The ABL Switch Control Inhibitor DCC-2036 Is Active against the Chronic Myeloid Leukemia Mutant BCR-ABL\textsuperscript{T315I} and Exhibits a Narrow Resistance Profile

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Abstract

Acquired point mutations within the BCR-ABL kinase domain represent a common mechanism of resistance to ABL inhibitor therapy in patients with chronic myeloid leukemia (CML). The BCR-ABL\textsuperscript{T315I} mutant is highly resistant to imatinib, nilotinib, and dasatinib, and is frequently detected in relapsed patients. This critical gap in resistance coverage drove development of DCC-2036, an ABL inhibitor that binds the switch control pocket involved in conformational regulation of the kinase domain. We evaluated the efficacy of DCC-2036 against BCR-ABL\textsuperscript{T315I} and other mutants in cellular and biochemical assays and conducted cell-based mutagenesis screens. DCC-2036 inhibited autophosphorylation of ABL and ABL\textsuperscript{T315I} enzymes, and this activity was consistent with selective efficacy against Ba/F3 cells expressing BCR-ABL (IC\textsubscript{50} 19 nmol/L), BCR-ABLT315I (IC\textsubscript{50} 63 nmol/L), and most kinase domain mutants. Ex vivo exposure of CML cells from patients harboring BCR-ABL or BCR-ABL\textsuperscript{T315I} to DCC-2036 revealed marked inhibition of colony formation and reduced phosphorylation of the direct BCR-ABL target CrkL. Cell-based mutagenesis screens identified a resistance profile for DCC-2036 centered around select P-loop mutations (G250E, Q252H, Y253H, E255K/V), although a concentration of 750 nmol/L DCC-2036 suppressed the emergence of all resistant clones. Further screens for resistance due to BCR-ABL compound mutations (two mutations in the same clone) identified BCR-ABLE255V / T315I as the most resistant mutant. Taken together, these findings support continued evaluation of DCC-2036 as an important new agent for treatment-refractory CML.

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Introduction

Use of ABL inhibitors to block the activity of the oncogenic BCR-ABL tyrosine kinase in the treatment of chronic myeloid leukemia (CML) serves as a model for molecularly targeted cancer therapies. The ABL inhibitor imatinib has an extensive and impressive clinical track record in CML, with newly diagnosed, chronic phase patients demonstrating 5-year overall and progression-free survival rates of 89% and 93%, respectively (1). Most patients treated with imatinib experience durable responses, although discontinuation of therapy due to intolerance or resistance is necessary in a subset of patients, particularly in advanced disease (2).

Resistance to imatinib is most commonly explained by acquired point mutations in the kinase domain of BCR-ABL, which impair drug binding (3). Mutations at over 50 residues conferring varying degrees of imatinib resistance have been reported clinically (4). The more potent ABL inhibitors nilotinib and dasatinib have proven largely successful in imatinib-refractory CML patients harboring this type of resistance, with the key exception of the BCR-ABL\textsuperscript{T315I} mutant. Consequently, the addition of targeted ABL inhibitors with activity against BCR-ABL\textsuperscript{T315I} will be critical to further controlling drug resistance in CML (3).

A recent approach to addressing this gap in resistance coverage utilized DCC-2036, an inhibitor that avoids direct binding contact at T315 and binds a transiently formed switch control pocket of ABL, resulting in stabilization of an electrostatic ion pair (R386 of the activation loop and E282 of the C-helix) critical for maintaining the catalytically inactive kinase conformation. DCC-2036 exhibits a long

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off-rate for binding both ABL and ABL T315I, and demonstrates additional highly selective activity for FLT3, TIE2, and SRC-family kinases. DCC-2036 also showed significant efficacy and improved survival in a murine bone marrow transplantation model of BCR-ABL T315I-driven CML (5).

Here, we evaluate the efficacy of DCC-2036 against BCR-ABL T315I and other mutants in CML cell lines and primary cells, and establish the anticipated resistance profile for DCC-2036 using cell-based screens.

Materials and Methods

**ABL autophosphorylation assays**

Kinase autophosphorylation assays with tyrosine-dephosphorylated ABL and ABL T315I (Invitrogen) were performed alone or with DCC-2036 (0.2–3.125 nmol/L) or imatinib (625–3,125 nmol/L) as described (6).

**Cell lines**

Certified Ba/F3, K562, KYO1, LAMA, HEL, CMK, and Marimo cells were obtained from the American Type Culture Collection and grown in the recommended culture medium. Ba/F3 transfectants expressing native BCR-ABL or BCR-ABL with a single kinase domain mutation were generated and maintained as described (7). The Ba/F3 BCR-ABL T315A cell line was a gift from N. Shah (UCSF). None of the cell lines used in this study was cultured for longer than 6 months from initial purchase or characterization. No further authentication of cell line characteristics was done.

**Cell proliferation assays**

Parental Ba/F3 cells and Ba/F3 cells expressing native or mutant BCR-ABL (4 × 10³ per well) were incubated alone or with DCC-2036 (0.2–15,625 nmol/L) for 72 hours. Proliferation measurements and IC₅₀ value determinations were done as described (7). Identical experiments were carried out for CML (K562, KYO1, LAMA) and non-CML cell lines (HEL, CMK, Marimo).

**Immunoblot analyses of CrkL phosphorylation**

For cell line experiments, Ba/F3 cells expressing BCR-ABL, BCR-ABL T255V, or BCR-ABL T315I (5 × 10⁶ cells/well) were cultured 4 hours in complete media alone or with DCC-2036 (0.2–3.125 nmol/L) or imatinib (0.2–3.125 nmol/L) as described (8). For primary cell experiments, following informed consent, peripheral blood mononuclear cells from a newly diagnosed CML patient and from an accelerated phase were cultured 4 hours in complete media alone or with DCC-2036 (0.2–3.125 nmol/L) or imatinib (625–3,125 nmol/L) as described (6).

Hematopoietic colony formation assays

To assess granulocyte/macrophage colony formation (CFU-GM), mononuclear cells from bone marrow of a newly diagnosed CML patient, an accelerated phase patient harboring BCR-ABL T315I, or a healthy donor were obtained following informed consent and plated alone or with DCC-2036 (50 or 500 nmol/L) or imatinib (2,000 nmol/L) in triplicate (5 × 10⁴ cells/plate) in IMDM/methylcellulose as described (9). Results are reported as percentage of colonies relative to untreated.

**Cell-based resistance screens**

For DCC-2036 screens starting from Ba/F3 cells expressing native BCR-ABL, cells were treated overnight with N-ethyl-N-nitrosourea (ENU; 50 μg/mL) and resuspended in complete media (1 × 10⁵ cells/well) supplemented with DCC-2036 (50–1,250 nmol/L) as described (10). DCC-2036 (160 nmol/L) was also evaluated in dual combinations with imatinib (2,000 nmol/L), nilotinib (500 nmol/L), or dasatinib (25 nmol/L). Wells exhibiting outgrowth were expanded, sequenced, and analyzed for mutations (Mutation Surveyor; SoftGenetics) as described (6). Similar experiments were conducted starting from Ba/F3 BCR-ABL T315I cells treated with DCC-2036 (250–750 nmol/L), and from a pooled mix of equal cell numbers of all Ba/F3 BCR-ABL cell lines treated with a cocktail of ABL kinase inhibitors [DCC-2036 (250 nmol/L), nilotinib (500 nmol/L), and dasatinib (25 nmol/L)].

**Results and Discussion**

We established that DCC-2036 (Fig. 1A) directly inhibits the catalytic activity of ABL and ABL T315I by evaluating kinase autophosphorylation activity. Although both imatinib and DCC-2036 attenuated activity of ABL, only DCC-2036 blocked tyrosine autophosphorylation (Fig. 1B). Unlike imatinib, nilotinib, and dasatinib, the binding mode of DCC-2036 to ABL or ABL T315I does not require a hydrogen bond to the side chain hydroxyl of native T315 and avoids a steric clash with mutated I315. On binding, DCC-2036 induces and stabilizes an aspartate-phenylalanine-glycine motif (DFG)-out, catalytically inactive conformation of the kinase domain, precluding phosphorylation of activation loop residue Y393, a critical event preceding full catalytic activation of ABL kinase (5).

Cellular assays demonstrated that DCC-2036 selectively inhibits most clinically relevant imatinib-resistant mutants (Fig. 1C). DCC-2036 inhibited growth of Ba/F3 cells expressing BCR-ABL IC₅₀ 19 nmol/L with approximately 16-fold higher potency than imatinib and, of key importance, cells expressing the imatinib-, nilotinib-, and dasatinib-resistant BCR-ABL T315I mutant IC₅₀ 63 nmol/L. The selectivity of DCC-2036 for BCR-ABL–positive cells was evidenced by its marked inhibition of CML cell lines compared with non-CML leukemia lines (Fig. 1C). Sensitivity of BCR-ABL mutants to DCC-2036 segregated into 3 categories: (a) IC₅₀ ≤ 50 nmol/L: 1/14; M351T, (b) IC₅₀ 100 nmol/L: 8/14; M244V, G250E, Q252H, Y253F, T315A, T315I, F317L, H396P, and (c) IC₅₀ > 100 nmol/L: 5/14; Y253H, E255K, E255V, F317V, F359V. Among these, BCR-ABL T255V was least sensitive to DCC-2036 (IC₅₀ 410 nmol/L).
Immunoblot analyses examining the ability of DCC-2036 to block tyrosine phosphorylation of the direct BCR-ABL substrate CrkL revealed greater inhibition in cells expressing BCR-ABL or BCR-ABL T315I than BCR-ABL E255V (Fig. 1D). These findings suggest that, although DCC-2036 exhibits activity against the T315I mutant, select mutations of the P-loop such as E255V may be more problematic. Notably, BCR-ABL E255V is highly resistant to imatinib and confers moderate resistance to both nilotinib and dasatinib in vitro (7), and has been reported in clinical failures of each of these therapies (4, 11, 12).

As follow-up to the efficacy of DCC-2036 observed in BCR-ABL–positive cells, particularly the BCR-ABL T315I mutant, we evaluated DCC-2036 against mononuclear cells from a newly diagnosed CML chronic phase patient and an accelerated phase patient harboring BCR-ABL T315I. Ex vivo exposure of primary BCR-ABL T315I cells to DCC-2036 sharply reduced CrkL phosphorylation, whereas imatinib, nilotinib, and dasatinib were ineffective (Fig. 2A). All inhibitors reduced CrkL phosphorylation in primary cells from the newly diagnosed CML patient (Fig. 2B), although imatinib (1 μmol/L) showed limited effect. CrkL phosphorylation is a clinical biomarker of BCR-ABL activity, and its inhibition in primary CML cells has been correlated with degree of response achieved on therapy (13).

Although complete pharmacodynamic data for DCC-2036 have not yet been reported, our results demonstrate that DCC-2036 is active in clinical isolates from CML patients harboring BCR-ABL or BCR-ABL T315I. This is corroborated by colony formation data for primary CML cells, wherein exposure of cells from the same BCR-ABL T315I CML patient to DCC-2036 substantially reduced outgrowth of CML cells, with no toxicity toward mononuclear cells from a healthy individual (Fig. 2C).

Given the unique binding characteristics of DCC-2036, we screened for resistance-conferring mutations specific to DCC-2036 but susceptible to other ABL inhibitors. Results from a cell-based resistance screen for BCR-ABL mutants persisting in the presence of DCC-2036 revealed a concentration-dependent
No resistant subclones were recovered with dual combinations of DCC-2036 and clinically achievable concentrations of nilotinib (500 nmol/L) or dasatinib (25 nmol/L). These findings are similar to those from studies with another ABLT315I inhibitor, SGX393 (9), and suggest that ABL inhibitor cocktails that include an ABLT315I inhibitor such as DCC-2036 may represent a rational therapeutic approach to mitigating resistance. As the immediate clinical application of an ABLT315I inhibitor is in refractory CML patients harboring this mutation, we performed resistance screens starting from Ba/F3 cells expressing BCR-ABL T315I to identify BCR-ABL compound mutations (2 mutations in the same clone) conferring increased resistance to DCC-2036. Such mutations have been reported in clinical failures to dasatinib or nilotinib salvage therapy, suggesting potential for selection on sequential treatment with ABL inhibitors (14–17). The compound mutation-based resistance profile for DCC-2036 narrowed predominantly to BCR-ABL T315I/A253V / Y253F (83.3% of recovered mutants at 750 nmol/L; Fig. 3C; Supplementary Table S3). An additional mutant featuring substitution of the baseline isoleucine at residue 315 for methionine (I315M) was also recovered. To our knowledge, mutation of the gatekeeper residue (either the native threonine or mutant isoleucine) to methionine has not been observed in resistance to other ABL tyrosine kinase inhibitors. DCC-2036 forms hydrogen bonds with the nearby ATP hinge.
DCC-2036 Inhibits BCR-ABL<sup>T315I</sup>

### Dual-combination studies

Starting from Native BCR-ABL:

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Starting from BCR-ABLT315I:

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### Single-agent DCC-2036

Starting from Native BCR-ABL:

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Starting from BCR-ABLT315I:

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**Figure 3.** DCC-2036 suppresses resistance alone or in ABL inhibitor combinations and exhibits few BCR-ABL compound mutant vulnerabilities. A, BCR-ABL mutants recovered from cell-based resistance screens for DCC-2036, starting from Ba/F3 BCR-ABL cells. ENU-mutagenized cells were plated with inhibitor, monitored for outgrowth, expanded, and sequenced for mutations. Bars represent frequency of a given mutant among all recovered clones at a given inhibitor concentration; percent of wells demonstrating outgrowth and number of clones sequenced is indicated. B, BCR-ABL mutants recovered in the presence of dual combinations of DCC-2036 and imatinib, nilotinib, or dasatinib, starting from Ba/F3 BCR-ABL cell lines. C, BCR-ABL compound mutants recovered in the presence of DCC-2036, starting from Ba/F3 BCR-ABL<sup>T315I</sup> cells. See also Supplementary Tables S1, S2, and S3.
site residue M318 and the K271-E286 salt bridge, allowing for accommodation of the bulky isoleucine substitution in BCR-ABL\textsuperscript{T315I}. Electrostatic interaction with E282 aids in stabilizing the E282-R386 switch control pair interaction and, consequently, the inactive kinase conformation (5). One explanation for the resistance of the mutant featuring methionine at residue 315 may be that the methionine sidechain impinges on DCC-2036 binding. Alternatively, introducing methionine at the gatekeeper position may induce the ABL kinase domain to adopt an active conformation.

To both broaden the screen for BCR-ABL compound-mutant-mediated resistance and evaluate efficacy of ABL inhibitor cocktails in this setting, we carried out a similar screen starting from a pooled mix of Ba/F3 BCR-ABL mutant cell lines (representing >70% of clinically observed imatinib-resistant mutations; ref. 4) using a combination of DCC-2036 (250 nmol/L), nilotinib (500 nmol/L), and dasatinib (25 nmol/L; Supplementary Fig. 1A). Strikingly, only 3 compound mutations were recovered: G250E/T315I, E255V/T315A, and E255V/T315I. Among these, the BCR-ABL\textsuperscript{E255V/T315I} mutant has been observed clinically and reported to confer high level resistance to multiple other ABL\textsuperscript{T315I} inhibitors (Supplementary Table S4; refs. 8, 15). Thus, although ABL inhibitor cocktails that include an ABL\textsuperscript{T315I} inhibitor may prove an effective strategy in minimizing resistance, certain BCR-ABL compound mutations are predicted to be recalcitrant to such an approach.

Our investigation of the switch control inhibitor DCC-2036 reveals substantial activity in CML cells, including cells expressing BCR-ABL\textsuperscript{T315I}. DCC-2036 is undergoing phase 1 evaluation for use in imatinib-refractory CML (NCT00827138; www.clinicaltrials.gov), and our results suggest that it may provide a treatment option for relapsed patients with a T315I mutation. DCC-2036 adds to a small set of ABL\textsuperscript{T315I} inhibitors currently in development, each of which targets the BCR-ABL\textsuperscript{T315I} mutant differently. Recent approaches include: dodging I315 via a carbon–carbon triple bond (ponatinib, formerly AP24534; ref. 8; pivotal phase 2 trial; NCT01207440; www.clinicaltrials.gov), utilizing a modified nilotinib–dasatinib hybrid structure to avoid gatekeeper mutations (HG-7–85-01; ref. 18; preclinical), and combining ATP-competitive and allosteric ABL inhibitors (GNT-2; refs. 19, 20: preclinical). Although disease eradication remains on the horizon, the much anticipated, imminent clinical availability of ABL\textsuperscript{T315I} inhibitors represents an important step toward maximal disease control.

References


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