SUPPORTING ONLINE MATERIAL

Materials and Methods

Creation of imatinib-resistant mutant BCR-ABL-expressing retroviral vectors.

Oligonucleotides containing mutant kinase domain sequences detected in clinical samples at the time of acquired imatinib resistance were synthesized (Operon, Inc., Valencia, CA). A plasmid harboring the entire coding sequences of BCR-ABL, BS P210 BCR-ABL, was used as the template for site-directed mutagenesis (QuickChange XL, Stratagene, La Jolla, CA) with various mutant oligonucleotides. Clones were confirmed to contain the desired mutation by DNA sequence analysis. For desired amino acid mutations M244V, G250E, Q252H, Q252R, Y253F, Y253H, E255K, E255V, T315I and F317L (convention: ABL Ia numbering system), the region spanning restriction enzyme sites KpnI and BsrG1 was sequenced to ensure that no unintentional mutations were introduced during the cloning process. For amino acid mutations M351T, E355G, F359V, and H396R, the regions spanning restriction enzyme sites BsrG1 and AatII were verified. The region spanning restriction enzyme sites AatII and BclI was verified by sequence analysis for amino acid mutation F486S. Fragments containing desired amino acid mutations were prepared by restriction digestion with KpnI and BsrGI (mutants M244V, G250E, Q252H, Q252R, Y253F, Y253H, E255K, E255V, T315I and F317L), BsrGI and AatII (mutants M351T, E355G, F359V, and H396R), or AatII and BclI (mutant F486S), and subsequently re-introduced into the corresponding region of BS P210 BCR-ABL. Proper introduction of mutant sequences was verified by DNA sequence analysis. The 7.0 kb EcoRI fragment containing BCR-ABL with the desired

kinase domain mutation was excised and introduced into the EcoRI site of the retroviral vector Murine Stem Cell Virus (MSCV) PURO (Clontech, Palo Alto, CA).

Creation of Ba/F3 stable cell lines expressing mutant BCR-ABL isoforms. Stocks of retrovirus were generated by transiently co-transfecting the human embryonic kidney cell line 293-T with MSCV constructs engineered to express various BCR-ABL kinase domain mutations along with the ecotropic packaging plasmid Ecopack (kindly provided by R. van Etten) by the calcium phosphate transfection method. Supernatants collected between 24 and 48 hours post-transfection were passed through a 0.45 micron filter and used to infect the interleukin-3 (IL-3)-dependent murine lymphoid cell line Ba/F3. After 24 hours, the media was changed to include IL-3 (1 ng/ml) and 2 ug/ml puromycin. After seven days of selection in puromycin, cells were washed once with phosphate buffered saline (PBS) and plated in media devoid of IL-3. Pools of puromycin-resistant, IL-3-independent Ba/F3 cells were propagated and used for biochemical and biological experiments.

Biochemical assessment of BCR-ABL kinase inhibition by BMS-354825. Ba/F3 cells expressing various isoforms of BCR-ABL were plated in RPMI containing 10 % fetal calf serum at 1 x 10^6 cells per ml. Cells (2 ml) were exposed in 6-well dishes to varying concentrations of BMS-354825 obtained from a stock solution prepared by dissolving the compound in dimethyl sulfoxide (DMSO) (10 mg/ml). 5 ul aliquots of stock solution were stored at –20^{o}C. After two hours of drug exposure, cells were harvested, washed once with PBS, and lysed in 100 ul 1% Triton lysis buffer supplemented with 10 ul/ml

protease and phosphatase inhibitors (inhibitor cocktails II and III, Calbiochem). Each lysate (15 ul) was electrophoresed on an 8 % SDS-polyacrylamide gel. BCR-ABL protein was identified with anti-ABL antibody AB-3 (Oncogene Research Products, Cambridge, MA). Phosphotyrosine was detected using an anti-phosphotyrosine antibody (4G10; Upstate Biotechnologies, Waltham, MA). Experiments with imatinib (kindly provided by Novartis Pharmaceuticals, Basel, Switzerland) were performed in a similar fashion.

Assessment of cell growth inhibition mediated by BMS-354825. 5 x 10^4 Ba/F3 cells/ml were incubated, in triplicate, in RPMI supplemented with 10 % fetal calf serum and varying concentrations of BMS-354825 or vehicle. After 48 hours, viable cells were quantitated by trypan blue dye exclusion.

Generation of luciferase-expressing BCR-ABL Ba/F3 cell lines. The retroviral vector pMMPlucneo, which encodes a fusion of the firefly luciferase and neomycin genes (a kind gift of A. Kung) was transiently transfected with Ecopack into 293-T cells and virus was subsequently harvested as described above. Ba/F3 cells expressing luciferase were selected in the presence of 1 mg/ml G418, and documented to express luciferase with a reporter assay. Ba/F3-lucneo cells were then superinfected with virus harboring either non-mutant *BCR-ABL* or imatinib-resistant mutant isoforms T315I and M351T. Selection was performed in the presence of puromycin and 1 ng/ml IL-3. Subsequently, IL-3 was removed from the media. Populations of puromycin-resistant, IL-3-independent cells were used for bioluminescence experiments.

***In vivo* experiments**. $1x10^6$ luciferase-expressing Ba/F3 cells harboring mutant or non-mutant BCR-ABL isoforms were injected into the tail vein of SCID mice (ten mice per Ba/F3 subtype for survival analysis). After 72 hours (day 3), mice were treated twice daily with either vehicle or 1 mg/kg BMS-354825 resuspended in a 50:50 mixture of water and propylene glycol. Treatment was administered by gavage daily for up to 20 days, depending upon survival. On day 13, a subset of mice were randomly chosen to be anesthetized, infused with luciferin, and imaged. On day 28, the experiment was terminated. Histologic sections of spleen and bone marrow revealed no abnormalities attributable to the drug. Mice with disease were sacrificed according to the guidelines of the UCLA Animal Research Committee.

Colony-forming unit assays and RT-PCR analysis of colonies. Bone marrow was harvested from clinical subjects who had signed a research consent form approved by the UCLA Institutional Review Board. Viably frozen Ficoll-Hypaque-purified mononuclear cells were thawed and grown overnight in Iscove's media supplemented with 10% fetal calf serum, l-glutamine, pen-strep, and stem cell factor (100 ug/ml) at a density of $5x10^5$/ml. After 24 hours, viable cells were quantitated and plated in Methocult media (Cell Signal Technologies, Beverly, MA) at $1x10^4$and $1x10^5$ cells per plate in the presence of 5 nM BMS-354825 or vehicle. Experiments were performed in triplicate. On day 11, erythroid blast-forming units (BFU-E) and granulocyte-macrophage colony forming units (CFU-GM) were quantitated. On day 14, colonies were isolated with a pipet tip, and RNA was isolated using a Qiagen Rneasy kit. A primer complementary to

the region of ABL approximately 300 nucleotides downstream of the BCR-ABL mRNA (5'-CGGCATTGCGGGACACAGGCCCATGGTACC) junction was annealed to purified RNA as previously described (Ref *S1*). cDNA was synthesized using mouse Moloney leukemia virus (MMLV) reverse transcriptase, and subjected to 40 cycles of PCR using either a BCR (5-TGACCAACTCGTGTGTGAAACT) or ABL type Ia 5' primer (GGGGAATTCGCCACCATGTTGGAGATCTGCCTGA) as a control for the quality of RNA. A small number of samples failed to amplify with the ABL Ia primer, and these were discarded from further analysis. PCR products were visualized on a 2 % agarose/Tris-Acetate-EDTA (TAE) gel.

Fig. S1. Effect of orally administered BMS-354825 on BCR-ABL signaling in vivo. **(A)** Schematic depiction of single dose BMS-354825 administration experiment. One million Ba/F3 cells were injected into each mouse. (**B)** Representative luminometric analysis of a cohort of mice eight days following injection with Ba/F3-BCR/ABL/WT cells. A control mouse in the upper right reveals the absence of detectable background signal. (**C)** Western blot analysis of whole cell extracts prepared from the spleens of mice. Time elapsed since the administration of a single dose of BMS-354825 (5, 10, or 15 mg/kg) is shown in hours. The positions of CRKL and phospho-CRKL are depicted.

Fig. S2. BMS-354825 substantially inhibits colony forming unit activity of bone marrow progenitors from CML patients but not from healthy volunteers. Representative results are shown.

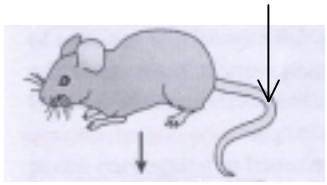
Supporting References

S1. A. Hernandez *et al.*, *Exp. Hematol.* **18**, 1142-4 (1990)

Figure S1

A

Ba/F3-BCR-ABL/WT-luciferase cells injected i.v. into tail vein of SCID mice



8 days

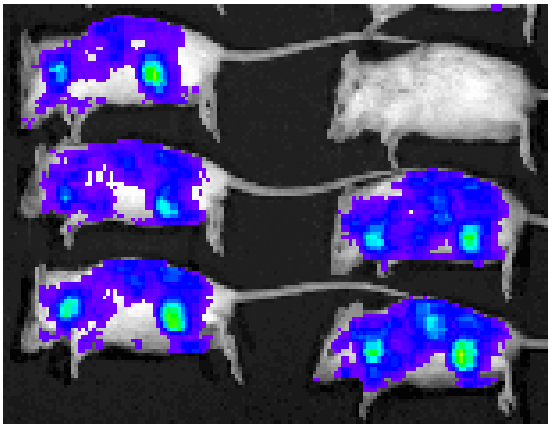
bioluminometry

BMS-354825
(single dose)

harvest spleen at 1,3,5,7 hours after drug administration

analyze CRKL phosphorylation

B



C

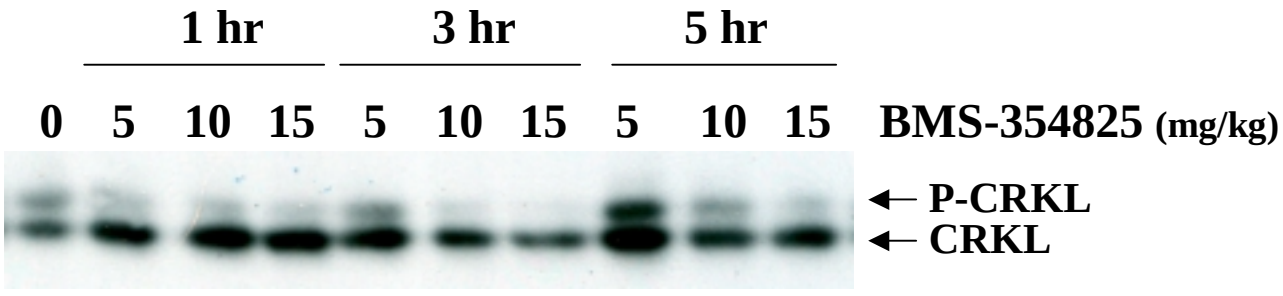


Figure S2