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ORIGINAL ARTICLE

Bruton's tyrosine kinase is not essential for Bcr-Abl-mediated transformation of lymphoid or myeloid cells

M MacPartlin[1], AM Smith[2], BJ Druker[1,3], LA Honigberg[2] and MW Deininger[1]

[1]Center for Hematologic Malignancies, Oregon Health & Science University Cancer Institute, Portland, OR, USA; [2]Pharmacyclics, Sunnyvale, CA, USA and [3]Howard Hughes Medical Institute, Portland, OR, USA

Bcr-Abl, a constitutively active tyrosine kinase, is the cause of chronic myeloid leukemia (CML) and a subset of acute lymphoblastic leukemias (ALL). Bruton's tyrosine kinase (BTK), a member of the Tec family of tyrosine kinases with a crucial role in B-cell development, is consistently tyrosine phosphorylated in Bcr-Abl expressing murine pre B cells. BTK has been implicated in Bcr-Abl-mediated B-cell transformation and resistance to imatinib, implying that inhibiting BTK may be therapeutically beneficial. We decided to test whether BTK is a critical node in Bcr-Abl transformation and potential drug target in imatinib-resistant Bcr-Abl-positive cells. We depleted BTK in Ba/F3 and 32D cells expressing native and kinase domain (KD) mutant (E255K and T315I) Bcr-Abl, using shRNA. BTK levels were reduced to <10% of controls. However, no differences in viability and cell proliferation were observed and the response to imatinib was not altered. Consistent with this, proliferation and viability were unaffected by inhibition of BTK with reversible (PC-005) and irreversible (PCI-31523) small molecule inhibitors. Lastly, BTK inhibition did not affect the ability of Bcr-Abl to transform primary murine hematopoietic cells in colony forming and B-cell transformation assays. Collectively this data argues against a critical role for BTK in Bcr-Abl-mediated leukemogenesis.
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Introduction

Bcr-Abl is a chimeric tyrosine kinase whose constitutive activity is critical to the pathogenesis of chronic myeloid leukemia and Philadelphia chromosome (Ph)-positive B-cell acute lymphoblastic leukemia (B-ALL).[1–3] Infection of murine hematopoietic cells with BCR-ABL retrovirus induces myeloproliferative disease or B-ALL, depending on the experimental conditions.[4] Bcr-Abl is known to activate multiple signaling pathways, whose relevance to leukemogenesis is incompletely understood. Using phosphoproteomics we have profiled tyrosine phosphorylated peptides in Ba/F3 cells expressing native and kinase domain mutant Bcr-Abl.[5] Among others we observed consistent phosphorylation of Bruton's tyrosine kinase (BTK) on tyrosine 223 (Y223). BTK is critical to normal B-cell development.[6,7] Upon B-cell receptor stimulation, Src family tyrosine kinases are activated, which rapidly phosphorylate tyrosine 551 (Y551) in the activation loop of BTK, followed by the subsequent autophosphorylation of Y223 in the SH3 domain.[8,9] It is thought that phosphorylation of Y223 may play a role in modulating key protein–protein interactions required for B-cell development.[10–13] Consistent with our findings, another study found that ABL phosphorylates BTK at Y223, but not Y551.[14] Furthermore, a recent study postulated a critical role for BTK in Bcr-Abl-mediated transformation of B-cells[15] and a gene expression study found an association between high levels of BTK and imatinib resistance.[16] This raises the question whether BTK may be a critical signaling node and thus potential drug target in Bcr-Abl-positive leukemia, particularly in patients with imatinib resistance. To validate this assumption, we decided to verify the relevance of BTK for Bcr-Abl-mediated transformation.

Correspondence: Dr MW Deininger, Center for Hematologic Malignancies, Oregon Health and Science University Cancer Institute, 3181 SW Sam Jackson Park Road, Mailcode L592, Portland, OR 97239, USA.
E-mail: deininge@ohsu.edu

Materials and methods

Kinase inhibitors

PCI-31523 is an irreversible BTK inhibitor referred to as compound 4 in Pan *et al.*[17] PC-005 is a reversible inhibitor of BTK, Src-family kinases and Abl described in Supplementary Figure 4.

Cell culture

Ba/F3 cells expressing native Bcr-Abl and the kinase domain point mutants E255K and T315I have been described previously.[18] The lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine (Invitrogen) 1 U/ml penicillin (Invitrogen) and 1 μg/ml streptomycin (Invitrogen) and 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA). 293T and 293FT cells[19] were maintained in Dulbecco's modified Eagles medium (DMEM, Invitrogen) supplemented with 10% FBS 1 U/ml penicillin, 1 μg/ml streptomycin and 2 mM L-glutamine. All cells were grown at 37 °C in 5% CO_2. Viable cell numbers upon treatment with tyrosine kinase inhibitors were measured in triplicate using methlythiazoletetrazolium assays as described previously.[18]

Immunoblotting

Cells were collected by centrifugation and lysed in phosphate-buffered saline containing 1% NP40, 1 mM EDTA, 1 mM Na_3VO_4, 0.5 mM PMSF and 1 μg/ml aprotinin. Lysates were separated by SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) (50–200 μg protein/lane). Blots were probed with specific antibodies to test for expression of Bcr-Abl (8E9; Pharmingen, San Jose, CA, USA), Abl phosphorylated on tyrosine-245 (2861, Cell Signaling Technology, Danvers, MA, USA), BTK (05-415, Upstate/Millipore, Billerica, MA, USA) and

Determination of BTK active site occupancy using a fluorescent probe

Cells were lysed by freeze/thaw in D-PBS and total protein was quantitated by BCA Protein Assay (Pierce, Rockford, IL, USA). Lysates were diluted in D-PBS to a final concentration of 0.9 mg/ml and incubated with 2.5 μM of the BTK probe PCI-33380 in a 37 °C water bath for 1 hr. PCI-33380 is a Bodipy-FL-conjugate of the pure R enantiomer of PCI-31523 that binds irreversibly to BTK through a covalent interaction. When cell lysates are exposed to probe after cells are treated with the covalently binding inhibitor PCI-31523, probe binding is inhibited, demonstrating occupancy.[20] Labeling was halted by the addition of LDS sample buffer and sample reducing agent (Invitrogen). Samples were heated at 70 °C for 10 min prior to gel electrophoresis. Fifteen micrograms of total cell lysate was run on a NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen) in the dark and Bodipy-FL labeled BTK was visualized by scanning the gel with a Molecular Dynamics Typhoon scanner (Ex = 532 nm and Em = 555 nm). Gels were then blotted (Invitrogen iBlot) and total BTK was detected using anti-BTK (BD Biosciences, San Jose, CA, USA) and standard immunoblotting techniques.

Production of retrovirus and determination of viral titer

The MSCV-p210$^{Bcr-Abl}$-IRES-GFP (MIG) construct has been described previously.[21] For the production of native, E255K and T315I retrovirus for use in the bone marrow (BM) transduction experiments, 293T cells were transiently co-transfected using Fugene6 (Roche, Nutley, NJ, USA) with the MIG construct of choice and the Ecopac vector helper virus DNA (a kind gift of Dr Richard Van Etten, Tuft's University, Boston, MA, USA). Viral supernatants were harvested at 72 h post-transfection, filtered through a 0.45 μm filter, flash frozen in liquid nitrogen and stored for determination of viral titer or use. Relative viral titer was determined by infection of NIH3T3 cells. Briefly, 1×10^5 cells were plated in 30 mm tissue culture plates. Twenty-four hours later cells were exposed to 100, 200 and 400 μl of viral supernatant in 2 ml total volume of media with 4 μg/ml polybrene (Sigma). Forty-eight hours post infection NIH3T3 cells were harvested with trypsin (Invitrogen) and washed twice in D-PBS. GFP (green fluorescent protein) expression was analyzed by FACS (fluorescent-activated cell sorter, FACSAria, BD Biosciences) to determine relative viral titers, as described previously.[5] In all cases viral titers were in the linear range.

Production of lentivirus expressing shRNA and cell line viral transduction

The five pLKO.1. plasmids (TRCN0000023692 (BTK1), TRCN0000023690 (BTK2), TRCN0000023691 (BTK3), TRCN0000023689 (BTK4), TRCN0000023693 (BTK5) expressing short hairpin RNA (shRNA) against BTK were purchased from Open Biosystems, Huntsville, AL, USA. For the production of lentivirus expressing shRNA against BTK, 293FT cells were transiently co-transfected using Fugene6 (Roche) with the pLKO.1 constructs and the ViraPower Packaging Mix (Invitrogen). As a control, scrambled shRNA virus was included (MISSION Non-Target shRNA Control pLKO1 Vector, Sigma-Aldrich). Viral supernatants were harvested at 72 h post-transfection, filtered through a 0.45 μm filter, flash frozen in liquid nitrogen and stored at −80 °C. Ba/F3 and 32D cells were infected with lentivirus using 10^6 cells in 3 ml of media, seeded in a single well of a 6-well plate. One milliliter of viral supernatant, 30 μl of 1 M HEPES and 2 μl of polybrene (10 μg/μl) were added to each well. Cells were then co-sedimented at 30 °C for 90 min at 2500 rpm. The cells were then allowed to recover overnight and the following day 2.5 μg/ml puromycin was added for the selection of lentivirally transduced cells.

Colony forming assays

For myeloid progenitor colony formation assays, BM was harvested from 6–10 week old female Balb/c mice. The BM was subjected to 24 h of prestimulation in Iscove's modified Dulbecco medium (IMDM, Invitrogen) supplemented with 15% heat-inactivated FBS, 5% WEHI conditioned media, 6 ng/ml rm IL-3 (StemCell Technologies, Vancouver, BC, USA), 10 ng/ml rm IL-6 (StemCell Technologies) and 50 ng/ml rm SCF (StemCell Technologies).[22] After 24 h of prestimulation equal numbers of cells were transferred to 6-well plates and exposed to matched viral supernatants in the presence 2 μg/ml polybrene. Cells were then co-sedimented at 30 °C for 90 min at 2500 rpm. After 4 h the media was replaced with fresh prestimulation media. At 48 h a second round of co-sedimentation was performed. After an additional 4 h adsorption period the cells were washed twice in IMDM to remove cytokines, counted and plated in triplicate in MethoCult (M3234, without cytokines or M3534, with cytokines, StemCell Technologies). Inhibitors were re-dosed every 2 days. Colony formation was scored at day 10.

B-cell transformation assays

For the analysis of the transformation of primary BM B-lymphoid progenitors, BM from Balb/c mice was subjected to red cell lysis using ammonium chloride solution (StemCell Technologies).[23] The cells were subjected to a single round of transduction and co-sedimentation with matched retroviral stock for native Bcr-Abl and vector control in DMEM with 10% FBS and 2 μg/ml polybrene and incubated overnight in the presence of viral supernatant. Inocula of 10^5, 5×10^4 and 10^4 cells were plated in triplicate in Whitlock/Witte media (RPMI 1640 supplemented with 5% FBS, 200 μM L-glutamine, 50 μM 2-mercaptoethanol and penicillin/streptomycin) in 24-well plates with 10^6 untransduced BM cells/well as feeders.[5] Cells were cultured for 4 weeks and fed twice weekly by replacing 0.5 ml of supernatant with fresh media and fresh inhibitor. Cultures were scored as positive for transformation when the number of nonadherent cells exceeded 1×10^6 per milliliter of culture medium.

Results

Recently, we reported global phosphotyrosine profiles of Ba/F3 cells expressing native and kinase domain mutant Bcr-Abl in comparison with parental Ba/F3 cells.[5] A BTK-derived peptide corresponding to KVVALyDYMPMNANDLQLR (amino acid 218–236) was consistently phosphorylated on tyrosine 223 in Bcr-Abl expressing cells, but not in parental Ba/F3 cells, implicating BTK as a potential downstream mediator of Bcr-Abl. To test whether BTK is relevant to growth and viability of Bcr-Abl expressing cells we downregulated BTK expression using lentivirally delivered shRNA. Initial screening experiments were carried out to select the optimal shRNA sequence. Immunoblot analysis of Ba/F3 and 32D cells expressing empty vector, native and kinase domain mutant (E255K and T315I) Bcr-Abl revealed a >90% reduction of BTK expression with BTK1 and BTK5, as shown for Ba/F3 p210 cells (Figure 1a). Control experiments with scrambled shRNA confirmed the

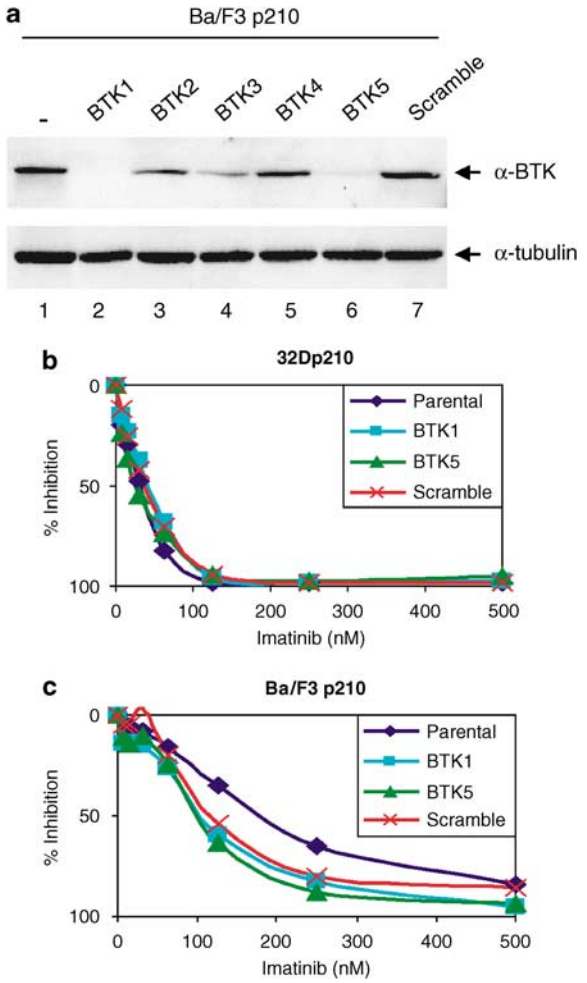

Figure 1 Lentivirally-mediated downregulation of BTK and its effect on Bcr-Abl dependent proliferation. (**a**) Lentiviruses BTK1-5 were produced expressing short hairpin RNA (shRNA) against BTK, as well as a control scramble non-specific shRNA lentivirus (MISSION Non-Target shRNA Control Vector). These viruses were used to infect a panel of Ba/F3 and 32D cell lines. Stably infected cells (Ba/F3 p210 shown) were lysed and examined by immunoblot for knockdown of BTK. (**b** and **c**) Cells infected with BTK1, BTK 5 and scramble shRNA were treated with imatinib (0, 7.8, 15.6, 31.2, 62.5, 125, 250 and 500 nM) and viable cells were measured by methlythiazoletetrazolium (MTT) assay after 72 h.

specificity of the effects for BTK (Figure 1a, lane 7). Cell lines with practically complete knockdown of BTK (shRNAs 1 and 5) were used in subsequent experiments.

In our first set of experiments we compared the growth rates of Ba/F3 and 32D cells expressing p210 Bcr-Abl, with and without simultaneous expression of BTK1, BTK5 or scrambled shRNA. No significant differences in growth rates were observed (Figures 1b and c), suggesting that BTK is not critical for cell growth of Bcr-Abl transformed lymphoid or myeloid cell lines. To determine whether BTK expression modulates sensitivity to ABL inhibitors, the same cell lines were exposed to graded concentrations of imatinib. Downregulation of BTK did not affect cell proliferation or viability in the presence of imatinib compared to cells infected with scrambled shRNA (Figures 1b and c). Identical experiments were performed on Ba/F3 cells expressing E255K and T315I mutants of Bcr-Abl, with similar results (Supplementary Figure 1). These data suggest that BTK is neither required for proliferation of Bcr-Abl-positive cells nor does it modulate the response to imatinib.

To confirm these observations we treated the identical panel of cell lines with two BTK inhibitors: PCI-31523, a selective irreversible inhibitor and PC-005, a reversible inhibitor of BTK and Abl (Pharmacyclics, Sunnyvale, CA, USA).[17,20] Due to the lack of suitable and good quality antibodies we were unable to directly test BTK Y223 phosphorylation inhibition. Instead we used an active site probe consisting of a BTK inhibitor, PCI-33380, a Bodipy-FL-conjugate of the pure R enantiomer of PCI-31523 that selectively labels BTK. This probe binds BTK at the same site as PCI-31523 and PC-005 and can be visualized by denaturing gel electrophoresis of lysates, followed by fluorescent scanning. To assay occupancy of the active site, cell lysates were prepared from Ba/F3 cells expressing either native or T315I Bcr-Abl and BV173 cells (human Ph-positive pre-B-cell line) treated with graded concentrations of PCI-31523. The lysates were then incubated with the conjugated probe and analyzed as described in the Materials and methods. There was complete occupancy of the active site in cells exposed to PCI-31523, the irreversible inhibitor (Figure 2a). Unsurprisingly, treatment with PC-005 did not affect probe binding, presumably due to displacement of the reversible inhibitor by the irreversible probe (Figure 2b).

Despite potently inhibiting BTK, PCI-31523, did not affect the proliferation of any of the cell lines, with IC_{50} values consistently above 5 μM (Figures 3a and c and Supplementary Figure 2a). However, upon treatment of the cells with the reversible inhibitor, PC-005, cells expressing Bcr-Abl showed a significant decrease in proliferation. Specifically, the IC_{50} was 827 nM in Ba/F3 cells expressing Bcr-Abl, 613 nM in 32D cells expressing Bcr-Abl and 718 nM in BV173 cells (Figures 3b and d and Supplementary Figure 2b). In contrast, no effect was seen in cells expressing the T315I mutant of Bcr-Abl (Figures 3b and d). The resistance of T315I expressing cells against PC-005 was reminiscent of combined ABL/SRC inhibitors such as dasatinib and suggested that the effects of PC-005 may be mediated by inhibition of Bcr-Abl rather than BTK.[24] Indeed a PC-005 dose-dependent decrease in Bcr-Abl tyrosine phosphorylation was observed by immunoblot analysis of BV173 cell lysates (Figure 4a) and Ba/F3 cell lysates expressing native Bcr-Abl (Figure 4b) but not T315I mutant Bcr-Abl (Figure 4c). This supports the conclusion that the effects of PC-005 are the result of inhibiting Bcr-Abl activity. *In vitro* inhibition of selected kinases by PC-005 was examined and IC_{50} values were determined by the Reaction Biology Hotspot assay, with staurosporine as a control (Supplementary Figure 4). This demonstrated an IC_{50} value of 3.49 nM for inhibition of Abl, further supporting our observations. Upon treatment of the cells with PCI-31523, the irreversible inhibitor, no such effects on Bcr-Abl phosphorylation were observed (Figures 4a and b).

Exogenous cytokines are required for normal murine BM to form myeloid colonies in semi-solid media, whereas Bcr-Abl expression induces cytokine-independent colony formation. Therefore, to assess whether BTK is required for transformation of primary myeloid cells, we examined colony formation of murine BM infected with a Bcr-Abl retrovirus in the presence of a concentration gradient of PCI-31523 and PC-005. In the presence of cytokines comparable numbers of colonies were observed from the empty vector and Bcr-Abl transduced cells (Figures 5a and b). As expected, only the Bcr-Abl transduced cells formed significant numbers of colonies in the absence of cytokines (Figures 5c and d). PCI-31523 did not affect colony formation. However, upon titration of PC-005 on the plates, a significant decrease in colony formation was seen

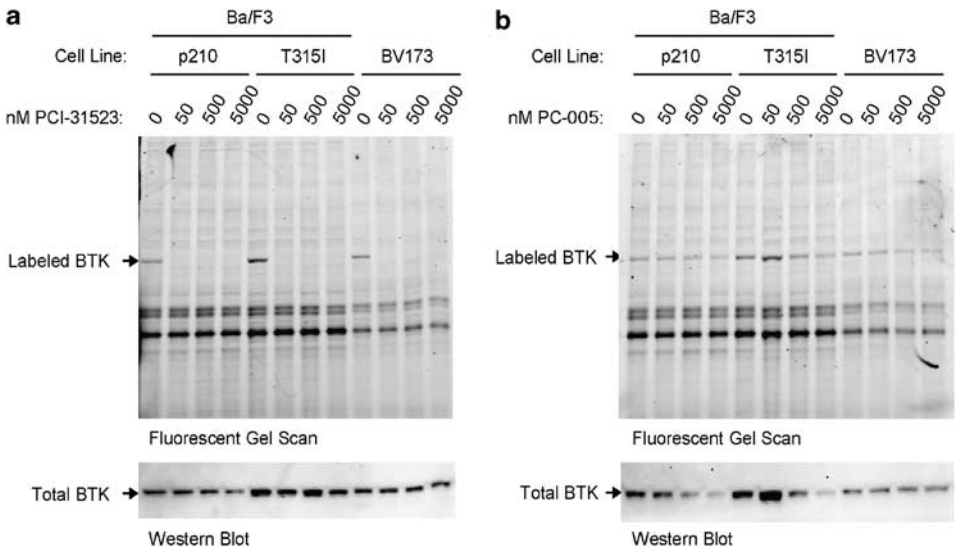

Figure 2 Probe labeling of cell lysates and demonstration of PCI-31523 binding to BTK. (**a**) Ba/F3 p210, 32D p210 and BV173 cells lines were exposed to PCI-31523 for 4 h and cell lysates were then subjected to probe labeling with PCI-33380, the fluorescent analog of PCI-31523 followed by denaturing gel electrophoresis. Fluorescent gel scan shows inhibition of probe labeling by pre-treatment with PCI-31523 and western blot confirms the presence of BTK in all lanes. (**b**) The experiment was repeated with the reversible inhibitor PC-005.

in the Bcr-Abl transduced cells grown in the absence of cytokines (Figure 5d). Thus, as with cell lines, the BTK/Abl inhibitor PC-005 is anti-proliferative, whereas the BTK selective inhibitor is not.

To assess the role of BTK in the transformation of primary B-lymphoid progenitor cells by Bcr-Abl, murine BM was retrovirally infected with native Bcr-Abl or the empty MIGR1 (Mig) retroviral vector as a control.[21] Cells were plated in triplicate in Whitlock–Witte cultures at various dilutions. The day of initial outgrowth and lowest dilution supporting outgrowth were used as a semi-quantitative measure of transformation potency (Figure 6).[5,25] Wells were scored as positive when the number of non-adherent cells reached 10^6 per well. As expected the empty vector failed to transform primary B cells. In contrast, expression of native Bcr-Abl induced rapid outgrowth of B cells (Figure 6a and b). Outgrowth was unaffected by 50 nM of PCI-31523 (Figure 6c), an inhibitor concentration that resulted in nearly complete occupancy of BTK (Figure 4c). Higher concentrations of PCI-31523 (500 nM) led to a reduction of B-cell outgrowth and a complete block was observed at 5 μM (Supplemental Figures 3c and d). With PC-005 we found a marginal reduction of outgrowth at 50 nM (Figure 6d), with complete inhibition at concentrations of 500 nM and above, concentrations where significant inhibition of Bcr-Abl is seen (Supplemental Figure 3a and b).

Discussion

Imatinib is a highly effective therapy for the treatment of early chronic myeloid leukemia.[26–28] However, drug resistance due to kinase domain mutations or other mechanisms is frequent in patients with accelerated or blastic phase or with Ph+ ALL.[29–32] Second-line ABL inhibitors like dasatinib or nilotinib have promising activity in patients with imatinib resistance. Unfortunately, responses in patients with blast crisis or Ph+ ALL are usually only transient. In many cases failure to second-line ABL inhibitors is caused by the T315I mutant, which is resistant to all Bcr-Abl inhibitors that are currently available for clinical use. Targeting essential pathways downstream of Bcr-Abl is a logical approach to address this clinical challenge.

BTK, a Tec family kinase with a central role in B-cell development, has been implicated as a critical mediator of B-cell transformation by Bcr-Abl. Thus, Feldhahn *et al*.[15] described Bcr-Abl as mimicking a constitutively active pre-B-cell receptor in pre-B lymphoblastic leukemia cells. The authors concluded that BTK is contributing to Bcr-Abl driven survival signaling in these cells, independent of pre-B-cell receptor dependent signals. Furthermore, a microarray study of patients with Ph+ ALL found a correlation between high levels of BTK mRNA and imatinib resistance.[16] Lastly, using phosphoproteomics we have detected phosphorylation of tyrosine 223 of BTK in Ba/F3 cells expressing Bcr-Abl, consistent with active BTK.[5] These data suggest that BTK may be a critical node and potential drug target in imatinib-resistant Bcr-Abl-positive cells.[15,16,33]

In a first series of experiments, we used shRNA to down-regulate BTK in Ba/F3 and 32D cells expressing native Bcr-Abl and the kinase domain mutants E255K and T315I. However, in contrast to the report by Feldhahn *et al*.[15] we saw no effect on the proliferation of these cells in culture (Figure 1). Moreover, sensitivity to imatinib was unaffected by the lack of BTK. The reasons for the discrepancy with the published data are not immediately obvious. To extend our observations, we treated the cell lines with two small molecule inhibitors of BTK. PCI-31523, a selective irreversible inhibitor that did not inhibit Abl phosphorylation in cells, had no effect on the proliferation of Bcr-Abl-positive cells (Figure 3). Using a fluorescent BTK probe, we confirmed that cellular BTK was fully occupied by PCI-31523 in these experiments (Figure 2). In contrast, PC-005, a less selective and reversible inhibitor, led to a decrease in proliferation of Bcr-Abl positive cells (Figure 3). However, this anti-proliferative effect was correlated with inhibition of Bcr-Abl rather than BTK, as evidenced by the complete resistance of cells expressing the T315I mutant (Figure 4c). We also tested LFM-A13, an established BTK inhibitor with a reported IC$_{50}$ of

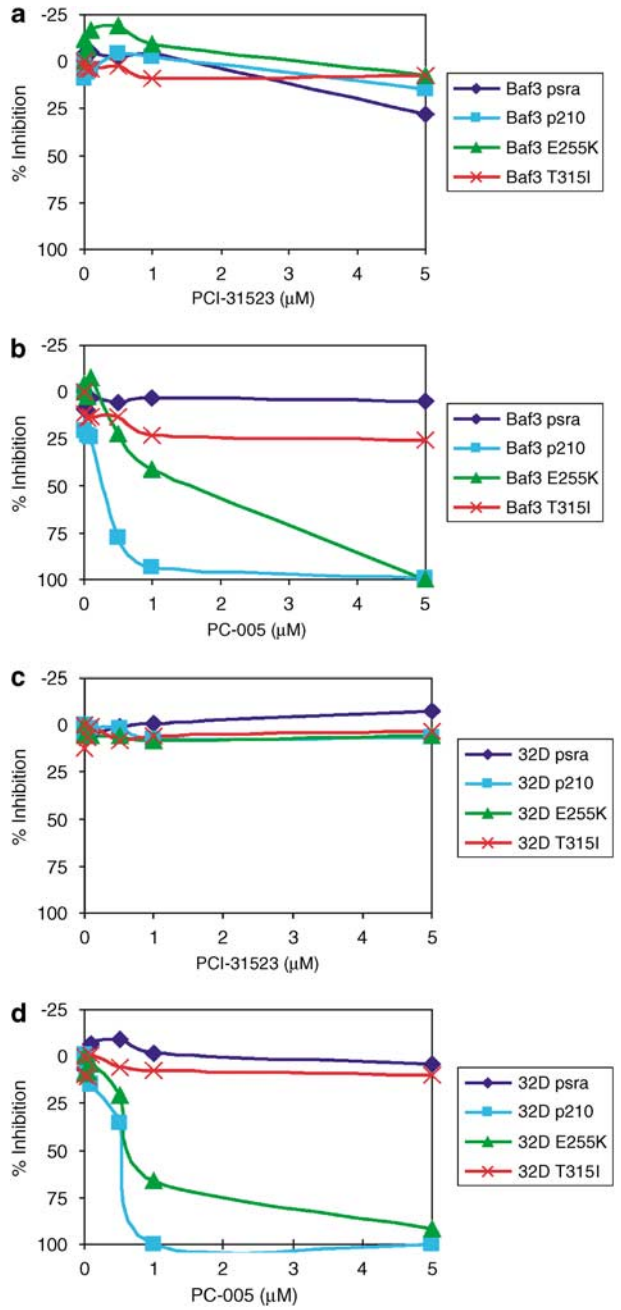

Figure 3 Cell proliferation in the presence of BTK inhibitors PCI-31523 and PC-005. (**a** and **b**) Ba/F3 cells expressing empty vector, native Bcr-Abl (p210) and the kinase domain (KD) mutants E255K and T315I were exposed to a range of inhibitor concentrations (0, 10, 50, 100, 500, 1000 and 5000 nM) and viable cells were measured by methlythiazoletetrazolium (MTT) assay after 72 h. (**c** and **d**) Identical assays were performed with 32D cells expressing native or kinase domain mutant Bcr-Abl.

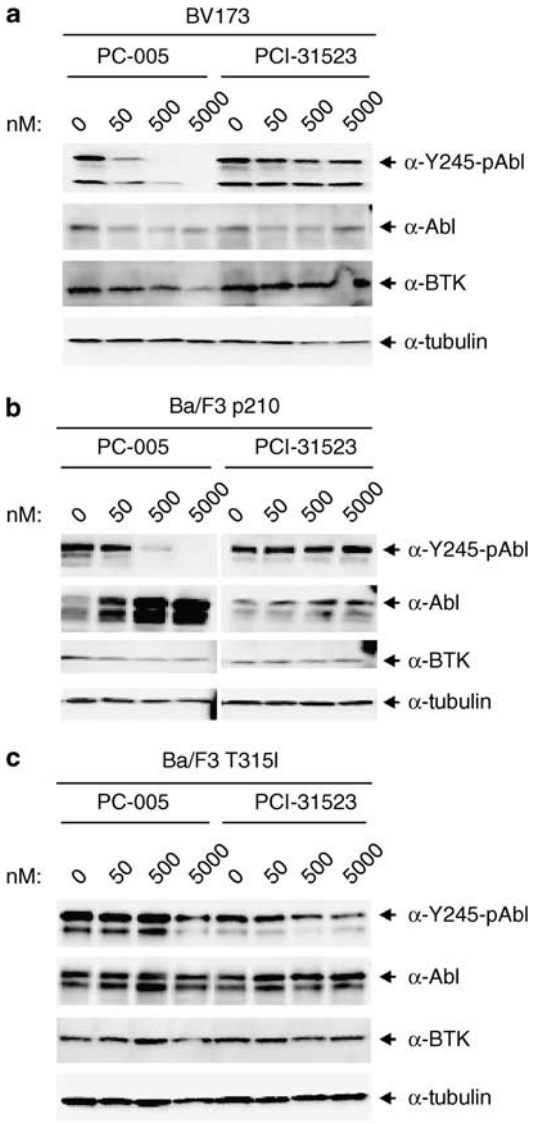

Figure 4 Immunoblot analysis of PC-005 and PCI-31523 inhibitor activity. (**a**, **b** and **c**) BV173, Ba/F3 p210 and Ba/F3 T315I cells, respectively, were treated with inhibitor (4 h at the concentration shown) and cell lysates were analyzed by immunoblot for Abl tyrosine 245 phosphorylation of Bcr-Abl.

17.2 μM.[34] However, we did not see any anti-proliferative effect at concentrations below 100 μM (data not shown). Any effects at higher concentrations are more consistent with non-specific toxicity than an effect related to BTK inhibition. These results appear to be, along with our siRNA (small interfering RNA) data, discordant with the data published by Feldhahn *et al*.[15] However, no drug concentrations are given in that paper, making a direct comparison impossible. To address the question

of a critical role of BTK for Bcr-Abl-mediated transformation of primary cells, we assayed Bcr-Abl-induced myeloid colony formation and B-cell transformation. Consistent with our cell line data, myeloid colony formation was unaffected by inhibition of only BTK (with the irreversible inhibitor PCI-31523), but reduced in the presence of PC-005, which inhibits ABL and BTK (Figure 5). Similarly, B-cell transformation was unaffected at concentrations of PCI-31523 (50 nM) that produced a complete block of BTK as shown by a specific fluorescence assay (Figures 2a and 6c). In summary, our data provide conclusive evidence that transformation of myeloid as well as B-lymphoid cells by Bcr-Abl does not depend on BTK, suggesting that the therapeutic effects of a pure BTK inhibitor in the setting of imatinib resistance would be minimal. However, it remains possible that BTK would assume a critical role upon simultaneous inhibition of another signaling pathway

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