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A Coiled-Coil Mimetic Intercepts BCR-ABL1 Dimerization in Native and Kinase-Mutant Chronic Myeloid Leukemia

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Abstract

Targeted therapy of chronic myeloid leukemia is currently based on small-molecule inhibitors that directly bind the tyrosine kinase domain of BCR-ABL1. This strategy has generally been successful, but is subject to drug resistance due to point mutations in the kinase domain. Kinase activity requires transactivation of BCR-ABL1 following an oligomerization event, which is mediated by the coiled-coil (CC) domain at the N-terminus of the protein. Here, we describe a rationally engineered mutant version of the CC domain, called CC^{mut3}, which interferes with BCR-ABL1 oligomerization and promotes apoptosis in BCR-ABL1-expressing cells, regardless of kinase domain mutation status. CC^{mut3} exhibits strong pro-apoptotic and anti-proliferative activity in cell lines expressing native BCR-ABL1, single kinase domain mutant BCR-ABL1 (E255V and T315I) or compound mutant BCR-ABL1 (E255V/T315I). Moreover, CC^{mut3} inhibits colony formation by primary CML $CD34^+$ cells *ex vivo*, including a sample expressing the T315I mutant.

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Conflict of Interest

The authors declare no competing financial interests.

Supplementary information is available at Leukemia's website.

Author Contributions

DWW designed and performed the research, collected data, analyzed and interpreted data and wrote the manuscript; AME assisted with patient sample experiments and virus preparation, provided key guidance in experimental design and wrote the manuscript. DWW and AME contributed equally. BJB and GDM assisted with cell line experiments; MSZ generated the Ba/F3 cell lines and assisted with preparation of figures; KRR processed patient samples; TO provided T315I patient samples and edited the manuscript; MWD supported the research on patient samples and cell lines and edited the manuscript; CSL provided key guidance in experimental design and support, and edited the manuscript.

These data suggest that targeting BCR-ABL1 with CC mutants may provide a novel alternative strategy for treating patients with resistance to current targeted therapies.

Keywords

CML; BCR-ABL1; Coiled-coil domain; TKI-resistant; CC^{mut3}; Kinase mutation; Compound mutant

Introduction

Chronic myeloid leukemia (CML) is caused by BCR-ABL1, the product of a reciprocal translocation t(9;22)(q34;q11)[1, 2], resulting in a shortened chromosome 22, also known as the Philadelphia chromosome. BCR-ABL1 is a constitutively active tyrosine kinase and the target of small-molecule inhibitors, including the first clinical tyrosine kinase inhibitor (TKI), imatinib[3–5]. Overall, imatinib has demonstrated considerable efficacy in CML, with high rates of complete hematologic and cytogenetic responses that have translated into improved progression-free and overall survival compared to non-TKI therapies such as interferon-α[6–11]. Although many imatinib responses are durable, some patients develop kinase domain mutations that confer resistance to imatinib and are associated with clinical relapse[12]. These mutations impair imatinib binding and restore BCR-ABL1 kinase activity[13]. To overcome this type of resistance, the second-generation TKIs, dasatinib, nilotinib, and bosutinib, and most recently the third-generation TKI, ponatinib, were developed[14]. Second generation TKIs are active against most imatinib-resistant BCR-ABL1 mutants, with the exception of T315I ($BCR\text{-}ABL1^{T315I}$)[15]. In contrast to imatinib and second generation TKIs, ponatinib is effective against the T315I mutant, representing a major therapeutic breakthrough[16, 17]. Thus far, no single mutation (except for T315M, observed emanating from an initial T315I mutant in one patient following ponatinib therapy) has been shown to confer resistance to ponatinib[18]; however, multiple mutations in the same BCR-ABL1 molecule, referred to as compound mutations, can confer resistance to ponatinib both *in vitro*[19] and in patients with clinical resistance to approved TKIs[17, 18].

Rational therapy of CML has generally focused on targeting the BCR-ABL1 catalytic site, but kinase domain mutations that impair or block drug binding limit the scope of this approach[20]. The N-terminal coiled-coil (CC) dimerization domain of BCR-ABL1 has been shown to be critically important for BCR-ABL1 kinase activity and could thus represent an alternative therapeutic target[21, 22]. A peptidomimetic to block dimerization has been explored by several groups. Ruthardt et al. reported that introduction of a peptidomimetic of helix α2 of the CC dimerization region reduced BCR-ABL1 phosphorylation and inhibited the proliferation of cells expressing native and mutant BCR-ABL1[23]. However, the isolated native helix 2 alone was inactive in cells expressing the T315I mutant[23, 24].

We recently described two iterations of a mutant CC (called CC^{mut2} and CC^{mut3}) with preferential specificity toward hetero-oligomerization with the CC region of BCR-ABL1 over homo-oligomerization with itself[25, 26]. This construct is similar to the Ruthardt helix α2 mimetic, but contains the full-length CC domain. Additionally, CC^{mut3} incorporates engineered mutations to enhance binding specificity within helix α2 and demonstrates

inhibitory activity against cells expressing native BCR-ABL1 or the T315I mutant[25–27]. Here, we have studied the effects of CC^{mut3} against kinase domain mutant variants of BCR-ABL1 in both cell lines and primary $CD34^+$ cells from newly diagnosed and TKI-resistant CML patients.

Materials and Methods

DNA Constructs

pmCherry-EV (empty vector) and pmCherry-CC^{mut3} have been described[26]. The lentiviral control vector pCDH-EF1-copGFP-EV was adapted from pCDH-CMV-MCS-EF1-copGFP (System Biosciences (SBI), Mountain View, CA, USA). The CMV promoter and multiple cloning sites were excised using SpeI and XbaI with compatible cohesive ends. The CMV fragment was removed using gel purification and the resulting DNA was ligated to form the final construct.

To make pCDH-EF1-copGFP-CC^{mut3}, sections of the construct were amplified separately by PCR and joined using overlap extension PCR. First, EF1-copGFP was amplified from the SBI parent plasmid with a 5′-SpeI and 3′-BamHI site using the following primers: 5′-CAACTAGTAAGGATCTGCGATCGCTCC-3′ and 5′-CCAT CTGAGTCCGGAGCGAGATCCGGTGGAGC-3. ′ CC^{mut3} was amplified from pEGFP-CC^{mut3} as described[26] using primers containing a 5′-BamHI site, a terminal TAG stop signal and a sequence complementary to the polyA signal on the 3′ overhang: 5′-CTCAGATGGATCCTTATGGTGGACCCGGTGGGCTTCG-3′ and 5′-GTTATCTAGATCTACCGGTCATAGCTCTTCTTTTCC-3′. Finally, the polyA signal from pEGFP-C1 (Clontech Laboratories, Mountain View, CA, USA) was amplified to include a 5′ complementary sequence to CC^{mut3}, and a 3′-SalI restriction site: 5′-GACCCGGTAGATCTAGATAACTGATCATAATC-3′ and 5′-GCTTACATGCGG CCGCGTCGACTGTGGGAGGTTTTTTAAAGC-3. ′ PCR products were combined in two steps, first by combining the CC^{mut3}-polyA and then by adding EF1-copGFP by overlap extension PCR. The PCR product was digested with SpeI and SalI and ligated to the SpeI and SalI-digested pCDH-CMV-MCS-EF1-copGFP vector (SBI). The lentiviral packaging plasmid psPAX2 was purchased from Cellecta, Inc. (Mountain View, CA, USA), and the viral envelope plasmid pVSV-G was purchased from Clontech Laboratories.

Cell Lines and Patient Samples

Stable Ba/F3 cells transduced with native (p210) BCR-ABL1[28], the kinase domain mutants BCR-$ABL1^{T315I}$, BCR-$ABL1^{E255V}$, or the compound mutant BCR-$ABL1^{E255V/T315I}$ were cultured as described[29, 30]. Briefly, cells were cultured in RPMI 1640 with 10% FBS, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine, and 0.1% gentamycin (complete RMPI medium). Additionally, 0.1% MycoZap™ (Lonza Bio, Basel, Switzerland) was added to prevent mycoplasma contamination. The non-transduced parental Ba/F3 cell line was grown in RPMI 1640 supplemented with 20% WEHI-3B conditioned medium as a source of murine IL-3[31]. To introduce CC^{mut3} or empty vector (EV), Ba/F3 cells were transfected with plasmid DNA using the Amaxa nucleofection system (Lonza, Basel, Switzerland) using program X-001 following the manufacturer's instructions. Cells were sorted on a BD

FACSAria cytometer (BD Biosciences, San Jose, CA, USA) for double-positive cells expressing mCherry and GFP prior to use in experiments.

Mononuclear cells (MNCs) from peripheral blood of patients with newly diagnosed or TKI-resistant CML were separated by Ficoll (Nycomed, Oslo, Norway), and the $CD34^+$ fraction was isolated using an autoMACS Pro Separator (Miltenyi Biotech, San Diego, CA, USA). Purity was determined to be >90% by flow cytometric analysis using a Guava 6HT flow cytometer (Millipore, Billerica, MA, USA). $CD34^+$ progenitors were maintained at 1×10^6 cells/mL in RPMI 1640 containing 20% FBS and 5 μL/mL StemSpan CC100 (Stem Cell Technologies, Vancouver, BC, Canada). Sanger sequencing was used to confirm BCR-ABL1 genotype. All patients gave their informed consent in accordance with the Declaration of Helsinki, and all studies with human specimens were approved by The University of Utah Institutional Review Board (IRB).

Lentivirus Generation and Infection

293FT cells (Life Technologies, Grand Island, NY, USA) were grown in DMEM with 10% FBS, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine, 0.1 mM MEM-non-essential amino acids, and 1 mM sodium pyruvate (Life Technologies, Grand Island, NY, USA). Cells were passaged every 2–3 days in T75 flasks, and grown to 65% confluence in T175 flasks for transfection. For lentivirus generation, cells were co-transfected with the experimental construct pCDH-EF1-copGFP-EV or pCDH-EF1-copGFP-CC^{mut3}, pVSV-G and psPAX2 using the Profection® mammalian transfection system (Promega, Madison, WI, USA) according to manufacturer's instructions. After 48 h, viral particles were complexed with polyethylene glycol overnight, pelleted, and concentrated to 100X in RPMI 1640. Lentiviral titers were determined as described[32]. Primary CML cells were infected with lentivirus at a multiplicity of infection of 5 for each construct at 24 and again at 48 h following harvest (fresh cells) or thaw (frozen cells), respectively. Cells were sorted on a BD FACSAria cytometer for GFP-positive cells after 72 h prior to use in experiments.

Cell Proliferation Assays

Cell proliferation was assessed using a methanethiosulfonate-based viability assay (MTS assay) utilizing CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) according to manufacturer instructions. Briefly, 5×10^3 viable cells were suspended in 100 μL complete RPMI medium per well in 96-well plates. Three independent samples were seeded in duplicate for each time point. Cell growth was assessed by MTS assay at 72 and 96 h. Readings at 490 nM were taken on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA) after a 3 h incubation with MTS reagent. In patient sample experiments, cell proliferation was assessed by trypan blue exclusion. TKI treatments in cells were at the following concentrations for the listed patient-samples: imatinib (0 or 2.5 μM) for newly diagnosed (ND) CML samples; ponatinib (0, 10 nM) for CML samples harboring BCR-$ABL1^{T315I}$.

Apoptosis Assays

For analysis of apoptosis and necrotic cell death, cells were pelleted and resuspended in Annexin V-binding buffer (BD Biosciences), stained with anti-Annexin V-APC and 7-AAD

(BD Biosciences or Life Technologies) and analyzed on a BD FACSCanto flow cytometer. In addition to the APC and 7-AAD channels, GFP- and mCherry-positive cells were also recorded.

Colony Forming Assays

Following selection of transfected cells by cell sorting, methylcellulose colony assays were performed by plating in 0.9% methylcellulose as described (Stem Cell Technologies; M3234 for Ba/F3 BCR-ABL1 native and mutant lines; M3434 for Ba/F3 parental cells; H4230 for CML patient samples)[25, 33]. mCherry-positive Ba/F3 cells (1.1×10^3 cells/plate) or GFP-positive primary CML $CD34^+$ cells (1×10^3 cells/plate) were seeded per dish in duplicate. In the case of CML patient samples, cells were plated in the presence of 1× StemSpan CC100 cytokine cocktail (StemCell Technologies). All cells were plated with or without the indicated TKI in three or more independent experiments. Plates were incubated at 37°C in a 5% CO_2 humidified incubator. Colonies were counted 7–14 days later in an area of 50 μm^2 per dish using an inverted microscope.

Statistics

Data are expressed as the means ± SEM from at least 3 independent experiments unless otherwise stated. Briefly, significant differences between groups in Ba/F3 cell proliferation experiments (n=3 in technical duplicates) were assessed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) using a two-way ANOVA with Bonferroni's multiple comparisons test. A two-tailed student's t-test was used to determine significant differences in Ba/F3 colony forming experiments (n=3 in technical duplicates) and flow cytometric analysis of apoptosis (n=3). One-way ANOVA and Tukey's multiple comparision's test was used for primary ND CML cell colony forming assays (n=4 in technical duplicates). A p-value of <0.05 was considered significant for all experiments.

Results

CC^{mut3} expression inhibits proliferation, increases apoptosis, and impairs survival of cell lines expressing native BCR-ABL1

We first investigated the antiproliferative effects of CC^{mut3} in Ba/F3 cells expressing native BCR-ABL1 and in unmanipulated parental Ba/F3 cells. Expression of the CC^{mut3} construct was confirmed by immunoblot analyses (Supplemental Figure 1a). Following transfection with EV or the CC^{mut3} construct, proliferation was measured by MTS assay at 72 and 96 h. No difference between EV or CC^{mut3} was observed in parental Ba/F3 cells at either time point (Figure 1a), whereas proliferation of Ba/F3 cells expressing native BCR-ABL1 was reduced by >2-fold at 96 h (Figure 1b). To determine whether CC^{mut3} promoted apoptosis, we measured Annexin V and 7-AAD at 72 h in BCR-ABL1 cells with CC^{mut3} or EV. While there was no effect on the parental cell line (Figure 1c), BCR-ABL1-expressing cells showed an approximately 3-fold increase of apoptotic cells when transfected with CC^{mut3} compared to the EV control (Figure 1d). Finally, we tested the effect of CC^{mut3} on survival of parental and native BCR-ABL1-expressing Ba/F3 cells by colony formation assays. Similar to its effects on cell proliferation, CC^{mut3} nearly eliminated colony forming ability compared to the EV in BCR-ABL1-expressing cells, with no effect on parental Ba/F3

controls (Figures 1e and 1f). These data confirm that our CC^{mut3} mimetic is effective in the Ba/F3 cell line system expressing the BCR-ABL1 oncoprotein.

CC^{mut3} inhibits proliferation, increases apoptosis, and impairs survival of cell lines harboring BCR-ABL1 single kinase domain mutants

Next we tested the effects of CC^{mut3} on Ba/F3 cells expressing BCR-ABL1 mutants associated with clinical imatinib failure [34, 35]. Ba/F3 cells engineered to express either BCR-ABL^{E255V} or BCR-ABL^{T315I} were transfected with EV or CC^{mut3} constructs. Expression of the CC^{mut3} construct was again confirmed by immunoblot analyses (Supplemental Figure 1a). At 96 h, single mutants showed an approximately 3-fold reduction of proliferation when transfected with CC^{mut3} compared to EV, whereas a lesser, yet significant difference was noted at the 72 h time point (Figures 2a and 2b). Both BCR-$ABL1^{E255V}$ and BCR-$ABL1^{T315I}$ cells demonstrated a 6–8-fold increase in apoptosis ($AnnexinV^{+}/7AAD^{-}$) after transfection with CC^{mut3} when compared to the EV after 72 h (Figures 2c and 2d). Lastly, we assessed the effects of CC^{mut3} on survival of Ba/F3 cells harboring single BCR-ABL1 mutants by plating in colony formation assays. CC^{mut3} expression produced a >10-fold reduction of colony forming ability in both BCR-$ABL1^{E255V}$ (Figure 2e) and BCR-$ABL1^{T315I}$ cells (Figure 2f) compared to the EV controls. Altogether, these data demonstrate that CC^{mut3} not only inhibits growth of cells harboring non-mutated BCR-ABL1, but also inhibits growth of cells harboring clinically relevant BCR-ABL1 kinase domain single mutants.

CC^{mut3} exerts anti-apoptotic and survival inhibitory effects on cell lines harboring a ponatinib-resistant BCR-ABL1 compound mutant

Compound mutations are arising as a clinical problem in patients undergoing sequential TKI therapy[18, 36, 37]. To determine whether CC^{mut3} also has growth inhibitory effects on cells harboring BCR-ABL1 compound mutants, we introduced CC^{mut3} or the EV into Ba/F3 cells expressing the highly TKI-resistant BCR-$ABL1^{E255V/T315I}$ compound mutant. Expression of the CC^{mut3} construct was again confirmed by immunoblot analyses (Supplemental Figure 1a). Importantly, CC^{mut3} significantly reduced proliferation of compound mutant cells at 96 h (Figure 3a), although the effects were far less pronounced than that observed in the single kinase domain mutant cells (Figures 2a and 2b). However, CC^{mut3} effectively induced apoptosis of Ba/F3 cells expressing BCR-$ABL1^{E255V/T315I}$ (Figure 3b) to a similar degree seen in single kinase mutants 72 h following transfection (Figures 2c and 2d). Importantly, CC^{mut3} expression in cells harboring the BCR-$ABL1^{E255V/T315I}$ compound mutant reduced colony forming ability by approximately 50% compared to EV controls (Figure 3c). Thus, these data extend the effects of CC^{mut3} to include not only native and single kinase domain mutant BCR-ABL1, but also cells harboring highly TKI-resistant compound mutant clones.

$CD34^{+}$ cells from CML patients harboring native or T315I mutant BCR-ABL1 are sensitive to CC^{mut3}

Lentivirus expressing CC^{mut3} or EV (Supplemental Figure 1b–c) was used to infect $CD34^{+}$ cells isolated from blood or bone marrow of ND CML patients, and cultured in complete RPMI 1640 with cytokines. For comparison, EV-expressing cells were also treated with 2.5 μM imatinib. CC^{mut3} reduced cell growth by more than 2-fold as determined by trypan blue

exclusion, a significant reduction compared to inhibition by imatinib (Figure 4a). Cells were also assessed for colony formation by plating in methylcellulose supplemented with growth factors. The effects of CC^{mut3} on colony formation were similar to that of imatinib, inhibiting CML $CD34^+$ cells from four independent newly diagnosed patient samples by ~60% (Figure 4b).

Additionally, we investigated the effect of CC^{mut3} expression on primary human $CD34^+$ CML cells expressing BCR-$ABL1^{T315I}$ as determined by Sanger sequencing of two samples (R1 and R2) obtained from the same individual at two time points approximately 6 months apart, during which the patient was treated with ponatinib (Supplemental Table 1). Ponatinib was used to assess sensitivity to inhibition of BCR-ABL1 catalytic activity. In the initial sample (R1) obtained when the patient was in accelerated phase CML, 10 nM ponatinib and CC^{mut3} reduced colony formation by 47% and 76%, respectively (Figure 5a). In contrast, while cells obtained at the time of blastic transformation (R2) were insensitive to treatment with ponatinib, CC^{mut3} was still able to reduce colony formation by 25% (Figure 5b). Importantly, Sanger sequencing confirmed that the mutation status had not changed compared to the initial sample. Altogether, these data suggest that like ponatinib[16, 18], CC^{mut3} has activity in CML patients harboring the T315I mutant.

Discussion

TKIs are an effective and generally well-tolerated therapy for CML[38]. However, a subset of patients fail TKIs due to drug resistance or intolerance[14, 39–42]. BCR-ABL1 kinase-dependent resistance is often the product of BCR-ABL1 kinase domain mutations that impair or prevent TKI binding to the catalytic site[12, 43, 44], which has led to the development of second and third generation inhibitors. Because kinase domain-targeted inhibitors are subject to resistance arising from mutations in this domain, we examined the ability of CC^{mut3}, a coiled-coil dimerization domain inhibitor[26], to impair growth and viability of CML cells by disrupting oligomerization, the key event necessary for autophosphorylation and activation of BCR-ABL1 kinase (Figure 6)[22, 45]. CC^{mut3} was computationally designed to include mutations that not only enhance interactions with BCR-ABL1, but also to incorporate charge-charge repulsions that destabilize CC^{mut3} homodimer formation. The net result is the preferential heterodimerization between CC^{mut3} and BCR-$ABL1$[25, 26].

Previous studies demonstrated that CC^{mut3} inhibits proliferation and induces apoptosis in K562 CML cells[26]. Here, we report the effects of CC^{mut3} against native and mutant BCR-ABL1, using murine pro-B cells (Ba/F3) engineered to express native and kinase domain mutant BCR-ABL1, as well as primary CML $CD34^+$ cells obtained from newly diagnosed or therapy-resistant patients. We found that CC^{mut3} reduced proliferation and colony formation and increased apoptosis of CML cell lines and patient samples expressing native BCR-ABL1 (Figures 1b, 1d, 1f, and 4); CC^{mut3} had no measurable toxicity in BCR-ABL1-negative cells (Figures 1a, c, e). Importantly, this is the first evidence of CC^{mut3} efficacy in primary CML patient samples.

While all TKIs currently act directly on the kinase domain, we demonstrate that alternative mechanisms of BCR-ABL1 inhibition can circumvent mutation-driven TKI resistance.

Consistent with native BCR-ABL1 inhibition by CC^{mut3}, the CC mimetic is effective in single BCR-ABL1 kinase domain mutants. We found a significant inhibition of growth and viability introduced by CC^{mut3} in BCR-$ABL1^{E255V}$ and BCR-$ABL1^{T315I}$ (Figures 2a and 2b), as well as a >5-fold increase in apoptosis (Figures 2c and 2d). Moreover, we observed a marked reduction of colony forming potential in both Ba/F3 cells expressing BCR-$ABL1^{E255V}$ and BCR-$ABL1^{T315I}$ (Figures 2e and 2f) and in a sample from a BCR-$ABL1^{T315I}$ CML patient following CC^{mut3} expression (Figure 5a). However, CC^{mut3} showed only minimal activity in a longitudinal sample obtained after the patient had developed resistance to ponatinib and progressed to the blastic phase of disease (Figure 5b). These data suggest that this patient may have developed a BCR-ABL1 kinase-independent mechanism of resistance[46].

The emergence of compound mutations in the kinase domain that confer resistance to multiple TKIs is of increasing clinical importance[18]. To this end, we examined CC^{mut3} in compound-mutant BCR-$ABL1^{E255V/T315I}$ cells, which are resistant to ponatinib, the most advanced tyrosine kinase inhibitor in clinical use[19]. Importantly, CC^{mut3} significantly increased apoptosis and reduced colony formation of BCR-$ABL1^{E255V/T315I}$-expressing cells (Figure 3b, 3c), with a lesser effect on cell proliferation (Figure 3a). The cause of the comparably weaker activity of CC^{mut3} in cell proliferation assays is unknown and remains to be determined in structural studies. Taken together, we show that CC^{mut3} expression is not only effective against CML cells expressing native and single kinase domain mutant BCR-ABL1, but also against cells harboring compound-mutant BCR-ABL1 that are resistant to multiple TKIs, providing proof of principle that targeting the dimerization domain of BCR-ABL1 can overcome kinase domain mutation-based TKI resistance (Figure 6). Furthermore, we speculate that CC^{mut3} will not be prone to mutational escape routes seen with traditional kinase inhibitors (the selection or genesis of mutant BCR-ABL1 molecules which are TKI-resistant), because any mutations in BCR-ABL1 that would reduce binding to the CC^{mut3} would also reduce the ability of BCR-ABL1 to dimerize, thereby precluding autophosphorylation and resulting in a monomeric, auto-inhibited kinase[45]. This may translate into a lower likelihood of clinical resistance due to point mutations. Another as yet hypothetical advantage of blocking dimerization may be the inhibition of BCR-ABL1 functions that are kinase-independent, yet require formation of BCR-ABL1 dimers or multimers. These functions persist upon TKI-mediated inhibition of BCR-ABL1 and may contribute to the innate TKI resistance of primitive CML cells.

In contrast to small molecule drugs, peptides present considerably greater drug delivery challenges[47]. In the present study, CC^{mut3} was transcribed in cells following lentiviral infection with an expression construct. Direct application of this therapy could include intramedullary injection of lentivirus encoding CC^{mut3} as explored in hemophelia therapy[48]. However, a challenge with this approach in cancer is to achieve 100% transduction efficiency. Therefore, we are currently formulating CC^{mut3} as a stapled peptide for therapeutic use in future *in vivo* studies. Stapled peptides improve drug delivery by their resistance to degradation, improved cell permeation, and increased *in vivo* half-life compared to conventional peptide therapeutics[49, 50]. Addition of a leukemia-specific cell-penetrating peptide motif might allow targeting of this peptide to CML stem cells[51]. For

instance, recent reports have identified antigens specifically or preferentially expressed on primitive CML cells, such as CD25, CD26 and the interleukin-1 receptor associated protein (IL1RAP)[52, 53]. Therefore, an antibody-CC^{mut3} peptide conjugate could be formulated for leukemic stem cell targeting. Combination of BCR-ABL1 inhibition by CC^{mut3} along with inhibition of stem cell survival or self-renewal pathways may result in robust eradication of the CML stem cell[54].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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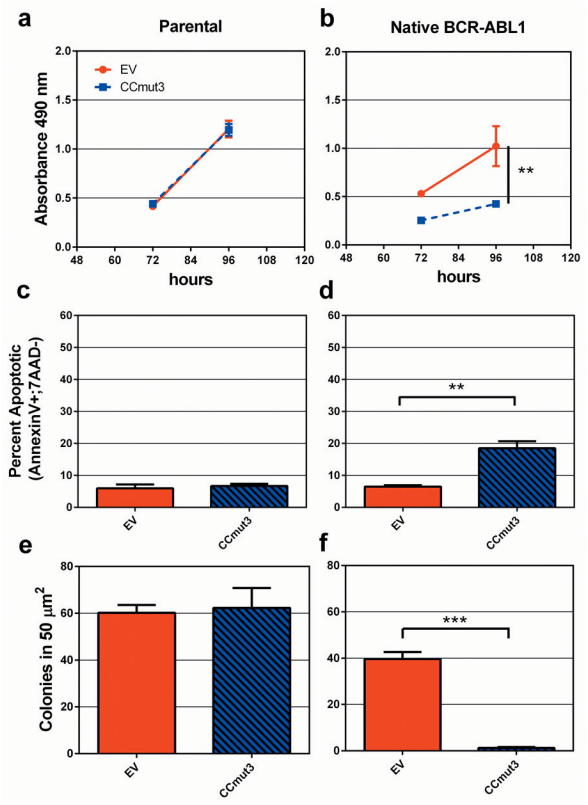

Figure 1. CC^{mut3} reduces proliferation, increases apoptosis, and impairs survival of Ba/F3 cells expressing native BCR-ABL1, with no effect on parental Ba/F3 cells

(**a, b**) Proliferation of Ba/F3 parental cells were not affected by expression of the CC^{mut3} (blue boxes) compared to EV controls (red circles) (**a**), whereas CC^{mut3} treatment of Ba/F3 cells expressing native BCR-ABL1 significantly reduced proliferation at 96 h compared to controls **(b)**. (**c, d**) Apoptotic cell populations at 72 h were quantified following flow cytometric analyses of transfected (GFP^{+}) cells. Panels indicate apoptotic populations (Annexin V-positive/7-AAD-negative) of Ba/F3 parental cells (**c**), and Ba/F3 cells expressing native BCR-ABL1 (**d**). (**e, f**) Colony forming ability of Ba/F3 parental cells were not affected by expression of the EV control or CC^{mut3} (**e**), while colony formation by Ba/F3 cells expressing native BCR-ABL1 was greatly reduced in the CC^{mut3} treatment group compared with EV (**f**) (n=3). All graphs display mean ± S.E.M. **p<0.01, ***p<0.001.

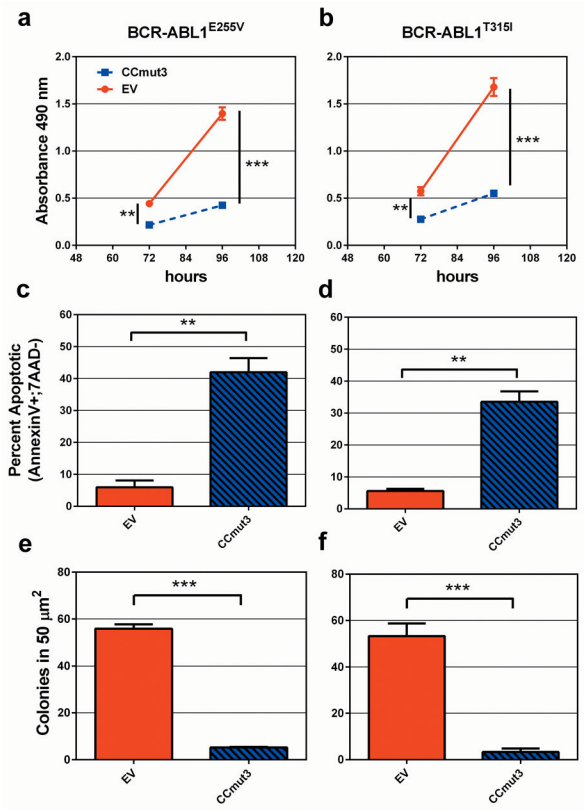

Figure 2. CC^{mut3} inhibits BCR-ABL1-driven proliferation and increases apoptosis in single-mutant BCR-ABL1

(**a, b**) A significant reduction of proliferative capacity was observed following CC^{mut3} (blue boxes) but not EV expression (red circles) of Ba/F3 cells expressing the single BCR-ABL1 mutants E255V (**a**) or T315I (**b**) at both 72 and 96 h (n=3). (**c, d**) An increase of apoptosis was observed in Ba/F3 BCR-ABL1^{E255V} (**c**) and Ba/F3 BCR-ABL1^{T315I} (**d**) cells at 72 h. (**e, f**) Colony formation of Ba/F3 mutants E255V (**e**) and T315I (**f**) represented significantly fewer colonies per unit area CC^{mut3} compared to EV groups (n=3). Graphs display mean ± S.E.M. **p<0.01, ***p<0.001.

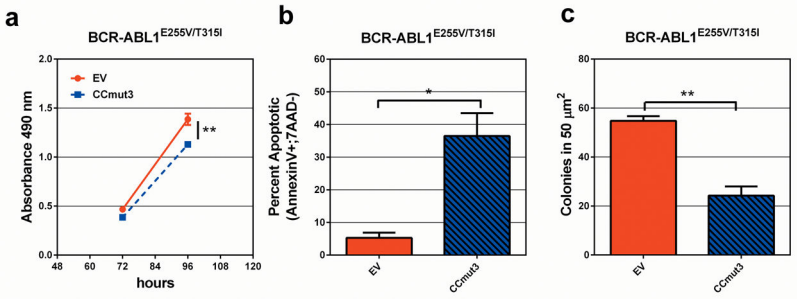

Figure 3. CCmut3 enhances apoptosis, and reduces colony forming ability of compound-mutant BCR-ABL1 cells

(**a**) Ba/F3 cells expressing the BCR-ABL1$^{E255V/T315I}$ compound mutant demonstrate a small yet significant growth reduction at 96 h but not 72 h with CC^{mut3} treatment compared to EV controls (n=3). (**b**) Enhanced apoptosis was evident in Ba/F3 BCR-ABL1$^{E255V/T315I}$ cells when expressing CC^{mut3} compared to EV controls (n=3). (**c**) Colonies per unit area were again reduced in the compound mutant cell line (Ba/F3 BCR-ABL1$^{E255V/T315I}$) by CC^{mut3} compared to EV controls (n=3). Graphs display mean ± S.E.M. *p<0.05, **p<0.01.

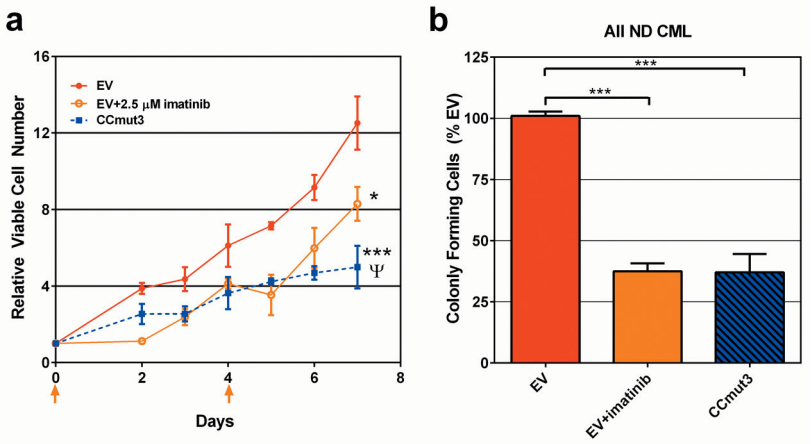

Figure 4. Cells from newly diagnosed CML patients are sensitive to CC^{mut3} lentiviral therapy *ex vivo*

(**a**) Equal number of cells were seeded on day 0 for each group and plotted as fold-change of starting cell number compared to controls on days 2–7. Imatinib was added on days 0 and 4 (indicated by orange arrows) to the EV+2.5 μM imatinib group only (n=3). *p<0.05 compared to EV, ***p<0.001 compared to EV, –p<0.05 compared to EV+2.5 μM imatinib group. (**b**) Colony forming cells were assessed 14 days following seeding in methylcellulose. Individual patient samples were counted in duplicate and normalized to the EV control. 2.5 μM imatinib and CC^{mut3} are equally effective in reducing colony number (n=4). Graphs display mean ± S.E.M. ***p<0.001.

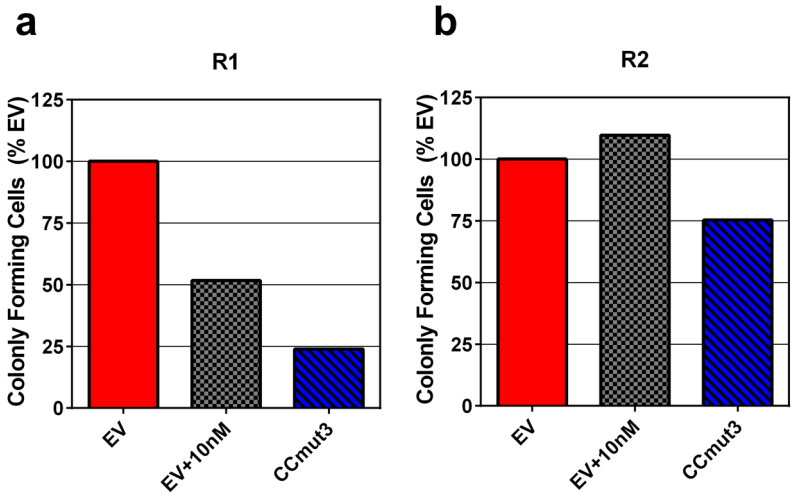

Figure 5. CC^{mut3} is effective in a patient sample from a CML patient in accelerated phase harboring BCR-$ABL1^{T315I}$, with a less pronounced effect upon blastic transformation

Colony forming assays were set up with two longitudinal samples from the same patient, R1 and R2, separated by a 6-month interval. Each sample was thawed and lentivirally transduced with CC^{mut3} or the EV control. Following cell sorting for transduction, each sample was plated in triplicate. (**a**) R1 displays sensitivity to 10 nM ponatinib (gray checked bars), but even greater sensitivity to transduction with CC^{mut3} (blue checked bars) compared to untreated EV-transduced controls (red solid bar). (**b**) R2 is a sample from the same individual after ponatinib failure and transformation to blast crisis (see Supplemental Table 1). While ponatinib treatment minimally increases colony forming ability, CC^{mut3} treatment resulted in a ~25% reduction in colony formation.

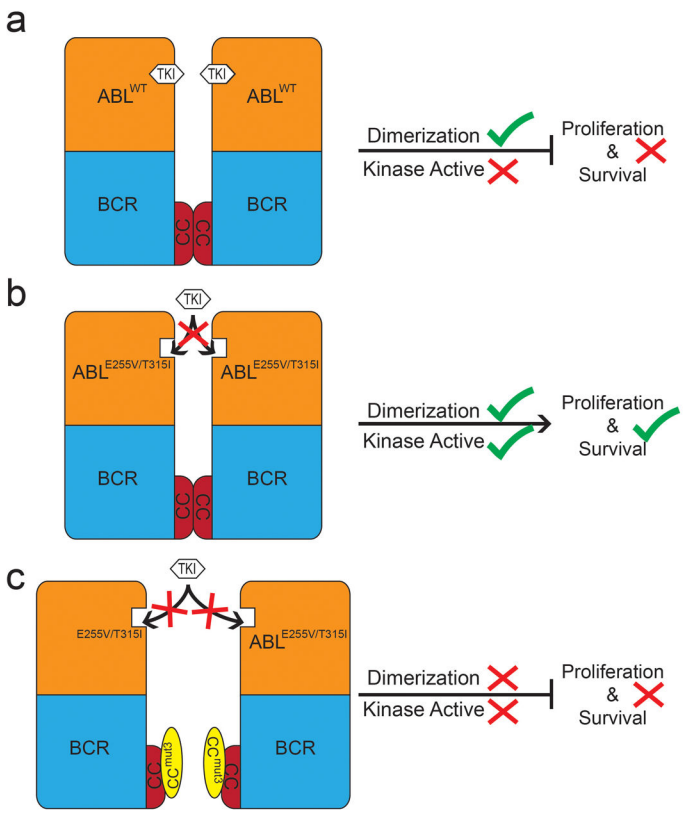

Figure 6. BCR-ABL1 inhibition state in native and mutant forms by TKI or by CC^{mut3}

(**a**) TKIs bind to the catalytic site of the BCR-ABL1 fusion protein. Proliferation and survival are blocked through kinase inhibition, irrespective of dimerization. (**b**) In compound mutant BCR-ABL$^{E255V/T315I}$, TKIs are unable bind to the catalytic site. Dimerization and kinase activity remain intact. (**c**) CC^{mut3} expression leads to competitive disruption of dimerization, preventing transphosphorylation, autophosphorylation and kinase activation of native, single and compound mutant BCR-ABL1.