Cancer Cell
Article

Transient Potent BCR-ABL Inhibition Is Sufficient to Commit Chronic Myeloid Leukemia Cells Irreversibly to Apoptosis

Neil P. Shah,[1,*] Corynn Kasap,[1] Christopher Weier,[1] Minna Balbas,[2] John M. Nicoll,[4] Eric Bleickardt,[5] Claude Nicaise,[5] and Charles L. Sawyers[2,3]
[1]Division of Hematology/Oncology, Department of Medicine, UCSF School of Medicine, San Francisco, CA 94143, USA
[2]Human Oncology and Pathogenesis Program
[3]Howard Hughes Medical Institute
Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
[4]Division of Hematology/Oncology, Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA
[5]Bristol-Myers Squibb Oncology, Wallingford, CT 06492, USA
*Correspondence: nshah@medicine.ucsf.edu
DOI 10.1016/j.ccr.2008.11.001

SUMMARY

The BCR-ABL inhibitor dasatinib achieves clinical remissions in chronic myeloid leukemia (CML) patients using a dosing schedule that achieves potent but transient BCR-ABL inhibition. In vitro, transient potent BCR-ABL inhibition with either dasatinib or imatinib is cytotoxic to CML cell lines, as is transient potent EGFR inhibition with erlotinib in a lung cancer cell line. Cytotoxicity correlates with the magnitude as well as the duration of kinase inhibition. Moreover, cytotoxicity with transient potent target inhibition is equivalent to prolonged target inhibition and in both cases is associated with BIM activation and rescued by BCL-2 overexpression. In CML patients receiving dasatinib once daily, response correlates with the magnitude of BCR-ABL kinase inhibition, thereby demonstrating the potential clinical utility of intermittent potent kinase inhibitor therapy.

INTRODUCTION

A general assumption in the development of small-molecule tyrosine kinase inhibitors (TKIs) for cancer therapy is that clinical success is likely to require prolonged target inhibition. For this reason, drug discovery efforts generally select compounds with longer half-lives for clinical development. These drugs are typically administered on a schedule that is predicted to result in continuous target inhibition, with the dose defined by tolerability as well as clinical activity. The oral ABL kinase inhibitor imatinib, which is now a frontline therapy for chronic myeloid leukemia (CML), confers prolonged inhibition of the BCR-ABL kinase as measured in the peripheral blood cells of CML patients 24 hr after a single dose. Imatinib has a half-life of 18 hr, as well as a long-acting metabolite with a half-life of 40 hr (Druker et al., 2001; Peng et al., 2004). Therefore, the dose and schedule commonly used for CML therapy produces nearly continuous target inhibition. Other approved TKIs are known to have similarly long half-lives in patients (erlotinib 36 hr, gefitinib 48 hr, lapatinib 24 hr, sunitinib 40–60 hr, sorafenib 25–48 hr) and are thus predicted to provide prolonged target inhibition (Burris et al., 2005; Cohen et al., 2004; Goodman et al., 2007; Johnson et al., 2005; Kane et al., 2006).

Dasatinib is a second-generation ABL kinase inhibitor with greater potency relative to imatinib in vitro that is effective in CML patients who fail imatinib therapy (Shah et al., 2004; Talpaz et al., 2006). Pharmacokinetic analysis performed during clinical development revealed a short serum half-life for dasatinib

SIGNIFICANCE

All previously approved tyrosine kinase (TK) inhibitors have prolonged half-lives in patients, resulting in continuous target inhibition. Here we show that the BCR-ABL inhibitor dasatinib can achieve deep clinical remissions in chronic myeloid leukemia patients using a dosing schedule that achieves transient potent BCR-ABL inhibition. Transient potent EGFR inhibition is similarly effective against a lung cancer cell line in vitro. The kinetics of cell death and activation of the proapoptotic protein BIM observed with transient potent kinase inhibition are identical to prolonged target inhibition. These findings demonstrate a critical reliance upon tonic TK activity for cell survival in two TK-associated malignancies and provide compelling rationale for the clinical development of compounds capable of achieving potent kinase inhibition, irrespective of biological half-life.

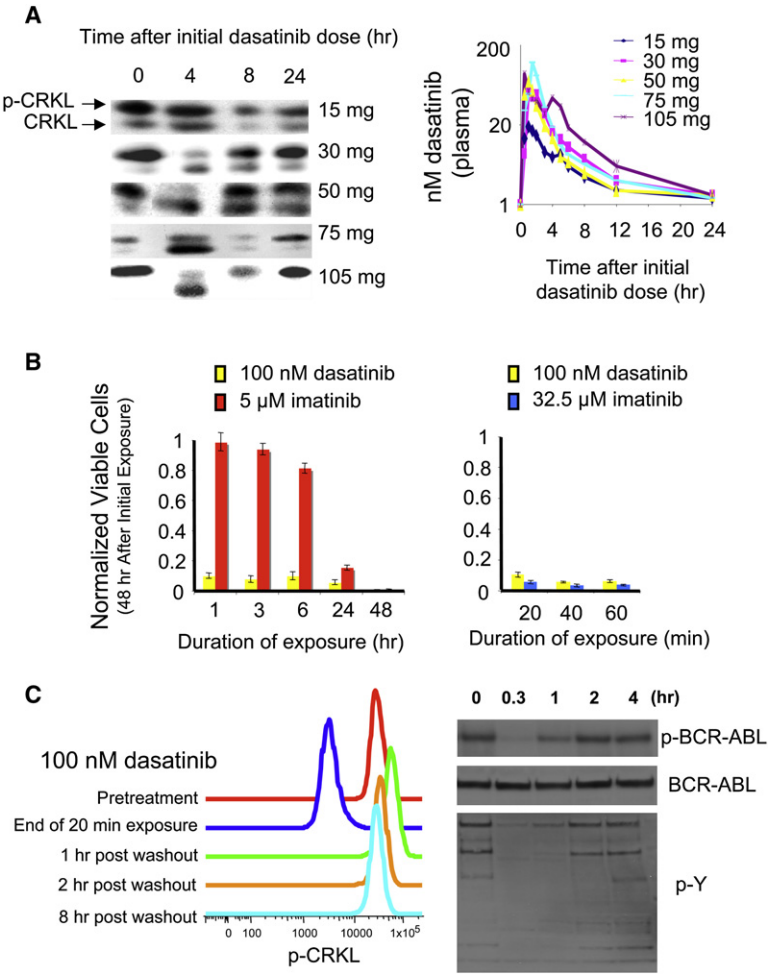

Figure 1. Transient but Potent BCR-ABL Inhibitor Therapy Is Sufficient to Kill CML Cells

(A) Pharmacokinetic and pharmacodynamic assessment of dasatinib in chronic myeloid leukemia (CML) patients. Left: immunoblot using a pan-CRKL antibody of peripheral blood mononuclear cell (PBMC) whole-cell lysates obtained from five CML patients at the times listed following ingestion of the initial dose of dasatinib. Right: plasma concentrations of dasatinib determined by high-pressure liquid chromatography (HPLC) in the same five CML patients at the times listed following ingestion of the initial dose of dasatinib.

(B) Normalized number of viable K562 cells following varying durations of exposure to the concentrations of dasatinib and imatinib indicated. Viable cell count was performed in triplicate 48 hr after initial exposure to tyrosine kinase inhibitor. Error bars represent standard deviation.

(C) Transient exposure to dasatinib achieves short-term tyrosine kinase inhibition. Left: BCR-ABL kinase activity as assessed by FACS-based phospho-CRKL analysis using a phospho-CRKL-specific antibody in K562 cells exposed to 100 nM dasatinib for 20 min. Right: phosphotyrosine content before and after exposure of K562 cells to 100 nM dasatinib for 0.3 hr as assessed by anti-p-BCR-ABL antibody (Y177) or anti-phosphotyrosine (p-Y) antibody (pY100). Anti-ABL antibody serves as loading control. Time following initiation of drug exposure is listed.

(approximately 3–5 hr) relative to imatinib (Brave et al., 2008). To avoid the potential for long periods of uninhibited BCR-ABL kinase activity, the phase I dosing schedule was amended to include patients treated twice daily in parallel with those treated once daily. Surprisingly, equivalent clinical response rates were observed in 40 patients treated either once or twice daily, including nearly 50% with complete cytogenetic remission (Philadelphia chromosome undetectable in a standard metaphase analysis) (Talpaz et al., 2006). Recently, a randomized clinical trial comparing once-daily to twice-daily dosing of dasatinib in CML showed equivalency in obtaining clinical responses and improved tolerability and progression-free survival in patients treated once daily, leading to a change in the approved frequency of administration from twice daily to once daily (Shah et al., 2008).

The clinical experience with dasatinib in CML challenges the conventional wisdom that TKIs must achieve nearly continuous target inhibition for maximal clinical impact. We sought to determine the relationship between time and depth of kinase inhibition in vitro as well as in clinical trial experience and to characterize the molecular mechanism whereby potent transient kinase inhibition achieves cytotoxicity in cancer cells associated with activated tyrosine kinases.

RESULTS

Efficacy of Transient Kinase Inhibition

The clinical success of once-daily dasatinib coupled with its short serum half-life implies that intermittent inhibition of BCR-ABL kinase activity is sufficient for clinical response. To formally address this question, we determined the magnitude of BCR-ABL inhibition in patients treated once daily with dasatinib at various times after administration of the initial dose. A number of strategies have been used to measure BCR-ABL kinase activity in the clinic, including tyrosine phosphorylation of downstream BCR-ABL substrates such as CRKL and STAT5 as well as phosphotyrosine content of BCR-ABL. CRKL phosphorylation has emerged as the most compelling readout to date based on the specificity of this signal for BCR-ABL activity as well as the reproducibility and reliability of the assay (Druker et al., 2001; Gorre et al., 2001). Phospho-BCR-ABL content has been technically challenging to measure in patient samples due to protein degradation after extraction of primary CML cells, whereas STAT5 is problematic due to a number of BCR-ABL-independent stimuli that can lead to its phosphorylation. In dasatinib-treated patients from five different dose cohorts treated in a phase I study (Talpaz et al., 2006), CRKL phosphorylation was diminished 4 hr after the initial dose in a dose-dependent manner but was largely restored by 8 hr, coincident with the decline in dasatinib serum levels measured in these same patients (Figure 1A). Therefore, as predicted by its pharmacokinetic profile, dasatinib achieves transient inhibition of BCR-ABL when taken once daily.

To model the clinical experience of transient BCR-ABL inhibition in vitro, we treated CML cells (K562) with 100 nM dasatinib or

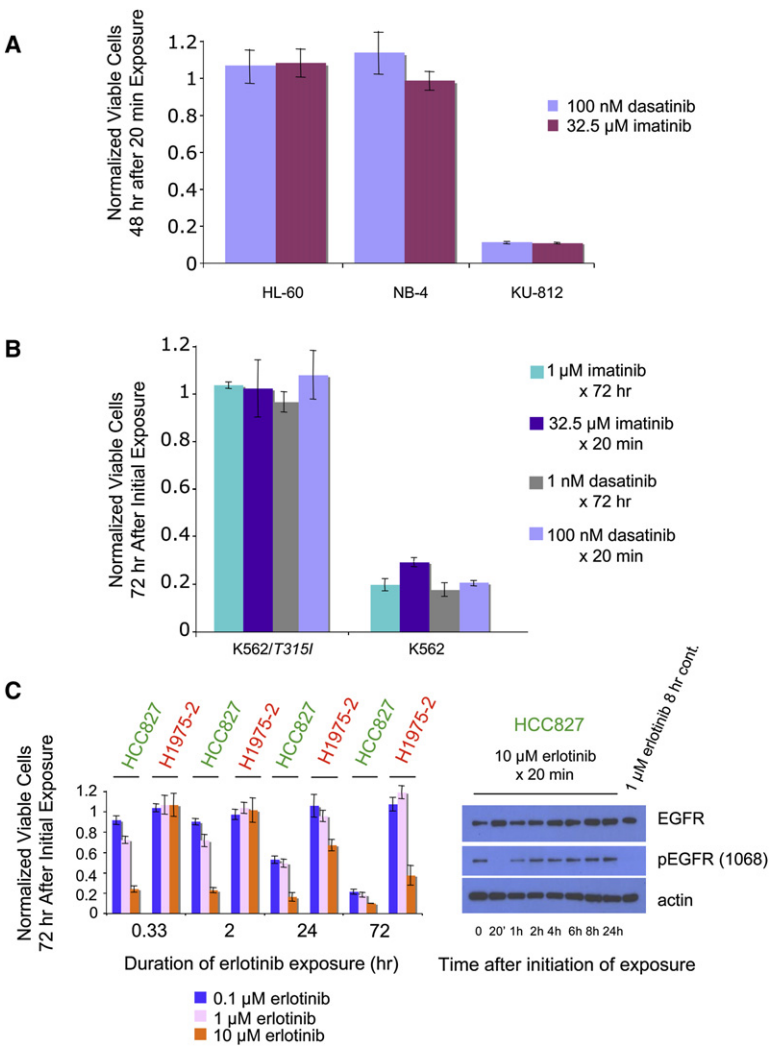

Figure 2. Cytotoxicity of High-Dose Pulse ABL and EGFR Kinase Inhibitor Treatment Is Target Dependent

(A) Normalized cell viability (assessed at 48 hr in triplicate) of BCR-ABL-negative (HL-60, NB-4) and BCR-ABL-positive (KU-812) cell lines following high-dose pulse exposure to dasatinib or imatinib.

(B) Normalized cell viability (assessed at 48 hr in triplicate) in BCR-ABL-positive K562 cells and in BCR-ABL/T315I K562 cells following continuous low-dose or high-dose pulse dasatinib or imatinib.

(C) Left: normalized cell viability (assessed at 72 hr in triplicate) of erlotinib-sensitive (HCC827) and -insensitive (H1975-2) non-small cell lung cancer cell lines exposed to the indicated concentrations of erlotinib for the indicated durations of time. Right: immunoblot of phospho-EGFR (Y1068) recovery following washout of 10 μM erlotinib. Total EGFR and actin immunoblots confirm adequate protein loading.

Error bars represent standard deviation.

5 μM imatinib for different time intervals ranging from 1 to 24 hr, followed by drug washout and replating of cells in media without drug. These TKI concentrations were chosen to mirror peak serum levels achieved in patients using clinically effective doses. Remarkably, when compared with mock-treated cells, only about 10% of the cells treated with dasatinib for just 1 hr were alive when assessed at 48 hr, whereas 24 hr of treatment was required to achieve comparable levels of cytotoxicity with imatinib (Figure 1B, left panel). However, a higher imatinib concentration (32.5 μM), chosen to match the in vitro ABL-inhibitory potency of 100 nM dasatinib, was as effective as dasatinib in killing CML cells after 1 hr of treatment. Indeed, similar levels of cytotoxicity (~90%) were seen with both drugs after exposure times as short as 20 min (Figure 1B, right panel), which we subsequently refer to as "high-dose pulse" therapy. BCR-ABL kinase activity, measured by flow cytometry using a phospho-CRKL-specific antibody, recovered within hours of drug washout after high-dose pulse dasatinib treatment, documenting that potent BCR-ABL inhibition was indeed transient as observed in CML patients treated once daily. Similar results were obtained by direct assessment of overall BCR-ABL phosphotyrosine content (using a global phosphotyrosine antibody) or specifically at Y177 (Figure 1C).

Dasatinib inhibits a broader range of kinases than imatinib (Carter et al., 2005), and both drugs are likely to have additional off-target effects when used at high concentrations, raising the possibility that the cytotoxicity observed after high-dose pulse therapy is not specific. Two lines of evidence suggest that this is not the case. First, high-dose pulse therapy did not impair the growth of two BCR-ABL-negative human myeloid leukemia cell lines, HL-60 and NB-4, but was cytotoxic in another BCR-ABL-positive leukemia cell line, KU-812 (Figure 2A). In addition, K562 cells engineered to express the BCR-ABL/T315I mutation, which confers a high degree of resistance to both imatinib and dasatinib (Shah et al., 2004), were no longer sensitive to high-dose pulse therapy (Figure 2B). As expected, we saw no modulation of CRKL phosphorylation in the T315I-expressing K562 cells or in BCR-ABL-negative leukemic cell lines after exposure to high-dose dasatinib or imatinib (data not shown).

Notably, the cytotoxicity of high-dose pulse TKI treatment is not unique to BCR-ABL-containing CML cell lines, as similar results were obtained with the EGFR inhibitor erlotinib in EGFR-dependent lung cancer cell lines. HCC827 lung cancer cells, which harbor an EGFR-activating exon 19 deletion as well as EGFR amplification (Amann et al., 2005), were equally sensitive to 20 min treatment with 10 μM erlotinib as to continuous exposure to 1 μM erlotinib for 72 hr. Similar to phospho-CRKL levels in CML cells treated with ABL inhibitors, phospho-EGFR levels were substantially reduced after 20 min but recovered within hours of drug washout (Figure 2C). As with the ABL inhibitors, the effect of high-dose pulse exposure appears to be specific as evidenced by the observation that high-dose pulse erlotinib had no effect on H1975-2 lung cancer cells, which contain the erlotinib-resistant EGFR T790M kinase domain mutation in addition to the activating L858R mutation (Pao et al., 2005). As expected, no change in EGFR phosphorylation was observed in H1975-2 cells after high-dose pulse erlotinib (data not shown).

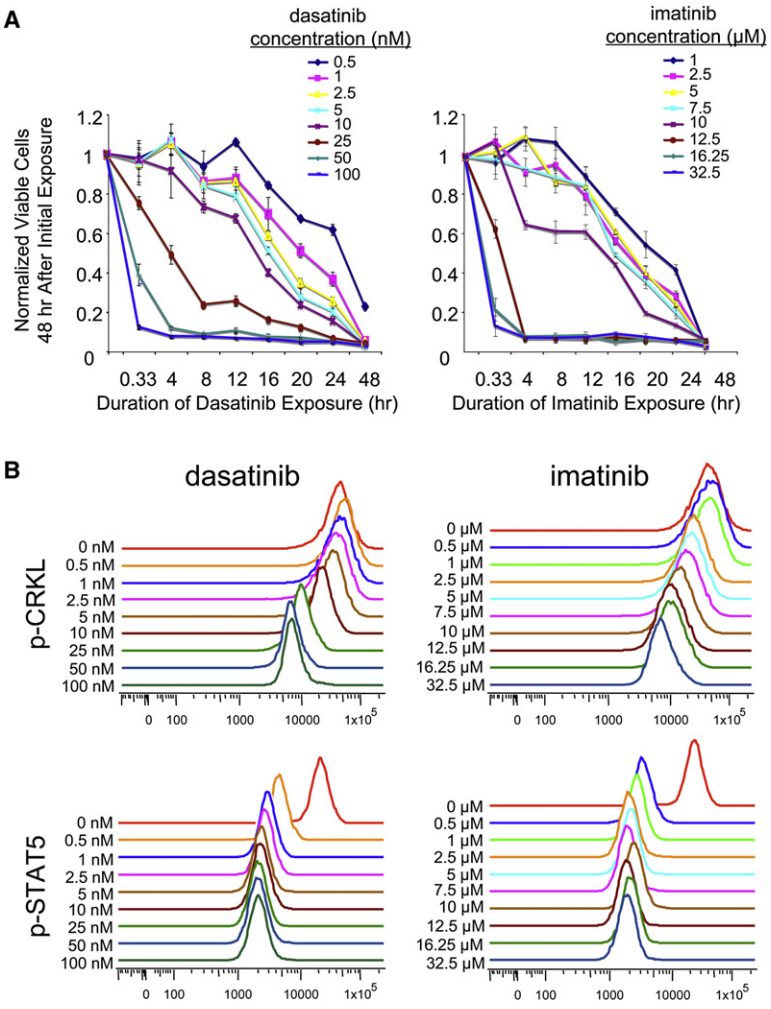

Figure 3. Magnitude and Duration of Kinase Inhibition Determines Cytotoxicity

(A) Normalized cell viability (assessed at 48 hr) of K562 cells exposed to a range of concentrations of dasatinib (left) and imatinib (right) for varying durations of time in triplicate. Error bars represent standard deviation.

(B) Assessment of BCR-ABL kinase activity in K562 cells through analysis of phospho-CRKL and phospho-STAT5 following treatment for 20 min with varying concentrations of dasatinib (left) and imatinib (right).

Magnitude and Duration of Kinase Inhibition Determines Cytotoxicity

Although the 100 nM concentration of dasatinib used in the high-dose pulse therapy experiments mimics peak serum levels achieved in patients, much lower concentrations (~0.5–1 nM IC_{50}) induce cytotoxicity in vitro with prolonged exposure (Shah et al., 2004). Since the effect of high-dose pulse ABL inhibitor therapy is BCR-ABL dependent, this begs the question of whether low concentrations might achieve similar levels of cytotoxicity after transient exposure or whether duration of treatment is an important variable under these conditions. To address this question, we exposed CML cells to eight different concentrations of either imatinib or dasatinib for treatment times varying from 20 min to 48 hr. Cytotoxicity was scored at 48 hr regardless of treatment duration.

The results establish a relationship between concentration and treatment duration (Figure 3A). Cytotoxicity approached 100 percent in cells exposed continuously to 0.5 or 1 nM dasatinib but was substantially diminished with shorter exposure times. Similar results were observed using concentrations of imatinib (1 μM) that match those used in earlier studies of continuous imatinib exposure in CML cells (Druker et al., 1996). Of note, 12 hr of treatment at these concentrations was insufficient to induce cell death (measured at 48 hr), whereas longer treatment periods led to an increasingly larger fraction of dead cells. In contrast, high concentrations of either drug (50–100 nM dasatinib or 12.5–32.5 μM imatinib) were nearly 100% cytotoxic after much shorter treatment intervals (from 20 min to 4 hr). Intermediate concentrations (2.5–25 nM dasatinib, 2.5–10 μM imatinib) showed progressively more cytotoxicity with extended treatment times. These data establish a relationship between concentration and treatment duration such that high-dose pulse therapy and low-dose continuous therapy confer equivalent cytotoxicity to CML cells.

To determine whether the concentration-dependent effect of ABL inhibitors on cytotoxicity is correlated with kinase inhibition, we used several assays to assess BCR-ABL kinase activity in K562 cells 20 min after drug exposure over the range of concentrations studied above. Phospho-CRKL content measured by flow cytometry was reduced in a concentration-dependent manner in cells treated with either dasatinib or imatinib, but this effect was clearly measurable only at higher concentrations (≥10 nM dasatinib, ≥2.5 μM imatinib). Surprisingly, we were unable to detect a substantial reduction in phospho-CRKL content at lower concentrations, even though these same concentrations confer substantial antiproliferative activity after prolonged continuous exposure. Similar results were obtained when phospho-CRKL was measured at later time points (up to 24 hr after drug exposure) or by western blot analysis using a pan-CRKL antibody that quantifies phospho-CRKL through mobility shift analysis of CRKL isoforms (data not shown), arguing against an accumulation of BCR-ABL inhibition over time as the explanation for this apparent paradox. In contrast, phospho-STAT5 content as measured by flow cytometry was substantially reduced by both drugs at the very lowest concentrations tested, consistent with published western blot data (Kindler et al., 2003), with apparent saturation of the assay at the second or third concentration level. The phosphotyrosine content of BCR-ABL, as measured by western blot using a global phosphotyrosine antibody, was similarly reduced in a dose-dependent manner (see Figure S1 available online). In summary, BCR-ABL kinase activity is inhibited in a concentration-dependent fashion over the range of concentrations studied here, indicating that the cytotoxicity of high-dose pulse exposure to ABL TKIs correlates with the magnitude of target inhibition.

The fact that two well-studied BCR-ABL pathway substrates displayed strikingly different abilities to measure BCR-ABL kinase inhibition was unexpected. Phospho-STAT5 appears to be highly sensitive to the lowest concentrations used here, which correspond to the IC_{50}s for growth inhibition caused by continuous drug exposure, whereas phospho-CRKL detects kinase inhibition at the highest concentrations where suppression of the phospho-STAT5 signal is saturated. One potential explanation for these differences could be in the relative pools of phosphorylated versus nonphosphorylated STAT5 and CRKL in CML cells at baseline. If only a small fraction of STAT5 molecules are phosphorylated in K562 cells at steady state, then relatively modest inhibition of BCR-ABL activity could be sufficient to dramatically shift the entire pool of phospho-STAT5. To address this possibility, we exposed cells to the nonspecific tyrosine phosphatase inhibitor pervanadate for 30 min in an attempt to maximally increase the pools of phosphorylated STAT5 and CRKL. Flow cytometric analysis showed a substantial (10-fold) increase in basal phospho-STAT5 content with relatively modest effects on phospho-CRKL content (Figure S2), consistent with the hypothesis that a much greater fraction of CRKL is phosphorylated (as previously demonstrated by western blot analysis) in CML cells relative to STAT5. The mechanism for these differences in the sizes of these phosphosubstrate pools is unknown but could be based on differential efficiency of substrate phosphorylation (CRKL, but not STAT5, is physically associated with BCR-ABL) (Ren et al., 1994; Senechal et al., 1998) or differential action of STAT5 versus CRKL phosphatases. Regardless, these data reveal unappreciated complexities in using substrate phosphorylation to measure target inhibition and argue for evaluation of multiple endpoints to ensure that an adequate dynamic range is assessed.

Rapid and Irreversible Engagement of the BIM/BCL-2 Pathway by High-Dose Pulse Therapy

The fact that 20 min of a high concentration of ABL kinase inhibitor treatment was sufficient to confer cytotoxicity at 48 hr suggested that high-dose pulse therapy leads to irreversible induction of a cell death program. Despite allowing 7 days for recovery in imatinib-free medium, no viable cells were detected following high-dose pulse imatinib (Figure S3). To search for potential differences in the onset of cell death, we measured caspase-3 activation and annexin V staining at various times after treatment with high-dose pulse versus low-dose continuous dasatinib. The earliest evidence for cell death detected by either assay was at 24 hr, with no clear difference between the two treatment regimens. Furthermore, continuous exposure of cells to a high concentration of dasatinib revealed similar kinetics of cell death, indicating that no further gain in cytotoxicity is achieved through continuous potent target inhibition (Figure S4). We therefore turned to earlier measures of engagement of the apoptotic pathway, such as the BH3-only protein BIM, whose expression is increased within hours of continuously applied imatinib treatment (Kuroda et al., 2006). As expected, BIM protein levels were increased and migrated with faster mobility (due to dephosphorylation) as early as 2 hr after continuous treatment with 1 nM dasatinib, and this was maintained for 48 hr. At later time points, levels of the antiapoptotic proteins BCL-2 and BCL-X_L were modestly reduced, whereas no change was observed in

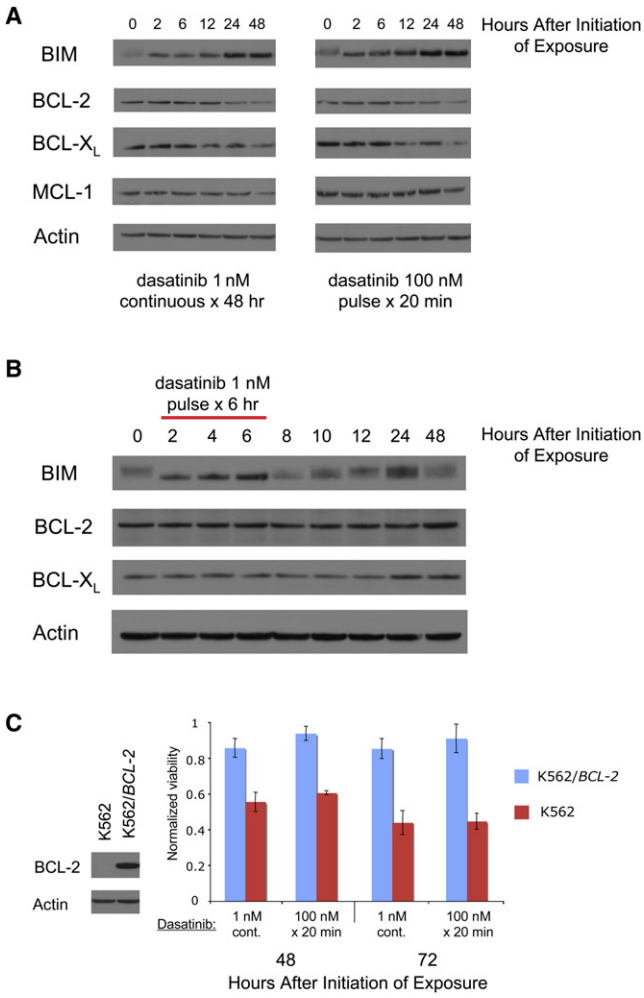

Figure 4. Kinetics of BIM Induction after High-Dose Pulse versus Low-Dose Continuous Dasatinib Exposure

(A) Kinetic assessment of select proapoptotic and antiapoptotic proteins in K562 cells treated with low-dose continuous (left) or high-dose pulse (right) dasatinib. Lysates were prepared at varying times after initiation of drug exposure as indicated and examined by immunoblot using antibodies specific for BIM, BCL-2, BCL-X_L, MCL-1, and actin.

(B) Effect of low-dose dasatinib treatment on select proapoptotic and antiapoptotic proteins in K562 cells treated for 6 hr.

(C) Left: immunoblot of K562/BCL-2 whole-cell extract demonstrating production of BCL-2 protein. Right: effect of ectopic BCL-2 overexpression on normalized cell viability of K562 cells treated with either 1 nM dasatinib continuously or 100 nM dasatinib for 20 min. Percentage of viable cells was determined (in triplicate) 48 and 72 hr after initial drug exposure and normalized to mock-treated cells. Error bars represent standard deviation.

MCL-1 levels (Figure 4A). Essentially identical results were obtained using high-dose pulse dasatinib, indicating that the apoptotic machinery is equally engaged early by both treatment regimens, and with imatinib (Figure S5).

It is unlikely that BIM induction at 2 hr is sufficient to induce cell death based on our earlier time-course studies (Figure 3A) showing that at least 8 hr of low-dose dasatinib treatment is required for substantial cytotoxicity. We therefore conducted a low-dose pulse experiment (6 hr of treatment with 1 nM dasatinib, which

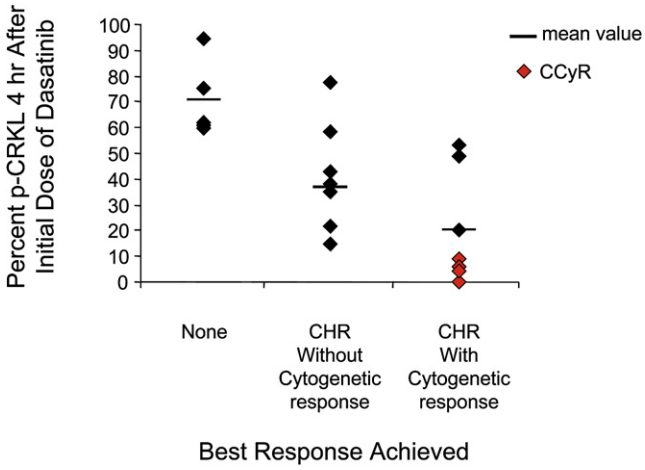

Figure 5. Correlation between BCR-ABL Inhibition 4 hr after Initial Dose of Dasatinib and Best Observed Outcome at that Dose of Drug

Each diamond represents the value obtained for an individual patient. CHR refers to complete hematologic response. Cytogenetic response is defined as a decline in the number of BCR-ABL-positive bone marrow metaphases following initiation of dasatinib. CCyR refers to complete cytogenetic response (no evidence of BCR-ABL in at least 20 bone marrow metaphases). The phosphorylated and nonphosphorylated forms of CRKL were detected by immunoblot using a pan-CRKL antibody as in Figure 1A and quantified by ImageQuant. Percent phospho-CRKL (% p-CRKL) was determined using the equation p-CRKL/(p-CRKL + CRKL) × 100 = % p-CRKL. By Kruskal-Wallis one-way analysis of variance, the overall null hypothesis that the distribution is the same among the three response groups was rejected (p = 0.0012). Comparing the p-CRKL values of patients with complete cytogenetic response to all others yields a two-sided p value of 0.0004 (rank-sum test).

does not confer substantial cytotoxicity) to examine the reversibility of BIM induction. Indeed, BIM levels and mobility were restored to levels seen in untreated cells within 2 hr of drug removal (Figure 4B), indicating that BIM induction is fully reversible after low-dose treatment but irreversible after high-dose treatment. Consistent with published data on imatinib (Kuroda et al., 2006), cell death induced by continuous exposure of CML cells to dasatinib was BCL pathway dependent, as shown by rescue of viability in K562 cells engineered to overexpress BCL-2. Notably, the cytotoxicity of high-dose pulse dasatinib was also fully reversed by BCL-2 overexpression (Figure 4C), suggesting that the two treatment regimens kill cells by similar mechanisms.

BCR-ABL Kinase Inhibition Is Associated with Clinical Response

An important clinical implication of the high-dose pulse therapy experiments is that the magnitude of BCR-ABL inhibition should impact the degree of clinical response for a drug with the short half-life of dasatinib. Specifically, the immunoblot and flow cytometry studies in CML cell lines suggest that >90% BCR-ABL inhibition is required for the irreversible cytotoxicity of high-dose pulse therapy. To determine whether this relationship exists in patients, we measured CRKL phosphorylation in peripheral blood 4 hr after the first dose in the 21 chronic-phase CML patients treated in the phase I clinical trial who received once-daily dasatinib (Talpaz et al., 2006) and correlated the degree of inhibition with clinical outcome (Figure 5). There was a statistically significant correlation between reduction of CRKL phosphorylation and magnitude of clinical response (hematologic or cytogenetic; p = 0.0012). Notably, the patients with the best responses (complete cytogenetic remission) had >90% reduction in CRKL phosphorylation (p = 0.0004).

DISCUSSION

Early in the development of targeted cancer therapies, much attention was devoted to the identification of compounds with relative specificity for the intended drug target and pharmacologic properties that maximize the probability of sustained target engagement using various measurements of target inhibition. These principles have resulted in the clinical success of inhibitors against a range of targets such as ABL, KIT, EGFR, HER2, and VEGFR, all of which are dosed using schedules derived to achieve sustained kinase inhibition while avoiding toxicity. Based on this rationale, the clinical development of dasatinib initially proceeded using a twice-daily dosing schedule due to its relatively short half-life, with the goal of providing continuous target coverage. However, we now know that the clinical benefit of dasatinib is equivalent when dosed once daily, and, importantly, tolerability is superior to the twice-daily schedule (Shah et al., 2008).

The clinical experience with dasatinib begs the question of whether sustained target inhibition should remain a driving principle in the development of targeted cancer therapies or whether intermittent target inhibition may be sufficient. Here we demonstrate that potent inhibition of BCR-ABL kinase activity is indeed transient in CML patients treated once daily but that maximal clinical benefit is associated with maximal BCR-ABL inhibition. We show that the same principle applies to CML cell lines grown in culture and extends more broadly to other kinase-dependent tumors such as *EGFR* mutant lung cancer. Of note, a small single-institution study of intermittent (weekly) high-dose therapy with erlotinib reported at least one clinical response in an *EGFR* mutant lung cancer patient (Milton et al., 2006). By examining a broad range of doses and treatment intervals, we found that both the magnitude and duration of target inhibition are linked to cytotoxicity in these kinase-dependent models, suggesting that an area under the curve (AUC) of target engagement (conceptually analogous to an AUC of drug exposure) could be a primary determinant of biological sensitivity.

The 20 min high-dose pulse exposure described here represents an extreme application of the transient therapy concept and raises several questions, including whether bioactivity is solely explained by inhibition of the intended target. Despite the fact that the high concentrations of dasatinib we selected can be achieved clinically, many additional targets are certainly inhibited at these doses. But several lines of evidence argue for specificity of the biological effect: (1) cytotoxicity is observed only in kinase-dependent models expressing the relevant target; (2) sensitive cells are rescued from cell death by drug-resistant mutants of the target (T315I for BCR-ABL, T790M for EGFR); and (3) similar bioactivity is seen with chemically distinct inhibitors, which would be expected to differ in their off-target activities at high doses. For these reasons, we favor a model whereby the biological activity of high-dose pulse therapy is explained by the potent suppression of the intended kinase target. In the absence of a pure inhibitor, we cannot exclude the possibility

that efficacy is explained by combined effects on the target kinase as well as other kinases.

Another consideration with the high-dose pulse model relates to the recovery of kinase activity after drug washout. Although we document restoration of BCR-ABL (and EGFR) phosphotyrosine content within hours, we cannot exclude the possibility of a small degree of residual kinase inhibition that is undetectable by these assays. Similarly, patients treated with once-daily dasatinib show rapid recovery of BCR-ABL activity (relative to imatinib), but a small amount of residual inhibition may persist since the pharmacokinetic profile shows a delayed terminal elimination phase (similar to most oral small-molecule drugs). The remarkable finding—in the cell culture model and in patients—is that potent target inhibition remains effective even if it is achieved only transiently.

The mechanism of cell death induced by high-dose pulse therapy needs further exploration but appears to be remarkably similar to that observed with low-dose continuous therapy. With both regimens, similar increases in BIM protein levels are observed within 2 hr of drug exposure, and levels of BCL-2 and BCL-X_L are gradually reduced with similar kinetics. One important distinction is that BIM induction by low-dose exposure is reversible after at least 6 hr of treatment, whereas induction by high-dose exposure is irreversible after only 20 min. This result is consistent with a model whereby a threshold level of kinase inhibition must be exceeded to commit cells to death and that threshold is determined by both the magnitude and duration of target inhibition. The kinetics of BIM induction (sustained versus unsustained) might serve as an early biomarker for defining this threshold. In the context of larger discussions on the phenomenon of oncogene addiction (Weinstein, 2002), it has been proposed that kinase inhibitors are effective against oncogene-addicted cancers due to the more rapid decay of oncogene-mediated prosurvival versus proapoptotic signals following pharmacologic inactivation of the oncogene (Sharma et al., 2006). Evidence for the "oncogenic shock" model comes from cells exposed continuously to concentrations of kinase inhibitors comparable to the low-dose continuous treatments used here. It is possible that the cytotoxicity of high-dose pulse exposure could be explained by a similar oncogenic shock model.

From the clinical perspective, the fact that efficacy was not compromised in the examples studied here by high-dose pulse therapy raises the question of whether a broader range of doses and schedules should be considered in early-stage clinical trials of targeted agents, particularly for compounds whose toxicity profile might preclude continuous therapy. As illustrated by the differential sensitivity of phospho-CRKL and phospho-STAT5 at reflecting BCR-ABL inhibition, the pharmacodynamic biomarkers used to evaluate different dosing schedules will need to be chosen carefully after consideration of several different downstream readouts. With robust quantitative measures of target inhibition in hand, it should also be possible to optimize individual patient doses, balancing target inhibition with toxicity, in pursuit of the goal of rational personalized cancer medicine.

EXPERIMENTAL PROCEDURES

Western Immunoblot of Cell Lysates from CML Patients

As specified by protocol, blood was obtained for pharmacokinetic and pharmacodynamic studies from consenting CML patients enrolled in a UCLA Institutional Review Board-approved phase I study of dasatinib. Blood samples for pharmacodynamic studies were collected on cycle 1/day 1 at the following time points relative to drug administration: predose, 4 hr, 8 hr, and 24 hr (just prior to the day 2 dose) postdose. Peripheral blood mononuclear cell (PBMC) isolation, preparation of whole-cell extracts, and CRKL immunoblotting were performed as described previously (Gorre et al., 2001).

Pharmacokinetic Analysis of Dasatinib

Blood samples were collected on cycle 1/day 1 at the following time points relative to drug administration: predose, 30 min, 1 hr, 1.5 hr, 2 hr, 3 hr, 4 hr, 5 hr, 6 hr, 8 hr, and 24 hr (just prior to the day 2 dose) postdose. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was utilized for the quantitation of dasatinib in 0.1 ml of human EDTA plasma using a Sciex API 3000 mass spectrometer (Applied Biosystems). After the addition of phosphoric acid containing internal standard (200 ng/ml of plasma) to 0.1 ml of each quality control (QC) sample and calibration standard, the samples were loaded onto an Oasis HLB μElution 96-well solid-phase extraction plate (Waters). The compounds were eluted with 50 μl of methanol. After the addition of water to the methanol eluate and vortexing, the extracts were injected into the LC-MS/MS system. Chromatographic separation was achieved isocratically on a Phenomenex Luna phenyl-hexyl analytical column (2 × 50 mm, 3 μm, Phenomenex). The mobile phase contained formic acid, water, methanol, and ammonium acetate. Detection was by positive ion electrospray-tandem mass spectrometry. The standard curves, which ranged from 1 to 1000 ng/ml, were fitted to a 1/x weighted quadratic regression model and converted to nM units. The intra-assay precision was within 4.2% coefficient of variation (CV), and interassay precision was within 3.0% CV for both analytes. The assay accuracy was within ±4.5% of the nominal values for analytical QC samples. For both analytes, at the lower limit of quantitation (LLOQ) of 1 ng/ml, the deviations of the predicted concentrations from the nominal value for all six LLOQ samples were within ±9.0%.

Quantification of BCR-ABL Kinase Inhibition in CML Patients 4 hr after Dasatinib Administration

Lysates prepared from PBMCs isolated 4 hr after ingestion of the initial dose of dasatinib were subject to immunoblotting with anti-CRKL antibody. The phosphorylated and nonphosphorylated forms of CRKL were detected using ECL (GE Healthcare). Autoradiographs (Kodak) were imaged using a digital imager (Bio-Rad) to generate TIFF files. The TIFF files were then analyzed with ImageQuant v2003 software (GE Healthcare). Background was corrected. Percent phospho-CRKL (% p-CRKL) was determined using the equation p-CRKL/(p-CRKL + CRKL) × 100 = % p-CRKL.

Tyrosine Kinase Inhibitors

Dasatinib was provided by Bristol-Myers Squibb, imatinib was provided by Novartis Pharmaceuticals, and erlotinib was purified from tablets. Stocks of 20 mM dasatinib and 10 mM imatinib and erlotinib were prepared in DMSO and stored at −80°C.

Cell Lines, Drug Exposures, and Viability Determination

Cell lines were propagated in RPMI supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin. Exponentially growing cells were plated at 2 × 10^5 cells/ml and exposed in medium to varying concentrations of TKIs prepared from stock solutions. For pulse conditions, cells were washed three times with a volume of medium that consisted of 50% of the volume of the drug exposure and replated in fresh medium without inhibitor at a final concentration of 2 × 10^5 cells/ml. Viability was assessed at 24, 48, and 72 hr after initial exposure using trypan blue exclusion on a Vi-CELL XR (Beckman Coulter, Inc.), and cell counts were normalized to mock-treated controls. Experiments were performed in triplicate. K562/T315I and K562/BCL-2 cells were created as follows. Cotransfection of MSCV-puroBCR-ABL/T315I (Gorre et al., 2001) or pMIG/BCL-2 (Addgene Inc., deposited by S. Korsmeyer) with pCL-Ampho into 293FT cells was performed, and the supernatant was removed after 48 hr and filtered through a 0.45 μm filter. Spinoculation of K562 cells was followed with selection of cells by puromycin (for MSCV-puroBCR-ABL/T315I) or by sorting GFP-positive cells (for pMIG/BCL-2). HCC827 and H1975-2 cells, kindly provided by W. Pao (Memorial Sloan-Kettering Cancer Center), were cultured in 5% serum.

Phosphoflow Analyses

Flow cytometry was performed with a LSR II (BD Biosciences) using standard methods. Briefly, for phosphoprotein analysis, 5×10^5 cells per sample were fixed using 3.2% paraformaldehyde and washed by adding 2 ml phosphate-buffered saline (PBS) followed by gentle resuspension and centrifugation. Cell pellets were aspirated and resuspended in methanol and stored overnight at −20°C. Cells were then rehydrated after a PBS wash by resuspending in PBS with 4% FBS (incubation buffer). Cells were blocked after centrifugation and aspiration by adding 0.5 μg Fcy III/II receptor antibody (BD Biosciences) and incubating for 15 min at 4°C. Cells were stained with 50 μl of an antibody mix containing 1 μl primary phospho-CRKL antibody (Cell Signaling Technology) in incubation buffer per sample; staining proceeded for 20 min at room temperature. Cells were washed with buffer, aspirated, and stained with 0.125 μg anti-rabbit R-phycoerythrin secondary antibody (BioSource) and 10 μl primary conjugated phospho-STAT5 antibody (BD Biosciences) for 20 min at room temperature. Cells were washed once with PBS prior to collection. Annexin V and active caspase-3 staining was performed according to the manufacturer (BD Biosciences.) Approximately 30,000 ungated events were collected for each sample on a LSR II (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.). All phosphoflow analyses were performed at least in triplicate and yielded similar results.

Phosphatase Experiments

Vanadate solutions were prepared using equimolar concentrations of sodium orthovanadate (Sigma-Aldrich) and hydrogen peroxide (Fisher Scientific), and cells were incubated in the presence of vanadate for 90 min before fixation and flow analysis.

Immunoblot of Cell Line Extracts

At the indicated time points, 5×10^6 cells were harvested, washed twice with PBS, and lysed in 200 μl cell extraction buffer (Omnia) supplemented with 10 μl/ml protease and phosphatase inhibitors (inhibitor cocktails II and III, Calbiochem). Each lysate (30 μg) was electrophoresed on a 10% Bis-Tris polyacrylamide gel using MES running buffer (NuPAGE). BIM, BCL-2, BCL-X_L, MCL-1, and actin were identified with Cell Signaling Technology antibodies. Phospho-BCR-ABL was assessed using a phosphospecific antibody for Y177 (Cell Signaling Technology), and BCR-ABL protein was identified with anti-ABL antibody AB-3 (Oncogene Research Products) following electrophoresis of cell lysates on a 3%–8% Tris-acetate gel. CRKL was identified with anti-CRKL antibody (Santa Cruz Biotechnology). Phosphotyrosine was detected using anti-phosphotyrosine antibodies (4G10, Upstate Biotechnologies; PY100, Cell Signaling Technology) as described previously (Gorre et al., 2001).

SUPPLEMENTAL DATA

The Supplemental Data include five figures and can be found with this article online at http://www.cancercell.org/supplemental/S1535-6108(08)00368-1.

ACKNOWLEDGMENTS

We thank E. Landaw for statistical analysis; K. Shannon, S. Oakes and J. Debnath for helpful discussions; and M. Chalukya for sample processing. This work was supported by the Doris Duke Charitable Foundation, the V Foundation, the Edward A. Ageno Leukemia Research Fund, the Sandler Program in Basic Sciences, the Howard Hughes Medical Institute, and Arthur Kern.

E.B. and C.N. are employees and shareholders of Bristol-Myers Squibb Oncology.

Received: April 28, 2008
Revised: September 10, 2008
Accepted: November 3, 2008
Published: December 8, 2008

REFERENCES

Amann, J., Kalyankrishna, S., Massion, P.P., Ohm, J.E., Girard, L., Shigematsu, H., Peyton, M., Juroske, D., Huang, Y., Stuart Salmon, J., et al. (2005). Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer. Cancer Res. *65*, 226–235.

Brave, M., Goodman, V., Kaminskas, E., Farrell, A., Timmer, W., Pope, S., Harapanhalli, R., Saber, H., Morse, D., Bullock, J., et al. (2008). Sprycel for chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia resistant to or intolerant of imatinib mesylate. Clin. Cancer Res. *14*, 352–359.

Burris, H.A., 3rd, Hurwitz, H.I., Dees, E.C., Dowlati, A., Blackwell, K.L., O'Neil, B., Marcom, P.K., Ellis, M.J., Overmoyer, B., Jones, S.F., et al. (2005). Phase I safety, pharmacokinetics, and clinical activity study of lapatinib (GW572016), a reversible dual inhibitor of epidermal growth factor receptor tyrosine kinases, in heavily pretreated patients with metastatic carcinomas. J. Clin. Oncol. *23*, 5305–5313.

Carter, T.A., Wodicka, L.M., Shah, N.P., Velasco, A.M., Fabian, M.A., Treiber, D.K., Milanov, Z.V., Atteridge, C.E., Biggs, W.H., 3rd, Edeen, P.T., et al. (2005). Inhibition of drug-resistant mutants of ABL, KIT, and EGF receptor kinases. Proc. Natl. Acad. Sci. USA *102*, 11011–11016.

Cohen, M.H., Williams, G.A., Sridhara, R., Chen, G., McGuinn, W.D., Jr., Morse, D., Abraham, S., Rahman, A., Liang, C., Lostritto, R., et al. (2004). United States Food and Drug Administration Drug Approval summary: Gefitinib (ZD1839; Iressa) tablets. Clin. Cancer Res. *10*, 1212–1218.

Druker, B.J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G.M., Fanning, S., Zimmermann, J., and Lydon, N.B. (1996). Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat. Med. *2*, 561–566.

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.

Goodman, V.L., Rock, E.P., Dagher, R., Ramchandani, R.P., Abraham, S., Gobburu, J.V., Booth, B.P., Verbois, S.L., Morse, D.E., Liang, C.Y., et al. (2007). Approval summary: sunitinib for the treatment of imatinib refractory or intolerant gastrointestinal stromal tumors and advanced renal cell carcinoma. Clin. Cancer Res. *13*, 1367–1373.

Gorre, M.E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P.N., and Sawyers, C.L. (2001). Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science *293*, 876–880.

Johnson, J.R., Cohen, M., Sridhara, R., Chen, Y.F., Williams, G.M., Duan, J., Gobburu, J., Booth, B., Benson, K., Leighton, J., et al. (2005). Approval summary for erlotinib for treatment of patients with locally advanced or metastatic non-small cell lung cancer after failure of at least one prior chemotherapy regimen. Clin. Cancer Res. *11*, 6414–6421.

Kane, R.C., Farrell, A.T., Saber, H., Tang, S., Williams, G., Jee, J.M., Liang, C., Booth, B., Chidambaram, N., Morse, D., et al. (2006). Sorafenib for the treatment of advanced renal cell carcinoma. Clin. Cancer Res. *12*, 7271–7278.

Kindler, T., Breitenbuecher, F., Kasper, S., Stevens, T., Carius, B., Gschaidmeier, H., Huber, C., and Fischer, T. (2003). In BCR-ABL-positive cells, STAT-5 tyrosine-phosphorylation integrates signals induced by imatinib mesylate and Ara-C. Leukemia *17*, 999–1009.

Kuroda, J., Puthalakath, H., Cragg, M.S., Kelly, P.N., Bouillet, P., Huang, D.C., Kimura, S., Ottmann, O.G., Druker, B.J., Villunger, A., et al. (2006). Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic. Proc. Natl. Acad. Sci. USA *103*, 14907–14912.

Milton, D.T., Azzoli, C.G., Heelan, R.T., Venkatraman, E., Gomez, J.E., Kris, M.G., Krug, L.M., Pao, W., Rizvi, N.A., Dunne, M., and Miller, V.A. (2006). A phase I/II study of weekly high-dose erlotinib in previously treated patients with nonsmall cell lung cancer. Cancer *107*, 1034–1041.

Pao, W., Miller, V.A., Politi, K.A., Riely, G.J., Somwar, R., Zakowski, M.F., Kris, M.G., and Varmus, H. (2005). Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med. *2*, e73.

Peng, B., Hayes, M., Resta, D., Racine-Poon, A., Druker, B.J., Talpaz, M., Sawyers, C.L., Rosamilia, M., Ford, J., Lloyd, P., and Capdeville, R. (2004).

Pharmacokinetics and pharmacodynamics of imatinib in a phase I trial with chronic myeloid leukemia patients. J. Clin. Oncol. *22*, 935–942.

Ren, R., Ye, Z.S., and Baltimore, D. (1994). Abl protein-tyrosine kinase selects the Crk adapter as a substrate using SH3-binding sites. Genes Dev. *8*, 783–795.

Senechal, K., Heaney, C., Druker, B., and Sawyers, C.L. (1998). Structural requirements for function of the Crkl adapter protein in fibroblasts and hematopoietic cells. Mol. Cell. Biol. *18*, 5082–5090.

Shah, N.P., Tran, C., Lee, F.Y., Chen, P., Norris, D., and Sawyers, C.L. (2004). Overriding imatinib resistance with a novel ABL kinase inhibitor. Science *305*, 399–401.

Shah, N.P., Kantarjian, H.M., Kim, D.-W., Réa, D., Dorlhiac-Llacer, P.E., Milone, J.H., Vela-Ojeda, J., Silver, R.T., Khoury, H.J., Charbonnier, A., et al. (2008). Intermittent target inhibition with dasatinib 100 mg once daily preserves efficacy and improves tolerability in imatinib-resistant and -intolerant chronic-phase chronic myeloid leukemia. J. Clin. Oncol. *26*, 3204–3212.

Sharma, S.V., Gajowniczek, P., Way, I.P., Lee, D.Y., Jiang, J., Yuza, Y., Classon, M., Haber, D.A., and Settleman, J. (2006). A common signaling cascade may underlie "addiction" to the Src, BCR-ABL, and EGF receptor oncogenes. Cancer Cell *10*, 425–435.

Talpaz, M., Shah, N.P., Kantarjian, H., Donato, N., Nicoll, J., Paquette, R., Cortes, J., O'Brien, S., Nicaise, C., Bleickardt, E., et al. (2006). Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. N. Engl. J. Med. *354*, 2531–2541.

Weinstein, I.B. (2002). Cancer. Addiction to oncogenes–the Achilles heal of cancer. Science *297*, 63–64.