyr 452 Gab2 was rapidly decreased in Bcr–Abl cells after treatment with TG (Figure 4d). We note that PI-3 kinase activation as measured by a commercial kit was also decreased within 60–120 min of Jak2 inhibition (not shown). These results indicate that Jak2 inhibition rapidly inhibited both Ras and PI-3 kinase activation.

We determined whether STAT5 activation would also be downregulated by this more potent Jak2 inhibitor. Jak2 inhibition rapidly decreased tyrosine phosphorylation of pTyr levels of STAT5 (Figure 4e), which is consistent with downregulation of STAT5 transcriptional activity. It remains to be determined whether inactivation of Bcr–Abl by Jak2 interferes with Bcr–Abl’s ability to activate STAT5.

Jak2 knockdown reduced levels of tyrosine phosphorylation of Shc and Gab2

The reduction of Grb2 binding to Bcr–Abl and the resultant decrease in Ras activation prompted an examination of Jak2 effects on phosphorylation of Shc. It is known that pTyr Shc (at the YvnV sequence) also binds Grb2 leading to Ras activation. Knockdown of Jak2 strongly decreased levels of pTyr Shc in CML cell line K562-R (Figure 4f) and BV173 cells (not shown). Thus, Jak2 inhibition not only reduced levels of Grb2 binding to Bcr–Abl, but Jak2 knockdown also reduced the alternate

Figure 4 Jak2 inhibition by TG reduced activation of the Ras pathway and PI-3 kinase pathways. (a) Jak2 inhibition of 32Dp210 cells reduced binding of Grb2 to anti-Abl immunoprecipitates. (b) Jak2 inhibition by TG reduced levels of Ras GTP in 32Dp210 cells. These assays were performed with a commercial kit as described by the manufacturer. It involves IP/westerns with their RAS GTP antibody. The kit allows for generation of internal positive and negative controls; the third control is untreated Bcr–Abl+ cells. (c) Quantitation of RAS GTP levels following treatment of 32Dp210 cells with 10 μM TG. The intensity values of the RAS GTP bands were divided by the intensity of the actin band. (d) Jak2 inhibition by TG rapidly reduced levels of pTyr (452 YxxM) Gab2 in 32Dp210 cells. pTyr 452 is believed to bind to regulatory subunit of the PI-3 kinase, which is required for activation of PI-3 kinase. (e) Jak2 inhibition by TG rapidly decreased tyrosine phosphorylation of STAT5 (residue 694). (f) Knockdown of Jak2 reduced pathways related to the Bcr–Abl/Jak2 pathway in CML cell line K562-R. Jak2-specific short hairpin RNA was induced by treatment of cells with doxycycline as in Figure 1. The lysate was western blotted with the antibodies listed.
Jak2 knockdown also reduced levels of pTyr Gab2, which binds to Grb2, thereby reducing the activation of the PI-3 kinase pathway in K562-R cells. The levels of pMEK1 and 2, pSer 9 of GSK3 and pTyr STAT5 were also reduced by Jak2 knockdown (Figure 4b) in BV173 cells (not shown). Jak2 controls the Gab2/PI-3 kinase, Akt and GSK3 through its ability to induce phosphorylation of Gab2 and would control Ras activation and downstream MEK activation by Jak2’s ability to phosphorylate Tyr177 of Bcr–Abl and Tyr 239/240 of Shc. We note that Jak2 knockdown did not decrease either Lyn kinase or total Grb2 levels (Figure 4f). Our findings indicate that Lyn kinase is not part of the Bcr–Abl/GRB2 network complex. Thus, prolonged Jak2 inhibition would seriously depress levels of activated Ras and PI-3 kinase activation in Bcr–Abl+ leukemia cells.

A new pan Jak kinase inhibitor, inhibits Jak2 effects on Bcr–Abl and Tyr177 phosphorylation

We tested the effects of a more potent analog of the Jak2 inhibitor AG490 (WP1193, Figure 5a) for its ability to reduce Bcr–Abl and to inhibit phosphorylation of Tyr177 of Bcr–Abl. WP1193 inhibited the phosphorylation of Tyr177 housed within the Bcr peptide with 50% inhibition point of <2.5 μM (Figure 5b). Like AG490, WP1193 inhibited tyrosine phosphorylation of Jak2 in cells (Figure 5c) and also inhibited tyrosine phosphorylation of Jak1 and Jak3 (not shown); WP1193 also inhibited the autophosphorylation of Jak2 in vitro (Supplementary Figure 2b). It has been reported that Jak1 kinase interacts with Jak2 leading to the strengthening of the downstream effects of cytokine signaling through Jak2. WP1193 rapidly reduced levels of Bcr–Abl and pTyr177 Bcr–Abl within several Bcr–Abl+ cell lines including T315I cells and cells from blast crisis CML patients (Figures 5c–e). WP1193 appeared to be more potent than TG (compare Figures 5c–e with Figures 3b–d).

**Figure 5.** A new Jak2 inhibitor WP1193 rapidly reduced levels of pTyr177 Bcr–Abl, Bcr–Abl protein and levels of pTyr Bcr–Abl in 32Dp210 cells. (a) Structure of WP1193 and AG490. AG490 is a known Jak kinase inhibitor. (b) Jak2 inhibitor WP1193 inhibits the ability of recombinant Jak2 (b1-H1-JH2) to phosphorylate Tyr177 Bcr peptide. The same methods were used as described in Figure 2. (c) Top panel: Jak2 inhibitor WP1193 rapidly inhibited phosphorylation of Tyr177 of Bcr–Abl and reduced levels of Bcr–Abl in 32Dp210 cells. 32Dp210 cells were lysed and immunoprecipitated with anti-Abl p6D monoclonal antibody as described in Figure 2a. Bottom panel: 32Dp210 cells were treated for up to 1 h with 10 μM WP1193; lysates were made and immunoprecipitated with anti-Jak2 antibody. Western blots were probed with anti-pTyr 4G10. (d) Jak2 inhibition by WP1193 causes rapid reduction of pTyr177 Bcr–Abl and the Bcr–Abl protein in BaF3 cells expressing IM-resistant form of Bcr–Abl containing the T315I mutation. (e) Jak2 inhibition by WP1193 caused rapid reduction of pTyr177 Bcr–Abl in cells from a blast crisis CML patient. Monocytes from patients were isolated by Histopaque (Sigma Chem Co.,) separation and grown for 24 h in culture medium without growth factors before WP1193 treatment. (f) The level of the Y177F Bcr–Abl mutant is decreased in amount similar to wild-type Bcr–Abl as a result of Jak2 inhibition. 32D cells were transfected with either Y177F BCR–ABL mutant or wild-type BCR–ABL. Cells were allowed to grow in the absence of IL-3 for about 3 weeks. Cells were treated with 10 μM WP1193 for 30 min. Lysates were analyzed by western blotting with anti-Abl 8E9, anti-Tyr 177 Bcr, anti-Jak2 pTyr 1007/8 and anti-Jak2 antibodies. (g) Levels of Grb2 were rapidly reduced in anti-Abl immune complexes containing Bcr–Abl. (h) Levels of Ras GTP were rapidly reduced in 32Dp210 cells treated with Jak2 inhibitor WP1193. Experiments were performed as in Figure 4b. (i) Quantitation of Ras GTP activity in 32Dp210 cells treated with Jak2 inhibitor WP1193. Quantitation was done as shown in Figure 4c.

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The estimated point of 50% inhibition of phosphorylation of Tyr177, and Bcr–Abl reduction for WP1193 was between 2.0 and 3.0 μM in whole cells, respectively (Supplementary Figure 2h). Overall, the pan Jak inhibitor, although much less potent in Jak2 kinase assays than TG101209 (estimated 50% inhibition point of about 2 μM for WP1193 compared with 0.01 μM for TG (compare Figure 5b with Supplementary Figure 1d), WP1193 was similar if not more potent at reducing levels of Bcr–Abl and pTyr177 compared with TG101209 (compare inhibition by WP1193 and TG101209 in Figures 5c–e and Figures 3b–d, respectively).

Like TG, WP1193 was able to reduce binding of Grb2 to Bcr–Abl complexes while reducing levels of pTyr177 Bcr–Abl (Figure 5g). WP1193 rapidly reduced RAS GTP levels (Figures 5h and i) and pTyr Gab2, and STAT5 levels (Supplementary Figure 2c, e, respectively). WP1193 was a potent inhibitor of the Jak2 kinase in a test tube kinase assay (Supplementary Figure 2b) but did not inhibit the Bcr–Abl kinase (Supplementary Figure 2f) whereas IM, as expected, inhibited the Bcr–Abl kinase (Supplementary Figure 2g).

Tyr177 Y to F mutant behaves as wild-type Bcr–Abl with respect to Jak2 inhibition
We compared the disappearance of Y177F Bcr–Abl mutant with wild-type Bcr–Abl in 32D cells transduced with either wild-type or mutant BCR–ABL. The results indicate that Jak2 inhibition by WP1193 for 30 min caused similar levels of Bcr–Abl disappearance in both mutant and wild-type forms (Figure 5f).

Moreover, as expected, Tyr177 phosphorylation was not detected in the Y177F mutant (Figure 5f). These results support the concept that Tyr177 is just one of possibly several Jak2 phosphorylation sites (Tyr360 being another, see Supplementary Table 1), and that phosphorylation of these sites is necessary to maintain Bcr–Abl in a functional state.

Jak2 inhibition reduced tumorigenicity in mouse models
As WP1193 was a more potent Jak2 inhibitor than TG, we tested the effects of WP1193 on the growth of tumors induced by IM-resistant K562-R cells. K562-R cells16 contain activated Lyn kinase, which maintains the leukemic state of the K562-R cells despite the presence of IM. Therefore, we tested the inhibitory effects of WP1193 on the growth of solid tumors induced by K562-R in a nude mouse model. Solid tumors were allowed to form for 12 days following injection of K562-R cells, and treatment with WP1193 was initiated at 12 days through day 22 (Figure 6a). The volume of solid tumors was determined following injection of WP1193 at 30 mg/kg of mouse body weight every 48 h. Solid tumor growth was significantly reduced (P-value of 0.007) over this time period (Figures 6a and b). Injection of these mice with WP1193 by intraperitoneal (i.p.) had little effect on the weights of spleen and liver tissue (Supplementary Figure 3c, d).

We next compared the effects of WP1193 at 30 mg/kg (i.p.) on the oncogenic effects of IM-resistant T315I Bcr–Abl + 32D cells

**Figure 6** Jak2 inhibition by WP1193 inhibited solid tumor formation caused by IM-resistant K562 cells and strongly reduced tumors caused by Bcr–Abl T315I. (a) WP1193 inhibits solid tumor formation in nude mice injected subcutaneously (s.c) with 5 million K562-R cells. Five mice were injected with WP1193 (30 mg/kg) every 48 h beginning 12 days after injection of cells through 22 days. (b) Quantitation of tumor weight from five mice. (c and d) WP1193 reduced the weight of liver and spleens (right spleen in figure) in leukemic injected with Bcr–Abl T315I + BaF3 cells (~2.5 million cells). Mice were injected every 48 h by the i.p. route with WP1193 beginning 1 day after cell inoculation and killed 1 day after the seventh injection. (e) Reduction of spleen weight by WP1193 in mice injected with Bcr–Abl T315I BaF3 cells. (f) Model describing how Jak2 regulates signaling in CML cells.
Jak2 inhibition induced apoptosis in CD34+ cells from blast crisis IM-resistant CML patients and cells from chronic phase IM-resistant CML patients

We harvested CD34+ progenitor cells from a patient with blast crisis CML. Fractionated cells were treated with either IM or TG. After 48h, cells were analyzed by annexin/PI flow cytometry. TG at 5 μM concentration induced 80% apoptosis whereas IM at 5–10 μM concentration had little apoptotic-inducing activity (Supplementary Figure 4a); (the same patient cells used in Figure 3f). All of our previous studies had shown that Jak2 inhibition20,21 induced apoptosis in cells from blast crisis CML patients. We also examined cells from chronic phase CML patients who were resistant to IM. Treatment of the chronic phase cells with 2.5–10 μM TG induced high levels of apoptosis whereas IM treatment had little effect in these samples (Supplementary Figure 4b-d). Cells from one chronic phase patient were partially sensitive to IM but these cells were almost completely killed by 5 μM TG (Supplementary Figure 4b). Cells from an accelerated phase CML patient, although resistant to IM, were quite sensitive to Jak2 inhibition with either TG or WP1193 (Supplementary Figure 4e). We note that BaF3 cells expressing the T315I form of Bcr–Abl, although resistant to IM as expected, were quite sensitive to TG at 5 μM and above (Supplementary Figure 4f).

Discussion

Our previous findings indicate that Jak2 is a critical signaling molecule in CML20,21. The most pertinent of these findings is that AG490, an inhibitor of Jak2, induced apoptosis in IM-resistant Bcr–Abl+ cell lines including BaF3 cells expressing the gatekeeper IM-resistant mutant T315I11 of Bcr–Abl.20 To pursue the effects of Jak2 inhibition further, we performed experiments with Jak2-specific short hairpin RNAs in three different CML cell lines and in 32Dp210 cells expressing Jak2-specific small interfering RNAs. We made a surprising finding that Jak2 knockdown caused a disappearance of Bcr–Abl from the lysate. The mechanism of this Jak2 inhibition effect on Bcr–Abl is unknown, but is under study.

Another effect of Jak2 inhibition in Bcr–Abl+ cells is the reduction of phosphorylation of Tyr177 of Bcr–Abl. Recombinant Jak2 (JH1) readily phosphorylated Tyr177 in a Bcr peptide, and this phosphorylation was strongly inhibited by a selective Jak2 inhibitor TG101209 but not by IM. Tyr177 phosphorylation in this system was also inhibited by a new Jak2 inhibitor, WP1193. Although Jak2 inhibition leads to reduction of pTyr 177 Bcr–Abl in Bcr–Abl+ cell lines and in cells from blast crisis patients, this whole-cell effect is less clear as Jak2 inhibition also decreased levels of the Bcr–Abl protein. However, in vitro immune complex kinase assays showed that Jak2 inhibition did not reduce levels of Bcr–Abl in immune complexes but strongly inhibited phosphorylation of Tyr177. Thus, our hypothesis is that Jak2 inhibition decreases phosphorylation of Tyr177 within Bcr–Abl and possibly other Tyr residues within Bcr–Abl. In this regard, there are eight consensus Jak2 phosphorylation sites (YxxV/L/I) within the Bcr portion of Bcr–Abl (b3a2) of which Tyr177 is one such site (Supplementary Table 1). We propose that decreases in Tyr phosphorylation renders Bcr–Abl insoluble in the non-ionic detergent extraction buffer normally used to solubilize Bcr–Abl. This insolubility may be caused by the destruction of the network structure that maintains leukemic signaling in Bcr–Abl+ cells.22 We have shown that another Jak2 inhibitor, which also inhibits the Bcr–Abl kinase, also causes rapid disappearance of Bcr–Abl. Importantly, this dual kinase inhibitor (ON 044580) causes the disruption of the HSP90 structure that houses Bcr–Abl and Jak2 within and other signaling components of the Bcr–Abl/Jak2 pathway.22

Of interest, CD34+ cells from CML blast crisis patients and from CD34+ cord blood cells transduced with BCR–ABL also showed reduction of Bcr–Abl and pTyr177 Bcr–Abl levels upon Jak2 inhibition, indicating that the dominant effects of Jak2 inhibition also occur in early progenitor cells.

Downregulation of Ras activation and the initial stage of PI-3 kinase activation (reduction of pTyr Gab2), and the inhibition of Tyr phosphorylation of STAT5 following Jak2 inhibition suggests that the Jak2 tyrosine kinase is the critical tyrosine kinase that drives major signaling pathways in the leukemic cell. These results partially explain why Jak2 inhibition can overcome IM resistance (Supplementary Figure 4, 5).20,21 What remains is whether the tyrosine kinase function of Bcr–Abl is needed to activate the Jak2 kinase. Our studies indicate that Bcr–Abl alone does not lead to Jak2 activation, as addition of the IL-3 receptor is required.10

It is of interest that the Jak2 kinase seems to be involved in phosphorylation of Tyr 360 of Bcr–Abl. Our studies with Bcr serine kinase indicate that Tyr 360 is a critical regulator of Bcr serine/threonine kinase activity,32,33 and that phosphorylation of Tyr360 of Bcr downregulates its serine/threonine kinase activity.12,13 Bcr resembles kinases such as pyruvate kinase, which is known to require a free tyrosine residue to maintain its kinase activity.34 The fact that Y177F mutant of Bcr–Abl is similar to wild-type Bcr–Abl in response to Jak2 inhibition, suggests that Tyr177 is one of several possible Jak2 phosphorylation sites (including Tyr360) in Bcr–Abl necessary to maintain functional Bcr–Abl levels (Figure 5f).

Transmission of downstream signals induced by IL-3 may be enhanced by Jak1 interaction with Jak2.30 In this regard, we found that a putative pan Jak kinase inhibitor (WP1193) appeared to be more potent in reducing levels of Bcr–Abl and reducing levels of pTyr177 in Bcr–Abl+ cells compared with the selective Jak2 kinase inhibitor (TG101209).35 We note that WP1193, despite its considerably less in vitro potency for phosphorylating Tyr177 in kinase assays compared with TG (approximately 100-fold), still maintained similar or greater potency to reduce Jak2 effects in intact cells expressing Bcr–Abl. It is possible that WP1193’s greater in vivo potency may be because of its ability to inhibit Jak1 as well Jak2 inside Bcr–Abl+ cells, although other explanations are possible.

Because of the apparent increased overall potency of WP1193 compared with TG101209, we chose to test WP1193 for its effects on inhibition of Bcr–Abl’s oncogenic effects in mouse tumor models. The results indicate that WP1193 was a potent inhibitor of solid tumor formation induced by CML line K562-R and a strong inhibitor of oncogenic effects of IM-resistant T315I in nude mice. Moreover, WP1193 had no
observable toxic effects on mice injected with WP1193 only over a 2-week period.

Importantly, Jak2 inhibition was effective in apoptosis induction in IM-resistant blast crisis cells, IM-resistant accelerated phase cells, IM-resistant chronic phase and CD34+ progenitors from accelerated and blast crisis patients.

We conclude from these experiments that Jak2 is the main tyrosine kinase that controls signaling in Bcr–Abl+ cells, as Jak2 appears to use the Bcr–Abl protein as a platform to activate the Ras and PI-3 kinase pathways by phosphorylating Tyr 177 within the Bcr portion of Bcr–Abl. Jak2 inhibition also strongly reduced STAT5 tyrosine phosphorylation, possibly because Jak2 inhibition may reduce functional Bcr–Abl levels. A model summarizes our current and past findings on Jak2 functions in Bcr–Abl+ leukemia (Figure 6f). Importantly, leukemia cells expressing either IM-resistant forms of Bcr–Abl or having other forms of drug resistance (such as in blast crisis) undergo apoptosis on exposure to Jak2 inhibitors\textsuperscript{20,21} (Supplementary Figure 4), suggesting that Jak2 inhibitors have potential for treatment of drug-resistant CML.

Conflicts of interest

WP is a senior inventor on patent covering WP1193 and has financial interest in (Moleculin, LLC) that licensed his patent from MD Anderson Cancer Center. He is also a member of Moleculin’s SAB and has an SRA covering discovery on novel inhibitors.

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