tion where ectopic Shh protein is applied to the anterior limb bud (*26*). Recognizable posterior digits (albeit not as perfect as with a ZPA graft) are formed in response to Shh protein beads, which may imply that there is an induction of a *Gremlin*-refractory cell population by exceptionally high levels of Shh signaling adjacent to the beads. Alternatively, the termination of growth of these limbs could reflect the limited amount of Shh protein in the bead. Another series of experiments seemingly relevant to the current study involves grafts of irradiated ZPA cells. However, these experiments need to be reevaluated with modern tools (supporting online material text).

We focused on *Fgf4* as the AER component of the Shh-Gremlin-Fgf feedback loop. As noted above, *Fgf8*, *Fgf9*, and *Fgf17* are also expressed in the AER. *Fgf4*, *Fgf9*, and *Fgf17* have been shown to depend on Shh for their transcription (*5*), whereas Gremlin is indirectly responsible for maintaining *Fgf8* expression by affecting AER organization (*12*). Hence, the loss of expression and/or down-regulation of all four of these genes is likely to result when the expansion of the posterior limb bud exceeds the ability of Shh to maintain *Gremlin*. This provides a unique mechanism for terminating the activity of two key signaling centers in the limb, with important implications for the little-understood properties of regulative development and size control during embryogenesis.

References and Notes

1. L. Niswander, S. Jeffrey, G. R. Martin, C. Tickle, *Nature* **371**, 609 (1994).
2. J. F. Fallon *et al.*, *Science* **264**, 104 (1994).
3. E. Laufer, C. E. Nelson, R. L. Johnson, B. A. Morgan, C. Tabin, *Cell* **79**, 993 (1994).
4. R. D. Riddle, R. L. Johnson, E. Laufer, C. Tabin, *Cell* **75**, 1401 (1993).
5. X. Sun *et al.*, *Nature Genet.* **25**, 83 (2000).
6. A. Zuniga, A.-P. G. Haramis, A. P. McMahon, R. Zeller, *Nature* **401**, 598 (1999).
7. J. Capdevila, T. Tsukui, C. R. Esteban, V. Zappavigna, J. C. I. Belmont, *Mol. Cell* **4**, 839 (1999).
8. D. R. Hsu, A. N. Economides, X. Wang, P. M. Eimon, R. M. Harland, *Mol. Cell* **1**, 673 (1998).
9. Y. Ganan, D. Macias, R. D. Basco, R. Merino, J. M. Hurle, *Dev. Biol.* **196**, 33 (1998).
10. S. Pizette, L. Niswander, *Development* **126**, 883 (1999).
11. C. Chiang *et al.*, *Dev. Biol.* **236**, 421 (2001).
12. M. K. Khokha, D. Hsu, L. J. Brunet, M. S. Dionne, R. M. Harland, *Nature Genet.* **34**, 303 (2003).
13. J. J. Sanz-Ezquerro, C. Tickle, *Development* **127**, 4811 (2000).
14. A. Hornbruch, L. Wolpert, *Nature* **226**, 764 (1970).
15. J. J. Sanz-Ezquerro, C. Tickle, *Curr. Biol.* **13**, 1830 (2003).
16. R. D. Dahn, J. F. Fallon, *Science* **289**, 438 (2000).
17. R. Merino *et al.*, *Development* **126**, 5515 (1999).
18. B. D. Harfe *et al.*, *Cell*, in press.
19. Materials and methods are available as supporting material on *Science* Online.
20. P. M. Lewis *et al.*, *Cell* **105**, 599 (2001).
21. V. Marigo, M. P. Scott, R. L. Johnson, L. V. Goodrich, C. J. Tabin, *Development* **122**, 1225 (1996).
22. H. Driesch, in *Foundations of Experimental Embryology*, B. H. Willier, J. H. Oppenheimer, Eds. (Hafner, New York, 1892).
23. S. J. Lee, A. C. McPherron, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 9306 (2001).
24. E. Hafen, H. Stocker, *PLoS Biol.* **1**, 319 (2003).
25. M. Richardson, J. Jeffrey, C. J. Tabin, *Evol. Dev.* **6**, 1 (2004).
26. Y. Yang *et al.*, *Development* **124**, 4393 (1997).

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Overriding Imatinib Resistance with a Novel ABL Kinase Inhibitor

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Resistance to the ABL kinase inhibitor imatinib (STI571 or Gleevec) in chronic myeloid leukemia (CML) occurs through selection for tumor cells harboring BCR-ABL kinase domain point mutations that interfere with drug binding. Crystallographic studies predict that most imatinib-resistant mutants should remain sensitive to inhibitors that bind ABL with less stringent conformational requirements. BMS-354825 is an orally bioavailable ABL kinase inhibitor with two-log increased potency relative to imatinib that retains activity against 14 of 15 imatinib-resistant BCR-ABL mutants. BMS-354825 prolongs survival of mice with BCR-ABL–driven disease and inhibits proliferation of BCR-ABL–positive bone marrow progenitor cells from patients with imatinib-sensitive and imatinib-resistant CML. These data illustrate how molecular insight into kinase inhibitor resistance can guide the design of second-generation targeted therapies.

Imatinib (STI571 or Gleevec) is a small-molecule inhibitor of the BCR-ABL tyrosine kinase that produces clinical remissions in chronic myeloid leukemia (CML) patients with minimal toxicity (*1*, *2*). Imatinib is now frontline therapy for CML, but resistance is increasingly encountered. Clinical resistance is primarily mediated by mutations within the kinase domain of BCR-ABL and, to a lesser extent, by amplification of the *BCR-ABL* genomic locus (*3*). Crystallographic studies revealed that imatinib binds to the adenosine triphosphate (ATP)–binding site of ABL only when the activation loop of the kinase is closed and thus stabilizes the protein in this inactive conformation (*4*). In addition, the normally smooth contour of the phosphate-binding loop of ABL is distorted by imatinib binding, adding further to the unique conformational requirements for optimal kinase inhibition. These conformation-specific binding requirements contribute to imatinib's selectivity, particularly with regard to the closely related kinase SRC, which imatinib does not inhibit. Structural studies of the pyrido[2,3-d]pyrimidine class of dual SRC-ABL inhibitors show that these compounds also bind to the ATP-binding site in ABL, but without regard for the position of the activation loop, which can be in the active or inactive conformation (*5*).

To date, mutations at 17 different amino acid positions within the BCR-ABL kinase domain have been associated with clinical resistance to imatinib in CML patients (*6*–*11*). The majority of amino acid substitutions are believed to cause resistance by impairing the ability of the kinase to adopt the specific closed conformation to which imatinib binds, although a small fraction directly interfere with drug binding (*9*). This insight raises the possibility that other small-molecule ABL kinase inhibitors, such as those that also inhibit SRC, might have activity against imatinib-resistant BCR-ABL mutants. Indeed, promising in vitro activity against a limited number of imatinib-resistant BCR-ABL isoforms has been seen for two compounds from the pyrido[2,3-d]pyrimidine class of dual SRC-ABL inhibitors (PD166326 and PD180970) (*12*, *13*).

BMS-354825 [N-(2-chloro-6-methylphenyl)-2-(6-(4-(2-hydroxyethyl)piperazin-1-yl)-2-

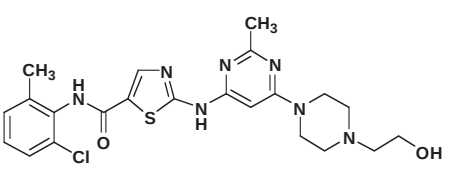

Fig. 1. Chemical structure of BMS-354825.

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methylpyrimidin-4-ylamino)thiazole-5-carboxamide] is a synthetic small-molecule inhibitor of SRC-family kinases (Fig. 1). The compound is a potent ATP-competitive inhibitor in biochemical assays with broad-spectrum antiproliferative activity against hematological and solid tumor cell lines (*14*). Structure-activity relationships leading to the identification of BMS-354825 and a description of the preparation, kinase selectivity profile, and pharmacokinetic profile of this compound will be described elsewhere. On the basis of prior structural insights from other dual SRC-ABL inhibitors, we reasoned that BMS-354825 may impose less stringent conformational requirements on ABL for kinase inhibition, and we therefore assessed its activity against imatinib-resistant BCR-ABL mutants (*15*). As expected, BMS-354825 was more potent than imatinib at inhibiting nonmutated BCR-ABL (BCR-ABL/WT) kinase activity in a cell-based assay (Fig. 2A). In addition, the kinase activity of 14 out of 15 different clinically relevant, imatinib-resistant BCR-ABL isoforms was successfully inhibited in the low nanomolar range (Fig. 2B). The growth of Ba/F3 cells (a murine pro-B cell line) expressing each of the various isoforms was similarly inhibited (Fig. 2C). The only imatinib-resistant BCR-ABL isoform that was clearly resistant to BMS-354825 was the T315I mutant, which retained kinase activity even in the presence of μM concentrations of the compound. Within the 1 to 10 nM range, we observed subtle yet highly reproducible differences in the sensitivity of certain BCR-ABL isoforms. For example, three- to fivefold higher concentrations of BMS-354825 were required to inhibit the growth of Ba/F3 cells expressing the F317L mutant, whereas the Q252R mutant was consistently more sensitive than nonmutated BCR-ABL. These findings are reminiscent of the relatively modest levels of imatinib resistance conferred by certain BCR-ABL mutations (*16*) and may provide clues toward the structural basis of BMS-354825 binding to ABL. Furthermore, these varying sensitivities may help guide decisions about the minimum serum concentrations of BMS-354825 required to provide therapeutic benefit in individual imatinib-resistant CML cases, depending on the specific imatinib-resistant mutation(s) observed.

To assess its potential as a therapeutic agent, we studied BMS-354825 in a mouse model of imatinib-resistant, BCR-ABL–dependent disease. Severe combined immunodeficient (SCID) mice were injected intravenously with Ba/F3 cells expressing different BCR-ABL isoforms as well as the firefly luciferase gene. Untreated mice harboring Ba/F3 cells expressing nonmutant or imatinib-resistant mutant BCR-ABL developed aggressive disease, with massive liver and splenic infiltration, typically resulting in death in ~15 days (*17*). To assess the ability of BMS-354825 to inhibit BCR-ABL in vivo, we assessed BCR-ABL kinase activity in splenocyte lysates prepared at various time points after administration of a single dose of 5, 10, or 15 mg/kg of BMS-354825 by oral gavage (fig. S1). Before dosing, mice were documented to have extensive disease by bioluminescence imaging. Phosphorylation of the adapter protein CRKL, a known BCR-ABL substrate (*3*, *18*), was inhibited for up to 7 hours by doses of 10 or 15 mg/kg, but recovered to baseline levels after 12 hours (fig. S1) (*19*). On the basis of a series of such pharmacodynamic experiments, we selected a dose of 10 mg/kg twice daily for efficacy studies. Mice were dosed with BMS-354825 or vehicle alone by gavage for 2 weeks, beginning 3 days after injection of Ba/F3 cells, and disease burden was then assessed by bioluminescence imaging (Fig. 3A). All vehicle-treated mice developed progressive disease. In contrast, BMS-354825–treated mice harboring nonmutant BCR-ABL or the clinically common imatinib-resistant mutation M351T (which accounts for 15 to 20% of CML resistance cases) appeared healthy with no evidence of weight loss, lethargy, or ruffled fur and showed greater than one log lower levels of bioluminescent activity after 2 weeks of therapy. Consistent with our in vitro studies, mice harboring T315I did not significantly respond to the compound.

To determine if the observed reduction in disease burden achieved by BMS-354825 was biologically meaningful, we investigated the effect of BMS-354825 on survival of mice injected with Ba/F3 cells expressing various isoforms of BCR-ABL (wild-type, T315I, and M351T). Mice harboring Ba/F3 cells expressing either nonmutated BCR-ABL or the M351T isoform of BCR-ABL showed significantly prolonged survival when treated with BMS-354825. No clinically significant improvement in survival of mice harboring the T315I isoform was detected (Fig. 3B). Additionally, spleen weights of BMS-354825–treated mice harboring wild-type BCR-ABL or M351T were normal, compared to a more-than-tenfold increase observed in untreated animals (*19*).

To assess the safety and efficacy of BMS-354825 on human bone marrow progenitors, we tested the compound in in vitro colony-forming-unit (CFU) assays. At a concentration of 5 nM, BMS-354825 did not inhibit growth of bone marrow progenitors isolated from healthy volunteers, but it inhibited by 60 to 80% the growth of bone marrow progenitors isolated from CML patients with either imatinib-sensitive (nonmutant BCR-ABL) or imatinib-resistant (M351T) disease (fig. S2). Furthermore, greater than 75% of the blast-forming unit–erythroid (BFU–E) and CFU–granulocyte-monocyte (GM) colonies from CML patient marrow samples that did

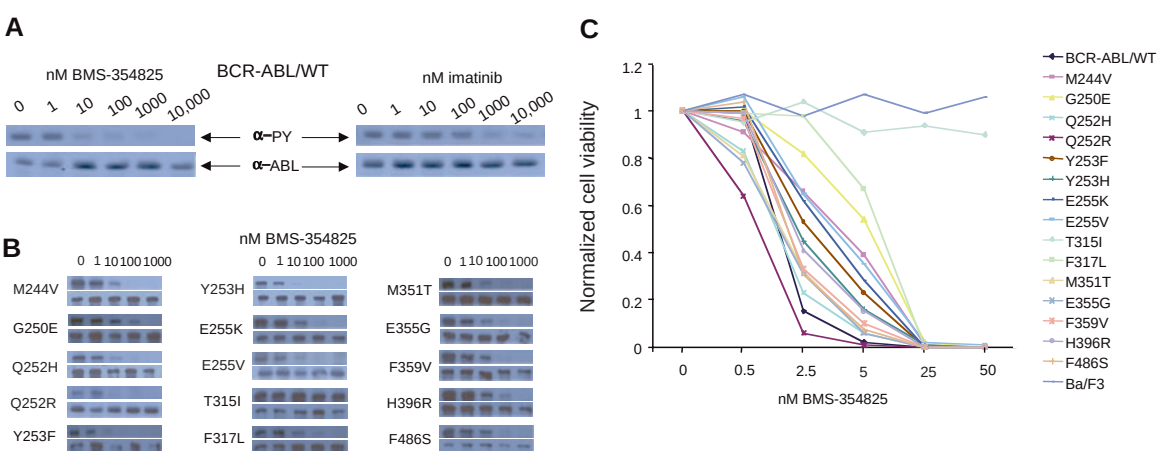

Fig. 2. Effect of BMS-354825 on the BCR-ABL kinase activity and cell growth of Ba/F3 cells expressing various imatinib-resistant BCR-ABL isoforms. (**A**) Assessment of relative sensitivity of BCR-ABL kinase activity to BMS-354825 and imatinib. Ba/F3 cells expressing nonmutant BCR-ABL were exposed to the indicated concentrations of BMS-354825 and imatinib. After 90 min, whole cell lysates were prepared. Western blots were analyzed with antibody to phosphotyrosine (α-PY) and to ABL (α-ABL). (**B**) Western blot analysis of imatinib-resistant Ba/F3 cells expressing mutant BCR-ABL and exposed to BMS-354825. Whole cell lysates were prepared from Ba/F3 cells expressing the indicated BCR-ABL isoforms after 2 hours of incubation with BMS-354825 and subjected to Western blot analysis with either antibody to phosphotyrosine or to ABL. (**C**) In vitro analysis of cell-growth inhibition of imatinib-resistant Ba/F3 cells expressing the indicated *BCR-ABL* mutants. Viable cell counts were performed in triplicate after 48 hours of exposure to BMS-354825 and normalized to the dimethyl sulfoxide–treated sample.

grow in BMS-354825 did not express *BCR-ABL* mRNA as determined by polymerase chain reaction (PCR) analysis, indicating potent selection for growth of rare normal progenitors present in these leukemic marrow samples (Fig. 4). These results mirror those previously reported for imatinib against human CML cells (*20*) and suggest that BMS-354825 may also be highly selective for leukemic versus normal hematopoietic cells.

These findings illustrate how structural insights into the mechanism of resistance to a clinically effective kinase inhibitor can guide the selection of second-generation targeted agents for subsequent clinical investigation. The primary consequence of nearly all the ABL kinase domain mutations associated with imatinib resistance is the impairment of kinase domain flexibility, such that the kinase domain is unable to assume the optimal inactive conformation required for imatinib binding. Inhibitors with less stringent conformation requirements for binding to the ABL kinase domain are therefore predicted to retain activity against many imatinib-resistant mutants. In vitro studies of at least three other ATP–binding site SRC inhibitors against a subset of ABL mutants support this hypothesis (*12*, *13*, *21*), but the in vivo activity of these compounds is unknown. On the basis of the preclinical results described here, the clinical safety and efficacy of BMS-354825 is currently being evaluated in a phase I clinical trial in CML patients with imatinib resistance.

If BMS-354825 should prove to be safe and effective in the clinic, one might envision the use of combination kinase inhibitor therapy as treatment for CML to optimize tumor response and to delay or prevent the emergence of drug-resistant clones, analogous to the combination antiviral therapy used for human immunodeficiency virus. One shortcoming of this proposed strategy is the essentially universal resistance of the T315I mutant to all known SRC inhibitor chemotypes. The homologous threonine residue in the kinase domain of the platelet-derived growth factor receptor has also been implicated in imatinib resistance (*22*) and has been experimentally modified in a range of kinases to create alleles that can be selectively inhibited by an otherwise inert ATP analog (*23*). Collectively, these findings suggest that this residue performs a gatekeeper function for ATP-competitive small-molecule kinase inhibitors. Other strategies are clearly required to address resistance caused by the T315I allele. As this mutation accounts for only 15 to 20% of imatinib-resistant cases, a substantial fraction of imatinib-resistant CML patients could benefit from BMS-354825 if low nanomolar concentrations of the drug can be safely achieved in humans.

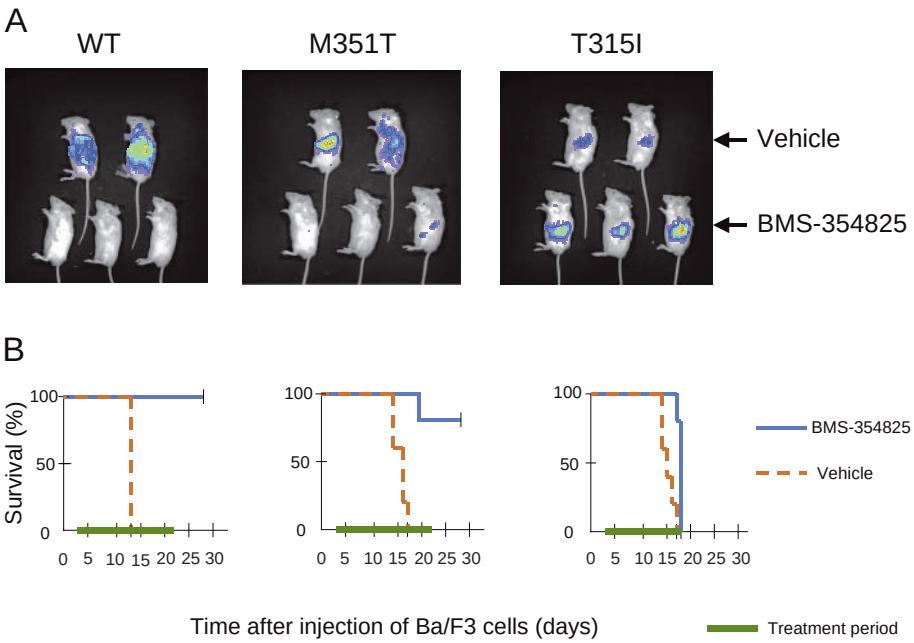

Fig. 3. Effect of BMS-354825 in a mouse model of imatinib-resistant BCR-ABL–dependent hematopoietic disease. (**A**) In vivo assay of growth inhibition of imatinib-resistant mutant *BCR-ABL*–expressing Ba/F3 cells. SCID mice were treated with a 50:50 mixture of propylene glycol and water (Vehicle) or BMS-354825, beginning 3 days after infusion of the Ba/F3 cells. Images were obtained after luciferin injection on day 13. Luciferase activity was primarily detected in the spleen. (**B**) Kaplan-Meier survival analysis of BMS-354825–treated SCID mice harboring BCR-ABL-WT, M351T, and T315I isoforms. The lone mouse with the M351T isoform that did not survive appeared healthy on the previous day, and there was no evidence of disease at the time of death. These experiments were repeated twice with similar results.

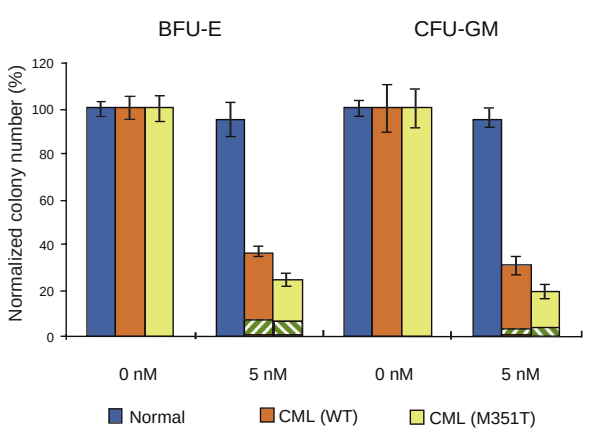

Fig. 4. Effect of BMS-354825 on a bone marrow progenitor CFU assay. Mononuclear cells purified from normal bone marrow or from imatinib-sensitive (WT) or imatinib-resistant (M351T) CML patients were assessed for colony formation. On day 11, BFU-E and CFU-GM were quantitated. Error bars represent the standard error of the mean. The proportion of colonies positive for the BCR-ABL transcript as assessed by PCR is depicted by the green and white striped boxes. Ninety to 100% of colonies isolated from CML patients that grew in the absence of BMS-354825 contained the *BCR-ABL* transcript. Experiments were performed in triplicate.

References and Notes

1. B. J. Druker *et al.*, *N. Engl. J. Med.* **344**, 1031 (2001).
2. S. G. O'Brien *et al.*, *N. Engl. J. Med.* **348**, 994 (2003).
3. M. E. Gorre *et al.*, *Science* **293**, 876 (2001).
4. T. Schindler *et al.*, *Science* **289**, 1938 (2000).
5. B. Nagar *et al.*, *Cancer Res.* **62**, 4236 (2002).
6. N. von Bubnoff, F. Schneller, C. Peschel, J. Duyster, *Lancet* **359**, 487 (2002).
7. S. Branford *et al.*, *Blood* **99**, 3472 (2002).
8. C. Roche-Lestienne *et al.*, *Blood* **100**, 1014 (2002).
9. N. P. Shah *et al.*, *Cancer Cell* **2**, 117 (2002).
10. A. Hochhaus *et al.*, *Leukemia* **16**, 2190 (2002).
11. H. K. Al-Ali *et al.*, *Hematol. J.* **5**, 55 (2004).
12. P. La Rosee, A. S. Corbin, E. P. Stoffregen, M. W. Deininger, B. J. Druker, *Cancer Res.* **62**, 7149 (2002).
13. D. R. Huron *et al.*, *Clin. Cancer Res.* **9**, 1267 (2003).
14. L. J. Lombardo *et al.*, in preparation.
15. Materials and methods are available as supporting material on *Science* Online.
16. M. Azam, R. R. Latek, G. Q. Daley, *Cell* **112**, 831 (2003).
17. M. Deng, G. Q. Daley, *Blood* **97**, 3491 (2001).
18. T. Oda *et al.*, *J. Biol. Chem.* **269**, 22925 (1994).
19. N. P. Shah *et al.*, unpublished data.
20. B. J. Druker *et al.*, *Nature Med.* **2**, 561 (1996).
21. M. Warmuth *et al.*, *Blood* **101**, 664 (2003).
22. J. Cools *et al.*, *N. Engl. J. Med.* **348**, 1201 (2003).
23. K. Shah, Y. Liu, C. Deirmengian, K. M. Shokat, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3565 (1997).

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