Cell
P R E S S

Nilotinib and MEK Inhibitors Induce Synthetic Lethality through Paradoxical Activation of RAF in Drug-Resistant Chronic Myeloid Leukemia

Leisl M. Packer,[1] Sareena Rana,[1] Robert Hayward,[1] Thomas O'Hare,[2] Christopher A. Eide,[3] Ana Rebocho,[1] Sonja Heidorn,[1] Matthew S. Zabriskie,[2] Ion Niculescu-Duvaz,[4] Brian J. Druker,[3] Caroline Springer,[4] and Richard Marais[1,*]
[1]Division of Cancer Biology, The Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, UK
[2]Division of Hematology and Hematologic Malignancies, Huntsman Cancer Institute, University of Utah, Salt Lake City, UT 84112-5550, USA
[3]Division of Hematology and Medical Oncology, Oregon Health & Science University Knight Cancer Institute, Howard Hughes Medical Institute, Portland, OR 97239, USA
[4]Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, 15 Cotswold Road, Sutton, Surrey SM2 5NG, UK
*Correspondence: richard.marais@icr.ac.uk
DOI 10.1016/j.ccr.2011.11.004

SUMMARY

We show that imatinib, nilotinib, and dasatinib possess weak off-target activity against RAF and, therefore, drive paradoxical activation of BRAF and CRAF in a RAS-dependent manner. Critically, because RAS is activated by BCR-ABL, in drug-resistant chronic myeloid leukemia (CML) cells, RAS activity persists in the presence of these drugs, driving paradoxical activation of BRAF, CRAF, MEK, and ERK, and leading to an unexpected dependency on the pathway. Consequently, nilotinib synergizes with MEK inhibitors to kill drug-resistant CML cells and block tumor growth in mice. Thus, we show that imatinib, nilotinib, and dasatinib drive paradoxical RAF/MEK/ERK pathway activation and have uncovered a synthetic lethal interaction that can be used to kill drug-resistant CML cells in vitro and in vivo.

INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloproliferative disease characterized by myeloid cell expansion in the bone marrow and blood (O'Dwyer and Druker, 2001). CML accounts for about 15% of adult leukemias, and there are about 5,000 cases each year in the United States. The largely asymptomatic chronic phase of CML can last several years and is followed by an accelerated phase that indicates disease progression, leading eventually to a life-threatening acute phase called blast crisis. CML has complex pathophysiology, but its diagnosis depends on the presence of the Philadelphia chromosome, a chromosome 9/chromosome 22 translocation that fuses *BCR* (encoding breakpoint cluster region) to *ABL*, which encodes the Abelson tyrosine kinase. The normal function(s) of BCR is unclear, but ABL is a cytosolic/nuclear tyrosine kinase that regulates stress responses, cell growth, and differentiation. Critically, fusion of

ABL to BCR generates a constitutively active kinase that drives transformation and leukemogenesis by phosphorylating substrates such as CRKL and STAT5 and activating pathways such as NF-κB and RAS/RAF/MEK/ERK (Deininger et al., 2000).

The clinical management of CML was revolutionized by imatinib, a small molecule ABL inhibitor (Druker et al., 2001). Imatinib mediates remission in the majority of patients with CML, but patients can develop resistance through acquired point mutations that block imatinib binding to BCR-ABL. Fortunately, most imatinib-resistant BCR-ABL mutants are sensitive to nilotinib and dasatinib, next-generation drugs that provide vital second-line treatments (Kantarjian et al., 2010). However, substitution of threonine 315 in ABL for isoleucine (BCR-ABL[T315I]) generates a protein that is resistant to all three drugs, and this mutant remains a persistent clinical problem for long-term management of CML. Pan-ABL inhibitors effective against BCR-ABL[T315I] are undergoing clinical trials (reviewed in O'Hare

Significance

Acquired drug resistance through BCR-ABL-dependent and BCR-ABL-independent mechanisms is a persistent problem for the treatment of chronic myeloid leukemia (CML). We show that some frontline CML drugs are RAF inhibitors, and they, therefore, drive paradoxical activation of BRAF and CRAF in drug-resistant CML cells. This leads to an unexpected dependency on the pathway, and accordingly, nilotinib and MEK inhibitors mediate synthetic lethal killing of these cells in vitro and in vivo. Thus, our study shows that paradoxical activation of BRAF and CRAF can drive unexpected biological responses in CML and provides an intriguing strategy that may prevent the emergence of drug resistance in patients with CML.
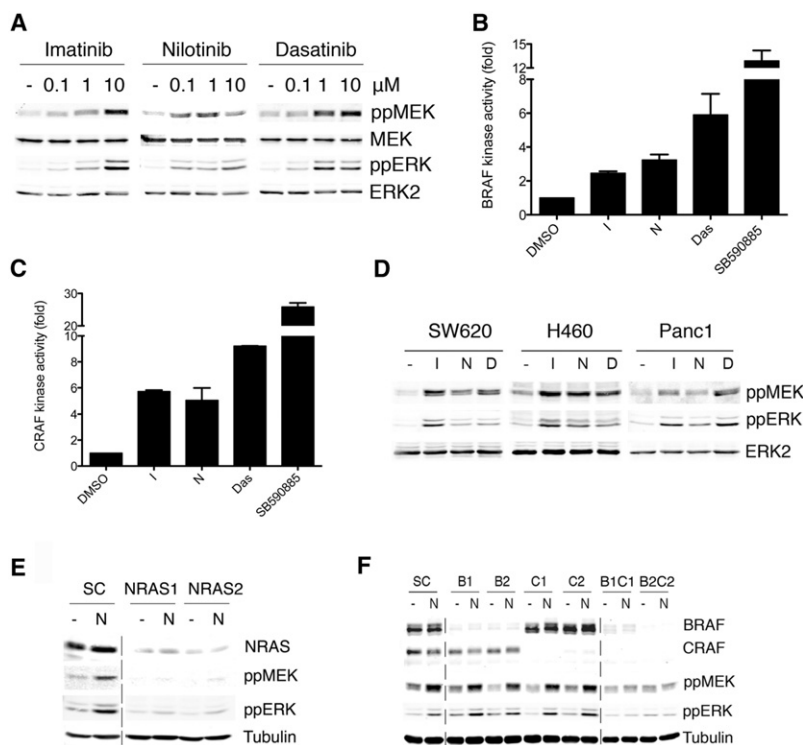
Figure 1. Imatinib, Nilotinib, and Dasatinib Activate RAF, MEK, and ERK in Cells Harboring RAS Mutations

(A) Western blot for phospho-MEK (ppMEK), MEK, phospho-ERK (ppERK), and ERK2 (loading control) in D04 cells treated with DMSO (−), imatinib, nilotinib, or dasatinib at the indicated concentrations (μM).

(B) Endogenous BRAF kinase activity in D04 cells treated with imatinib (I; 5 μM), nilotinib (N; 1 μM), dasatinib (5 μM), or SB590885 (0.1 μM) for 3 hr. Data represent means of triplicate measurements with error bars to represent SD.

(C) Endogenous CRAF kinase activity in D04 cells treated with imatinib (I; 5 μM), nilotinib (N; 1 μM), dasatinib (5 μM), or SB590885 (0.1 μM) for 3 hr. Data represent means of triplicate measurements with error bars to represent SD.

(D) Western blot for phospho-MEK (ppMEK), phospho-ERK (ppERK), and ERK2 (loading control) in SW620, H460, and Panc-1 cells treated with DMSO (−), imatinib (I; 10 μM), nilotinib (N; 1 μM), or dasatinib (D; 5 μM).

(E) Western blot for NRAS, phospho-MEK (ppMEK), phospho-ERK (ppERK), and tubulin (loading control) in D04 cells transfected with nonspecific control (SC) or two NRAS (NRAS1, NRAS2) siRNAs. The cells were treated with DMSO (−) or nilotinib (N; 1 μM) after 48 hr.

(F) Western blot for BRAF, CRAF, phospho-MEK (ppMEK), phospho-ERK (ppERK), and tubulin (loading control) in D04 cells transfected with nonspecific control (SC), or two different BRAF (B1, B2) or two different CRAF (C1, C2) siRNAs, or combinations thereof. After 72 hr the cells were treated with DMSO (−) or nilotinib (N; 1 μM) as indicated. The dotted line in (E) and (F) shows where discontinuous sections of the same blot were joined.

See also Figure S1.

et al., 2011), but compound mutants (two or more mutations in the same protein) are resistant to all current ABL inhibitors and may represent a future obstacle for CML management (O'Hare et al., 2009; Eide et al., 2011). Furthermore, patients can develop resistance that is mediated by BCR-ABL-independent mechanisms, and for these patients treatment options are limited (Bixby and Talpaz, 2011).

The RAS/RAF/MEK/ERK pathway promotes CML cell survival (Goga et al., 1995). RAS is a small membrane bound G protein, and RAF, MEK, and ERK are sequentially activated protein kinases. There are three *RAS* genes (*HRAS*, *KRAS*, and *NRAS*) in humans, and together, they are mutated in about 30% of human cancers. There are also three *RAF* genes (*ARAF*, *BRAF*, and *CRAF*), and *BRAF* is mutated in about half of melanomas and at a lower frequency in several other cancers (Wellbrock et al., 2004). BRAF inhibitors such as vemurafenib (PLX4032, RG7204) mediate dramatic responses in BRAF mutant melanoma patients, but not in BRAF wild-type patients (Flaherty et al., 2010), validating mutant BRAF as a therapeutic target in melanoma. However, these drugs also reveal an unexpected paradox because whereas they inhibit MEK and ERK in cells expressing oncogenic BRAF, they activate MEK and ERK in cells expressing oncogenic RAS (Halaban et al., 2010; Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). This is because in the presence of oncogenic RAS, BRAF inhibition drives BRAF binding to CRAF, resulting in BRAF acting as a scaffold to facilitate CRAF hyperactivation by stimulating critical events such as serine 338 (S338) phosphorylation (Hatzivassiliou et al., 2010; Heidorn et al., 2010). Paradoxical activation of the pathway can also be achieved by CRAF inhibition, which drives CRAF homodimerization in which a drug-bound partner facilitates the activation of the drug-free partner through scaffold functions or conformational changes (Poulikakos et al., 2010). Thus, under some circumstances RAF inhibitors drive paradoxical activation of BRAF and CRAF to accelerate tumorigenesis by hyperactivating MEK and ERK (Hatzivassiliou et al., 2010; Heidorn et al., 2010).

Here, we investigated if other kinase inhibitors can also drive paradoxical activation of RAF, MEK, and ERK and investigated the underlying mechanisms and potential clinical consequences.

RESULTS

Imatinib, Nilotinib, and Dasatinib Activate RAF, MEK, and ERK in RAS Mutant Cells

To initiate our study, we treated D04 cells, a melanoma line that expresses NRAS^{Q61L}, with a variety of protein kinase inhibitors and investigated their effects on the MEK/ERK pathway by measuring MEK and ERK phosphorylation by western blot. The majority of compounds tested did not affect MEK or ERK phosphorylation (see Figure S1A available online), but surprisingly, imatinib, nilotinib, and dasatinib stimulated robust MEK and ERK phosphorylation at concentrations as low as 100 nM (Figure 1A). Because the peak plasma/serum concentrations of imatinib, nilotinib, and dasatinib are ∼5 μM, 4 μM, and 90 nM, respectively (Weisberg et al., 2007; Druker et al., 2001), these data show that the drugs activate this pathway at physiologically relevant concentrations.

Imatinib, nilotinib, and dasatinib also activated BRAF and CRAF in D04 cells, albeit much less efficiently than SB590885 (Figures 1B and 1C), a BRAF selective inhibitor (Takle et al., 2006). We show that imatinib, nilotinib, and dasatinib also activated MEK and ERK in SW620 (KRAS^{G12V}) colorectal carcinoma cells, Panc1 (KRAS^{G12D}) pancreatic carcinoma cells, and H460 (KRAS^{Q61H}) lung cancer cells (Figure 1D), but not in BRAF^{V600E} expressing A2058 or A375P melanoma cells (Figure S1B). We used RNA interference (RNAi) to show that NRAS depletion blocked MEK and ERK activation in D04 cells (Figure 1E), whereas BRAF or CRAF depletion did not (Figure 1F). However, when BRAF and CRAF were both depleted, MEK and ERK activation was blocked (Figure 1F).

Imatinib, Nilotinib, and Dasatinib Induce Paradoxical Activation of the MEK/ERK Pathway by Inhibiting BRAF and CRAF

The data above show that imatinib, nilotinib, and dasatinib activate BRAF, CRAF, MEK, and ERK in RAS mutant, but not BRAF mutant, cells. We, therefore, examined directly if this was driven by the paradoxical mechanism(s) previously described. First, we show that although imatinib, nilotinib, and dasatinib activated BRAF and CRAF in cells (Figures 1B and 1C), they inhibited BRAF and CRAF in vitro (Figure 2A), their IC$_{50}$ values determined to be 1,630, 1,700, and 119 nM, respectively, for BRAF and 515, 745, and 61 nM, respectively, for CRAF.

We next examined if these drugs drove RAF dimerization. Endogenous CRAF was immunoprecipitated and western blotted for endogenous BRAF. Imatinib, nilotinib, and dasatinib all induced robust BRAF binding to CRAF in cells expressing oncogenic RAS (D04, SW620, H460, and Panc1 cells; Figures 2B and 2C), but not in cells expressing oncogenic BRAF (A2058 or A375 cells; Figure S2A). Mutations that prevented BRAF (BRAF^{R188L}) or CRAF (CRAF^{R89L}) binding to RAS (Fabian et al., 1994) blocked BRAF binding to CRAF (Figures 2D and 2E), confirming that BRAF and CRAF must bind to RAS in order to dimerize. We also examined if BRAF and CRAF formed homodimers. We expressed myc-epitope or HA-epitope tagged versions of BRAF or CRAF in D04 cells, immunoprecipitated the myc-tagged proteins and western blotted for the HA-tagged proteins, and show that both BRAF and CRAF homodimers were formed in D04 cells (Figures 2F and 2G).

To test directly if dimer formation was driven by drug binding to BRAF or CRAF, we used mutant versions of BRAF and CRAF in which the so-called gatekeeper residues were substituted with asparagine (BRAF^{T529N} and CRAF^{T421N}, respectively). We have previously shown that this mutation blocks drug binding to BRAF (Whittaker et al., 2010) and confirm here that both BRAF^{T529N} and CRAF^{T421N} were resistant to imatinib, nilotinib, and dasatinib (Figure 2A). Critically, BRAF^{T529N} and CRAF^{T421N} were severely impaired in their ability to form BRAF:CRAF heterodimers and BRAF:BRAF or CRAF:CRAF homodimers (Figures 2H–2J; Figure S2B).

Imatinib, Nilotinib, and Dasatinib Induce Paradoxical MEK/ERK Pathway Activation in Leukemia Cells Expressing BCR-ABL^{T315I}

The data above show that imatinib, nilotinib, and dasatinib are weak RAF inhibitors that drive formation of RAF hetero- and homodimers, and stimulate paradoxical activation of BRAF and CRAF in the presence of activated RAS. Previous studies have shown that imatinib activates ERK in leukemia cells expressing imatinib-resistant BCR-ABL (Yu et al., 2002; Suzuki et al., 2010; Mohi et al., 2004; Chu et al., 2004), so we tested if this was also driven through paradoxical activation of RAF. For this we used isogenic clones of murine Ba/F3 pro-B cells whose growth was driven by either BCR-ABL or BCR-ABL^{T315I} (Golub et al., 1996). We confirmed that imatinib, nilotinib, and dasatinib blocked BCR-ABL phosphorylation on tyrosine 245 (Y245) and CRKL phosphorylation on tyrosine 207 (Y207) in BCR-ABL Ba/F3 cells (Figure 3A). Furthermore, imatinib, nilotinib, and dasatinib blocked CRAF activity in these cells (Figure 3B), and consistent with this, they suppressed CRAF phosphorylation on S338 and blocked MEK and ERK activity (Figure 3A). In contrast, in BCR-ABL^{T315I} Ba/F3 cells imatinib, nilotinib, and dasatinib did not inhibit BCR-ABL or CRKL phosphorylation (Figure 3C). More importantly, in these cells all three drugs induced CRAF phosphorylation on S338 (Figure 3C) and activated CRAF (Figure 3D), MEK, and ERK (Figure 3C). Critically, we show that whereas imatinib, nilotinib, and dasatinib did not affect BRAF binding to CRAF in the BCR-ABL cells, they enhanced BRAF binding to CRAF in BCR-ABL^{T315I} Ba/F3 cells (Figures 3A and 3C).

We also compared responses in BV173 and BV173R cells. BV173 cells were derived from a blast crisis CML patient and express BCR-ABL endogenously, whereas BV173R cells were selected for imatinib resistance and express BCR-ABL^{T315I} (Pegoraro et al., 1983; Bartholomeusz et al., 2007). Imatinib, nilotinib, and dasatinib inhibited BCR-ABL and CRKL phosphorylation in BV173, but not BV173R, cells (Figure 3E). Furthermore, whereas imatinib, nilotinib, and dasatinib did not induce BRAF binding to CRAF and inhibited MEK and ERK in BV173 cells, they induced BRAF binding to CRAF and activated MEK and ERK in BV173R cells (Figure 3E).

RAS Signaling Is Critical to Paradoxical Activation of the RAF-ERK Pathway in CML Cells

The results above show that imatinib, nilotinib, and dasatinib block RAF/MEK/ERK signaling in BCR-ABL cells but induce unexpected paradoxical activation of this pathway in BCR-ABL^{T315I} cells. To investigate the mechanism(s) underlying this difference, we first examined RAS because of its critical role in RAF activation. Dominant-negative HRAS (HRAS^{S17N}) blocked ERK activation by nilotinib in BCR-ABL^{T315I} Ba/F3 cells (Figure 4A), and nilotinib blocked RAS activity in BCR-ABL, but not BCR-ABL^{T315I}, cells (Figure 4B). We also show that imatinib, nilotinib, and dasatinib did not induce BRAF binding to CRAF in K562 cells (which express BCR-ABL), but when these cells expressed HRAS^{G12V}, all three drugs induced BRAF binding to CRAF (Figure 4C). Note that imatinib, nilotinib, and dasatinib did not increase MEK and ERK phosphorylation in K562 cells expressing HRAS^{G12V} because the pathway is already saturated by the expression of HRAS^{G12V} (Figure 4C). Taken together, we conclude that RAS plays a critical role in paradoxical MEK/ERK pathway activation in BCR-ABL^{T315I} expressing cells.

We next examined cell responses to GNF-2, an allosteric inhibitor of BCR-ABL. As a control we show that GNF-2 blocked BCR-ABL, CRKL, CRAF, MEK, and ERK phosphorylation in
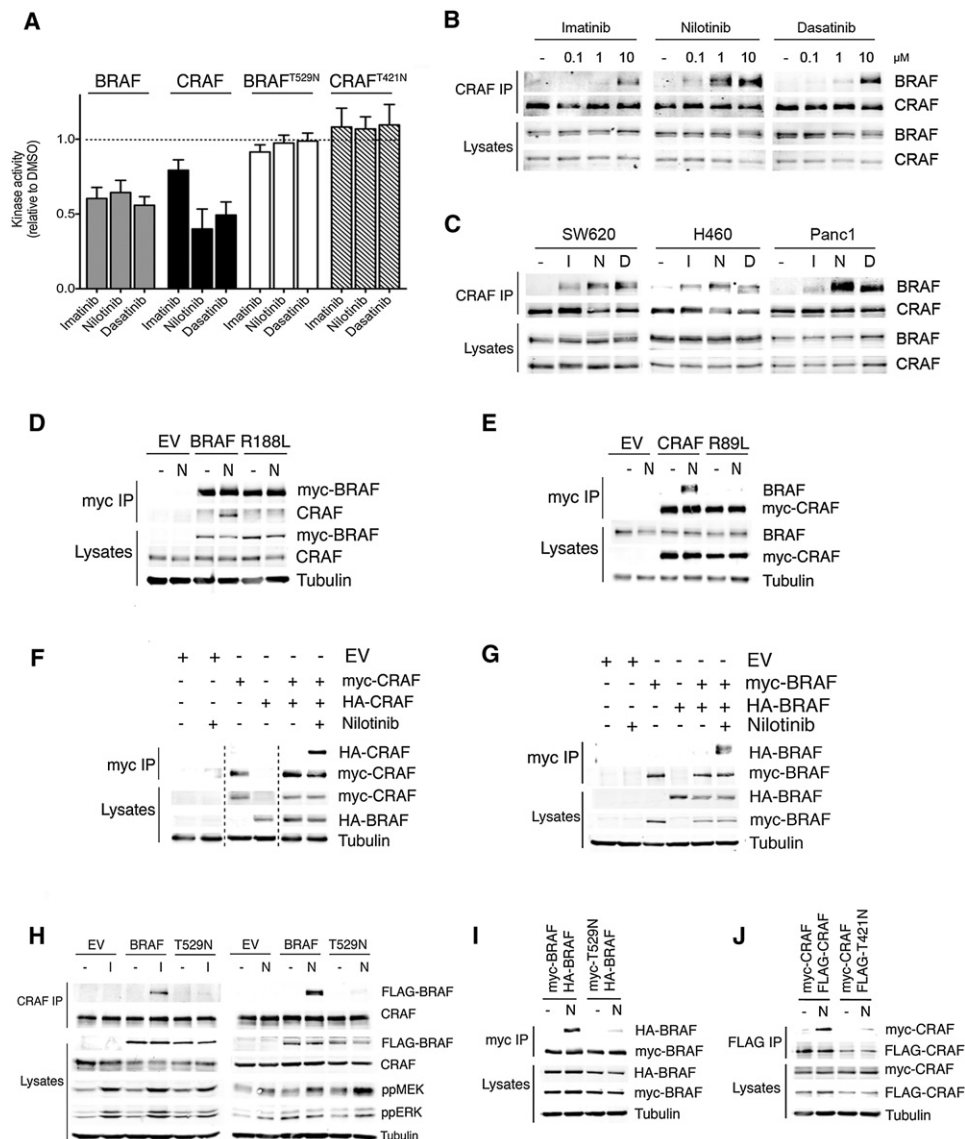
Figure 2. Imatinib, Nilotinib, and Dasatinib Drive Paradoxical Activation of BRAF and CRAF in RAS Mutant Cells

(A) RAF kinase assays. myc-epitope tagged BRAF, CRAF, BRAF^{T529N}, or CRAF^{T421N} were transiently expressed in COS cells. BRAF and BRAF^{T529N} were coexpressed with HRAS^{G12V}, and CRAF and CRAF^{T421N} were coexpressed with HRAS^{G12V} and SRC^{Y527F}. The myc-tagged proteins were captured, and their kinase activity was determined in the presence of imatinib (10 μM), nilotinib (1 μM), or dasatinib (5 μM). The mean of triplicate determinations is shown relative to DMSO-treated controls (dotted line). Error bars represent the SD.

(B) Western blots for endogenous BRAF and CRAF in CRAF immunoprecipitates (CRAF IP) or cell lysates from D04 cells treated with DMSO (−), imatinib, nilotinib, or dasatinib at the indicated concentrations.

(C) Western blots for endogenous BRAF and CRAF in CRAF immunoprecipitates (CRAF IP) or cell lysates from SW620, H460, and Panc1 cells treated with DMSO (−), imatinib (I; 10 μM), nilotinib (N; 1 μM), or dasatinib (D; 5 μM).

(D) Western blots for myc-tagged BRAF, endogenous CRAF, or tubulin (loading control) in myc immunoprecipitates (myc IP) or cell lysates from D04 cells expressing empty vector control (EV), myc-BRAF (BRAF), or myc-BRAF^{R188L} (R188L) and treated with DMSO (−) or nilotinib (N; 1 μM).

(E) Western blots for myc-tagged CRAF, endogenous BRAF, or tubulin (loading control) in myc immunoprecipitates (myc IP) or cell lysates from D04 cells expressing empty vector control (EV), myc-CRAF (CRAF), or myc-CRAF^{R89L} (R89L) and treated with DMSO (−) or nilotinib (N; 1 μM).

(F) Western blots for myc-tagged CRAF, HA-tagged CRAF, or tubulin (loading control) in myc immunoprecipitates (myc IP) or cell lysates from D04 cells expressing empty vector control (EV), myc-CRAF, or HA-CRAF and treated with DMSO (−) or nilotinib (+; 1 μM). The dotted line shows where discontinuous sections of the same blot were joined.

(G) Western blots for myc-tagged BRAF, HA-tagged BRAF, or tubulin (loading control) in myc immunoprecipitates (myc IP) or cell lysates from D04 cells expressing empty vector control (EV), myc-BRAF, or HA-BRAF and treated with DMSO (−) or nilotinib (+; 1 μM).

(H) Western blots for FLAG-tagged BRAF or FLAG-tagged BRAF^{T529N} (FLAG-BRAF), endogenous CRAF, phospho-MEK (ppMEK), phospho-ERK (ppERK), and tubulin (loading control) in CRAF immunoprecipitates (CRAF IP) or cell lysates from D04 cells expressing empty vector control (EV), FLAG-tagged BRAF (BRAF), or FLAG-tagged BRAF^{T529N} (T529N) and treated with DMSO (−), imatinib (I; 10 μM), or nilotinib (N; 1 μM).

BCR-ABL Ba/F3 cells and confirmed that BCR-ABL^{T315I} was resistant to GNF-2 by showing that it did not block BCR-ABL or CRKL phosphorylation in cells expressing this mutant (Figure 4D). Critically, GNF-2 did not inhibit BRAF activity in vitro (Figure 4E), and in BCR-ABL^{T315I} Ba/F3 cells it did not induce BRAF binding to CRAF, did not increase CRAF, MEK, or ERK phosphorylation (Figure 4D), and did not activate BRAF or CRAF (Figure 4F). We also performed apposite experiments with the BRAF selective inhibitors SB590885 and L779450. Neither agent inhibited BCR-ABL or CRKL phosphorylation in BCR-ABL Ba/F3 cells, and accordingly, they both stimulated BRAF binding to CRAF and CRAF, MEK, and ERK phosphorylation in these cells (Figure 4G). Thus, BCR-ABL inhibitors that do not inhibit BRAF do not activate the pathway in BCR-ABL^{T315I} cells, whereas BRAF inhibitors activate the pathway in BCR-ABL cells.

Taking these data together, we propose the following model. We posit that imatinib, nilotinib, and dasatinib are weak RAF inhibitors that drive paradoxical activation of BRAF and CRAF in the presence of activated RAS. Because RAS is activated downstream of BCR-ABL (Goga et al., 1995; Suzuki et al., 2010), when BCR-ABL is inhibited, so is RAS (Figure 4B), and although BRAF and CRAF are also inhibited, the lack of RAS activity means that they are not paradoxically activated. In contrast because BCR-ABL^{T315I} is resistant to these three inhibitors, RAS activity persists in the presence of the drugs, and consequently, they are able to drive paradoxical activation of BRAF and CRAF.

Nilotinib Synergizes with MEK Inhibition to Induce Synthetic Lethality in Drug-Resistant CML Cells In Vitro
We next investigated how paradoxical MEK/ERK pathway activation affected the growth of leukemia cells expressing BCR-ABL^{T315I}. As mentioned, imatinib, nilotinib, and dasatinib reach concentrations of ∼5 μM, 4 μM, and 90 nM, respectively, in patient plasma (Weisberg et al., 2007; Druker et al., 2001). We, therefore, examined the effects of imatinib and nilotinib at 3 and 1 μM, respectively, but because dasatinib only activated the RAF/MEK/ERK pathway at concentrations above 1 μM, we did not further examine the effects of this drug. As expected, BCR-ABL Ba/F3 cells were sensitive to imatinib and nilotinib, whereas BCR-ABL^{T315I} Ba/F3 cells were resistant (Figure 5A). The MEK inhibitor PD184352 did not inhibit the growth of BCR-ABL or BCR-ABL^{T315I} Ba/F3 cells, and PD184352 did not synergize with imatinib, to inhibit the growth of BCR-ABL^{T315I} Ba/F3 cells (Figure 5A). Importantly, whereas PD184352 and nilotinib did not synergize to inhibit the growth of the BCR-ABL Ba/F3 cells, they synergized to inhibit the growth of BCR-ABL^{T315I} Ba/F3 cells (Figure 5A).

These responses were accompanied by apposite responses in apoptosis. Thus, imatinib and nilotinib induced apoptosis in BCR-ABL, but not in BCR-ABL^{T315I} Ba/F3, cells (Figure 5B; Figure S3A). PD184352 did not induce apoptosis in either line

(Figure 5B; Figure S3A), and whereas it did not synergize with imatinib, it did synergize with nilotinib to induce apoptosis in BCR-ABL^{T315I} cells (Figure 5B; Figure S3A). We observed similar responses in BV173 and BV173R cells. Imatinib and nilotinib inhibited cell proliferation and induced apoptosis in BV173 cells, but not BV173R cells (Figure 5C; Figure S3B). PD184352 did not inhibit cell proliferation or induce apoptosis in either line, and whereas it synergized with nilotinib to inhibit cell proliferation and induce apoptosis in BV173R cells, we saw no such synergy with imatinib (Figure 5C; Figure S3B).

These data show that paradoxical activation of RAF leads CML cells to develop an unexpected dependence on MEK/ERK signaling, such that if MEK is inhibited, proliferation is inhibited and apoptosis induced. We support this model by showing that PD184352 synergized with the BRAF inhibitors SB590885 and L779450 to inhibit the growth of BCR-ABL Ba/F3 cells (Figure 5D), whereas GNF-2 did not synergize with PD184352 to inhibit the growth of BCR-ABL^{T315I} Ba/F3 cells (Figure 5E). Thus, BRAF inhibitors that did not inhibit BCR-ABL were able to drive paradoxical activation of RAF and synergy with MEK inhibitors to kill cells expressing BCR-ABL. Furthermore, GNF-2, which did not drive paradoxical activation of RAF, did not synergize with MEK to kill BCR-ABL^{T315I} Ba/F3 cells.

We further show that the pan-RAF inhibitors sorafenib and RAF265 did not inhibit BCR-ABL or CRKL phosphorylation in BCR-ABL^{T315I} Ba/F3 cells, and although they induced BRAF binding to CRAF, they inhibited, rather than activated, MEK and ERK (Figure 5F). Critically, even in the absence of PD184352, these agents inhibited proliferation and induced cell death in cells expressing BCR-ABL^{T315I} (Figures 5G and 5H). In line with our previous conclusions (Hatzivassiliou et al., 2010; Heidorn et al., 2010), we posit that because sorafenib and RAF265 are relatively potent pan-RAF inhibitors, they drive RAF dimerization but also inhibit the RAF proteins in the complexes that are formed. By simultaneously driving the paradoxical activation of RAF and inhibiting MEK/ERK signaling, they, therefore, inhibit proliferation and induce death in CML cells even in the absence of MEK inhibitors. Note also that the BRAF inhibitor PLX4720, which did not induce strong binding of BRAF to CRAF (Hatzivassiliou et al., 2010; Heidorn et al., 2010), only produced weak synergy with PD184352 to inhibit cell proliferation of these cells (Figure 5I). These data suggest that the formation of RAF dimers in the presence of RAF inhibitors is critical to the ability of these agents to synergize with PD184352 and kill the cells.

Nilotinib Synergizes with MEK Inhibition to Induce Synthetic Lethality in Cells Expressing Compound BCR-ABL Mutants
Next, we tested if similar responses occurred in cells expressing compound BCR-ABL mutants because clinical resistance to ABL inhibitors is mediated largely by T315I or compound

---

(I) Western blots for HA-tagged BRAF (HA-BRAF), myc-tagged BRAF (myc-BRAF), or tubulin (loading control) in myc immunoprecipitates (myc IP) or cell lysates from D04 cells expressing myc-BRAF, myc-BRAF^{T529N} (myc-T529N), or HA-BRAF and treated with DMSO (−), or nilotinib (N; 1 μM).
(J) Western blots for myc-tagged CRAF (myc-CRAF), FLAG-tagged CRAF (FLAG-CRAF), or tubulin (loading control) in FLAG immunoprecipitates (FLAG IP) or cell lysates from D04 cells expressing myc-CRAF, FLAG-CRAF, or FLAG-CRAF^{T421N} (FLAG-T421N) and treated with DMSO (−), or nilotinib (N; 1 μM).
See also Figure S2.
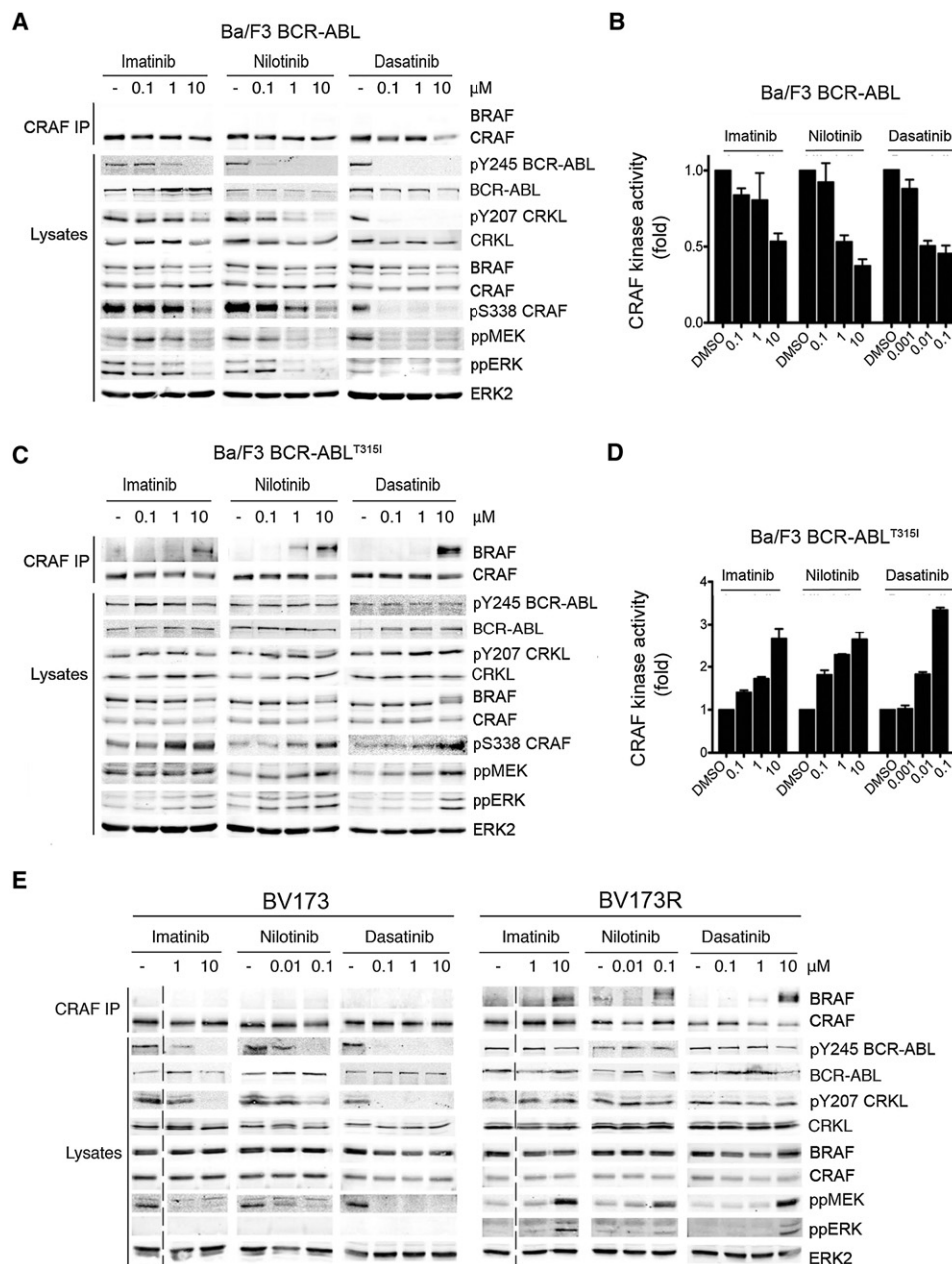
Figure 3. Imatinib, Nilotinib, and Dasatinib Induce Paradoxical RAF, MEK, and ERK Activation in Cells Expressing BCR-ABL^{T315I}

(A) Western blots for endogenous BRAF, CRAF, pY245 BCR-ABL, BCR-ABL, pY207 CRKL, CRKL, pS338 CRAF, ppMEK, ppERK, and ERK2 (loading control) in CRAF immunoprecipitates (CRAF IP) and cell lysates from Ba/F3 cells expressing BCR-ABL treated with DMSO (−), or the indicated concentrations of imatinib, nilotinib, and dasatinib.

(B) RAF kinase assays. Endogenous CRAF kinase activity in Ba/F3 cells expressing BCR-ABL treated with imatinib, nilotinib, or dasatinib at the indicated concentrations (μM). Data represent means of triplicate measurements with error bars to represent SD.

(C) Same as in (A) but with Ba/F3 cells expressing BCR-ABL^{T315I}.

(D) Same as in (B) but with BCR-ABL^{T315I} Ba/F3 cells.

(E) Western blots for endogenous BRAF, CRAF, pY245 BCR-ABL, BCR-ABL, pY207 CRKL, CRKL, ppMEK, ppERK, and ERK2 (loading control) in CRAF immunoprecipitates (CRAF IP) and cell lysates from BV173 and BV173R cells treated with DMSO (−) or the indicated concentrations of imatinib, nilotinib, and dasatinib. The dotted line indicates the joining of two discontinuous sections of the same blot.

mutants that emerge following sequential treatment with imatinib and then nilotinib or dasatinib (Shah et al., 2007). We show that in Ba/F3 cells expressing BCR-ABL$^{G250E/T315I}$, BCR-ABL$^{E255K/T315I}$, or BCR-ABL$^{E255V/T315I}$, nilotinib did not inhibit

BCR-ABL or CRKL phosphorylation, and induced BRAF binding to CRAF as well as MEK and ERK activation (Figure S3D). Furthermore, whereas nilotinib and PD184352 by themselves did not affect proliferation of cells expressing these compound
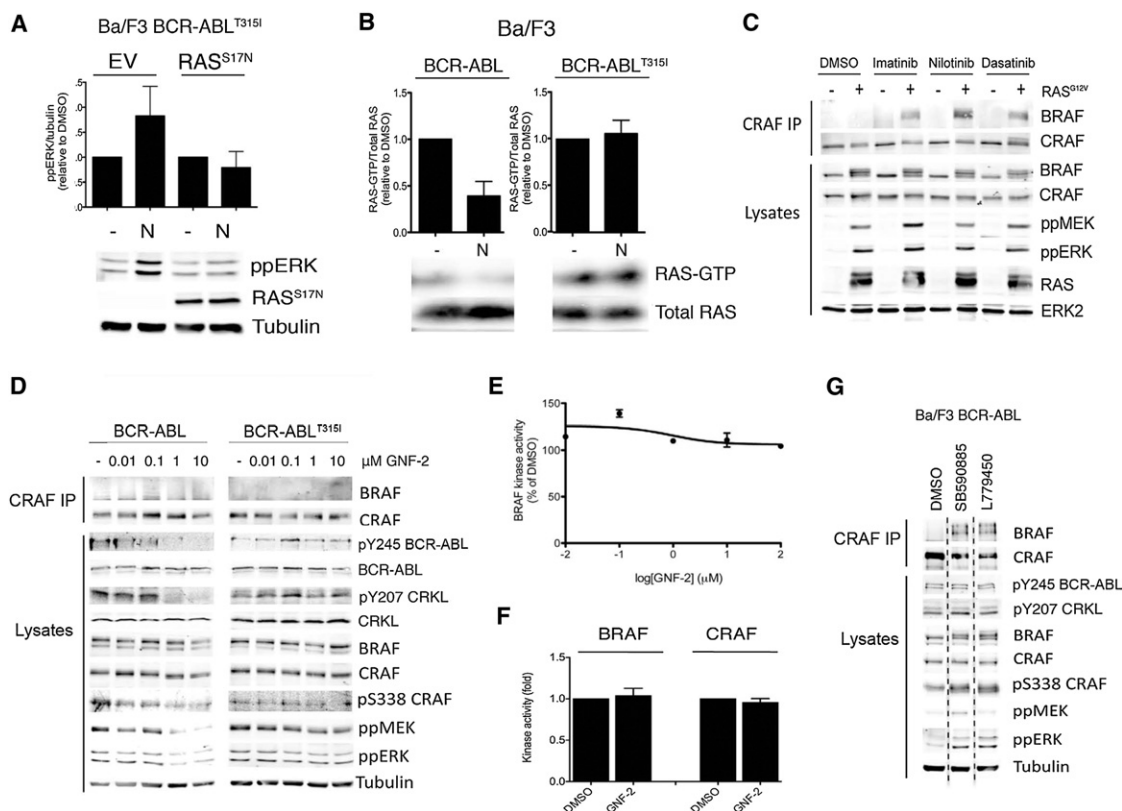
Figure 4. Active RAS Is Required for Paradoxical Activation of RAF/MEK/ERK Signaling

(A) Graph showing phospho-ERK quantification in BCR-ABL^{T315I} Ba/F3 cells expressing empty vector control (EV) or RAS^{S17N} and treated with DMSO (−) or nilotinib (N; 1 μM). The data were quantified by western blotting, a representative of which is below the graph and shows phospho-ERK (ppERK), myc-RAS^{S17N}, and tubulin (loading control). The ppERK levels are presented relative to tubulin and were calculated from triplicate determinations with error bars to represent the SD.

(B) Graph showing RAS-GTP quantification in BCR-ABL and BCR-ABL^{T315I} Ba/F3 cells treated with DMSO (−) or nilotinib (N; 1 μM, 60 min). The data were quantified by western blotting, a representative of which is below the graph and shows RAS-GTP and total RAS levels in the extracts. The RAS-GTP levels are shown relative to total RAS levels and are from triplicate experiments with error bars to represent the SD.

(C) Western blots for endogenous BRAF, CRAF, phospho-MEK (ppMEK), phospho-ERK (ppERK), RAS, and ERK2 (loading control) in CRAF immunoprecipitates (CRAF IP) and cell lysates from K562 cells expressing an empty vector control (−) or HRAS^{G12V} (+) treated with DMSO, imatinib (10 μM), nilotinib (1 μM), or dasatinib (5 μM).

(D) Western blots for BRAF, CRAF, pY245 BCR-ABL, BCR-ABL, pY207 CRKL, CRKL, pS338 CRAF, ppMEK, ppERK, and tubulin (loading control) in CRAF immunoprecipitates (CRAF IP) and cell lysates from BCR-ABL and BCR-ABL^{T315I} Ba/F3 cells treated with GNF-2 at the indicated concentrations.

(E) BRAF kinase assay. myc-epitope tagged BRAF was transiently expressed in COS cells with HRAS^{G12V}. myc-BRAF was captured, and its kinase activity was determined in the presence of various GNF-2 concentrations for 30 min. The results are the mean of triplicate determinations, and error bars represent SD.

(F) BRAF and CRAF kinase assays. Endogenous BRAF and CRAF kinase activities were measured in D04 cells treated with DMSO or GNF-2 (1 μM). The data are presented relative to DMSO and represent triplicate measurements with error bars to represent SD.

(G) Western blot for endogenous BRAF, CRAF, pY245 BCR-ABL, pY207 CRKL, pS338 CRAF, ppMEK, ppERK, and tubulin (loading control) in CRAF immunoprecipitates (CRAF IP) and cell lysates from BCR-ABL Ba/F3 cells treated with DMSO, SB590885 (0.3 μM), or L779450 (0.3 μM). The dotted line indicates where discontinuous sections of the same blot were joined.

BCR-ABL mutants, they synergized to induce synthetic lethality in these cells (Figure 5J).

Nilotinib Synergizes with MEK Inhibition to Induce Synthetic Lethality in Cells Whose Resistance Is BCR-ABL Independent

We also tested if similar responses occurred in CML cells whose resistance was mediated by non-BCR-ABL mechanisms. K562 cells were derived from a patient in terminal blast crisis, and K562R cells are a clone that is resistant due to overexpression of the SRC family kinase LYN (Donato et al., 2003). In K562 cells

nilotinib inhibited BCR-ABL and CRKL phosphorylation, suppressed RAS activity, and inhibited CRAF, MEK, and ERK phosphorylation (Figures S3E and S3F). Nilotinib also blocked BCR-ABL and CRKL phosphorylation in K562R cells (Figure S3E) but, nevertheless, did not inhibit RAS (Figure S3F) and did not block CRAF, MEK, or ERK phosphorylation (Figure S3E). Nilotinib induced apoptosis in K562 cells, but PD184352 did not kill these cells and did not enhance nilotinib-induced cell death (Figure 5K). In contrast, nilotinib and PD184352 alone did not affect the growth of K562R cells, but together, they synergized to induce death in these cells (Figure 5K).
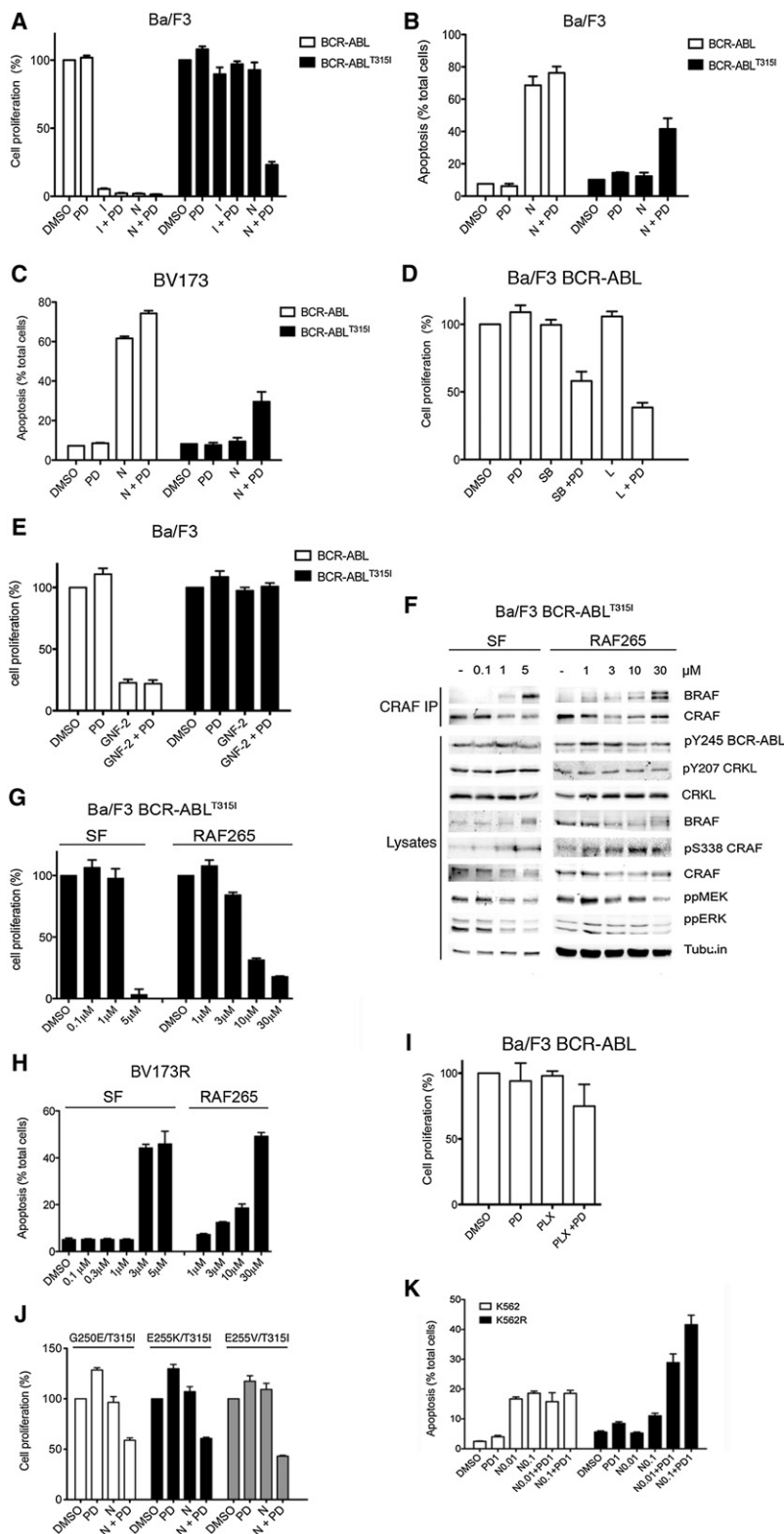
Figure 5. PD184352 Synergizes with Nilotinib to Induce Synthetic Lethality in Cells Expressing BCR-ABL^{T315I} In Vitro

(A) Cell proliferation was measured in BCR-ABL and BCR-ABL^{T315I} Ba/F3 cells treated with DMSO, PD184352 (PD; 2 μM), imatinib (I; 3 μM), nilotinib (N; 1 μM), or the indicated combinations for 4 days. Growth, determined in triplicate, is expressed as a percentage of the DMSO controls, and error bars represent SD.

(B) Percentage of apoptotic cells in BCR-ABL and BCR-ABL^{T315I} Ba/F3 cells treated with DMSO, PD184352 (PD; 2 μM), nilotinib (N; 1 μM), or the indicated combinations for 4 days. Apoptosis was detected by staining cells with annexin V and propidium iodide. The mean percentage of apoptotic cells is shown, as determined by triplicate samples, with error bars representing the SD.

(C) Percentage of apoptosis in BV173 and BV173R cells treated with DMSO, PD184352 (PD; 0.2 μM), nilotinib (N; 0.1 μM), or the indicated combinations for 6 days. The cells were fixed and stained with propidium iodide, and sub-G1 cells were measured by flow cytometry. The mean of triplicate samples is shown with error bars representing the SD.

(D) Cell proliferation was measured in BCR-ABL Ba/F3 cells treated with PD184352 (PD; 2 μM), SB590885 (SB; 0.3 μM), L779450 (L; 0.3 μM), or the indicated combinations for 72 hr. Cell growth determinations in triplicate are expressed relative to the DMSO control. Error bars represent the SD.

(E) Cell proliferation was measured in BCR-ABL and BCR-ABL^{T315I} Ba/F3 cells treated with PD184352 (PD; 2 μM), GNF-2 (1μM), or the indicated combination for 4 days. Cell growth determinations in triplicate are expressed relative to the DMSO control. Error bars represent the SD.

(F) Western blots for endogenous BRAF, CRAF, pY245 BCR-ABL, pY207 CRKL, CRKL, pS338 CRAF, ppMEK, ppERK, and tubulin (loading control) in CRAF immunoprecipitates (CRAF IP) and cell lysates from BCR-ABL^{T315I} Ba/F3 cells treated with the indicated concentrations (uM) of sorafenib (SF) and RAF265.

(G) Cell proliferation was measured in BCR-ABL^{T315I} Ba/F3 cells treated with the indicated concentrations of sorafenib (SF) and RAF265 for 72 hr. Growth, determined in triplicate, is expressed as a percentage of the DMSO controls, with error bars representing the SD.

(H) Percentage of apoptotic BV173R cells treated with the indicated concentrations of sorafenib (SF) and RAF265 for 72 hr. Cells were fixed and stained with propidium iodide, and sub-G1 cells were measured by flow cytometry. The mean of triplicate samples is shown, with error bars representing the SD.

(I) Cell proliferation was measured in BCR-ABL Ba/F3 cells treated with PD184352 (PD; 2 μM), PLX4720 (PLX; 1 μM), or the indicated combinations for 72 hr. Growth, determined in triplicate, is expressed as a percentage of the DMSO controls, and error bars represent SD.

(J) Cell proliferation was measured in BCR-ABL$^{G250E/T315I}$, BCR-ABL$^{E255K/T315I}$, and BCR-ABL$^{E255V/T315I}$ Ba/F3 cells treated with DMSO, PD184352 (PD; 2 μM), nilotinib (N; 1 μM), or the indicated combinations for 4 days. Growth, determined in triplicate, is expressed as a percentage of the DMSO controls, and error bars represent SD.

(K) Percentage of apoptotic K562 and K562R cells treated with PD184352 (PD; 1 μM), the indicated concentrations of nilotinib (N; uM), or combinations of both for 4 days. The cells were fixed and stained with propidium iodide, and sub-G1 cells were measured by flow cytometry. The mean percentage of apoptotic cells is shown, as determined by triplicate samples, along with error bars representing the SD.

See also Figure S3.
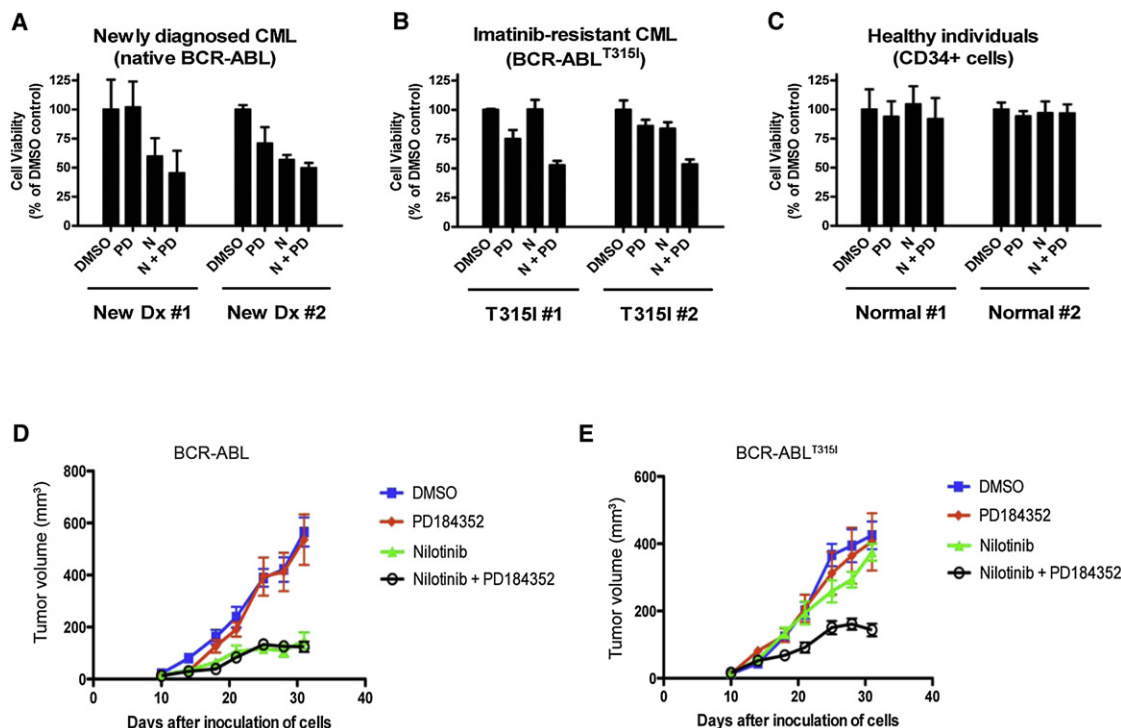
Figure 6. PD184352 Synergizes with Nilotinib to Inhibit Cell Proliferation of CML Primary Cells and Induce Synthetic Lethality in Cells Expressing BCR-ABL^{T315I} In Vivo

(A) Cell proliferation was measured in mononuclear cells harboring native BCR-ABL isolated from newly diagnosed patients with CML (n = 2) treated ex vivo with nilotinib (N; 1 μM), PD184352 (PD; 0.3 μM), or both for 96 hr. All treatment conditions were performed in quadruplicate; values are normalized to those of DMSO-treated control wells and represent the mean ± SE.

(B) As in (A) but with BCR-ABL^{T315I} expressing mononuclear cells isolated from patients with imatinib-resistant CML (n = 2).

(C) As in (A) but with CD34+ cells from healthy individuals (n = 2).

(D and E) The growth of tumor allografts formed by Ba/F3 cells expressing BCR-ABL (D) or BCR-ABL^{T315I} (E) grown in nude mice is shown. The mice were treated with vehicle, PD184352 (25 mg/kg), nilotinib (25 mg/kg), or both. The results show mean tumor volumes for groups of six animals, with error bars to represent the SE.

Nilotinib Synergizes with MEK Inhibition to Induce Synthetic Lethality in Primary Drug-Resistant CML Cells

We next determined if nilotinib and PD184352 also inhibited the growth of primary cells from patients with BCR-ABL-driven CML. Mononuclear cells derived from blood or bone marrow of patients with CML harboring native BCR-ABL or BCR-ABL^{T315I} and from healthy individuals were treated with nilotinib, PD184352, or both for 96 hr, and cell viability was measured. Consistent with the cell lines, nilotinib inhibited the proliferation of cells expressing BCR-ABL from newly diagnosed patients with CML, and PD184352 did not substantially enhance this effect (Figure 6A). In contrast, nilotinib and PD184352 alone did not affect the growth of BCR-ABL^{T315I} cells but synergized to inhibit growth of these cells (Figure 6B). As a control, we show that CD34+ hematopoietic cells from healthy individuals were resistant to all combinations of nilotinib and PD184352 (Figure 6C).

Nilotinib and PD184352 Induce Synthetic Lethality in Drug-Resistant CML Cells In Vivo

Finally, we tested the implications of our findings in vivo by examining how the drugs affected the growth of subcutaneously implanted Ba/F3 allografts expressing BCR-ABL or BCR-

ABL^{T315I}. The growth of BCR-ABL tumors was strongly suppressed by nilotinib, but not by PD184352, and PD184352 did not enhance the growth-inhibitory activity of nilotinib (Figure 6D). In contrast, BCR-ABL^{T315I} tumors were insensitive to both nilotinib and PD184352, but together, these drugs synergized to inhibit the growth of these tumors (Figure 6E).

Taking all of these data together, we conclude that nilotinib and PD184352 induced synthetic lethality in drug-resistant CML cells both in vitro and in vivo.

DISCUSSION

Building on our previous studies, we tested a panel of drugs for their ability to activate MEK and ERK in cells expressing oncogenic RAS. Most of the drugs were ineffective, but imatinib, nilotinib, and dasatinib activated MEK and ERK in a variety of lines. Critically, we show that these drugs are weak RAF inhibitors whose binding to BRAF and CRAF drives BRAF:CRAF heterodimer and BRAF and CRAF homodimer formation, leading to paradoxical activation of both BRAF and CRAF. We established an essential role for RAS in these responses by showing that its depletion blocked MEK/ERK activation, and if BRAF or CRAF was unable to bind to RAS, they did not form dimers.
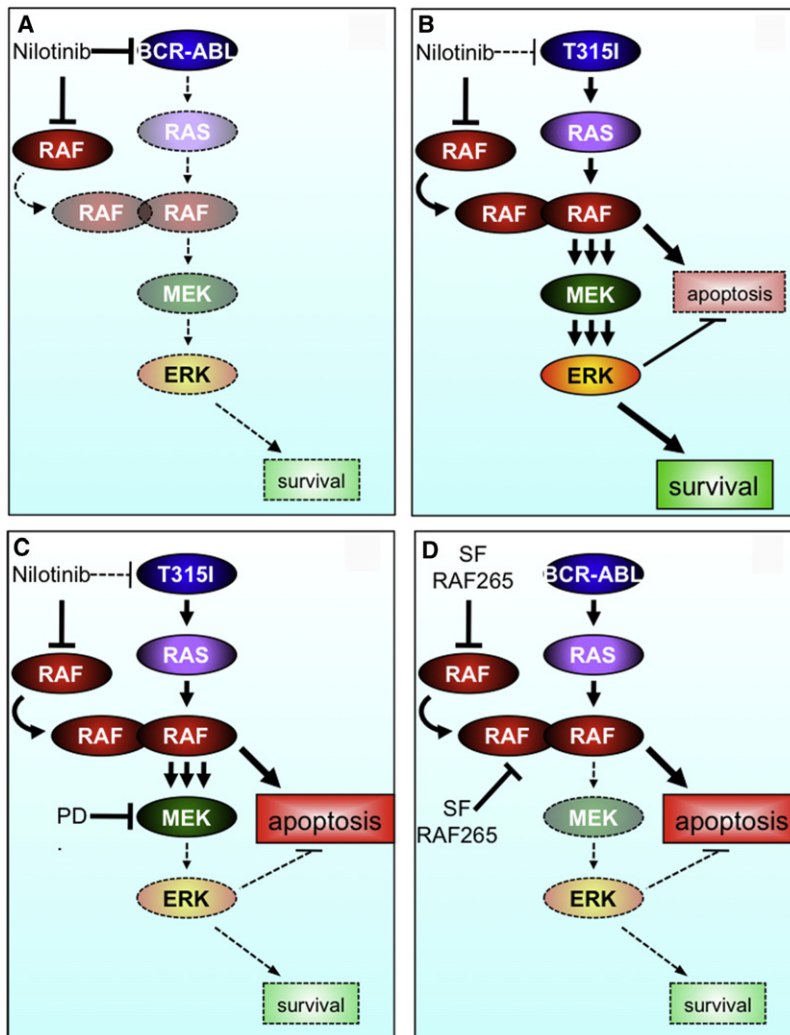
Figure 7. Model of Paradoxical RAF/MEK/ERK Activation in Drug-Resistant CML Cells by Nilotinib

(A) Nilotinib binds to and inhibits BCR-ABL, which inhibits downstream signaling, including RAS. Nilotinib also inhibits BRAF and CRAF, but because RAS is inactivated, this is without consequence.

(B) Nilotinib inhibits BRAF and CRAF but not BCR-ABL[T315I]. Consequently, RAS remains active and so nilotinib induces the formation of RAF dimers and activation of the RAF/MEK/ERK survival signal. We posit that these RAF complexes also activate a MEK/ERK-independent apoptotic signal, but this is overridden by the dominant survival signal.

(C) Nilotinib inhibits RAF in the presence of BCR-ABL[T315I], leading to paradoxical activation of RAF/MEK/ERK. MEK inhibition by PD184352 (PD) blocks the survival signal, allowing apoptosis to predominate.

(D) Pan-RAF drugs such as sorafenib (SF) and RAF265 inhibit both BRAF and CRAF with high potency. So, although they induce RAF dimers, they simultaneously inhibit RAF in these dimers, blocking MEK/ERK signaling, thereby favoring apoptosis.

in BCR-ABL expressing, but not BCR-ABL[T315I] expressing, cells. We further showed that dominant-negative RAS blocked MEK/ERK activation in BCR-ABL[T315I] cells and that imatinib, nilotinib, and dasatinib drove RAF dimerization in BCR-ABL cells when oncogenic RAS was ectopically introduced. These data establish that RAS plays a key role in these responses, and accordingly, we propose the following model. We posit that BCR-ABL inhibition leads to RAS inhibition, and so, although RAF is also inhibited, it is not paradoxically activated (Figure 7A). In contrast because BCR-ABL[T315I] is resistant to imatinib, nilotinib, and dasatinib, RAS activity persists in the presence of these drugs, and hence, the off-target inhibition of RAF leads to its paradoxical pathway activation (Figure 7B). This model also explains why BRAF inhibitors drive paradoxical activation of the pathway in BCR-ABL cells: they do not inhibit BCR-ABL, so do not inhibit RAS and, hence, can drive paradoxical activation of RAF. It also explains why BCR-ABL inhibitors such as GNF-2 do not drive paradoxical activation of the pathway: although they do not inhibit BCR-ABL[T315I] and, therefore, do not inhibit RAS, they are not BRAF/CRAF inhibitors and so cannot drive their paradoxical activation.

It has been reported that imatinib activates MEK and ERK in cells expressing imatinib-resistant BCR-ABL (Yu et al., 2002; Suzuki et al., 2010; Mohi et al., 2004; Chu et al., 2004), and our studies now provide a mechanistic explanation for those observations. More importantly, we show that whereas the growth of the drug-resistant cells was unaffected by nilotinib and PD184352 in vitro and in vivo, these drugs synergized to inhibit cell growth and induce apoptosis in vitro, and to suppress tumor growth in mice. Thus, we show that drug-resistant cells develop an unexpected dependency on MEK/ERK signaling when the pathway is paradoxically activated. We, therefore, posit that in these cells paradoxical activation of this pathway drives both

We also established a critical role for BRAF and CRAF by showing that depletion of both was necessary to block MEK and ERK activation by these drugs. Thus, although they only inhibit RAF weakly, imatinib, nilotinib, and dasatinib possess sufficient off-target activity to drive the formation of BRAF:CRAF dimers and stimulate paradoxical activation of the pathway. It has previously been shown that RAF inhibitors also drive paradoxical activation of BRAF and CRAF (Halaban et al., 2010; Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010), and our data show that imatinib, nilotinib, and dasatinib appear to mimic these effects. We, therefore, posit that like RAF inhibitors (Downward, 2011), imatinib, nilotinib, and dasatinib bind to monomeric RAF and induce RAF dimerization in which one partner is bound to drug, and the other is not. The drug-bound partner then acts as a scaffold, or induces a conformational change to facilitate activation of the drug-free partner.

We extended these observations to show that imatinib, nilotinib, and dasatinib drove paradoxical activation of the RAF/MEK/ERK pathway in drug-resistant leukemia cells. Critically, we showed that inhibition of BCR-ABL causes RAS inactivation

a MEK/ERK-dependent antiapoptotic signal and a MEK/ERK-independent proapoptotic signal (Figure 7B). Under normal conditions the antiapoptotic signal overcomes the proapoptotic signal (Figure 7B), but when MEK is inhibited, the proapoptosis signal predominates (Figure 7C).

It is unclear how MEK inhibition induces apoptosis under these conditions, but one possibility is that it is driven by the formation of the RAF dimers. Previous studies have shown that CRAF opposes cell death in a MEK/ERK-independent manner by sequestering the proapoptotic kinases ASK1, MST2, ROCK1, and RIP2 (O'Neill et al., 2004; Navas et al., 1999; Chen et al., 2001; Piazzolla et al., 2005). We posit that the recruitment of CRAF into homo- and heterodimers releases these binding partners, allowing them to induce apoptosis. Our preliminary experiments failed to establish a clear role for ASK1 and MST2 in the death of BCR-ABL^{T315I} cells, but the response of these cells to RAF inhibitors supports our model. We show that SB590885 and L779450 induced robust BRAF binding to CRAF and synergized with PD184352 to induce synthetic lethality (Figures 4G and 5D). In contrast, PLX4720, which induced weak BRAF binding to CRAF (Figure S3C), only weakly synergized with the MEK inhibitor to inhibit cell proliferation (Figure 5I). Furthermore, although sorafenib and RAF265 induced strong BRAF binding to CRAF, they simultaneously inhibited MEK signaling and were thus able to induce cell death without the need of a MEK inhibitor. It has been proposed that sorafenib induces apoptosis in imatinib-resistant leukemia cells by targeting multiple kinases (Rahmani et al., 2007; Kurosu et al., 2009), but our data suggest that pan-RAF inhibitors such as sorafenib induce apoptosis because they induce paradoxical activation of RAF and simultaneously inhibit MEK/ERK, thereby favoring the proapoptotic signal (Figure 7D).

Imatinib was approved for first-line treatment of CML over a decade ago and is generally well tolerated, but 20%–30% of patients do not achieve complete responses, and acquired resistance is a persistent clinical problem (Quintás-Cardama et al., 2009). Most imatinib-resistant BCR-ABL mutants remain sensitive to nilotinib and dasatinib providing vital second-line treatments (Saglio et al., 2010; Kantarjian et al., 2011), and both were recently approved as first-line CML drugs. However, BCR-ABL^{T315I} and the compound mutants that arise following long-term or sequential drug treatment are resistant to all three drugs (Shah et al., 2007), and some patients develop resistance that is mediated by BCR-ABL-independent mechanisms. Thus, new treatments are still required for relapsed patients, and agents active against BCR-ABL^{T315I} are undergoing clinical trials (O'Hare et al., 2011).

We propose that the synthetic lethality we describe could provide an approach to block the emergence of drug-resistance in patients. This is based on the observation that BCR-ABL cells are sensitive to nilotinib alone, whereas the resistant cells are sensitive to nilotinib plus the MEK inhibitor. Thus, if these drugs were to be combined, the primary disease would be treated by nilotinib and the resistant clones by nilotinib plus a MEK inhibitor. Thus, this combination has the potential to treat both the bulk disease and prevent the emergence of resistance. Critically, this synthetic lethality also occurred in K562R cells, where resistance was mediated by BCR-ABL-independent mechanisms, suggesting that our findings could have wide utility. In

this context it is intriguing to note a recent report where acute lymphoblastic leukemia resistance was shown to be mediated by EphB4 receptor tyrosine kinase overexpression that led to constitutive RAS activation and ERK hyperactivation following imatinib treatment (Suzuki et al., 2010). Importantly, the MEK inhibitor U0126 synergized with imatinib to inhibit proliferation of these cells, corroborating our model. Clearly, not all BCR-ABL drugs will mediate these responses. GNF-2 lacks off-target RAF activity, and dasatinib, which only inhibits RAF at levels above those that can be achieved in patients' blood, would not be suitable. We wish also to be clear that we are not proposing BRAF inhibitors for the treatment of CML patients, and indeed, we show that PLX4720 did not induce robust RAF dimerization or efficient synthetic lethality.

In summary CML is a heterogeneous disease characterized by the evolution of drug resistance. We have elaborated an unexpected synthetic lethality mediated by paradoxical activation of RAF in drug-resistant cells. Importantly, this response could provide approaches to extend clinical responses to nilotinib by preventing the emergence of the drug-resistant clones.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for detailed protocols.

Reagents

Expression vectors for epitope-tagged BRAF, BRAF^{T529N}, BRAF^{R188L}, CRAF, CRAF^{T421N}, CRAF^{R89L}, HRAS^{S17N}, and HRAS^{G12V} have been described (Marais et al., 1995, 1998). For western blotting the antibodies used were as follows: rabbit anti-ppMEK1/2 and rabbit anti-phospho-c-Abl (Tyr245), c-Abl, rabbit anti-phospho-CRKL (Tyr207), and rabbit anti-phospho-CRAF (Ser338) (Cell Signaling Technology); mouse anti-NRAS (C-20), rabbit anti-ERK2 (C-14), mouse anti-BRAF (F-7), and Crkl (Santa Cruz Biotechnology); mouse anti-FLAG, mouse anti-Tubulin, and mouse anti-ppERK1/2 (Sigma-Aldrich); and mouse anti-CRAF, mouse-anti RAS, and mouse anti-MEK1 (BD Transduction Laboratories). For immunoprecipitation the antibodies used were rabbit anti-myc (Abcam) and rabbit anti-CRAF (C-20; Santa Cruz Biotechnology). Imatinib, nilotinib, dasatinib, and sorafenib were from LC Laboratories (Woburn, MA, USA), GNF-2 from Sigma-Aldrich, SB590885 from Symansis (Timaru, New Zealand), L779450 from Tocris Bioscience (Ellisville, MO, USA), RAF265 from American Custom Chemicals (San Diego, CA, USA), and PD184352 was synthesized in house. All drugs were prepared in DMSO.

Cell Culture Techniques

Human cell lines were cultured in DMEM (A375, A2058, COS-7, Panc1, SW620, H460, K562) or RPMI (D04, BV173, K562R) supplemented with 10% fetal bovine serum. Drug treatments were performed for 3 hr, unless otherwise specified. For RNAi studies D04 cells were transfected with Lipofectamine (Invitrogen, Paisley, UK) and 10 nM of double-stranded siRNAs. See Supplemental Experimental Procedures for sequences. Transient and stable expression of proteins was as described (Wan et al., 2004). For immunoprecipitation, lysates were incubated with 5 μg CRAF C-20 or 3 μg myc antibody (Abcam), captured on Protein G Sepharose 4B beads (Sigma-Aldrich), and western blotted using standard protocols. FLAG-tagged BRAF was captured using Anti-FLAG-M2 Agarose beads (Sigma-Aldrich). Coupled RAF assays were performed as described (Wan et al., 2004). Cell proliferation was determined using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA), and apoptotic cells were detected using the FITC Annexin V Apoptosis Detection Kit (BD PharMingen) or propidium iodide staining of fixed cells followed by fluorescence-activated cell sorting (FACS) analysis. RAS capture assays were performed as described (Marais et al., 1998). BRAF and CRAF IC$_{50}$ determinations were performed using Z'-LYTE technology (Invitrogen) at 100 μM ATP.

Cell Viability Assays for Primary Cells

Clinical samples were obtained following informed consent and protocol approval by the Oregon Health & Science University (OHSU) Institutional Review Board. Mononuclear cells from newly diagnosed and imatinib-resistant CML patients harboring native BCR-ABL or BCR-ABL[T315I], respectively (n = 2/group), were isolated from bone marrow or peripheral blood by Ficoll centrifugation (GE Healthcare). Normal bone marrow CD34+ cells were obtained from Lonza. The OHSU Institutional Review Board considers the use of these materials exempt as human subjects. Cells were distributed in 96-well plates (4×10^4 cells/well) in the presence of PD184352 (0.3 μM) and nilotinib (1 μM) alone or in combination in serum-free IMDM media (Invitrogen) supplemented with 20% BIT 9500 serum substitute (STEMCELL Technologies), 40 μg/ml human low-density lipoprotein (Sigma-Aldrich), and 100 μM β-mercaptoethanol. Cell viability was assessed at 96 hr using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay.

In Vivo Approaches

All procedures involving animals were approved by the Animal Ethics Committees of the Institute of Cancer Research in accordance with National Home Office regulations under the Animals (Scientific Procedures) Act 1986 and according to the guidelines of the Committee of the National Cancer Research Institute (Workman et al., 2010). Nude mice were injected subcutaneously with 5×10^6 BCR-ABL[T315I] Ba/F3 or 2×10^7 BCR-ABL Ba/F3 cells. Tumors were allowed to establish for 7 days, size matched, and allocated to groups of six animals. Treatment was by oral gavage daily with vehicle (5% DMSO, 95% water), 25 mg/kg nilotinib, 25 mg/kg PD184352, or both. Tumor size was determined by caliper measurements of tumor length, width, and depth, and volume was calculated as volume = 0.5236 × length × width × depth (mm).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2011.11.004.

REFERENCES

Bartholomeusz, G.A., Talpaz, M., Kapuria, V., Kong, L.Y., Wang, S., Estrov, Z., Priebe, W., Wu, J., and Donato, N.J. (2007). Activation of a novel Bcr/Abl destruction pathway by WP1130 induces apoptosis of chronic myelogenous leukemia cells. Blood 109, 3470–3478.

Bixby, D., and Talpaz, M. (2011). Seeking the causes and solutions to imatinib-resistance in chronic myeloid leukemia. Leukemia 25, 7–22.

Chen, J., Fujii, K., Zhang, L., Roberts, T., and Fu, H. (2001). Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. Proc. Natl. Acad. Sci. USA 98, 7783–7788.

Chu, S., Holtz, M., Gupta, M., and Bhatia, R. (2004). BCR/ABL kinase inhibition by imatinib mesylate enhances MAP kinase activity in chronic myelogenous leukemia CD34+ cells. Blood 103, 3167–3174.

Deininger, M.W., Goldman, J.M., and Melo, J.V. (2000). The molecular biology of chronic myeloid leukemia. Blood 96, 3343–3356.

Donato, N.J., Wu, J.Y., Stapley, J., Gallick, G., Lin, H., Arlinghaus, R., and Talpaz, M. (2003). BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571. Blood 101, 690–698.

Downward, J. (2011). Targeting RAF: trials and tribulations. Nat. Med. 17, 286–288.

Druker, B.J., Talpaz, M., Resta, D.J., Peng, B., Buchdunger, E., Ford, J.M., Lydon, N.B., Kantarjian, H., Capdeville, R., Ohno-Jones, S., and Sawyers, C.L. (2001). Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N. Engl. J. Med. 344, 1031–1037.

Eide, C.A., Adrian, L.T., Tyner, J.W., Mac Partlin, M., Anderson, D.J., Wise, S.C., Smith, B.D., Petillo, P.A., Flynn, D.L., Deininger, M.W., et al. (2011). The ABL switch control inhibitor DCC-2036 is active against the chronic myeloid leukemia mutant BCR-ABLT315I and exhibits a narrow resistance profile. Cancer Res. 71, 3189–3195.

Fabian, J.R., Vojtek, A.B., Cooper, J.A., and Morrison, D.K. (1994). A single amino acid change in Raf-1 inhibits Ras binding and alters Raf-1 function. Proc. Natl. Acad. Sci. USA 91, 5982–5986.

Flaherty, K.T., Puzanov, I., Kim, K.B., Ribas, A., McArthur, G.A., Sosman, J.A., O'Dwyer, P.J., Lee, R.J., Grippo, J.F., Nolop, K., and Chapman, P.B. (2010). Inhibition of mutated, activated BRAF in metastatic melanoma. N. Engl. J. Med. 363, 809–819.

Goga, A., McLaughlin, J., Afar, D.E., Saffran, D.C., and Witte, O.N. (1995). Alternative signals to RAS for hematopoietic transformation by the BCR-ABL oncogene. Cell 82, 981–988.

Golub, T.R., Goga, A., Barker, G.F., Afar, D.E., McLaughlin, J., Bohlander, S.K., Rowley, J.D., Witte, O.N., and Gilliland, D.G. (1996). Oligomerization of the ABL tyrosine kinase by the Ets protein TEL in human leukemia. Mol. Cell. Biol. 16, 4107–4116.

Halaban, R., Zhang, W., Bacchiocchi, A., Cheng, E., Parisi, F., Ariyan, S., Krauthammer, M., McCusker, J.P., Kluger, Y., and Sznol, M. (2010). PLX4032, a selective BRAF(V600E) kinase inhibitor, activates the ERK pathway and enhances cell migration and proliferation of BRAF melanoma cells. Pigment Cell Melanoma Res. 23, 190–200.

Hatzivassiliou, G., Song, K., Yen, I., Brandhuber, B.J., Anderson, D.J., Alvarado, R., Ludlam, M.J., Stokoe, D., Gloor, S.L., Vigers, G., et al. (2010). RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature 464, 431–435.

Heidorn, S.J., Milagre, C., Whittaker, S., Nourry, A., Niculescu-Duvas, I., Dhomen, N., Hussain, J., Reis-Filho, J.S., Springer, C.J., Pritchard, C., and Marais, R. (2010). Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell 140, 209–221.

Kantarjian, H.M., Cortes, J., La Rosée, P., and Hochhaus, A. (2010). Optimizing therapy for patients with chronic myelogenous leukemia in chronic phase. Cancer 116, 1419–1430.

Kantarjian, H.M., Giles, F.J., Bhalla, K.N., Pinilla-Ibarz, J.A., Larson, R.A., Gattermann, N., Ottmann, O.G., Hochhaus, A., Radich, J.P., Saglio, G., et al. (2011). Nilotinib is effective in patients with chronic myeloid leukemia in chronic phase following imatinib resistance or intolerance: 24-month follow-up results. Blood 117, 1141–1145.

Kurosu, T., Ohki, M., Wu, N., Kagechika, H., and Miura, O. (2009). Sorafenib induces apoptosis specifically in cells expressing BCR/ABL by inhibiting its kinase activity to activate the intrinsic mitochondrial pathway. Cancer Res. 69, 3927–3936.

Marais, R., Light, Y., Paterson, H.F., and Marshall, C.J. (1995). Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. EMBO J. 14, 3136–3145.

Marais, R., Light, Y., Mason, C., Paterson, H., Olson, M.F., and Marshall, C.J. (1998). Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. Science 280, 109–112.

Mohi, M.G., Boulton, C., Gu, T.L., Sternberg, D.W., Neuberg, D., Griffin, J.D., Gilliland, D.G., and Neel, B.G. (2004). Combination of rapamycin and protein

tyrosine kinase (PTK) inhibitors for the treatment of leukemias caused by oncogenic PTKs. Proc. Natl. Acad. Sci. USA *101*, 3130–3135.

Navas, T.A., Baldwin, D.T., and Stewart, T.A. (1999). RIP2 is a Raf1-activated mitogen-activated protein kinase kinase. J. Biol. Chem. *274*, 33684–33690.

O'Dwyer, M.E., and Druker, B.J. (2001). Chronic myelogenous leukaemia—new therapeutic principles. J. Intern. Med. *250*, 3–9.

O'Hare, T., Shakespeare, W.C., Zhu, X., Eide, C.A., Rivera, V.M., Wang, F., Adrian, L.T., Zhou, T., Huang, W.S., Xu, Q., et al. (2009). AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. Cancer Cell *16*, 401–412.

O'Hare, T., Deininger, M.W., Eide, C.A., Clackson, T., and Druker, B.J. (2011). Targeting the BCR-ABL signaling pathway in therapy-resistant Philadelphia chromosome-positive leukemia. Clin. Cancer Res. *17*, 212–221.

O'Neill, E., Rushworth, L., Baccarini, M., and Kolch, W. (2004). Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. Science *306*, 2267–2270.

Pegoraro, L., Matera, L., Ritz, J., Levis, A., Palumbo, A., and Biagini, G. (1983). Establishment of a Ph1-positive human cell line (BV173). J. Natl. Cancer Inst. *70*, 447–453.

Piazzolla, D., Meissl, K., Kucerova, L., Rubiolo, C., and Baccarini, M. (2005). Raf-1 sets the threshold of Fas sensitivity by modulating Rok-alpha signaling. J. Cell Biol. *171*, 1013–1022.

Poulikakos, P.I., Zhang, C., Bollag, G., Shokat, K.M., and Rosen, N. (2010). RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. Nature *464*, 427–430.

Quintás-Cardama, A., Kantarjian, H.M., and Cortes, J.E. (2009). Mechanisms of primary and secondary resistance to imatinib in chronic myeloid leukemia. Cancer Contr. *16*, 122–131.

Rahmani, M., Nguyen, T.K., Dent, P., and Grant, S. (2007). The multikinase inhibitor sorafenib induces apoptosis in highly imatinib mesylate-resistant bcr/abl+ human leukemia cells in association with signal transducer and activator of transcription 5 inhibition and myeloid cell leukemia-1 down-regulation. Mol. Pharmacol. *72*, 788–795.

Saglio, G., Kim, D.W., Issaragrisil, S., le Coutre, P., Etienne, G., Lobo, C., Pasquini, R., Clark, R.E., Hochhaus, A., Hughes, T.P., et al; ENESTnd Investigators. (2010). Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. N. Engl. J. Med. *362*, 2251–2259.

Shah, N.P., Skaggs, B.J., Branford, S., Hughes, T.P., Nicoll, J.M., Paquette, R.L., and Sawyers, C.L. (2007). Sequential ABL kinase inhibitor therapy selects for compound drug-resistant BCR-ABL mutations with altered oncogenic potency. J. Clin. Invest. *117*, 2562–2569.

Suzuki, M., Abe, A., Imagama, S., Nomura, Y., Tanizaki, R., Minami, Y., Hayakawa, F., Ito, Y., Katsumi, A., Yamamoto, K., et al. (2010). BCR-ABL-independent and RAS / MAPK pathway-dependent form of imatinib resistance in Ph-positive acute lymphoblastic leukemia cell line with activation of EphB4. Eur. J. Haematol. *84*, 229–238.

Takle, A.K., Brown, M.J., Davies, S., Dean, D.K., Francis, G., Gaiba, A., Hird, A.W., King, F.D., Lovell, P.J., Naylor, A., et al. (2006). The identification of potent and selective imidazole-based inhibitors of B-Raf kinase. Bioorg. Med. Chem. Lett. *16*, 378–381.

Wan, P.T., Garnett, M.J., Roe, S.M., Lee, S., Niculescu-Duvaz, D., Good, V.M., Jones, C.M., Marshall, C.J., Springer, C.J., Barford, D., and Marais, R.; Cancer Genome Project. (2004). Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell *116*, 855–867.

Weisberg, E., Manley, P.W., Cowan-Jacob, S.W., Hochhaus, A., and Griffin, J.D. (2007). Second generation inhibitors of BCR-ABL for the treatment of imatinib-resistant chronic myeloid leukaemia. Nat. Rev. Cancer *7*, 345–356.

Wellbrock, C., Karasarides, M., and Marais, R. (2004). The RAF proteins take centre stage. Nat. Rev. Mol. Cell Biol. *5*, 875–885.

Whittaker, S., Kirk, R., Hayward, R., Zambon, A., Viros, A., Cantarino, N., Affolter, A., Nourry, A., Niculescu-Duvaz, D., Springer, C., et al. (2010). Gatekeeper mutations mediate resistance to BRAF-targeted therapies. Sci. Transl. Med. *2*, 35ra41.

Workman, P., Aboagye, E.O., Balkwill, F., Balmain, A., Bruder, G., Chaplin, D.J., Double, J.A., Everitt, J., Farningham, D.A., Glennie, M.J., et al; Committee of the National Cancer Research Institute. (2010). Guidelines for the welfare and use of animals in cancer research. Br. J. Cancer *102*, 1555–1577.

Yu, C., Krystal, G., Varticovksi, L., McKinstry, R., Rahmani, M., Dent, P., and Grant, S. (2002). Pharmacologic mitogen-activated protein/extracellular signal-regulated kinase kinase/mitogen-activated protein kinase inhibitors interact synergistically with STI571 to induce apoptosis in Bcr/Abl-expressing human leukemia cells. Cancer Res. *62*, 188–199.