ARTICLES

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Requirement of Src kinases Lyn, Hck and Fgr for *BCR-ABL1*-induced B-lymphoblastic leukemia but not chronic myeloid leukemia

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The Abl kinase inhibitor imatinib mesylate is the preferred treatment for Philadelphia chromosome–positive (Ph$^+$) chronic myeloid leukemia (CML) in chronic phase but is much less effective in CML blast crisis or Ph$^+$ B-cell acute lymphoblastic leukemia (B-ALL). Here, we show that Bcr-Abl activated the Src kinases Lyn, Hck and Fgr in B-lymphoid cells. *BCR-ABL1* retrovirus-transduced marrow from mice lacking all three Src kinases efficiently induced CML but not B-ALL in recipients. The kinase inhibitor CGP76030 impaired the proliferation of B-lymphoid cells expressing Bcr-Abl *in vitro* and prolonged survival of mice with B-ALL but not CML. The combination of CGP76030 and imatinib was superior to imatinib alone in this regard. The biochemical target of CGP76030 in leukemia cells was Src kinases, not Bcr-Abl. These results implicate Src family kinases as therapeutic targets in Ph$^+$ B-ALL and suggest that simultaneous inhibition of Src and Bcr-Abl kinases may benefit individuals with Ph$^+$ acute leukemia.

Human Ph$^+$ leukemias are caused by the *BCR-ABL1* oncogene and include CML and B-ALL. CML has a triphasic clinical course, with chronic and accelerated phases followed by a terminal blast crisis phase resembling acute leukemia, in which myeloid or lymphoid blasts fail to differentiate. Together, lymphoid blast crisis of CML and Ph$^+$ B-ALL account for 20% of adult cases and 5% of pediatric cases of acute lymphoblastic leukemia. One-half of adult and 20% of pediatric B-ALLs express the p210 isoform of Bcr-Abl, and the remainder express the p190 isoform[1,2]. Whereas the Abl tyrosine kinase inhibitor imatinib mesylate induces a complete hematologic response in nearly all individuals with chronic phase CML[3], imatinib is much less effective in treating CML blast crisis and Ph$^+$ B-ALL[2] owing to acquired resistance[4–7]. Drugs that target essential signaling molecules downstream of Bcr-Abl may help overcome or prevent imatinib resistance.

Bcr-Abl activates multiple signaling pathways, including Ras, MAPK, STAT, JNK/SAPK, PI-3 kinase, NF-kB and c-Myc[8]. Furthermore, in myeloid cell lines Bcr-Abl activates the Src family kinases Lyn and Hck[9]. Multiple domains of Bcr-Abl interact with and activate Src kinases independently of Bcr-Abl kinase activity[10–12], and studies with dominant-negative mutants and Src inhibitors suggest that Src kinases may contribute to the proliferation and survival of myeloid cell lines expressing Bcr-Abl *in vitro*[13–15]. Results in cell lines expressing Bcr-Abl often do not correlate with leukemogenesis *in vivo*[16], emphasizing the need to evaluate signaling pathways in an animal model of leukemia. We developed accurate and quantitative mouse models of chronic phase CML[17] and Ph$^+$ B-ALL[17,18]. Here, we use these models to show that Src kinases are required for the induction of B-ALL by Bcr-Abl but are dispensable for induction of CML-like myeloproliferative disease.

RESULTS

Bcr-Abl activates Lyn, Hck and Fgr in pre-B cells

Bcr-Abl interacts with and activates the Src kinases Lyn and Hck in myeloid cells[9]. We investigated whether Bcr-Abl also activates Src kinases in pre-B lymphoid cells. We found Bcr-Abl protein and abundant tyrosine-phosphorylated cell proteins in BL-2 cells (isolated from a mouse with Bcr-Abl-induced B-ALL) and in ENU48515 cells (an N-ethyl-N-nitrosourea (ENU)-induced pre-B leukemia mouse line) transduced with *BCR-ABL1* retrovirus, but not in vector-transduced cells (**Fig. 1a**). Of the eight Src family kinases expressed in hematopoetic cells (Blk, Fgr, Fyn, Hck, Lck, Lyn, c-Src and Yes), parental ENU48515 cells had moderate levels of constitutively activate Fyn and Lyn, and Bcr-Abl expression increased the activation of Lyn and also activated five other Src kinases (Blk, Fgr, Hck, Lck and c-Src), as indicated by increased tyrosine phosphorylation (**Fig. 1b**). Phosphorylation of Fyn was not increased with Bcr-Abl expression in ENU48515 cells, and Yes was not expressed. In primary leukemic cells isolated from peripheral blood of mice with

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Bcr-Abl-induced B-ALL, only Lyn, Hck and Fgr were prominently activated when compared with peripheral blood leukocytes from control mice (**Fig. 1c**), whereas the other Src kinases were activated weakly (Blk), not activated (Lck, c-Src and Fyn; data not shown) or not expressed (Yes; data not shown). *In vivo* activation of Lyn and Hck was confirmed by blotting with an antibody against the phosphorylated Tyr416 homolog (**Fig. 1c**). Hck was expressed at very low levels in normal peripheral blood lymphocytes but was induced by Bcr-Abl in both ENU48515 (**Fig. 1b**) and primary B-lymphoid leukemia cells (**Fig. 1c**). These results identify Lyn, Hck and Fgr as the principal Src kinases activated by Bcr-Abl in B-lymphoid cells.

Src kinases are required for B-ALL but not CML

We next investigated whether Lyn, Hck and Fgr are involved in leukemogenesis by Bcr-Abl by using triple knockout mice deficient in all three Src kinases in separate models of CML and Ph$^+$ B-ALL. Most recipients of *BCR-ABL1*-transduced bone marrow from wild-type C57Bl/6 donor mice treated with 5-fluorouracil (5-FU) develop myeloproliferative disease closely resembling the chronic phase of human CML. CML-like leukemia arises from multipotential stem or progenitor cells[17] between 5 and 7 weeks after transplantation[19]. A few recipients succumb to a mixture of CML-like disease and B-ALL after 8–12 weeks (**Fig. 2a**). Recipients of *BCR-ABL1*-transduced marrow from *Lyn*$^{-/-}$ *Hck*$^{-/-}$ *Fgr*$^{-/-}$ donor mice treated with 5-FU developed CML-like disease exclusively (**Fig. 2a**), with mean peripheral blood leukocyte counts twice those of recipients of wild-type marrow (data not shown). This result indicates that these Src kinases are not required for efficient induction of CML by Bcr-Abl. There was a slight (but statistically insignificant) delay in the time to morbidity or death of mice in the *Lyn*$^{-/-}$ *Hck*$^{-/-}$ *Fgr*$^{-/-}$ cohort, which was probably due to the complete absence of B-ALL in these recipients (**Fig. 2a**). Although the difference in incidence of B-ALL between the two groups was also not significant, it suggested that Src kinases might have a role in B-lymphoid leukemogenesis by Bcr-Abl. Although we observed a similar defect in development of histocytic sarcoma in the recipients of *BCR-ABL1*-transduced Src-deficient marrow, we did not pursue this finding because this disease has no Ph$^+$ counterpart in humans.

We confirmed a requirement for Src kinases in Ph$^+$ B-lymphoblastic leukemia in a distinct mouse model of B-ALL (**Fig. 2b**), where all recipients of *BCR-ABL1*-transduced bone marrow from wild-type donors not treated with 5-FU develop acute pre-B cell leukemia, originating from committed lymphoid progenitors, within 4–8 weeks after transplantation[17,18]. The malignant pre-B cells express B220 and CD19 and phenotypically resemble *de novo* Ph$^+$ B-ALL and lymphoid blast crisis of CML. In one experiment, recipients of *BCR-ABL1*-transduced marrow from *Lyn*$^{-/-}$ *Hck*$^{-/-}$ *Fgr*$^{-/-}$ donors not treated with 5-FU were completely resistant to induction of B-ALL. In a second experiment that used higher titer virus stocks, most recipients (60%) remained healthy without clinical or pathological evidence of leukemia (**Fig. 2b**).

Although triple Src knockout mice have developmental and proliferation defects in mature B-lymphoid cells and develop autoimmune disease with age[20], the mice have normal early B-lymphoid development and normal bone marrow donor and recipient function. Excluding the possibility that *Lyn*$^{-/-}$ *Hck*$^{-/-}$ *Fgr*$^{-/-}$ bone marrow is deficient in B-lymphoid progenitors or less susceptible to retroviral transduction, we found no significant difference between *Lyn*$^{-/-}$ *Hck*$^{-/-}$ *Fgr*$^{-/-}$ and wild-type bone marrow with respect to the percentage of cells expressing CD19, B220, Ig heavy chain, AA4.1, Gr-1, Mac-1, c-Kit or Sca-1 (data not shown), or with respect to the efficiency of retroviral transduction of B-lymphoid progenitors (**Fig. 2c**). These results indicate that one or more of these Src kinases are required for efficient induction of B-ALL by Bcr-Abl.

Several Src kinases contribute to B-cell transformation

To investigate the individual Src kinases involved in Bcr-Abl-induced B-lymphoid leukemogenesis, we used an *in vitro* assay for transformation of bone marrow B-lymphoid progenitors by Bcr-Abl[21], quantified by limiting dilution[22]. In this assay, p210 *BCR-ABL1* transformed B-lymphoid progenitors from *Lyn*$^{-/-}$ *Hck*$^{-/-}$ *Fgr*$^{-/-}$ bone marrow with much

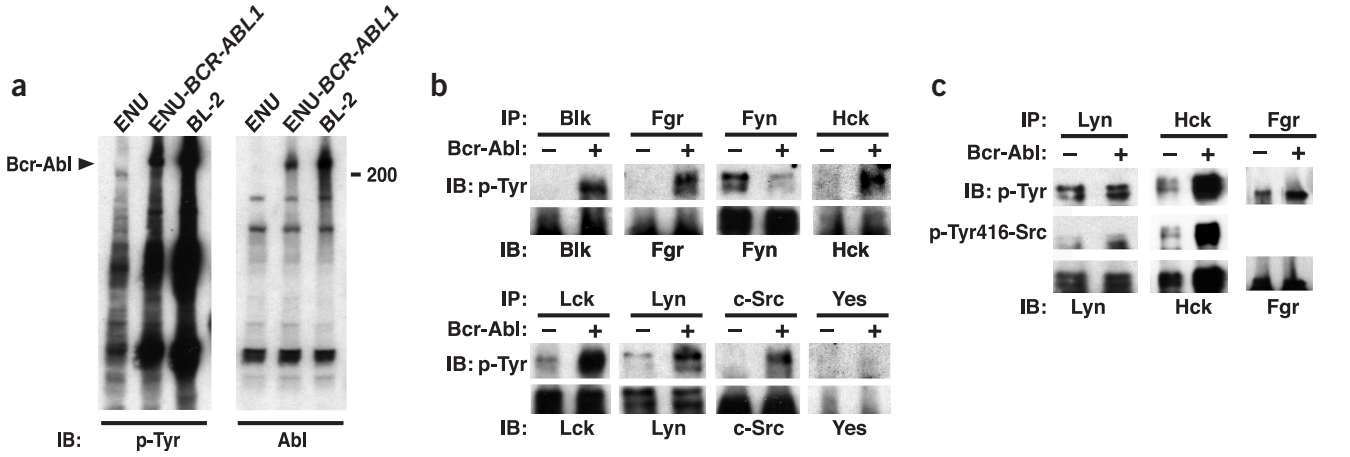

Figure 1 Activation of Src kinases by Bcr-Abl. (**a**) Documentation of tyrosine phosphorylation (left panel) and Bcr-Abl expression (right panel) in whole-cell extracts from ENU48515 cells transduced with retrovirus without an oncogene (ENU), ENU48515 cells transduced with *BCR-ABL1* retrovirus (ENU-*BCR-ABL1*) and BL-2 cells. IB, immunoblot. (**b**) Extracts from ENU (–) or ENU-*BCR-ABL1* cells (+) were immunoprecipitated (IP) with antibody against the indicated Src kinase and then immunoblotted (IB) with the same antibody (bottom panel of each set) or with antibody to phosphorylated tyrosine (p-Tyr; top panel of each set). (**c**) Extracts from primary B-lymphoid leukemic blasts expressing Bcr-Abl (+) or peripheral blood leukocytes from a normal mouse (–) were immunoprecipitated (IP) with antibodies against Lyn, Hck or Fgr and immunoblotted (IB) with the same antibody (bottom panel) or with antibody to phosphorylated tyrosine (p-Tyr; top panel). For Lyn and Hck immunoprecipitates, phosphorylation of the Tyr416 homolog was confirmed by blotting with antibody to Src specific to phosphorylated Tyr416 (p-Tyr416-Src; middle panels); this antibody does not react with Fgr.

lower efficiency than it transformed those from wild-type marrow (**Fig. 2d**), as shown by the growth of cultures initiated only at the highest density of transduced cells. To begin to identify the individual Src kinases involved, we backcrossed *Lyn*$^{-/-}$ *Hck*$^{-/-}$ *Fgr*$^{-/-}$ mice to triply heterozygous (*Lyn*$^{+/-}$ *Hck*$^{+/-}$ *Fgr*$^{+/-}$) mice and determined the susceptibility of bone marrow from littermates of different genotypes to *in vitro* transformation by *BCR-ABL1*. Whereas bone marrow from triply heterozygous mice was fully sensitive to transformation, marrow from *Lyn*$^{-/-}$ *Hck*$^{+/-}$ *Fgr*$^{-/-}$ and *Lyn*$^{+/-}$ *Hck*$^{-/-}$ *Fgr*$^{-/-}$ mice was nearly as defective as marrow lacking all three kinases. In contrast, marrow lacking only Fgr or Hck was completely sensitive to *BCR-ABL1* transformation (**Fig. 2d**). These results were complemented by leukemogenesis studies in which we transplanted *BCR-ABL1*-transduced bone marrow from the various mutant donors, including single *Lyn* knockout mice, into lethally irradiated recipients (**Fig. 2e**). *BCR-ABL1*-transduced bone marrow lacking only Lyn, Fgr or Hck induced B-ALL in recipients as efficiently as wild-type marrow, whereas marrow from *Lyn*$^{-/-}$ *Hck*$^{+/-}$ *Fgr*$^{-/-}$ and *Lyn*$^{+/-}$ *Hck*$^{-/-}$ *Fgr*$^{-/-}$ mice was profoundly defective for induction of B-ALL (**Fig. 2e**). Together, these results indicate that Lyn, Hck and Fgr have overlapping or partially redundant functions downstream of Bcr-Abl in B-lymphoid signaling, and that at least two of these Src kinases are required for efficient induction of B-lymphoid leukemia by Bcr-Abl.

CGP selectively inhibits Lyn, Hck and Fgr over Bcr-Abl

We tested whether the Src kinase inhibitor CGP76030 (CGP) could inhibit Lyn, Hck and Fgr in lymphoid cells expressing Bcr-Abl. CGP76030 reduced tyrosine phosphorylation of Lyn and Hck in BL-2 cells, and of Fgr (expressed at very low levels in BL-2 cells) in ENU *BCR-ABL1* cells, in a dose-dependent manner (**Fig. 3a**). The concentration required for 50% inhibition of phosphorylation (IC_{50}) was 0.73 μM, 6.38 μM and 0.36 μM, respectively. Previous studies in myeloid cell lines showed that CGP76030 may inhibit Bcr-Abl directly

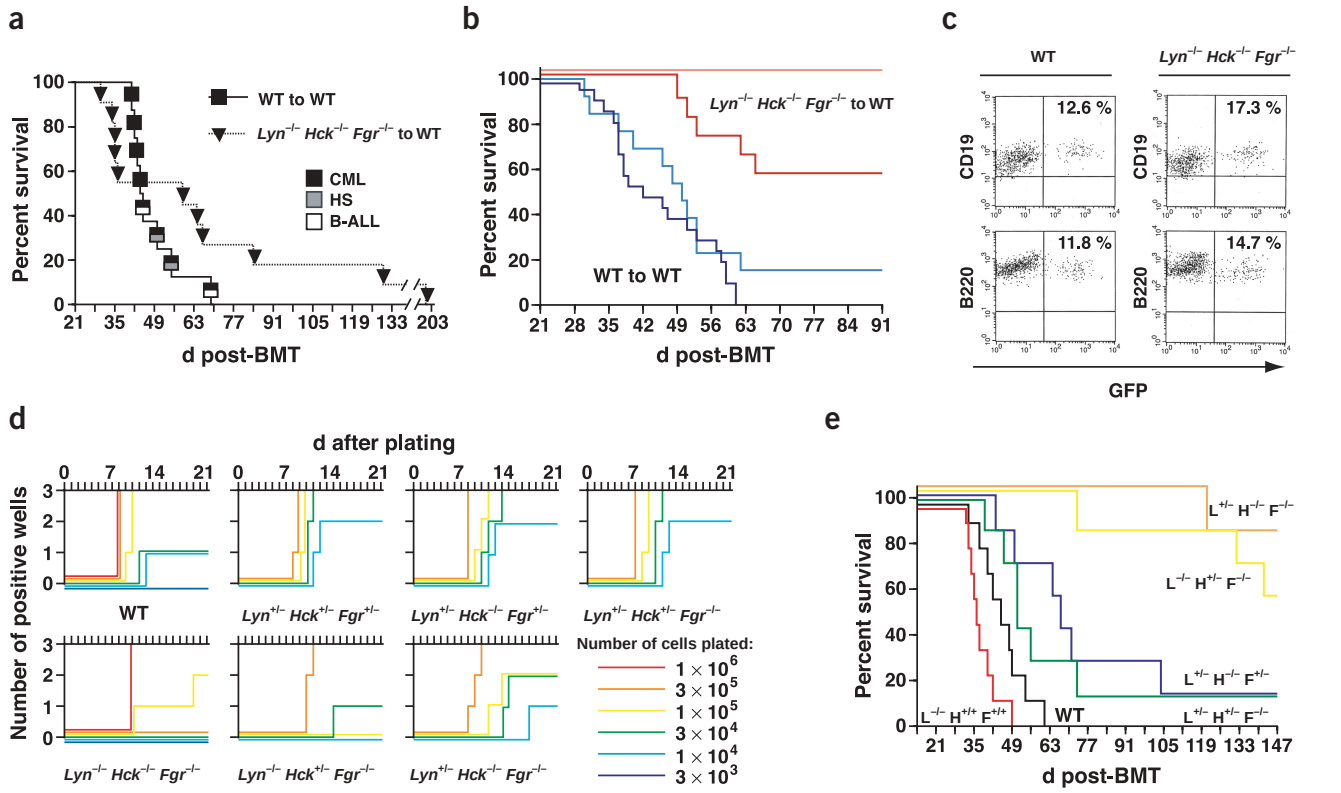

Figure 2 Src kinases are required for induction of B-ALL but not CML by Bcr-Abl. (**a**) Kaplan-Meier-style survival curve for recipients of *BCR-ABL1*-transduced bone marrow from wild-type (solid line with squares, $n = 8$) or *Lyn*$^{-/-}$ *Hck*$^{-/-}$ *Fgr*$^{-/-}$ C57Bl/6 donors (dotted line with triangles, $n = 11$) treated with 5-FU. Disease phenotype in each mouse is indicated by shaded symbols: CML-like disease, black; histocytic sarcoma (HS), gray; B-ALL, white. The differences in survival ($P = 0.319$, Mantel-Cox test) and incidence of B-ALL (2 of 8 wild-type recipients versus 0 of 11 Src mutant recipients, $P = 0.164$, Fischer's exact test) between the two groups were not significant. (**b**) Kaplan-Meier-style survival curves for recipients of *BCR-ABL1*-transduced bone marrow from wild-type (dark blue line, $n = 21$; light blue line, $n = 13$) or *Lyn*$^{-/-}$ *Hck*$^{-/-}$ *Fgr*$^{-/-}$ donors (dark red line, $n = 12$; light red line, $n = 10$) not treated with 5-FU. All diseased mice developed B-ALL exclusively. Two independent experiments carried out with viral stocks with matched low (light colors) and high (dark colors) titers are represented. The difference in survival between each wild-type and Src knockout arm is highly significant ($P < 0.0001$, Mantel-Cox test). (**c**) FACS analysis showed no significant difference in efficiency of retroviral transduction of CD19$^+$ (top panels) or B220$^+$ (bottom panels) B-lymphoid progenitors from bone marrow of wild-type and *Lyn*$^{-/-}$ *Hck*$^{-/-}$ *Fgr*$^{-/-}$ mice. Percentages of double-positive cells in each panel are indicated. (**d**) Bone marrow from the indicated donors (not treated with 5-FU) was transduced with *BCR-ABL1* retrovirus and plated at different cell numbers per well (indicated by colored lines) in triplicate wells. Nontransduced cells were added to 10^6 total cells to provide stromal support. Wells were scored as positive when the viable nonadherent cell number reached 10^6 per well. (**e**) Kaplan-Meier-style survival curves for recipients of *BCR-ABL1*-transduced bone marrow from donors with the indicated genotypes (L, Lyn; H, Hck; F, Fgr; WT, wild-type) not treated with 5-FU from **d**. All diseased mice developed B-ALL exclusively (wild-type and *Lyn*$^{-/-}$ *Hck*$^{+/+}$ *Fgr*$^{+/+}$cohorts, $n = 9$; all other cohorts, $n = 7$). The difference in survival between recipients of *BCR-ABL1*-transduced bone marrow from wild-type donors and donors lacking single Src kinases (*Lyn*$^{+/-}$ *Hck*$^{-/-}$ *Fgr*$^{+/-}$, *Lyn*$^{+/-}$ *Hck*$^{+/-}$ *Fgr*$^{-/-}$ and *Lyn*$^{-/-}$ *Hck*$^{+/+}$ *Fgr*$^{+/+}$) was not significant ($P \geq 0.01$), whereas the survival of recipients of marrow from donors lacking two Src kinases (*Lyn*$^{+/-}$ *Hck*$^{-/-}$ *Fgr*$^{-/-}$ and *Lyn*$^{-/-}$ *Hck*$^{+/-}$ *Fgr*$^{-/-}$) was significantly prolonged ($P < 0.001$, Mantel-Cox test). d post-BMT, d after bone marrow transplantation.

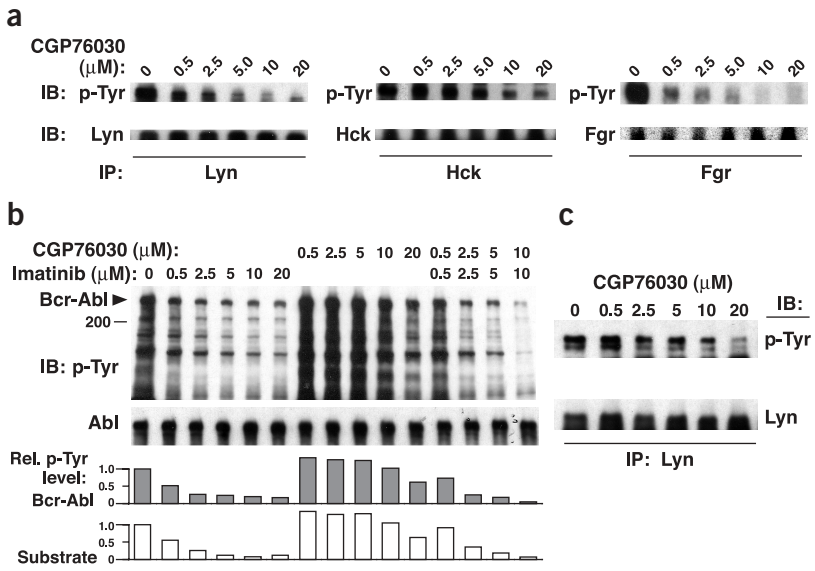

Figure 3 The Src kinase inhibitor CGP76030 inhibits activation of Lyn, Hck and Fgr in pre-B cells expressing Bcr-Abl. (**a**) BL-2 (left and middle panels) or ENU-*BCR-ABL1* (right panels) cells were treated with CGP76030 at the indicated concentrations. Extracts were immunoprecipitated (IP) with antibodies against Lyn, Hck or Fgr and immunoblotted (IB) with the same antibody (lower panels) or with antibody to phosphorylated tyrosine (p-Tyr; upper panels). (**b**) CGP76030 is a weak inhibitor of Bcr-Abl. BL-2 cells were treated with CGP76030 or imatinib alone or in combination at the indicated concentrations. Whole-cell extracts were analyzed by immunoblotting (IB) with antibodies to phosphorylated tyrosine (p-Tyr; top panel) or to Abl (bottom panel). The relative level of tyrosine phosphorylation of Bcr-Abl and total cellular substrates was determined by densitometry (expressed as arbitrary units relative to no drug) and indicated by the bar graphs below. (**c**) Inhibition of Lyn activation by CGP76030 in the absence of Bcr-Abl. Activation of Lyn was assessed in ENU-vector-transduced cells treated with the indicated concentrations of CBG76030, as described in **a**.

at higher concentrations[15]. Here, we found CGP76030 to be a weak inhibitor of Bcr-Abl in B-lymphoid cells, as we observed a significant inhibitory effect on Bcr-Abl auto- and trans-phosphorylation in BL-2 cells only at 20 μM CGP76030, whereas imatinib had a strong inhibitory effect at 0.5 μM (**Fig. 3b**). These results suggest that activation of Src kinases by Bcr-Abl (which does not require Bcr-Abl kinase activity[10,15]) induces Src kinase autophosphorylation, and that CGP76030 selectively inhibits Src kinases over Bcr-Abl when used at concentrations less than 10 μM. This was confirmed by using the parental ENU48515 cell line, which does not express Bcr-Abl but shows a moderate level of constitutive Lyn activation. CGP76030 inhibited Lyn phosphorylation in these cells (**Fig. 3c**), indicative of direct inhibition of Src kinases by this compound.

CGP inhibits B-ALL cells expressing Bcr-Abl *in vitro*

We also determined whether CGP76030 (CGP) could inhibit growth and survival of B-lymphoid cells expressing Bcr-Abl. CGP76030 decreased the viability (**Fig. 4a**) and induced apoptosis (**Fig. 4b**) of BL-2 cells in a dose-dependent manner after 24 and 48 h of treatment. We observed prominent effects on cell proliferation and survival at a CGP76030 concentration of 2 μM, well below the level associated with direct inhibition of Bcr-Abl[15]. In addition to having effects on cell survival, both CGP76030 and imatinib induced apoposis of BL-2 cells, in which Bcr-Abl expression was the initial transforming event (**Fig. 4c**). In contrast, ENU48515 cells do not depend on either Bcr-Abl or Src kinases for survival or proliferation, and hence neither drug induced apoposis of these cells (**Fig. 4c**).

CGP improves survival of mice with B-ALL but not CML

As Src kinases are required for induction of B-lymphoid leukemia by Bcr-Abl (**Fig. 2**), we hypothesized that they might be targets for therapy of Ph^+ B-ALL and investigated whether CGP76030 (CGP) could inhibit the development or clinical course of Bcr-Abl-induced leukemias in mice. Mice with p210 *BCR-ABL1*-induced B-ALL were

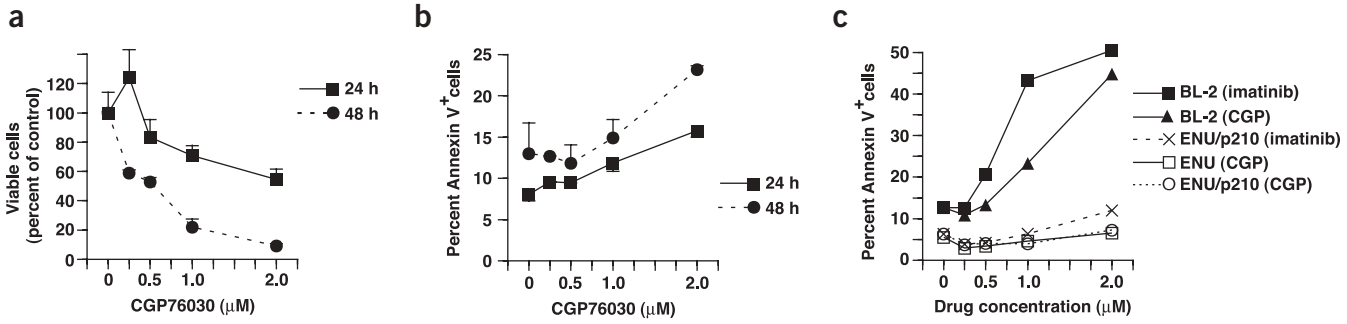

Figure 4 CGP76030 inhibits proliferation and induces apoptosis of pre-B cells expressing Bcr-Abl. (**a**) CGP76030 inhibits the proliferation of BL-2 cells. The number of viable cells at the indicated drug concentrations was determined by trypan blue exclusion at 24 h (solid lines) and 48 h (dotted lines) and expressed as percent of untreated cells. (**b**) CGP76030 induces apoptosis of BL-2 cells. The number of apoptotic cells was determined at the various drug concentrations by flow cytometric analysis of Annexin V staining at 24 h (solid lines) and 48 h (dotted lines) and expressed as percent of untreated cells. Values in **a** and **b** are mean ± s.d. (**c**) CGP76030 (CGP) has a selective inhibitory effect on BL-2 cells expressing Bcr-Abl versus parental and ENU48515 cells expressing Bcr-Abl. Imatinib was used as control. The number of apoptotic BL-2 and ENU48515 cells was determined as in **b** after 48 h of treatment at the indicated drug concentrations.

treated with CGP76030 or imatinib, alone or in combination (**Fig. 5**). Compared with a placebo, either drug alone significantly prolonged the survival of mice with *BCR-ABL1*-induced B-ALL, and CGP76030 in combination with imatinib was even more effective (**Fig. 5a**). Prolonged survival of drug-treated mice correlated with decreased peripheral blood leukocyte counts during therapy (**Fig. 5b**) and less splenomegaly at necropsy (**Fig. 5c**); the combination of CGP76030 and imatinib had the greatest effect in this regard. We assessed Bcr-Abl and Src family kinase activity in primary leukemia cells isolated from pleural effusion of drug-treated B-ALL mice (**Fig. 5d**). We found inhibition of Src kinase but not Bcr-Abl phosphorylation by CGP76030, whereas Bcr-Abl was inhibited by imatinib as expected. These results indicate that the *in vivo* concentrations of CGP76030 did not reach a level that had an inhibitory effect on Bcr-Abl and argue that the therapeutic effects of CGP76030 in Bcr-Abl-induced B-ALL are a consequence of direct inhibition of Src kinases, not of Bcr-Abl.

In contrast to its therapeutic efficacy in *BCR-ABL1*-induced B-ALL, treatment with CGP76030 did not significantly improve the survival of mice with *BCR-ABL1*-induced CML-like myeloproliferative disease (**Fig. 5e**), whereas treatment with imatinib resulted in a clear survival benefit, in agreement with previous findings[23]. The lack of a therapeutic effect of CGP76030 on *BCR-ABL1*-induced CML-like disease was also reflected by the peripheral blood leukocyte counts during therapy (**Fig. 5f**) and the spleen size at necropsy (**Fig. 5g**); both parameters were decreased by imatinib treatment but unaffected by CGP76030. These results suggest that inhibition of Src kinases would be beneficial for treatment of Ph^+ B-ALL but not CML.

We further evaluated the effect of CGP76030 on the development of Bcr-Abl-induced B-ALL at early stages of the disease. We observed fewer circulating leukemia blasts in drug-treated mice, with the combination of CGP76030 and imatinib having the most significant effects (**Fig. 6a**). The smaller spleen size in drug-treated mice with B-ALL correlated with lower numbers of leukemia blasts in the spleen (**Fig. 6b**) and preservation of the normal splenic follicular architecture (**Fig. 6c**).

The target of CGP76030 is Src kinases, not Bcr-Abl

We determined whether CGP76030 could inhibit growth and survival of pre-B lymphoid leukemia cells expressing the Bcr-Abl T315I mutant, which is resistant to inhibition by both imatinib[4] and CGP76030 (ref. 15). As expected, neither imatinib nor CGP76030 inhibited autophosphorylation of Bcr-Abl T315I (**Fig. 7a**). CGP76030 but not imatinib decreased the number of viable pre-B leukemia cells in a dose-dependent manner after 48 h of treatment (**Fig. 7b**). In parallel, CGP76030 but not imatinib induced apoptosis of pre-B cells

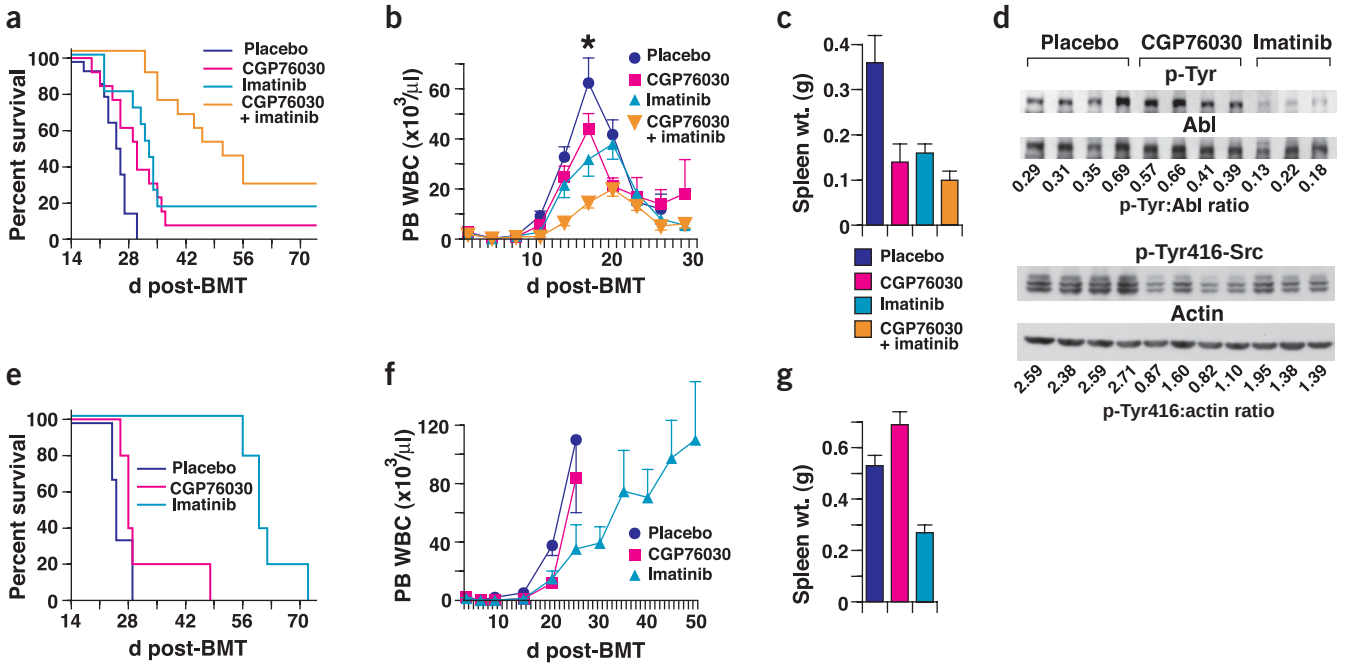

Figure 5 Src kinases are therapeutic targets in Bcr-Abl-induced B-ALL but not in CML. (**a–c**) Survival (**a**), peripheral blood (PB) leukocyte (white blood cell, WBC) counts (**b**) and spleen weights (**c**) of cohorts of mice with *BCR-ABL1*-induced B-ALL treated with vehicle alone (placebo, purple), CGP76030 (magenta), imatinib (blue) or both CGP76030 and imatinib (red). Peripheral blood leukocyte counts of both placebo- and drug-treated mice decrease around 18 d after transplantation owing to development of a malignant pleural effusion that is the principal cause of death (data not shown). (**a**) There was a significant difference in survival between placebo-treated mice ($n = 14$) and each drug-treated group ($P = 0.014$, 0.004 and <0.001 for CGP76030 ($n = 13$), imatinib ($n = 11$) and CGP76030 plus imatinib ($n = 13$), respectively; Mantel-Cox test). (**b**) The difference in leukocyte counts between placebo-treated ($n = 9$) and each drug-treated group on day 17 (asterisk) was statistically significant ($P = 0.047$, 0.007 and <0.001 for CGP76030 ($n = 8$), imatinib ($n = 7$) and CGP76030 plus imatinib ($n = 9$), respectively; unpaired *t*-test). (**c**) $n = 8$ for all groups except CGP76030 plus imatinib ($n = 9$). Values in **b** and **c** are mean ± s.e.m. (**d**) Tyrosine phosphorylation of Bcr-Abl (top panel) and Src family kinases (bottom panel) in leukemic cells from pleural effusion of placebo- and drug-treated B-ALL mice was assessed using antibodies to phosphorylated tyrosine (p-Tyr), to Abl and to phosphorylated Tyr416 (p-Tyr416-Src). Protein lysates were collected 3–4 h after drug administration. Actin was used as a loading control. (**e–g**) Survival (**e**), peripheral blood leukocyte (PB WBC) counts (**f**) and spleen weights (**g**) of cohorts of mice with *BCR-ABL1*-induced CML-like disease treated with placebo (purple), CGP76030 (magenta) or imatinib (blue). (**e**) The difference in survival between the placebo-treated ($n = 3$) and imatinib-treated ($n = 5$) groups was significant ($P = 0.0042$, Mantel-Cox test), whereas the difference between placebo- and CGP76030-treated ($n = 5$) groups was not ($P = 0.278$). In **f** and **g**, $n = 5$ for all groups. Values in **f** and **g** are mean ± s.e.m. d post-BMT, d after bone marrow transplantation.

expressing Bcr-Abl T315I at the same time point (**Fig. 7c**). These results indicate that blocking Src kinases with CGP76030 has an inhibitory effect on B-lymphoid transformation that is independent of any effect on Bcr-Abl.

DISCUSSION

Imatinib mesylate induces complete hematologic and cytogenetic remissions in most newly diagnosed individuals with chronic phase CML[3], but relatively few achieve molecular remission, suggesting that therapy with imatinib as a single agent will not be curative. In addition, imatinib is much less effective in CML blast crisis and Ph$^+$ acute lymphoblastic leukemia, due principally to development of drug resistance. The challenge for the future is to improve on current clinical results with kinase inhibitor therapy in CML, developing treatment strategies that can eradicate chronic phase CML and overcome or prevent resistance in advanced disease. One approach is to combine imatinib treatment with drugs targeting pathways downstream of Bcr-Abl that are essential to initiating or maintaining the leukemia. For example, several recent studies identified synergy against cell lines expressing Bcr-Abl between imatinib and drugs targeting the Ras[24] or phosphatidylinositol 3-kinase[25] pathways. Src kinases have been implicated in transformation and proliferation of myeloid cells by Bcr-Abl, but these studies used immortalized cell lines[13–15], in which transformation by Bcr-Abl does not correlate well with leukemogenesis[16]. For example, Tyr177 of Bcr-Abl is not required for transformation of 32D cells[26] but has a crucial role in CML[27].

Here, we used accurate and quantitative mouse models of chronic phase CML and Ph$^+$ acute B-lymphoblastic leukemia to investigate the role of Src family kinases in leukemogenesis by Bcr-Abl. We used mice lacking Lyn, Hck and Fgr, as these three Src kinases are the only ones expressed in early hematopoetic progenitor and myeloid cells[28]. Bcr-Abl induced CML-like disease in bone marrow lacking all three Src kinases. This result was complemented by treatment of leukemic mice with kinase inhibitor drugs; mice with CML-like disease responded to imatinib but not to the Src inhibitor CGP76030. We did not carry out genetic tests of the roles of other Src kinases in myeloid leukemogenesis by Bcr-Abl, but the lack of efficacy of CGP76030 (which inhibits all Src family members) in mice with Bcr-Abl-induced CML-like disease suggests that Src kinases other than Lyn, Hck and Fgr are not compensating for the lack of these three. Phosphorylation of Bcr-Abl Tyr177 is required for induction of CML-like disease[27,29] and Hck phosphorylates this site[10]. But we found that the amount of Grb2 associated with Bcr-Abl through phosphorylated Tyr177 was not decreased in cells lacking Lyn, Hck and Fgr (**Supplementary Fig. 1** online), suggesting that Bcr-Abl itself can phosphorylate Tyr177 in addition to previously identified autophosphorylation sites[22].

In contrast to CML, we observed a profound defect in induction of mouse B-ALL by Bcr-Abl in the absence of Lyn, Hck and Fgr. As in myeloid cells, Lyn, Hck and, to a lesser extent, Fgr are also the three main Src kinases activated by Bcr-Abl in pre-B lymphoid cells. The observation that Hck is both induced and activated by Bcr-Abl helps explain a functional role for Hck in Ph$^+$ B-ALL even though Hck is not highly expressed in nonmalignant B-lymphocytes. The development of B-ALL after considerable delay in a few recipients of *BCR-ABL1*-transduced Src mutant marrow when very high titer retrovirus stock was used may reflect the acquisition of additional genetic or epigenetic changes that compensate for lack of Src kinase signaling. As the disease induced in mice closely resembles both Ph$^+$ B-ALL and lymphoid blast crisis of CML, our results are suggestive of a general role for Src kinases

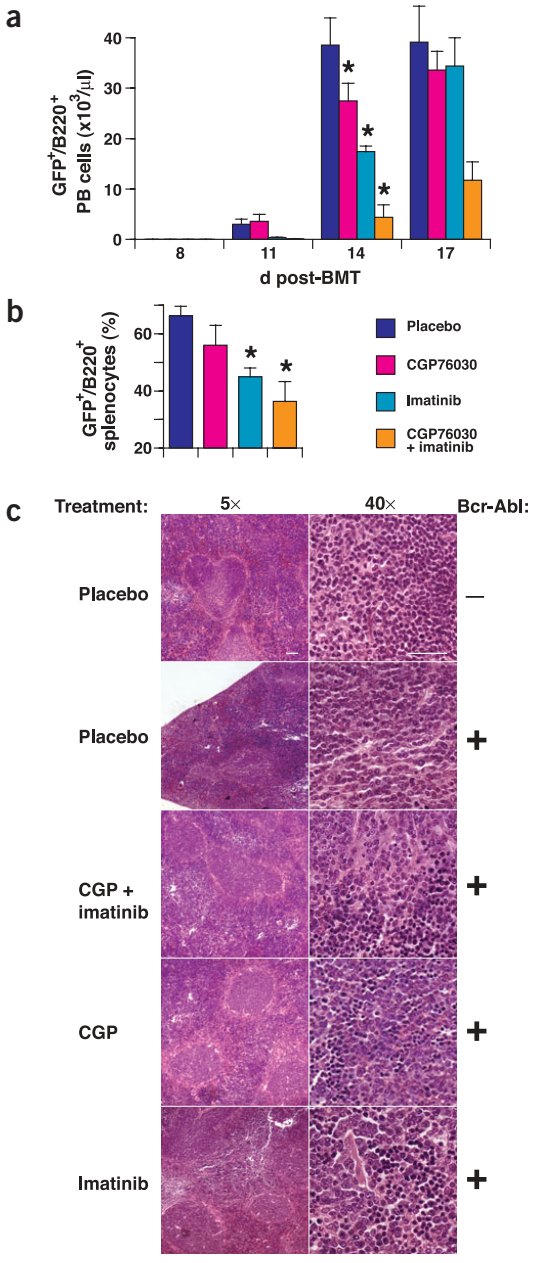

Figure 6 Flow cytometric and histopathological evaluation of the leukemic process in mice treated with kinase inhibitor. (**a**) The number of circulating leukemic blasts (calculated as percentage of B220$^+$ GFP$^+$ cells × white blood cell count) in cohorts of mice ($n = 5$) with B-ALL treated with placebo (purple), CGP76030 (magenta), imatinib (blue) or both CGP76030 and imatinib (red) was determined on days 8, 11, 14 and 17 after transplantation (d post-BMT). There was a significant difference (asterisks) in percentages of GFP$^+$ B220$^+$ cells in peripheral blood (PB) on day 14 between placebo-treated mice and each drug-treated group ($P = 0.030$, 0.019 and 0.002 for CGP76030, imatinib and CGP76030 plus imatinib, respectively; unpaired *t*-test). (**b**) The percentage of leukemic blasts in the spleen at day 17 after transplantation in cohorts of mice ($n = 5$) from **a**. There was a significant difference (asterisks) in the percentage of GFP$^+$ B220$^+$ splenocytes between placebo-treated mice and mice treated with imatinib or with CGP76030 plus imatinib ($P = 0.010$ and 0.036, respectively; unpaired *t*-test). (**c**) Photomicrographs of spleen sections from drug-treated mice at day 17 after transplantation stained with hematoxylin and eosin. Scale bars, 100 μM (left panels, magnification 5×) and 50 μM (right panels, magnification 40×). CGP, CGP76030.

in Ph+ acute leukemia. Modeling CML myeloid blast crisis in mice requires coexpression of Bcr-Abl and an oncogenic transcription factor such as NUP98-HoxA9 (ref. 30) or AML1-EVI1 (ref. 31), and the role of Src kinases in this disease will require further study.

These genetic experiments were complemented by drug treatment studies that confirmed the therapeutic efficacy of CGP76030, a tyrosine kinase inhibitor that inhibits Src kinases more efficiently than Bcr-Abl, in mice with Bcr-Abl-induced B-ALL. The combination of CGP76030 and imatinib was significantly more effective in treating these mice than was either drug alone. Although CGP76030 can inhibit Bcr-Abl at higher concentrations[15], we found evidence of direct inhibition of Src kinases in leukemic cells isolated from mice treated with CGP76030, whereas there was no appreciable inhibition of Bcr-Abl itself. In addition, we used the Bcr-Abl T315I mutant, which is resistant to both CGP76030 and imatinib[4,15], in a genetic strategy to verify the target of CGP76030. Whereas Src kinase inhibitor treatment of IL-3-dependent hematopoietic cells expressing the Bcr-Abl T315I mutant can either inhibit proliferation[15] or not[32], depending on the cell type, CGP76030 had potent antiproliferative and apoptosis-inducing activity on primary bone marrow–derived leukemic cells expressing Bcr-Abl T315I. These results argue that the principal target of CGP76030 in Ph+ B-lymphoid leukemia is Src kinases, and not Bcr-Abl. Together, our preclinical studies make a specific prediction: drugs targeting Src kinases may be useful for the therapy of Ph+ acute leukemia, particularly B-ALL, but will be ineffective in treating chronic phase CML. There may still be a rationale for dual kinase inhibitor therapy for individuals with chronic phase, as increased activation of Src kinases including Lyn have been observed in individuals with CML with acquired imatinib resistance[33].

Although we used the p210 form of Bcr-Abl rather than p190, which is more common in childhood Ph+ B-ALL, our results probably apply to p190-induced leukemia as well, because both forms of Bcr-Abl induce CML-like disease and B-ALL in the respective mouse models[17,18] and both activate Src kinases in cell lines[10]. As deficiency of any one of the three individual Src kinases does not impair B-lymphoid leukemogenesis by Bcr-Abl, our results indicate that Src kinases have partially overlapping signaling functions in Ph+ pre-B leukemia cells. This is reminiscent of redundant signaling by Src kinases in phagocytic cells[28]. We are currently generating mice lacking different pairs of Src kinases to investigate further the roles of the individual kinases in leukemogenesis.

These results imply that Bcr-Abl uses different signaling pathways to induce CML and Ph+ B-ALL. The cells that initiate mouse CML-like disease and B-ALL are distinct[17], and we showed previously that the Bcr-Abl SH2 domain is required for efficient induction of CML-like disease but not of B-ALL[18]. This study shows that Src kinase signaling also differs between CML and B-ALL. Several signaling pathways downstream of Src kinases in Ph+ leukemia have been proposed. Hck activates Stat5 in myeloid cells expressing Bcr-Abl [11], but Bcr-Abl can induce both CML-like disease and B-ALL in the absence of Stat5 (ref. 34). In 32D cells expressing Bcr-Abl T315I, CGP76030 treatment inhibited Akt activation but had no effect on Stat5 activation[15]. SHP-1 interacts physically with Bcr-Abl[35] and functionally with Lyn in myeloid[36] and lymphoid[37] cells and is another possible effector of Src kinases. The identity of the important substrates of Src kinases in B-lymphoid leukemia cells expressing Bcr-Abl is under investigation. The Bcr-Abl T315I mutant used in this study is an excellent reagent to identify signaling events in leukemic cells that are Src-dependent and should illuminate the pathophysiological basis of the differential requirement of Src kinases for Bcr-Abl-induced myeloid and B-lymphoid leukemia.

METHODS

Cell lines. We isolated the pre-B cell line ENU48515 from bone marrow of a male mouse treated with the germline mutagen ENU. ENU48515 cells express AA4.1, CD19 and CD45R (B220) but not immunoglobulin (Ig) heavy chain (data not shown). We transduced the ENU48515 line with parental MSCV-IRES-GFP retrovirus without *BCR-ABL1* or with MSCV-IRES-GFP virus carrying the *BCR-ABL1* fusion gene[19] and sorted the cells expressing GFP by flow cytometry to make control cell lines (ENU-empty vector) and cell lines expressing *BCR-ABL1* (ENU-*BCR-ABL1*). BL-2 is a pre-B cell line expressing *BCR-*

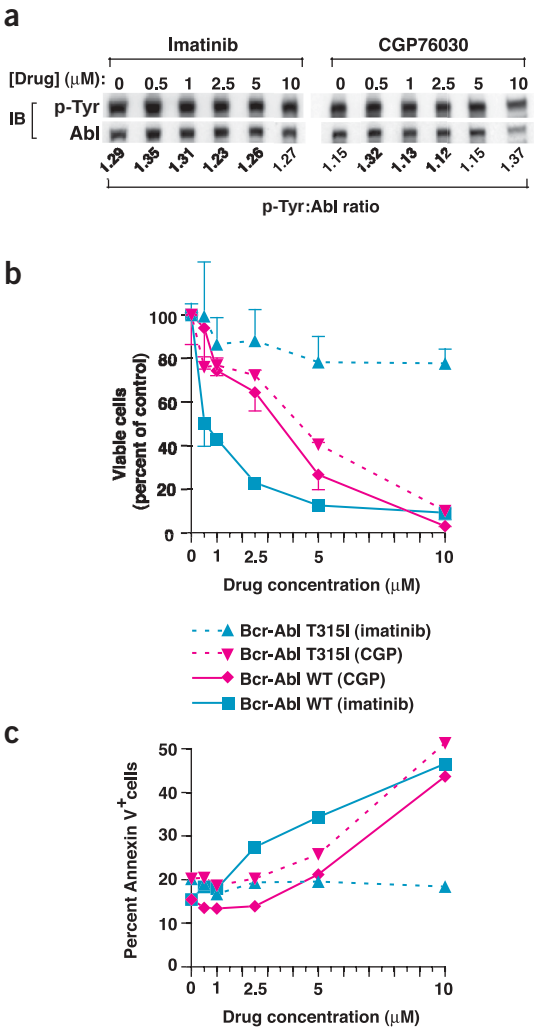

Figure 7 CGP76030 inhibits proliferation and induces apoptosis of pre-B cells expressing the Bcr-Abl T315I mutant that is resistant to inhibition by imatinib and CGP76030. (**a**) Autophosphorylation of Bcr-Abl T315I in drug-treated leukemic cells isolated from Whitlock-Witte bone marrow culture was assessed using antibodies to phosphorylated tyrosine (p-Tyr) and to Abl. The drugs were added to the culture 3 d after transduction with *BCR-ABL1* T315I retrovirus, and protein lysates were prepared 48 h later. (**b**) CGP76030 (CGP) but not imatinib inhibits the proliferation of pre-B cells expressing Bcr-Abl T315I. The number of viable cells at the indicated drug concentrations was determined by trypan blue exclusion at 48 h and expressed as percent of untreated cells. Values are mean ± s.d. (**c**) CGP76030 but not imatinib induces apoptosis of pre-B cells expressing Bcr-Abl T315I. The number of apoptotic cells was determined at the various drug concentrations by flow cytometric analysis of Annexin V staining at 48 h and expressed as percent of untreated cells.

ABL1 isolated from a Balb/c mouse with B-ALL induced by p210 *BCR-ABL1*. BL-2 cells are positive for CD19, B220, CD43 and BP-1 antigens and negative for AA4.1 and Ig heavy chain (data not shown). We grew both cell lines in RPMI 1640 medium containing 10% fetal calf serum and 50 μM 2-mercaptoethanol without exogenous cytokines.

Antibodies and western-blot analysis. We purchased antibodies against phosphorylated tyrosine, c-Abl and the Src kinases Blk, Fgr, Fyn, Hck, Lck, Lyn, c-Src and Yes from Santa Cruz Biotechnology. We prepared protein lysates by lysing cells in RIPA buffer and carried out immunoprecipitation and western blotting as described previously[38].

Bone marrow transduction and transplantation. We used the retroviral vector MSCV-IRES-eGFP[39] carrying the p210 *BCR-ABL1* cDNA to make high-titer, helper-free, replication-defective ecotropic virus stock by transient transfection of 293 cells using the kat system[40] as previously described[17]. We used 4–10-week-old wild-type C57Bl/6 (Taconic Farms and The Jackson Laboratory) and homozygous Src triple knockout ($Lyn^{-/-}$ $Hck^{-/-}$ $Fgr^{-/-}$) mice[28], backcrossed 15 generations into C57Bl/6, for leukemogenesis experiments. The phenotype of the triple knockout mice is similar to that of Lyn single knockout mice; they have developmental and proliferation defects in mature B-lymphoid cells and develop autoimmune disease with age[20]. In addition, phagocytic cells from mice lacking Lyn, Hck and Fgr have functional defects in Fc gamma and LPS receptor signaling[28]. But the mice have normal early B-lymphoid development and normal bone marrow donor and recipient function, and these abnormalities do not affect leukemogenesis. We induced CML-like disease[17] and B-ALL[18] as described previously. Briefly, to model CML, we transduced bone marrow from donor mice treated with 5-FU (200 mg kg^{-1}) twice with *BCR-ABL1* retrovirus by cosedimentation in the presence of IL-3, IL-6 and SCF. In a C57Bl/6 background, most recipients of wild-type *BCR-ABL1*-transduced marrow develop myeloproliferative disease closely resembling the chronic phase of human CML[19]. To model B-ALL, we transduced bone marrow from donors not treated with 5-FU without cytokines. Under these conditions, all recipients develop fatal acute B-lymphoblastic leukemia[18]. We prepared wild-type C57Bl/6 recipient mice by 1100 cGy gamma irradiation and transplanted a dose of 0.5×10^6 (CML) or 1.0×10^6 (B-ALL) cells by tail vein injection. We analyzed diseased mice by histopathological, flow cytometric and molecular analysis as described previously[17].

Drug treatment. For drug treatment studies, we used Balb/c mice, as this strain develops CML-like disease with 100% incidence when donors treated with 5-FU are used[17]. We dissolved CGP76030 in 50 μl of 1 N HCl and then diluted it in distilled water to 5 mg ml^{-1}. We dissolved imatinib in water directly at a concentration of 10 mg ml^{-1}. We administered the drugs orally in a volume less than 0.5 ml by gavage twice a day at 50 mg per kg body weight per dose for CGP76030 and 100 mg per kg body weight for imatinib, beginning 2 d after bone marrow transplantation and continuing until the morbidity or death of the leukemic mice.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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