Combined STAT3 and BCR-ABL1 Inhibition Induces Synthetic Lethality in Therapy-Resistant Chronic Myeloid Leukemia

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Abstract

Mutations in the BCR-ABL1 kinase domain are an established mechanism of tyrosine kinase inhibitor (TKI) resistance in Philadelphia chromosome-positive leukemia, but fail to explain many cases of clinical TKI failure. In contrast, it is largely unknown why some patients fail TKI therapy despite continued suppression of BCR-ABL1 kinase activity, a situation termed BCRABL1 kinase-independent TKI resistance. Here, we identified activation of signal transducer and activator of transcription 3 (STAT3) by extrinsic or intrinsic mechanisms as an essential feature of BCR-ABL1 kinase-independent TKI resistance. By combining synthetic chemistry, *in vitro* reporter assays, and molecular dynamics-guided rational inhibitor design and high-throughput

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screening, we discovered BP-5-087, a potent and selective STAT3 SH2 domain inhibitor that reduces STAT3 phosphorylation and nuclear transactivation. Computational simulations, fluorescence polarization assays, and hydrogen-deuterium exchange assays establish direct engagement of STAT3 by BP-5-087 and provide a high-resolution view of the STAT3 SH2 domain/BP-5-087 interface. In primary cells from CML patients with BCR-ABL1 kinase-independent TKI resistance, BP-5-087 (1.0 μM) restored TKI sensitivity to therapy-resistant CML progenitor cells, including leukemic stem cells (LSCs). Our findings implicate STAT3 as a critical signaling node in BCR-ABL1 kinase-independent TKI resistance, and suggest that BP-5-087 has clinical utility for treating malignancies characterized by STAT3 activation.

INTRODUCTION

Chronic myeloid leukemia (CML) is caused by the BCR-ABL1 tyrosine kinase, the result of the t(9;22)(q34;q11) translocation, which is cytogenetically visible as the Philadelphia chromosome (Ph). Targeting BCR-ABL1 with tyrosine kinase inhibitors (TKIs) such as imatinib induces complete cytogenetic responses in many patients with chronic phase CML (CP-CML)[1]. However, ~20-30% of CP-CML patients fail imatinib due to primary or acquired resistance[2], and TKI responses in patients with blastic phase CML (BP-CML) are not durable.

Point mutations in the *BCR-ABL1* kinase domain are the most commonly cited mechanism of TKI resistance[3, 4]. Beyond imatinib, the regulatory approval of four additional TKIs with differing point mutation susceptibilities renders this mechanism of resistance clinically addressable[5]. However, *BCR-ABL1* point mutations fail to explain many cases of clinical TKI failure, as many patients with resistance express exclusively native BCR-ABL1. In these cases, BCR-ABL1 kinase-independent mechanisms activate alternative signaling pathways that maintain survival despite BCR-ABL1 inhibition[6]. BCR-ABL1 kinase-independent resistance likely plays a key role in preventing disease eradication in patients responding to therapy, as imatinib inhibits BCR-ABL1 kinase activity but does not trigger cell death in primitive CML cells cultured *ex vivo*[7, 8].

Activation of signal transducer and activator of transcription 3 (STAT3) by bone marrow (BM)-derived factors protects CML cells upon TKI-mediated BCR-ABL1 inhibition[9, 10]. We now demonstrate that, in CML patients with BCR-ABL1 kinase-independent resistance, STAT3 is activated without a requirement for BM-derived factors, and represents a major signaling node conferring TKI resistance. We hypothesized that targeting STAT3 in addition to BCR-ABL1 would resensitize CML cells with kinase-independent resistance to TKI therapy. Using structure-activity relationship (SAR) studies and compound library screens, we identified BP-5-087, a potent and selective STAT3 inhibitor. Computational simulations and hydrogen-deuterium exchange assays confirmed binding of BP-5-087 to the STAT3 SH2 domain. Experiments on TKI-resistant CML cell lines and primary CML stem and progenitor cells reveal that BP-5-087 restores TKI sensitivity *in vitro* and *ex vivo*, with no toxicity to normal hematopoietic stem or progenitor cells. We conclude that targeting STAT3 with BP-5-087 may be useful for the treatment of kinase-independent TKI resistance and for other malignancies driven by STAT3 activation.

MATERIALS AND METHODS

Cell cultures and primary cells

Imatinib-resistant $K562^R$ and $AR230^R$ cells were derived by long-term culture in the presence of low-dose imatinib, followed by incremental increases in concentration (0.1-1.0 μM imatinib), and maintenance of a single clone in continual 1.0 μM imatinib (isogenic TKI-sensitive $K562^S$ and $AR230^S$ cells were used as controls). Mononuclear cells (MNCs) from peripheral blood (PB) of CP-CML patients or healthy donors were $CD34^+$ selected to >90% purity and kept overnight without cytokines prior to use in 96 hr inhibitor treatment assays. All donors gave informed consent and all studies were approved by The University of Utah Institutional Review Board (IRB). For additional information see Supplementary Materials and Methods.

Clonogenic assays

Methylcellulose colony assays were performed by plating CML cell lines or patient samples in 0.9% MethoCult (H4230; Stem Cell Technologies). For cell lines, 10^3 cells were plated in cytokine-free conditions +/− imatinib (1.0 μM) and/or the indicated STAT3 inhibitors. For patient samples, cells were treated for 96 hr in regular medium (RM) or HS-5 conditioned medium (CM), without additional cytokines, +/− the indicated inhibitors. Following culture, 10^3 viable $CD34^+$ cells were plated in the presence of rhIL-3 (20 ng/mL), rhIL-6 (20 ng/mL), rhFlt-3 ligand (100 ng/mL), and rhSCF (100 ng/mL).

Immunoblot analysis

For CML cell lines, 1.5×10^5 cells/mL were cultured in an equal volume of either RM or HS-5 CM and treated with TKI for 24-36 hr. For CML^{CD34+} cells, assays were performed with 10^6 cells/mL in RM or HS-5 CM containing 10% BIT9500 instead of FBS, in the absence of cytokines, and treated with TKI for 24 hr. For additional information see Supplementary Materials and Methods.

Pharmacologic inhibitors

Imatinib was a gift from Novartis. Dasatinib and nilotinib were purchased from Selleck. STAT3 inhibitors were produced as described in Chemical Methods. Proliferation was assessed by methanethiosulfonate (MTS)-based viability assay (CellTiter 96 AQueous One; Promega).

Fluorescence polarization (FP) assay

To assess STAT3 SH2 domain binding, a high-throughput FP assay was used as described[11].

Chemical Methods

Synthesis and characterization of STAT3 inhibitors was performed as described in Supplementary Materials and Methods. Briefly, all compounds were purified by silica gel column chromatography, with final molecules characterized by both ^1H and ^{13}C NMR and high resolution mass spectrometry. Inhibitor purity was evaluated by analytical reversed-

phase HPLC. Prior to biological testing, all compounds were subject to fluorescence polarization and/or luciferase inhibitor screening as described.

Luciferase inhibitor screen

To detect endogenous STAT3 activity, AR230[R] cells were transduced with the pGreenFire Lenti-Reporter system (pGF1; System Biosciences) harboring two sequential STAT3-inducible elements (SIE) or mutated negative control (NEG) sequences (Supplementary Figure 3b). AR230[R] cells (3×10^5) expressing either pGF1-SIE (AR230[R]-SIE) or pGF1-NEG (AR230[R]-NEG) were exposed to imatinib (1.0 μM) and/or STAT3 inhibitors (5-10 μM) for 6 hr, followed by detection of luciferase reporter activity. See Statistical Analyses and Supplementary Materials and Methods for details.

Docking simulations

The crystal structure of the STAT3B-DNA complex (PDB entry 1BG1) utilized for docking was prepared with Protein Preparation Wizard. Initial docking simulation was performed using Glide Extra Precision (GlideXP module, version 5.7) (Suite 2012: LigPrep, version 2.5, Schrödinger, LLC, New York, NY, 2012), followed by induced fit docking simulation[12]. Residues within 7 Å of the initial binding pose were optimized by side chain reorientations in Prime module. Receptor and ligand van der Waals spheres were scaled by a factor of 0.5 to allow unusual contacts, then refined by Prime module to readjust orientation. See Supplementary Materials and Methods.

STAT3 analysis by site-specific TRESI-MS/HDX

STAT3 residues 127-688 (pET15b_STAT3, provided by Dr. Rob Laister) was subcloned into pMAL-c5X (New England Biolabs) to generate an N-terminal MBP-tagged fusion. MBPSTAT3[(127-688)] was expressed in *E. coli* BL21(DE3) and purified by amylose-affinity chromatography. MBP-STAT3[(127-688)] samples were prepared for mass spectrometry (MS) by buffer exchange into 100 mM ammonium acetate (pH 7.5) on a Vivaspin 20 (GE Healthcare). BP-5-087 (200 mM) was dissolved in DMSO. MBP-STAT3[(127-688)] (80 μM) was incubated with or without BP-5-087 (600 μM) for 2 hr on ice. Site-specific time-resolved electrospray ionization mass spectrometry (TRESI-MS) and hydrogen-deuterium exchange (HDX) was conducted on a microfluidic device[13] as described in Supplementary Materials and Methods.

Long-term culture-initiating cell (LTC-IC) assays

Following 96 hr culture +/− imatinib (2.5 μM) and/or BP-5-087 (1 μM), in the absence of cytokines, 5×10^3 viable CD34^{+} cells were plated in MyeloCult (H5100; Stem Cell Technologies) on top of irradiated (80 Gy) M210B4 cells in duplicate LTC-IC assays as described[14, 15]. Following 6 weeks of culture, cells were trypsinized, plated into methylcellulose colony assays (H4435; Stem Cell Technologies), and scored after 18 days. Colony numbers were adjusted to reflect the total number of viable LTC-ICs present following the 96 hr culture. BCR-ABL1^{+} colonies were identified by qRT-PCR for *BCR-ABL1* mRNA[16].

Cytospin and immunofluorescence

CML^{CD34+} cells were cultured for 24 hr in the indicated conditions prior to cytospin. Cells were fixed, permeabilized, and incubated with rabbit anti-pSTAT3^{Y705} (Cell Signaling Technologies), followed by detection using an AlexaFluor 594-conjugated goat anti-rabbit IgG (Invitrogen). Slides were examined using a Nikon Eclipse E600 equipped with a CRI Nuance multispectral imaging system (model N-MSI-420-FL).

Statistical analyses

A two-tailed Student's t test was used for assays with identical cell lines and for immunoblot densitometry. Luminescence of SIE and NEG constructs were assessed in triplicate for 74 inhibitors and standardized to 6 measures of luciferase control for a given construct in each run. A total of three such runs were independently performed. Luciferase controls were assessed for normality in each construct/run. One construct in the third run had a wide bimodal distribution, and was hence excluded from analyses based on non-uniformity of controls. Average values for each inhibitor's effects on SIE and NEG constructs were calculated and plotted to identify those with the most potent (assessed by a high negative SIE luminescence value) and selective (assessed by a high NEG value) luciferase inhibition. Patient CML^{CD34+} colony data was analyzed using Welch's t-test for unequal variances. Data were considered statistically different when p values were <0.05. For MTS assays, three distinct runs each with 4 replicates per concentration were performed on unique plates with untreated controls. Median values for each concentration were calculated as a percentage of the plate's control. IC$_{50}$ values were calculated from a 4-parameter variable-slope logistic equation: $y = min + \frac{(max-min)}{\left(1 + 10^{((x - log\ IC_{50}) \cdot HillSlope)}\right)}$ and fit by Prism Software. Significant differences in IC$_{50}$ run values between inhibitors was calculated by Welch's t-test.

RESULTS

STAT3 is activated in BCR-ABL1 kinase-independent TKI resistance

TKI resistance in CML occurs through reactivation of BCR-ABL1 by kinase domain mutations, or through mechanisms allowing survival despite continued BCR-ABL1 inhibition, known as kinase-independent resistance. The latter may be caused through activation of alternative signaling pathways by *extrinsic*, BM-derived factors, or through *intrinsic*, cell-autonomous mechanisms. To model *extrinsic* resistance, we cultured CML^{CD34+} cells from newly diagnosed patients or parental TKI-sensitive cell lines (K562^{S} and AR230^{S}) in HS-5 CM[9, 10]. To model *intrinsic* resistance without BM-derived factors, we used CML^{CD34+} cells from patients with treatment failure on two or more TKIs, as well as TKI-resistant CML cell lines adapted for growth in the presence of 1.0 μM imatinib (K562^{R} and AR230^{R}). These cells demonstrate cross-resistance to nilotinib and dasatinib, thereby modeling resistance to multiple TKIs as observed in our patient samples (Supplementary Figures 1 and 2). All cells express exclusively native *BCRABL1* and therefore harbor no detectable kinase domain mutations. For details on primary samples see Supplementary Table 1.

TKI-sensitive or TKI-resistant CML cell lines and primary CML^{CD34+} cells were cultured in RM or HS-5 CM +/− imatinib. To identify active signaling pathways common to BCR-ABL1 kinase-independent resistance, phosphorylated versions of canonical signaling proteins were examined by immunoblot analyses. Regardless of TKI sensitivity, imatinib suppressed BCRABL1 kinase activity (Figure 1a; Supplementary Figure 3a), verifying kinase-independent resistance. As expected, in *extrinsic*, BM-derived TKI resistance, pSTAT3^{Y705} levels were elevated in parental CML^{CD34+} cells from newly diagnosed CML patients, as well as K562^S and AR230^S cells, when cultured in HS-5 CM compared to RM in the presence of imatinib (Figures 1a and 1b; Supplementary Figures 3b and 3c). In contrast, imatinib markedly reduced pSTAT5^{Y694} and pSTAT3^{S727} levels in both RM and HS-5 CM.

Similar to *extrinsic* resistance, pSTAT3^{Y705} levels were elevated in CD34$^+$ cells from TKI-resistant compared to treatment-naïve newly diagnosed CML patients, in the absence of exogenous cytokines (Figures 1a and 1b). Three of five samples from newly diagnosed patients had no detectable levels of pSTAT3^{Y705} when cultured in RM, whereas pSTAT3^{S727} and total STAT3 were readily detectable. In contrast, pSTAT3^{Y705} was highly detectable in all five TKI-resistant samples analyzed, in both the presence and absence of imatinib. However, not all samples were run on the same blot, precluding direct quantitative comparisons between newly diagnosed and TKI-resistant samples. In contrast to pSTAT3^{Y705}, imatinib reduced the levels of pSTAT5^{Y694} and pSTAT3^{S727} in both newly diagnosed and TKI-resistant CML cells, implying that these sites remain under the control of BCR-ABL1 (Figures 1a and 1b). In *intrinsically* TKI-resistant K562^R and AR230^R cell lines, imatinib suppression of BCR-ABL1 kinase activity also correlated with increased pSTAT3^{Y705} levels compared to parental K562^S and AR230^S controls under the same conditions (Supplementary Figure 3a). Importantly, pSTAT3^{Y705} activation was maintained in K562^R and AR230^R cells treated with imatinib, nilotinib, or dasatinib (Supplementary Figure 2). K562^R and AR230^R cells demonstrated increased levels of the SRC family kinases, pSRC^{Y416} and pLYN^{Y507}, which decreased with dasatinib in a concentration-dependent manner (Supplementary Figure 2). In both cell lines, dasatinib resulted in a partial reduction of pSTAT3^{Y705} that was not further reduced with escalating dasatinib concentrations, consistent with partial but not full dependence on SRC family kinases (Supplementary Figure 2). pJAK2$^{Y1007/1008}$ was also elevated in K562^R and AR230^R cells in the absence of TKIs, but were reduced to low levels in the presence of TKIs (Supplementary Figure 2), implying that JAK2 is not directly involved in STAT3 activation.

Altogether, we conclude that CML cells with *intrinsic* TKI resistance activate pSTAT3^{Y705} upon BCR-ABL1 inhibition in the absence of BM-derived factors, suggesting that pSTAT3^{Y705} represents a point of convergence for *extrinsic* and *intrinsic* resistance pathways promoting kinase-independent resistance.

STAT3 inhibition reduces survival of TKI-resistant CML cell lines and primary CML^{CD34+} progenitor cells

To determine whether STAT3 confers TKI resistance, we used retroviral or lentiviral delivery of shRNA targeting STAT3 (shSTAT3). shRNA-mediated knockdown of STAT3

and not STAT5 was confirmed by immunoblot analyses (Figures 2a and 2b). We initially showed that STAT3 is required for *extrinsic* resistance by infecting parental K562^S cells with a puromycin-selectable vector harboring shSTAT3 or scrambled control (shSCR), followed by culture in HS-5 CM or RM +/− imatinib. As expected, shSTAT3 reduced colony formation and increased apoptosis of K562^S cells following culture in HS-5 CM but not RM, thereby abolishing the protective effects of BM-derived factors (Supplementary Figures 4a and 4b). Consistent with STAT3 activation in *intrinsic* TKI resistance (Figure 1), shSTAT3 reduced the clonogenicity of K562^R and AR230^R cells by 50-60% in the presence of 1.0 or 2.5 μM imatinib, with no effect in TKI-sensitive parental controls (Figures 2a and 2b). We then functionally inhibited STAT3 using dominant-negative mutants (dnSTAT3)[17, 18], both a C-terminal truncated mutant[17] and a STAT3-Y705F mutant[18], yielding the same results. Similar to shSTAT3, dnSTAT3 reduced colony formation of K562^R and AR230^R cells by ~30-60%, without significant effects on parental TKI-sensitive controls, and this was correlated with reduction of pSTAT3^{Y705} but not pSTAT5^{Y694} (Figures 2c and 2d). We consistently observed a reduction of colony formation upon imatinib withdrawal from K562^R and AR230^R cells. This is reminiscent of previous observations in imatinib-resistant Ba/F3 cells, where increased cell death was observed following drug withdrawal[19].

To pharmacologically target STAT3 in TKI resistance, we used S3I-201.1066 (SF-1-066), a salicylic acid-based STAT3 inhibitor[20]. SF-1-066 was previously shown to interact with the STAT3 SH2 domain, to reduce phosphorylation at Y705, and to reduce DNA-binding in human breast and pancreatic cancer cells[21, 22]. We reasoned that this inhibitor, while not sufficiently potent for clinical applications, would facilitate direct pharmacologic targeting of STAT3 in primary BCR-ABL1 kinase-independent resistance. SF-1-066 (10 μM) in combination with imatinib (1.0 μM) reduced colony formation of TKI-resistant K562^R and AR230^R cells by ~60-70%, with no significant effects on parental TKI-sensitive controls (Figures 2e and 2f). These data confirm the selectivity of SF-1-066 for STAT3 over STAT5, since STAT5 inhibition is expected to kill TKI-sensitive CML cells[23, 24]. Next we tested the effect of SF-1-066 in CML^{CD34+} cells from newly diagnosed patients. Cells were cultured for 96 hr in HS-5 CM or RM, without additional cytokines, +/− SF-1-066 (10 μM) and/or imatinib (2.5 μM). In HS-5 CM, SF-1-066 in combination with imatinib reduced colony formation of CML^{CD34+} cells by 42.5% compared to imatinib alone, thereby abrogating its protective effects (Figure 2g). However, SF-1-066 resulted in slight inhibition of cells cultured in RM (Figure 2g), and also impaired colony formation by mononuclear cells (MNCs) from healthy individuals (Figure 2g). These results provide proof of principle for synthetic lethality by combined inhibition of STAT3 and BCRABL1 in primary kinase-independent TKI resistance. However, the high dose of SF-1-066 (10 μM) required to achieve an effect, along with inhibition of normal MNCs, prompted us to identify more potent and selective STAT3 inhibitors.

Design, synthesis, and biochemical validation of STAT3 inhibitors

To identify more potent and selective STAT3 inhibitors, we employed an iterative SAR study to interrogate the effects of functional group alterations on the activity of SF-1-066 (Supplementary Figure 5a). STAT3 SH2-domain binding was assessed using fluorescence

polarization (FP) assays, and cellular activity was evaluated in a luciferase reporter assay designed to quantify endogenous STAT3 transcriptional activity in TKI-resistant AR230^R cells. AR230^R cells were lentivirally transduced with a reporter construct harboring either two STAT3-inducible elements (AR230^R-SIE) or a negative control reporter with two mutated STAT3 binding sequences (AR230^R-NEG) (Supplementary Figure 5b). A difference in luminescence intensity in AR230^{R}SIE cells indicates a change in potency compared to SF-1-066, whereas a difference in AR230^R NEG cells indicates a change in selectivity.

We synthesized 74 putative STAT3 inhibitors and screened them in FP and/or luciferase reporter assays. We initially examined 10 STAT3 inhibitors based on SF-1-066 (**1**) and the more recent derivative, BP-1-102 (**2**)[25, 26], in our luciferase assay at 10 μM. These compounds possessed additional functionality on the sulfonamide nitrogen position, designated as R_1 (Supplementary Figure 5c)[27]. This initial screen revealed that substituting the sulfonamide sulfur position (designated as R_2) with a pentafluorobenzene group (such as **2a**) resulted in increased potency in AR230^R-SIE cells (Supplementary Figure 5d), but also demonstrated activity in the AR230^R-NEG cells, indicating a lack of selectivity. By contrast, the R_1=pentafluorobenzyl substituent (such as **1a**) increased inhibitor potency in AR230^R-SIE cells without sacrificing selectivity in AR230^R-NEG cells (Supplementary Figure 5d). To capitalize on this advance, we synthesized a library of 24 analogues incorporating the R_1=pentafluorobenzyl group, imparting structural diversity at the R_2 position (Figure 3a). Using FP for the initial screen, we selected molecules with 4-fluorobenzene and 4-trifluoromethylbenzene in the R_2 position for further modification. A focused library of compounds containing the best R_2 substituents was also synthesized to incorporate other promising R_1 substituents. Figure 3b summarizes the FP EC_{50} values and luciferase data for select compounds categorized by functional group. Compounds with enhanced potency and selectivity were identified based on a substantial difference ( ) in luminescence intensity for the AR230^R-SIE cells and little difference in the AR230^R-NEG cells (Figures 3b). Based on potency and selectivity, BP-5-087 (**16d**) was selected for testing in the context of BCR-ABL1 kinase-independent resistance.

BP-5-087 interacts with the SH2 domain of STAT3

To confirm binding to the STAT3 SH2 domain, we performed high resolution computational docking simulations. Modeling was first performed using the Glide Extra Precision (GlideXP) algorithm, and the estimated docking scores for BP-5-087 and SF-1-066 were −4.9 kcal/mol and −3.8 kcal/mol, respectively. A more negative docking score for BP-5-087 reflects a higher propensity for ligand binding. In the second stage of simulation, Glide induced-fit docking was used to consider the inherent flexibility of the STAT3 SH2 domain, which we observed as thermal fluctuations in X-ray crystallography experiments (Supplementary Figures 6a and 6b). This significantly lowered the docking scores for BP-5-087 (−9.6 kcal/mol) and SF-1-066 (−7.6 kcal/mol), with the corresponding binding poses displayed in Figures 4a-e. As expected, the salicylic acid moiety common to BP-5-087 and SF-1-066 occupied the site in which the charged phosphotyrosine (pY) of a second STAT3 monomer normally binds, engaging in hydrogen bonding with R609 and S613. Both compounds displayed similar interactions with the hydrophobic site including W623, V637,

and Y657, as well as hydrogen bonding with the amine group of K591. Although BP-5-087 and SF-1-066 both interact with T622, I634, and R595, the degree of induced fit observed in the two cases was remarkably distinct. Upon BP-5-087 binding, the side chain of R595 reorients, creating a more evident hydrophobic pocket. In addition, molecular modeling suggests that the R_1=2-methylbenzyl group present in BP-5-087 may provide a stabilizing intramolecular aromatic interaction with the R_2=4-trifluoromethylbenzene group, which may enhance inhibitor rigidity.

To further map the STAT3 amino acid residues important for compound action, binding of BP-5-087 to the STAT3 SH2 domain was experimentally confirmed by analyzing changes in site-specific deuterium uptake in STAT3 upon BP-5-087 binding. Specifically, we employed time-resolved electrospray ionization mass spectrometry (TRESI-MS) and hydrogen-deuterium exchange (HDX)[28] to characterize the structural transitions that occur to STAT3 upon BP-5-087 binding. In three independent replicates, these data confirmed that the binding epitope for BP-5-087 is indeed located within the STAT3 SH2 domain (Figures 4f and 4g). Fold change in deuterium uptake was analyzed for 68 peptic peptides of STAT3, generating a 71% sequencing coverage, and mapped onto the X-ray crystal structure of STAT3 (Figure 4f). Significant decreases in deuterium uptake clustered almost exclusively to the STAT3 SH2 domain, indicating exclusion of solvent molecules or the formation of new backbone hydrogen bonds in this region (Figure 4g). A number of significant decreases in deuterium uptake were observed in SH2 domain regions proximal to the predicted BP-5-087 sub-pockets, which may result from allosteric changes in STAT3 induced by drug binding. A model for the proposed mechanism of action for BP-5-087 is presented in Supplementary Figure 6c.

BP-5-087 targets TKI-resistant CML^{CD34+} stem and progenitor cells

Similar to the effects of shSTAT3 or dnSTAT3 on apoptosis of AR230^R cells (Supplementary Figure 7a), BP-5-087 (1.0 μM) increased apoptosis of AR230^R cells by 13.7% compared to SF-1-066, which had no significant effect even at 10 μM (Supplementary Figure 7b). Importantly, BP-5-087 had no effect on parental AR230^S cells (Supplementary Figure 7a) or CD34$^+$ progenitor cells from healthy individuals (Figure 5a; Supplementary Figure 7c), demonstrating a substantial improvement over the parent compound, SF-1-066.

We first assessed the effects of BP-5-087 in primary CML^{CD34+} cells from newly diagnosed patients cultured in HS-5 CM. BP-5-087 had little effect on treatment-naïve patient cells cultured in RM, indicating no off-target effects on STAT5. However, BP-5-087 in combination with imatinib reduced colony formation of cells grown in HS-5 CM by 56%, thereby abrogating its protective effects (Figure 5b). We next analyzed the effects of BP-5-087 and imatinib on STAT3 phosphorylation and subcellular localization in CML^{CD34+} progenitors by immunofluorescence. As expected, CML^{CD34+} cells from newly diagnosed patients showed high levels of nuclear pSTAT3^{Y705} when cultured in HS-5 CM, but not in RM (Figure 5c). In contrast, CML^{CD34+} cells from TKI-resistant patients demonstrated high levels of pSTAT3^{Y705} in the absence of BM-derived factors. In both

cases, BP-5-087 reduced the overall levels of nuclear $pSTAT3^{Y705}$, with the remaining signal located within the cytoplasm (Figure 5c).

To assess the effects of BP-5-087 in *intrinsic* TKI resistance, CML^{CD34+} cells from TKI-resistant patients were cultured in BP-5-087 or SF-1-066 (1-10 μM) +/− imatinib (2.5 μM), and analyzed for colony formation following drug exposure. BP-5-087 in combination with imatinib reduced colony formation and increased apoptosis of TKI-resistant CML^{CD34+} progenitors as low as 1.0 μM, a marked improvement in potency compared to SF-1-066 (Figures 5d and 5e). These data show that BP-5-087 exhibits increased potency over SF-1-066, without compromising selectivity, and without toxic effects to normal controls.

Ex vivo studies have shown that CML LSCs are not 'addicted' to BCR-ABL1 kinase activity and survive despite BCR-ABL1 inhibition[7, 8]. To assess STAT3 activation in the relevant stem and progenitor cell populations, we used CD38 to distinguish between primitive ($CD34^+38^-$) and mature ($CD34^+38^+$) CML progenitor cells (Figure 6a). FACS-sorted cells from newly diagnosed or TKI-resistant CML patients were treated with imatinib (2.5 μM) for 4 hr followed by immunofluorescence for $pSTAT3^{Y705}$. No significant $pSTAT3^{Y705}$ was detected in untreated $CD34^+38^-$ cells from newly diagnosed or TKI-resistant patients. However, in $CD34^+38$-cells from TKI-resistant patients, imatinib markedly induced nuclear and cytoplasmic $pSTAT3^{Y705}$, whereas levels remained low in samples from newly diagnosed patients (Figure 6a). To determine whether BP-5-087 targets this primitive cell population, we performed long-term culture-initiating cell (LTC-IC) assays on CML^{CD34+} cells from newly diagnosed and TKI-resistant patients. Following *ex vivo* exposure to BP-5-087 (1.0 μM) +/− imatinib (2.5 μM), cells were cultured on irradiated M210B4 stroma for 6 weeks and plated in colony forming assays as described[15, 29]. BP-5-087 had no effect on LTC-IC survival of normal cord blood $CD34^+$ cells (Figure 6b, *left*). In samples from newly diagnosed CML patients, BP-5-087 reduced the number of LTC-IC colonies alone and in combination with imatinib to 69.9% and 61.6% of untreated controls, respectively (Figure 6b, *middle*). In samples from TKI-resistant CML patients, neither BP-5-087 nor imatinib alone had any effect on LTC-IC survival, whereas dual treatment reduced LTC-IC colonies to 34.2% of controls (Figure 6b, *right*). All TKI-resistant LTC-ICs were positive for *BCR-ABL1*, consistent with the low number of normal LTC-ICs that characterizes advanced CML. Altogether, these data suggest that LSCs from CML patients with kinase-independent resistance activate STAT3 upon challenge with imatinib, and that BP-5-087 may be a novel therapeutic approach for eradicating this TKI-resistant stem cell population (Figure 7).

DISCUSSION

BCR-ABL1 kinase-independent TKI resistance is associated with constitutive activation of various signaling pathways, including SRC family kinases[30-33], STAT5[34], PI3K/AKT[35], and Wnt/β-catenin[36-38], but no uniform picture has emerged[6]. Furhermore, STAT3 activation by BM-derived factors confers TKI resistance to CML progenitor cells[10, 39]. Here, we demonstrate that STAT3 activation is a key feature of primary CML stem and progenitor cells with kinase-independent resistance. Using genetic, functional, and pharmacologic inhibition, we demonstrate that STAT3 inhibition in combination with BCR-

ABL1 reduces survival of TKI-resistant CML stem and progenitor cells, highlighting a critical role for STAT3. Previous reports have implicated STAT5 in TKI resistance[34, 40]. However, in our patient specimens, pSTAT5^{Y694} remained under the control of BCR-ABL1 kinase activity (Figure 1). Importantly, pSTAT3^{Y705} was the only signaling node activated in both the presence and absence of BM-derived factors (Figure 1; Supplementary Figures 3 and 8). These data are consistent with a model whereby STAT3 is initially activated in CML stem cells through interaction with the BM microenvironment. However, upon long-term TKI challenge, cell-autonomous resistance develops when malignant cells establish *intrinsic* mechanisms to further activate STAT3 without a requirement for BM-derived factors (Figure 7).

STAT3 activation is implicated in malignant transformation and drug resistance in a variety of cancers[41]. In some cases, inactivation of negative STAT3 regulators has been demonstrated[42, 43]. In others, STAT3 is activated by autocrine production of IL-6[44] or through acquired activating mutations[45, 46]. SRC family kinases are known to activate STAT3[47], and have also been linked to imatinib resistance in CML cell lines and patient samples[30-32, 48, 49]. In both K562^R and AR230^R cells, treatment with dasatinib resulted in partial reduction of pSTAT3^{Y705}, suggesting partial but not full dependence on SRC family kinases (Supplementary Figure 2). Since multiple mechanisms are known to activate STAT3, directly targeting STAT3 rather than upstream pathways is an attractive therapeutic approach[50]. Unlike classical enzyme active sites, the STAT3 transcription factor lacks a defined binding pocket, and relies on non-contiguous interactions across large surface areas for affinity with binding partners. The STAT3 SH2 domain is primarily hydrophobic, with a hydrophilic sub-pocket that binds to phosphotyrosine peptide sequences, most notably the one presented by its partner in the STAT3:STAT3 dimer. Precise placement of a small-molecule inhibitor within the STAT3 SH2 domain should therefore block SH2-dependent dimer formation, a step subsequent to phosphorylation by kinases such as JAK or SRC[51]. Incorporating drug-like characteristics into SH2 domain binders is challenging; however, development of a potent STAT3 inhibitor will have therapeutic value for treatment of many different diseases, including TKI-resistant CML. We developed a number of lead compounds to optimize STAT3 inhibitor potency and selectivity. Our high throughput screening system allowed us to evaluate STAT3 binding affinity in biochemical FP assays and in a cellular context with luciferase reporter assays (Supplementary Figure 5a). Beginning with the parent compound, SF-1-066[20], we used SAR-based drug design and compound library screening to identify BP-5-087 as a potent and selective salicylic acid-based STAT3 inhibitor with activity against TKI-resistant CML. Using a computational induced-fit docking approach, the enhanced potency of BP-5-087 was traced to reorientation of the R595 side chain within the binding site (Figure 4), resulting in optimized inhibitor affinity. Importantly, TRESI-MS/HDX experiments precisely mapped binding of BP-5-087 to the STAT3 SH2 domain.

BP-5-087 exerts effects on TKI-resistant CML stem and progenitor cells at 1.0 μM, representing a 10-fold or greater improvement in potency compared to SF-1-066, and a marked improvement to other recently published STAT3 inhibitors[26,52,53, 54,55,56,57]. The combination of BP-5-087 and imatinib was required to reduce survival of CML progenitors

and LTC-ICs from patients with kinase-independent resistance, suggesting that a situation of synthetic lethality is required to target these cells. The term synthetic lethality, while traditionally a genetics term, is more recently being used to describe combinatorial anticancer therapeutics[6]. In this particular case, the combined inhibition of both BCR-ABL1 and STAT3 is required to kill CML stem and progenitor cells with kinase-independent TKI resistance, while inhibition of only BCR-ABL1 or only STAT3 has very limited effects, consistent with a synthetically lethal situation.

In summary, our data unveil a novel mechanism of kinase-independent TKI resistance in primary CML stem and progenitor cells, and suggest that the STAT3 inhibitor, BP-5-087, intercepts survival signals that are *intrinsic* and *extrinsic* to the CML LSC. BP-5-087 may therefore have utility for the treatment of TKI-resistant CML and other diseases characterized by STAT3 activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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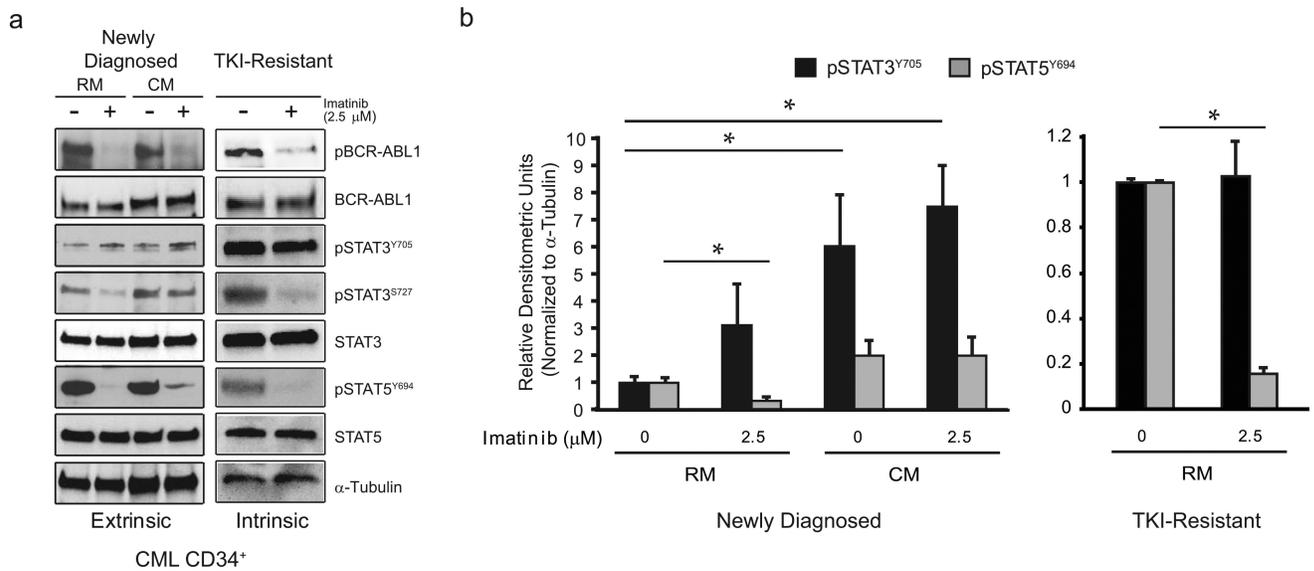
Figure 1. pSTAT3^{Y705} is activated in TKI-resistant CML cells in the presence of imatinib
(**a**) CML CD34$^+$ cells from newly diagnosed or TKI-resistant patients lacking BCR-ABL1 kinase domain mutations were cultured in RM or HS-5 CM with or without 2.5 μM imatinib for 24 hr followed by immunoblot with the specified antibodies. Activated pBCR-ABL1 was detected using a phosphotyrosine-specific antibody. The dose of imatinib was chosen to achieve near complete suppression of BCR-ABL1 kinase activity. pSTAT3^{Y705} was elevated in CD34$^+$ cells from newly diagnosed patients when cultured in HS-5 CM (n=5), and in CD34$^+$ cells from TKI-resistant patients (n=5) in the presence of imatinib. TKI-sensitive CD34$^+$ cells cultured in RM (n=5) were examined as controls. (**b**) Data presented in panel **a** are quantified by densitometry for pSTAT3^{Y705} and pSTAT5^{Y694} for both newly diagnosed (n=4) and TKI-resistant (n=5) patients. Error bars represent SEM. *p<0.05.
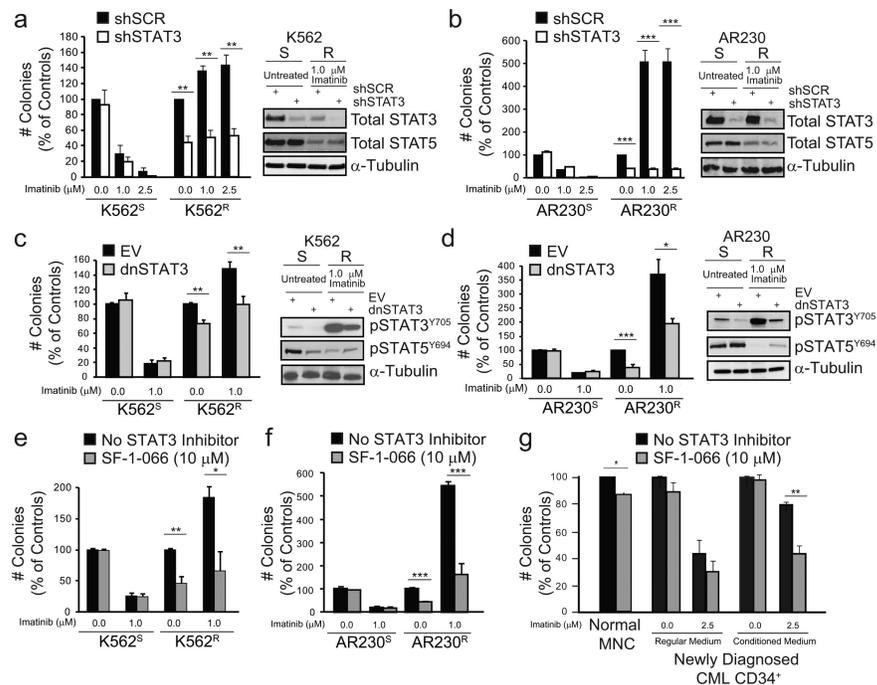
Figure 2. Inhibition of STAT3 reduces colony formation by TKI-resistant CML cells
(**a** and **b**) TKI-resistant CML cell lines were retrovirally transduced with shRNA targeting STAT3 (shSTAT3) or scrambled control (shSCR), and cultured in semisolid medium +/− imatinib (1.0-2.5 μM). STAT3 and not STAT5 knockdown was confirmed by immunoblot analyses (**a** and **b**, *right*). shSTAT3 reduced colony formation of K562^R (**a**, *left*, n=4) and AR230^R (**b**, *left*, n=4) cells in the presence of imatinib, with no effect on parental TKI-sensitive controls. (**c** and **d**) TKI-resistant CML cell lines were transduced with dominant-negative STAT3 mutants (dnSTAT3) or empty vector (EV) and cultured in semisolid medium +/− imatinib (1.0 μM). Inhibition of pSTAT3^{Y705} was confirmed by immunoblot analyses (**c** and **d**, *right*). dnSTAT3 reduced colony formation of K562^R (**c**, *left*, n=4) and AR230^R (**d**, *left*, n=3) cells with no effect on parental TKI-sensitive controls (n=2). (**e** and **f**) K562^R (**e**, n=4) and AR230^R (**f**, n=4) cells were incubated in methylcellulose semisolid medium with SF-1-066 (1-10 μM) +/− imatinib (1.0 μM). SF-1-066 reduced colony formation of only TKI-resistant and not TKI-sensitive cells. (**g**) Mononuclear cells (MNCs) from peripheral blood of normal donors (n=2) or CML^{CD34+} cells from newly diagnosed patients (n=4) were treated *ex vivo* with SF-1-066 (10 μM) +/− imatinib (2.5 μM) in RM or HS-5 CM for 96 hr followed by colony forming assays. All data are represented as percent of controls. Error bars represent SEM. *p<0.05; **p<0.01; ***p<0.001.
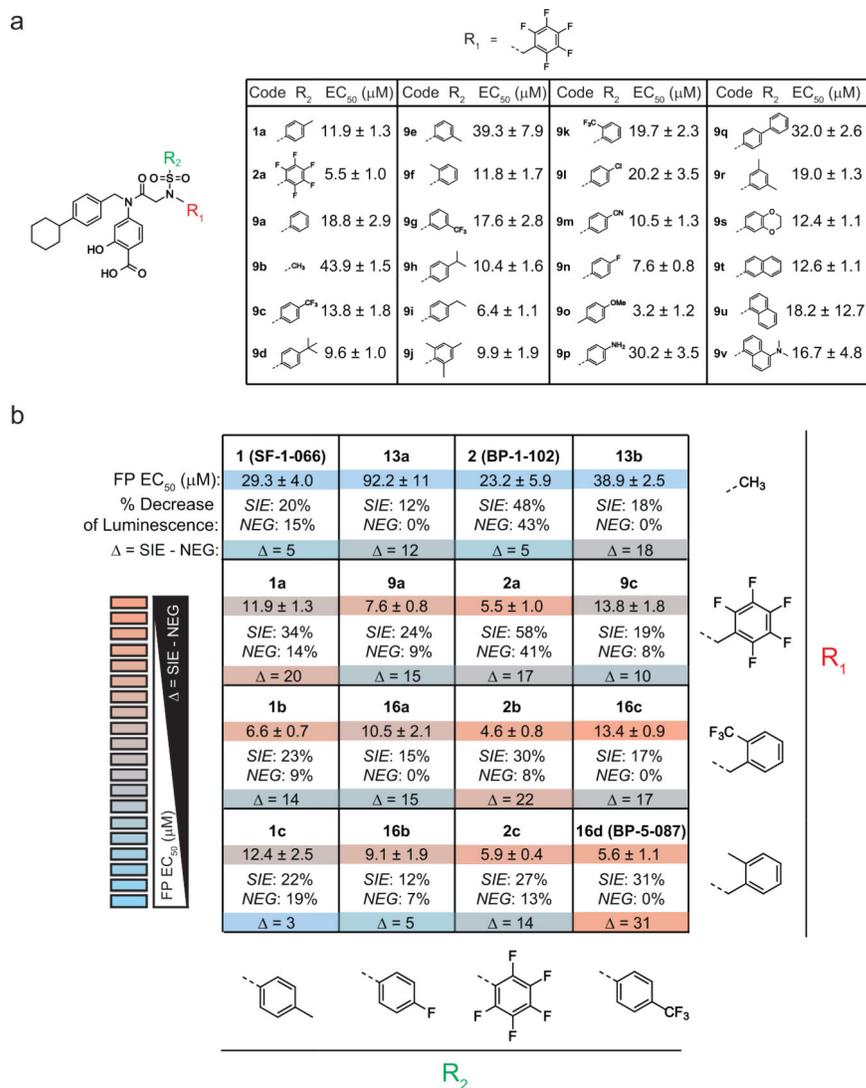
Figure 3. A STAT3 compound library screen identifies compounds with greater potency and selectivity than SF-1-066

(**a**) A library of 24 putative STAT3 inhibitors was synthesized to incorporate the R_1=pentafluorobenzyl group, imparting structural diversity at the R_2 position. Values represent the EC_{50} of each molecule in FP assays. (**b**) Subsequent STAT3 inhibitor libraries were compared by both FP and luciferase reporter assays. For the luciferase assay, TKI-resistant AR230[R] cells were transduced with a luciferase reporter harboring sequential STAT3-inducible elements (AR230[R]-SIE) or a mutated control sequence (AR230[R]-NEG) (see also Supplementary Figure 4b). For each compound, the table represents EC_{50} values as assessed by FP (*top*, n=3) and the percent inhibition that each compound achieved in AR230[R]-SIE versus AR230[R]-NEG cells at 5 μM in the presence of 1.0 μM imatinib (*bottom*, n=3).
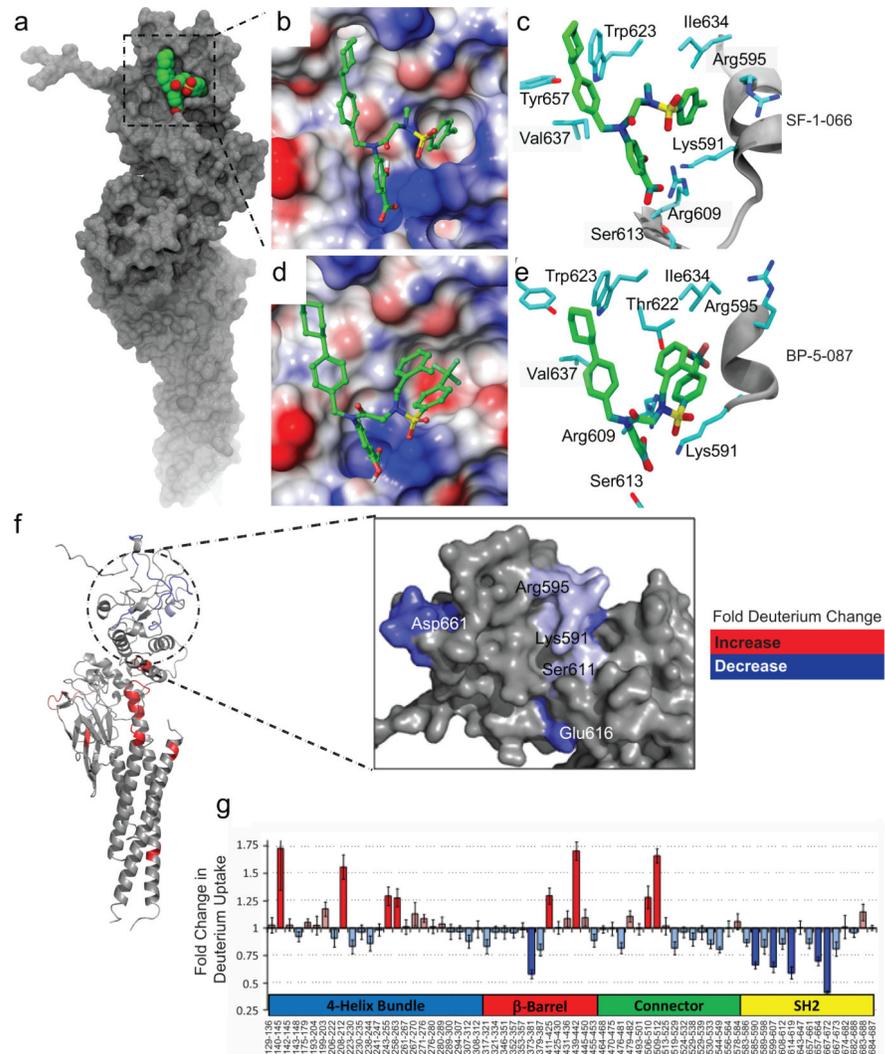
Figure 4. Computational modeling of STAT3 binding by BP-5-087

(**a**) The entire STAT3 protein is represented in grey with the SH2 domain in green and red within the boxed region. (**b** and **d**) The protein surface of the STAT3 SH2 domain bound to either SF-1-066 (**b**) or BP-5-087 (**d**) is represented depending on the electrostatic potential with color-coding ranging from red (negative charge) to blue (positive charge). (**c** and **e**) The amino acid residues of the STAT3 SH2 domain predicted to interact with SF-1-0-66 (**c**) or BP-5-087 (**e**) are also shown. Importantly, BP-5-087 reorients various residues within the binding pocket, which may optimize inhibitor complementarity. (**f**) Site-specific change in % deuterium uptake observed by TRESI-MS/HDX following BP-5-087 binding to STAT3 in a 7:1 molar ratio color-coded onto the STAT3 X-ray crystal structure (PDB ID:1BG1; *left*). The enlarged region depicts the SH2 domain on the surface of the predicted BP-5-087 binding site (*right*). (**g**) Relative changes in deuterium uptake observed by TRESI-MS/HDX following BP-5-087 binding to STAT3 and grouped by domain. Sequence coverage was 71%. Changes considered significant (>25%) are highlighted by darker colors. The most pronounced decreases in deuterium uptake were observed in peptic peptides that line the BP-5-087 salicylic acid-binding and trifluoromethylbenzene-binding sub-pockets of the SH2

domain. However, the HDX experiment sequence coverage did not extend to peptides lining the BP-5-087 cyclohexylbenzyl-binding sub-pocket. Data represent the average of three independent replicates. Error bars represent SEM.
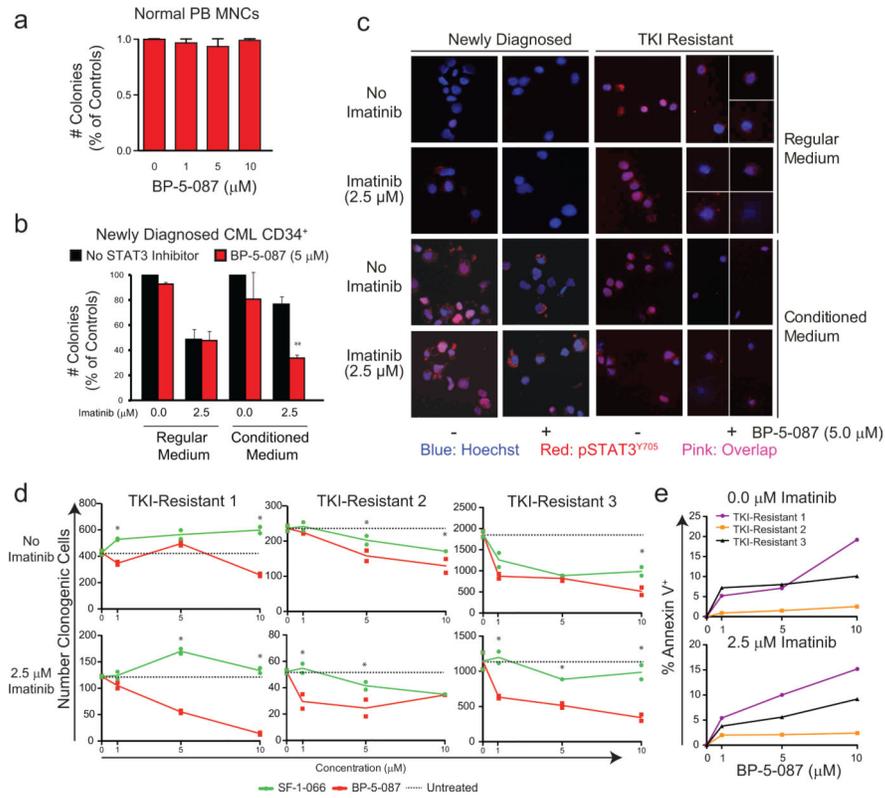
Figure 5. BP-5-087 impairs colony formation of TKI-resistant CML progenitor cells
(**a**) MNCs from healthy individuals (n=5) were plated in cytokine-supplemented
methylcellulose semisolid medium with the indicated concentrations of BP-5-087. (**b**)
CML[CD34+] cells from newly diagnosed patients (n=3) were treated *ex vivo* with BP-5-087 (5
μM) and/or imatinib (2.5 μM) for 96 hr followed by colony forming assays. Error bars
represent SEM. **p<0.01. (**c**) Aliquots of CML CD34+ cells from newly diagnosed and
TKI-resistant patients were harvested after 24 hr of treatment for immunofluorescence with
a pSTAT3[Y705] antibody. BP-5-087 reduced and excluded pSTAT3[Y705] from the nucleus in
primary cells with *intrinsic* or *extrinsic* TKI resistance, which correlated with a reduction in
colony forming ability. For TKI-resistant samples treated with BP-5-087, it was difficult to
obtain fields with more than two cells. Therefore, 2 fields are shown with white dividing
lines. One representative experiment is shown. Blue: Hoechst; Red: pSTAT3[Y705]; Pink:
Overlap. (**d** and **e**) CML[CD34+] cells from TKI-resistant (n=3) patients were treated *ex vivo*
with BP-5-087 (1-10 μM) and/or imatinib (2.5 μM) for 96 hr followed by colony forming
(**d**) and apoptosis (**e**) assays. *p<0.05. Error bars represent SEM.
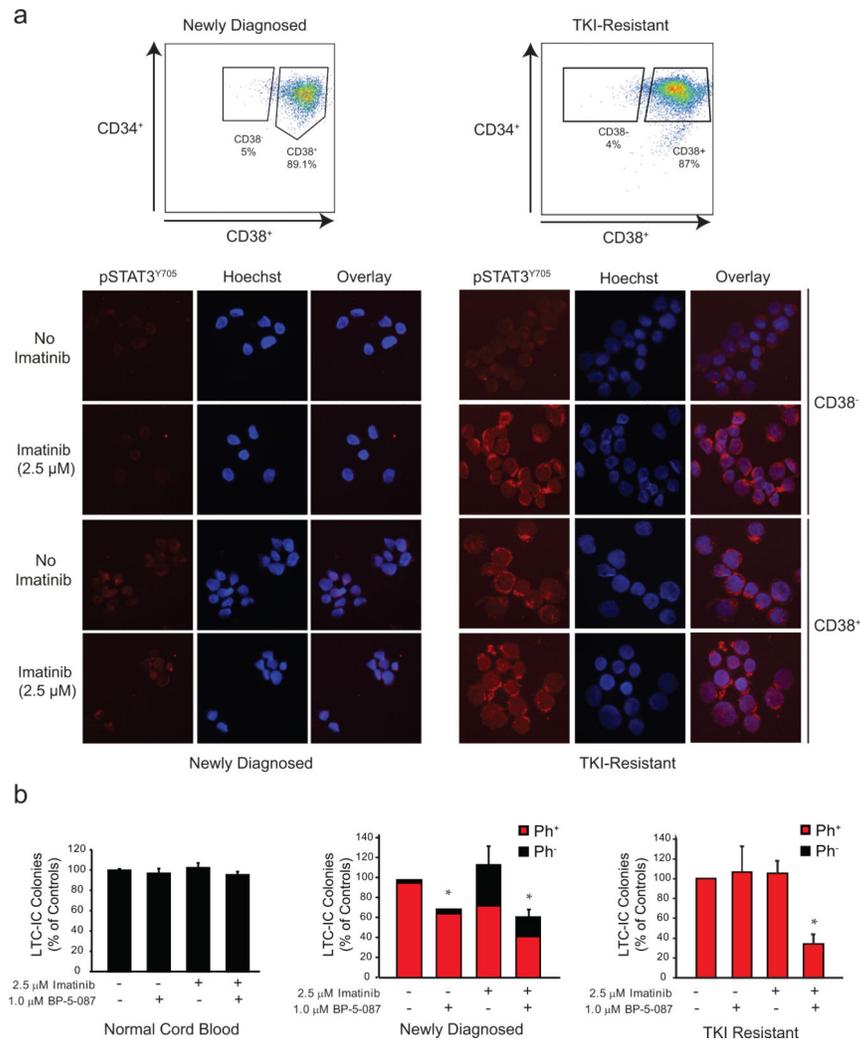
Figure 6. BP-5-087 reduces survival of CML LSCs

(**a**) CML^{CD34+} cells from newly diagnosed (n=3) or TKI-resistant (n=4) CML patients were sorted by FACS for primitive (CD34$^+$38$^-$) and mature (CD34$^+$38$^+$) cells followed by immunofluorescence with a pSTAT3^{Y705} antibody. In CD34$^+$38$^-$ cells, pSTAT3^{Y705} was only detectable in samples from TKI-resistant patients following exposure to imatinib. One representative experiment is shown. Blue: Hoechst; Red: pSTAT3^{Y705}; Pink: Overlap. (**b**) CD34$^+$ cells from normal cord blood (n=3, *left*), newly diagnosed (n=2, *middle*), or TKI-resistant (n=3, *left*) CML patients were treated *ex vivo* with BP-5-087 (1 μM) +/− imatinib (2.5 μM) in RM for 96 hr followed by plating in LTC-IC assays. Following 6 weeks of culture, remaining cells were plated in colony forming assays. Combined treatment with BP-5-087 and imatinib reduced LTC-IC survival in samples from newly diagnosed and TKI-resistant patients, but not normal cord blood. Bars represent percent of untreated controls. Ph$^+$ colonies are represented in red; Ph$^-$ colonies are represented in black. Error bars represent SEM. *p<0.05.
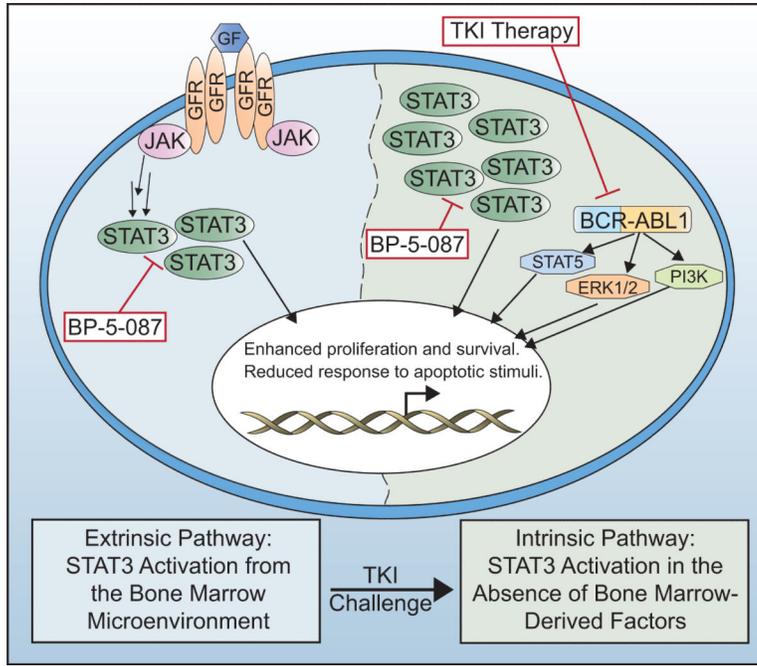
**Figure 7. Model of the molecular network regulating kinase-independent TKI resistance +/−
TKIs and the STAT3 inhibitor, BP-5-087**

In the absence of TKIs, BCR-ABL1 kinase activates canonical downstream signaling
pathways, including pSTAT3^{S727}, STAT5, ERK1/2, and PI3K, whereas pSTAT3^{Y705}
activation occurs through interaction with the BM microenvironment. Upon long-term
challenge with multiple TKIs, overt resistance develops when malignant cells establish
intrinsic mechanisms to further activate STAT3 without a requirement for BM-derived
factors. BP-5-087 is predicted to block STAT3 activation in both scenarios of TKI
resistance.