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MULTI-DOMAIN TARGETING OF BCR-ABL BY DISRUPTION OF OLIGOMERIZATION AND TYROSINE KINASE INHIBITION: TOWARDS ERADICATION OF CML

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Abstract

The oncoprotein Bcr-Abl, the causative agent of chronic myeloid leukemia (CML), requires homo-oligomerization via a coiled-coil domain to function.^{1–4} While tyrosine kinase inhibitors (TKIs) have shown great efficacy as treatment options for CML, their use may cause an acquisition of mutations in the tyrosine kinase domain which prevent TKI binding and lead to a loss in activity.⁵ Previously, we have shown that a rationally modified coiled-coil domain (CC^{mut3}) can disrupt this oligomerization, inhibit proliferation, and induce apoptosis in CML cells.⁶ Here, we show that using the most recently approved TKI, ponatinib (IclusigTM), in combination with CC^{mut3} allows a dose reduction of ponatinib and increased therapeutic efficacy *in vitro* measured by reduction in kinase activity, induction of apoptosis via caspase-3/7 and 7AAD/Annexin V assays, and reduced transformative ability measured by a colony forming assay. The combination was effective not only in cells containing wild-type Bcr-Abl (K562, Ba/F3-p210) but also cells with Bcr-Abl containing the T315I mutation (Ba/F3-p210-T315I). In addition, we report for the first time the ability of CC^{mut3} alone to inhibit the T315I mutant form of Bcr-Abl. This novel combination may prove to be more potent than single agent therapies and should be further explored for clinical use.

Keywords

CML; Bcr-Abl; CC^{mut3}; ponatinib; combination therapy; T315I

Introduction

CML is characterized by the presence of the Philadelphia (Ph) chromosome, created from a reciprocal translocation between the long arm of chromosome 9 and the short arm of chromosome 22. This unique genetic abnormality produces the cytosolic fusion protein, Bcr-Abl.^{1, 2} Signaling through survival and proliferation pathways including RAS, PI3K/AKT, and Src family kinases, Bcr-Abl is the driving force for the development and persistence of

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Supplemental Information

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CML.^{7, 8} Activation of this protein occurs via homo-oligomerization at the coiled-coil (CC) domain, located on the N-terminus of the Bcr portion (Figure 1). Upon homo-dimer and homo-tetramer formation, trans-autophosphorylation can occur most notably at the tyrosine kinase domain, among other locations.^{3, 4} This autophosphorylation is responsible for the constitutive tyrosine kinase activity present in the cell, the primary oncogenic driver responsible for CML disease manifestation.⁹

Currently, most clinically available drugs for CML treatment are designed to block this tyrosine kinase activity by binding to and inhibiting the tyrosine kinase domain.⁵ Although often effective, these drugs are non-curative and may result in the acquisition of mutations in the tyrosine kinase domain upon prolonged administration. Such mutations prevent the binding and activity of TKIs.^{10, 11} One such mutation in Bcr-Abl, T315I, evades binding to first generation (imatinib) and second generation (dasatinib and nilotinib) TKIs.¹² The recent development of ponatinib (Iclusig™ – 45mg, daily), a third generation TKI, has now provided a means by which to combat many resistant forms of Bcr-Abl, including those with the T315I mutation.^{13, 14} However, compound mutations (defined as two or more mutations in a single molecule), such as E255V/T315I, confer high-level resistance to all currently available TKIs.¹⁵ Indeed, patients whose molecular profile identifies compound mutations in Bcr-Abl (e.g. G250E/T315I, E255V/T315I) would also be expected to be resistant to ponatinib.¹⁶ It is thought that Bcr-Abl inhibitors with “broader mutational coverage ... may reduce the likelihood of a resistance mutation emerging and decrease the risk of disease progression.”¹⁷ In addition, because ponatinib shows some non-specificity of kinase inhibition, side effects include thrombocytopenia, rash, and arthralgia; ponatinib’s dose-limiting toxicity is pancreatitis, indicating a need for strategies that would reduce its dose^{18, 19}. With this in mind, the use of two Bcr-Abl-targeted drugs with distinct mechanisms of action may provide this broader mutational coverage and increase potency against Bcr-Abl.

We recently demonstrated that disruption of oligomerization of Bcr-Abl is an effective means of inhibiting its activity.^{6, 20} A mutant coiled-coil was designed to bind to and inhibit the Bcr-Abl coiled-coil domain (responsible for oligomerization). This mutated coiled-coil, termed CC^{mut3}, contains six mutated residues from the wild-type Bcr-Abl coiled-coil domain (C38A, K39E, S41R, L45D, E48R, Q60E) designed to both favor hetero-dimerization between CC^{mut3} and endogenous Bcr-Abl (CC^{mut3}:Bcr-Abl) and to disfavor homo-dimerization between two CC^{mut3} molecules (CC^{mut3}:CC^{mut3}). Previous work has validated CC^{mut3} as a potential therapeutic for CML.⁶ To devise a more potent therapy, combining CC^{mut3} with ponatinib was attempted as a “dual-hit” strategy (Figure 1). Using this strategy, both the oligomerization domain and the tyrosine kinase domain of Bcr-Abl can be targeted concurrently to provide an enhanced therapeutic effect.

In the following studies, the ‘dual-hit’ hypothesis was tested by treating CML cells with both CC^{mut3} and ponatinib (or each agent alone). Experiments were performed in cells containing the wild-type, unmutated form of Bcr-Abl (K562; Ba/F3-p210), as well as cells containing the T315I mutation in Bcr-Abl (Ba/F3-p210-T315I). Western blots measured the kinase activity of Bcr-Abl and its phosphorylation pattern regarding downstream targets. Colony forming assays were used to measure the inhibition of both transformative ability and proliferation of CML cells. Finally, the induction of apoptosis was studied by measuring the activation of intracellular caspases and by 7AAD and Annexin V staining. In most cases, CC^{mut3} in combination with ponatinib resulted in more potent inhibition of Bcr-Abl than either agent alone. We also report, for the first time, effective use of our CC^{mut3} inhibitor in T315I mutant cells.

Materials and Methods

Construct Design

The constructs pEGFP-CC^{mut3} and pmCherry-CC^{mut3} were subcloned as previously described.^{6, 28}

Ponatinib

Ponatinib HCl salt (AP24534) was obtained from ChemieTek (Indianapolis, IN, USA) and stored at -20°C as a 10 mM stock solution. Serial dilutions (100 μM and 10 nM) of stock solution were made prior to cell experiments.

Cell Lines, Transfection Method, and Ponatinib Treatment

Cells were maintained at 37°C and 5% CO_2 in a humidity-controlled incubator.

K562—K562 cells, human leukemia Bcr-Abl⁺ cells (gifted from Kojo Elenitoba-Johnson, University of Michigan), were grown in RPMI 1640 media supplemented with 10% FBS, 1% penicillin-streptomycin-glutamine, and 0.1% gentamicin (complete medium). The cells were passaged every two to three days, seeded at a density of 5.0×10^4 cells/mL. Transfections were carried out two days following cell passaging. 2.0×10^6 cells were collected and transfected with 6 μg of either pEGFP, pEGFP-CC^{mut3}, pmCherry, or pmCherry-CC^{mut3} according to the *Cell Line Nucleofector Kit V* protocol, program T-013, using the Amaxa Nucleofector II (Lonza Group, Basel, Switzerland). Immediately following transfection, cells were added to 10 mL RPMI complete medium and treated with ponatinib at 100 pM, 1 nM, or 10 nM doses.

Ba/F3—Ba/F3 cells, mouse pro B cells (gifted from Michael Deininger, University of Utah) transduced to express either p210-Bcr-Abl (Ba/F3-p210) or p210-Bcr-Abl containing the T315I mutation (Ba/F3-p210-T315I) were maintained in RPMI complete medium. Parental Ba/F3 cells without Bcr-Abl (also from Deininger), used as control, were grown in RPMI 1640 complete medium supplemented with IL-3 produced in WEHI-3 cells.²⁹ All groups of cells were passaged every two to three days, seeded at a density of 1.0×10^5 cells/mL. Transfection method (Amaxa, Kit V) included program X-001, 3.0×10^6 cells, and 4 μg DNA per transfection. In addition, immediately following transfection, transfected cells were incubated in plain RPMI 1640 for 20 minutes, as per optimized conditions. Cells were then added to 10 mL RPMI complete medium and treated with respective dose of ponatinib.

Kinase Activity (Western Blot)

Western blot was done as previously described.⁶ In short, 48 hours following transfection and treatment with ponatinib, 2.0×10^6 cells were collected from each transfection and treatment group, and subjected to at least one freeze-thaw cycle at -80°C . Next, cells were lysed using RIPA buffer with protease inhibitor (1:200) added and sonicated at 70% amplitude for two pulses of 5 seconds each. After electrophoresis and transfer, the membrane was probed using a combination of primary antibodies against phospho-c-Abl (Cell Signaling, #2861), phospho-STAT5 (Abcam, ab32364), phospho-CrkL (Cell Signaling, #3181) and GAPDH (Cell Signaling, #5174) as a loading control, followed by incubation with secondary HRP-conjugated antibody (Cell Signaling, #7074). Finally, blots were imaged using a FluorChem FC2 imager (AlphaInnotech) after addition of chemiluminescent substrate (WesternBrightTM Quantum Western blotting detection kit, Advanta). Assay was performed three separate times ($n=3$).

Colony Forming Assay

Both EGFP and EGFP-CC^{mut3} were transfected into separate groups of cells on day 0. One day following transfection, 1.0×10^6 cells per treatment group were collected and resuspended in 1.0 mL PBS. Through serial dilutions, 1.0×10^3 cells in IMDM (Isocove's modified Dulbecco's media) with 2% FBS were seeded into methylcellulose medium in the absence of cytokines (MethoCult H4230 for K562 cells, MethoCult M3234 for p210 and p210-T315I cells) or in the presence of cytokines (MethoCult GF M3434 for parental Ba/F3 cells). Ponatinib was then added in the correct molar amounts (0, 100 pM, 1 nM, or 10 nM) to the methylcellulose medium. Colonies formed were counted after 7 days of incubation. All reagents were purchased from Stem Cell Technologies, Vancouver, BC, Canada. Assay was run three separate times (n=3) in duplicate.

7AAD and Annexin V Staining

72 hours following transfection and treatment with ponatinib, 5 mL of cells from each treatment were pelleted and resuspended in 0.5 mL of 1× Annexin Binding Buffer (Invitrogen). Next, 0.5 µL of 1 mM 7-aminoactinomycin D (Invitrogen) was added to each sample and allowed to incubate for 45 minutes. Five minutes before flow cytometric analysis, 1.0 µL of Annexin V (APC) (Invitrogen) was added to each sample. Analysis was performed using the FACSCantoII analyzer with BD FACSDiva software. Fluorophores were excited/emitted at the following wavelengths: EGFP, 488/530nm; mCherry, 587/610nm; 7AAD, 488/660nm; and APC, 635/660nm. Untransfected cells were eliminated from analysis by gating for cells only showing EGFP or mCherry fluorescence. Percentage of apoptosis / necrosis was calculated by combining the transfected cells (EGFP-positive or mCherry-positive) that stained positively for 7AAD and those that stained positively for APC. Assays were run in triplicate (n=3).

Caspase-3/7 Assay

Caspase-3/7 Assay was performed as previously described.⁶ In short, 48 hours following transfection and treatment with ponatinib, 3.0×10^6 cells were pelleted and frozen at -80°C . After thawing, cells were resuspended in 50µL of EnzChek Caspase-3/7 lysis buffer (Invitrogen). Lysates were then mixed with 50µL of 2× AMC-DEVD substrate in a 96-well plate and allowed to incubate in the dark at room temperature for 30 min. Following incubation, fluorescence was measured on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA). Lysates from three separate transfections were analyzed on the same plate for caspase-3/7 activity (n=3).

Cell Proliferation

72 and 96 hours following transfection of either pmCherry or pmCherry-CC^{mut3}, trypan blue exclusion was used to determine proliferation/viability of cells.⁶ Cell counts were performed using a standard light microscope.

Statistical Analysis

Experiments were analyzed using one-way ANOVA with Tukey's post-test, or a Student's t-test, as mentioned in figure legends.

Results

Bcr-Abl phosphorylation and downstream signaling (STAT5 and CrkL) are diminished upon treatment with CC^{mut3} in combination with ponatinib

Western blots measuring kinase activity were performed using CC^{mut3} and ponatinib to examine the potential oligomeric disruption and signaling inhibition of endogenous Bcr-Abl.

A range of ponatinib between 1 nM and 100 nM was originally tested based on previous *in vitro* studies¹³ in order to determine the lowest dose of ponatinib that could be used in combination with CC^{mut3} (data not shown). The current effective *in vivo* physiologic therapeutic range of ponatinib is between 60–145 nM.³⁰

Because Bcr-Abl undergoes trans-autophosphorylation upon dimerization at the coiled-coil domain,³ oligomeric disruption in this case was measured according to the phosphorylation state of Bcr-Abl (Figure 2A). In addition, inhibition of Bcr-Abl signaling, thus inhibition of kinase activity, was measured by examining the phosphorylation states of Bcr-Abl downstream target STAT5 (Figure 2A) and direct substrate CrkL (Figure 2B). When comparing equal doses of ponatinib with or without CC^{mut3}, Bcr-Abl phosphorylation appears to most significantly decline when adding 1 nM of ponatinib in combination with CC^{mut3} (Figure 2A, lane 5 vs. lane 2). At this same dose, the phosphorylation of both STAT5 (Figure 2A, lane 5) and CrkL (Figure 2B, lane 6) is greatly diminished. Complete eradication of phosphorylation of STAT5 and CrkL appears at 10 nM ponatinib (Figure 2A, lane 3; Figure 2B, lane 7). One further lower dose of ponatinib, 100 pM, was also tested (Figure 2B, lanes 3 and 4); no significant difference in CrkL phosphorylation with the combination was observed at this dose. Because p-Bcr-Abl activity is obliterated when 10 nM ponatinib is used in combination with CC^{mut3} (Figure 2A, lane 6 vs. lane 3), we chose to reduce ponatinib to a sub-saturating dose of 1 nM in subsequent experiments.

CC^{mut3} plus 1 nM Ponatinib Treatment Activates Caspase-3/7

Following analysis of the phosphorylation state of the signaling pathways, apoptotic induction following treatment was measured. Here, the activity of the effector caspase-3/7 upon treatment was analyzed as a measure of apoptosis. In order to suggest that a combination of CC^{mut3} and ponatinib provides an enhanced effect over each treatment alone, CC^{mut3} plus ponatinib doses were compared against both (i) CC^{mut3} alone and (ii) EGFP with the corresponding ponatinib dose. Figure 3 shows that the combination consisting of CC^{mut3} and 1 nM ponatinib (last bar) results in significant induction of apoptosis compared to all other groups. Importantly, the enhanced apoptosis seen with this combination (last bar) is significantly greater than both CC^{mut3} alone (second bar) and EGFP with 1 nM ponatinib (fifth bar).

CC^{mut3} Combined with Ponatinib Causes Further Induction of Apoptosis and Necrosis in K562 Cells

Flow cytometry was utilized to measure apoptosis and necrosis of cells treated with our combination. 7-Aminoactinomycin D (7AAD), which binds the DNA of dead and dying cells no longer possessing an intact membrane, and Annexin V, which binds to the externalized apoptotic marker, phosphatidylserine, were used to determine apoptosis. To ensure that we tested the effects of the combination treatment, only cells showing EGFP fluorescence (thus, CC^{mut3}-positive or positive for control) were selected for and analyzed (ponatinib itself is known to freely enter cells¹³). As seen in Figure 4, the combination of CC^{mut3} and 1 nM ponatinib (Figure 4F) induces the highest percent of apoptosis and necrosis. Notably, this combination is higher than each of its individual components (Figure 4F vs Figures 4B and E). These results are summarized in Figure 4G.

Increased Apoptotic and Necrotic Effect from CC^{mut3} and Ponatinib Is Not K562 Cell Specific

To ensure that the combination induces apoptosis and necrosis in other cells harboring Bcr-Abl (besides human K562 cells), experiments were conducted in mouse Ba/F3 cells expressing the 210 kDa Bcr-Abl fusion protein (Ba/F3-p210). The full length p210 form of Bcr-Abl, the product of the Bcr-Abl hybrid gene, causes leukemic cell growth in

hematopoietic cell lines, and is known to induce leukemia in animal models.³¹ Ba/F3-p210 cells have been engineered to depend on Bcr-Abl for growth, and stably express EGFP along with Bcr-Abl.^{32, 33} Therefore, in these experiments, mCherry was used as a negative control and as the CC^{mut3} tag (instead of EGFP). Flow cytometry was again utilized to study apoptosis and necrosis, where the cell population that contained both EGFP (Bcr-Abl positive) and mCherry (transfection positive) was analyzed. Figure 5 shows the induction of apoptosis in Ba/F3-p210 cells, where the data agrees with the results also seen in K562 cells (Figure 4). Again, the combination including 1 nM ponatinib (Figure 5F) shows the highest induction of apoptosis and necrosis, higher than 1 nM ponatinib alone (Figure 5E) and also higher than CC^{mut3} alone (Figure 5B). These results are summarized in Figure 5G.

Treatment with CC^{mut3} and 1 nM Ponatinib Significantly Decreases the Transformative Ability (Oncogenic Potential) of K562 Cells

Transformative ability of K562 cells following treatment with our combination was tested using a colony forming assay, where outgrowth of colonies were a direct measure of oncogenic potential. Results are shown in Figure 6. With CC^{mut3} alone, it should be noted that this construct causes a 3-fold decrease in the amount of colonies formed compared to EGFP (Figure 6, second bar vs. first bar). With CC^{mut3} plus 1 nM ponatinib (Figure 6, last bar), the combination therapy shows a significant effect with a near six-fold decrease in the amount of colonies formed compared to EGFP (Figure 6, last bar vs. first bar), an over five-fold decrease compared to EGFP plus 1 nM ponatinib (last bar vs. fifth bar) and a two-fold decrease compared to the number of colonies formed when treated with CC^{mut3} alone (last bar vs. second bar). This highlights specifically the benefit of CC^{mut3} + Pon 1 nM compared to CC^{mut3} alone or EGFP + Pon 1 nM, indicated by asterisks in Figure 6.

In Ba/F3-p210 cells, however, the reduction in transformative ability seemed to be mainly mediated by treatment with CC^{mut3}. In Figure 7, CC^{mut3} was significantly lower than the EGFP control (second bar vs. first bar). Likewise, CC^{mut3} + Pon 100 pM or CC^{mut3} + Pon 1 nM were both significantly lower than ponatinib alone at either dose (fourth bar vs. third bar, sixth bar vs. fifth bar). Importantly, CC^{mut3} + Pon 100 pM and CC^{mut3} + Pon 1 nM were not significantly lower than CC^{mut3} alone. In other words, the combination did not enhance the reduction in oncogenic potential in this cell line.

Combination Treatment Enhances Induction of Apoptosis in Cells Containing the T315I Mutation

Bcr-Abl cells containing the T315I tyrosine kinase domain mutation are known to be resistant to most TKIs. Ponatinib is known to be effective against these cells, albeit with a higher *in vitro* IC₅₀.¹³ Because ponatinib is a pan-inhibitor of Bcr-Abl, higher dosing may lead to unintended inhibition of other receptor tyrosine kinases, potentially leading to increased side effects (*in vivo*).^{19, 34} Therefore, the combination was tested in Bcr-Abl-containing Ba/F3 cells which harbor the T315I mutation (Ba/F3-p210-T315I cells). Notably, the combinations of CC^{mut3} with both 1 nM and 10 nM ponatinib showed significantly higher induction of apoptosis/necrosis than corresponding ponatinib doses alone (Figure 8F vs. 8E, and 8H vs. 8G). Overall, CC^{mut3} with 10 nM ponatinib works more effectively than all other treatments (Figure 8H vs. 8A–G). All results are summarized in Figure 8I.

CC^{mut3} Drives the Reduction of Transformative Ability and Decrease in Proliferation of Cells Containing the T315I Mutation

Transformative ability of Ba/F3-p210-T315I cells treated with the CC^{mut3} and ponatinib combination was also tested (Figure 9). Again, CC^{mut3} alone reduced transformative ability, this time by ~40% compared to EGFP control (Figure 9, second bar vs. first bar). However, as seen in the Ba/F3-p210 experiment, the combination did not provide additional benefit

over either agent alone. Therefore, to support the evidence of anti-proliferative activity caused by CC^{mut3} alone, a cell proliferation assay was also performed. CC^{mut3} alone was also found to inhibit Ba/F3-p210-T315I cell proliferation (standard trypan blue exclusion assay, Figure S1), as measured at 72 hour and 96 hour time points.

Lastly, the CC^{mut3} and ponatinib combination did not have activity in the Ba/F3-parent cells (Bcr-Abl negative, IL-3 dependent cells, data not shown) as expected, which suggests that the effects seen were only due to the presence of Bcr-Abl.

Discussion

We previously demonstrated the inhibitory effect of coiled-coil mutants on Bcr-Abl activity.^{6, 20, 35} In this study, we sought to determine whether Bcr-Abl inhibition could be enhanced by a “dual-hit” strategy. Using this strategy, both the oligomerization domain and the tyrosine kinase domain of Bcr-Abl were targeted concomitantly. Both a novel coiled-coil therapeutic (CC^{mut3}) and a recently approved TKI (ponatinib) were used. Bcr-Abl was further inhibited when these two agents were administered in combination, leading to decreased oncogenic potential and enhanced apoptosis in both Bcr-Abl-positive myeloid K562 cells and Ba/F3-p210 lymphoid cells, including the T315I mutant Ba/F3-p210 cell line.

Kinase activity of Bcr-Abl, including auto-phosphorylation and phosphorylation of downstream targets (STAT5, CrkL), was reduced to an extent by each agent alone and further reduced in combination. The combination of CC^{mut3} and 10 nM ponatinib resulted in complete elimination of phosphorylation; therefore, a sub-saturating dose of 1 nM ponatinib was used for further studies. With the goal of reducing the IC₅₀ of ponatinib (3.9 nM in K562 cells¹³), a “dose-lowering capability” provided by CC^{mut3} may be important in managing adverse drug events caused by off-target kinase inhibition manifested by higher doses of ponatinib.^{19, 36}

In both K562 and Ba/F3-p210 cells, induction of apoptosis (activation of caspase-3/7, 7AAD/Annexin V binding) was enhanced using the combination of CC^{mut3} and 1 nM ponatinib. Likewise, transformative ability (oncogenic potential) was diminished using the combination at the same ponatinib concentration in K562 cells. However, in the Ba/F3-p210 cells, the diminished oncogenic potential seemed to be mediated solely by CC^{mut3}, with no enhanced effect upon the addition of ponatinib.

To test the effects of the combination against cells containing the T315I mutation in Bcr-Abl, a higher, 10 nM dose of ponatinib was included to account for the increased IC₅₀ seen in the Ba/F3-p210-T315I cell line (reported IC₅₀ of = 11 nM¹³, or 18 nM¹⁵). Apoptosis induction, as measured by 7AAD and Annexin V binding, showed an increase when treated with the combination at this 10 nM dose of ponatinib.

Finally, CC^{mut3} reduced transformative ability of cells containing the T315I mutation by 40% compared to EGFP control. *Importantly, this is the first report of a Bcr-Abl N-terminally targeted agent that is effective alone against T315I.* In this colony forming assay, the combination did not provide additional benefit over either agent individually. Additionally, CC^{mut3} alone also inhibits Ba/F3-p210-T315I cell proliferation (standard trypan blue exclusion assay, Figure S1), as measured at 72 hour and 96 hour time points. Ruthardt et al. have shown a benefit of using a truncated wild-type CC (helix 2) in combination with an allosteric inhibitor of Bcr-Abl.³⁷ However, their CC by itself was not effective against T315I Bcr-Abl, unlike our CC^{mut3}.

Because we inhibit Bcr-Abl at different domains, we could possibly be affecting different intracellular signal transduction pathways. Bcr-Abl, through its different domains, is known to interact with many intracellular proteins involved with cell proliferation, differentiation, apoptosis, and other functions.³⁸ Interestingly, we consistently observe a decrease of one of the STAT5 isoforms recognized by this STAT5 antibody with CC^{mut3} treatment (Figure 2A, lane 4, top p-STAT5 band disappears, presumably STAT5a), while a decrease in the lower STAT5 band is seen with ponatinib treatment (Figure 2A, lane 2, bottom p-STAT5 band reduced, presumably STAT5b).^{39–41} In future studies, it would be intriguing to explore other differential pathways that are differentially regulated by CC^{mut3} vs. ponatinib.

Resistance to TKIs is a clinical problem that has been recently addressed by the development of second and third-generation inhibitors. A particular problem that has evolved is the appearance of multiple mutations in Bcr-Abl that evade binding to any one single TKI. A particular *in vitro* compound mutation, E255V/T315I, cannot be effectively inhibited with current therapies (425 nM cell proliferation IC₅₀ for ponatinib¹⁵).¹⁶ Recently, Deininger et al. have analyzed double/compound mutations in CML patients. Notably, a G250E/T315I mutation was found in two out of 47 CML patients, neither of which had prior exposure to ponatinib. The authors further suggest that since “certain compound mutations such as G250E/T315I and E255V/T315I confer high-level resistance to ponatinib or DCC-2036 *in vitro*... these mutations may emerge as a significant clinical challenge in patients on third-line TKIs.”¹⁶ It should be noted that DCC-2036 is not a TKI but instead an allosteric switch control inhibitor of Bcr-Abl. Therefore, the use of small molecules as Bcr-Abl inhibitors may be limited due to the ability of Bcr-Abl to mutate to escape their binding. With CC^{mut3}, which uses multiple contact points and interactions with Bcr-Abl for binding, we hypothesize that Bcr-Abl mutational escape may be diminished due to the need for Bcr-Abl to evolve many mutations in its coiled-coil domain to evade binding to CC^{mut3}. In addition, if Bcr-Abl were to mutate sufficiently to evade CC^{mut3} binding, this could also potentially prevent itself from homo-dimerizing, resulting in a self-inactivation. Testing of CC^{mut3} alone and in combination with ponatinib in cell lines and patient samples containing compound Bcr-Abl mutations is a focus of our future studies.

While in these studies CC^{mut3} has been delivered via plasmid transfection, we are currently developing CC^{mut3} as a peptide therapy using various approaches to enhance stability, decrease degradation, and improve delivery. Our goal is to develop this into a peptide drug which can be easily administered to a patient with CML. If successful, this combination could be used after first or second generation TKI failure to avoid further mutations, or could even be used as a “personalized medicine approach” for CML where the Bcr-Abl mutational status of a patient is typed prior to, and/or during therapy. Finally, one could envision this combination therapy as the optimal frontline therapy for newly diagnosed CML patients due to its specificity for Bcr-Abl, potency, and reduced likelihood of mutational escape.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Targeting two distinct domains of Bcr-Abl. CC^{mut3} (left) targets the coiled-coil oligomerization domain of Bcr-Abl while ponatinib (right) targets the tyrosine kinase domain of Bcr-Abl. Bcr-Abl domains labeled as follows: CC = Coiled-Coil (Oligomerization Domain)⁴; S/T Kinase = Serine/Threonine Kinase²¹; DH/PH = Dbl Homology / Pleckstrin Homology^{22, 23}; Y-Kinase = Tyrosine Kinase; DB = DNA-Binding²⁴; AB = Actin-Binding²⁵⁻²⁷; SH3 = Src Homology 3; SH2 = Src Homology 2; NLS = Nuclear Localization Signal; NES = Nuclear Export Signal; G-actin = Globular Subunit of Actin; F-actin = Fibrous Subunit of Actin. Numbers above domains represent approximate amino acid residues.

Figure 2.

Western blots: Oligomeric disruption and decrease in Bcr-Abl downstream signaling in K562 cells. Upon 48 hour treatment of K562 cells with CC^{mut3} and ponatinib or EGFP (transfection control) and ponatinib, phosphorylation states of (A) Bcr-Abl and downstream target STAT5, or (B) direct Bcr-Abl substrate CrkL (including 100 pM dose of ponatinib) were assessed (n=3 for both (A) and (B), representative images shown).

Figure 3. Induction of apoptosis by measuring intracellular activation of caspase-3/7. 48 hours following transfection and treatment of K562 cells, activity of caspase-3/7 was analyzed via fluorescence (n=3). Values reported as overall means $\pm$ SD; one-way ANOVA with Tukey's post test, * p < 0.05, ** p < 0.01. Notable significance shown.

Figure 4. Induction of apoptosis and necrosis in K562 cells using CC^{mut3} and/or ponatinib measured by Annexin V (apoptotic) and 7AAD (necrotic) staining. 72 hours following transfection and treatment, K562 cells were gated for EGFP fluorescence and cellular apoptosis was assessed using Annexin V and 7AAD dyes. **A–F)** Representative individual contour plots from each transfection and treatment group showing only EGFP-gated cells. Q1 = 7AAD+/AnnexinV– (necrotic); Q2 = 7AAD+/AnnexinV+ (apoptotic); Q3 = 7AAD–/AnnexinV– (healthy); Q4 = 7AAD–/AnnexinV+ (apoptotic) **G)** Percentage of apoptosis and necrosis induced by each transfection and treatment group (plots A–F), n=3. Values reported as overall mean $\pm$ SD; one-way ANOVA with Tukey's post test, * $p < 0.05$, *** $p < 0.005$. Notable significance shown.

Figure 5.

Induction of apoptosis and necrosis measured by Annexin V and 7AAD staining in Ba/F3-p210 cells. 72 hours following transfection and treatment with ponatinib, Ba/F3-p210 cells were gated on EGFP (internal presence of Bcr-Abl) and mCherry (presence of control or CC^{mut3}) fluorescence, and the percentage of cells undergoing apoptosis and necrosis was assessed via flow cytometry. **A–F**) Representative individual scatterplots (as opposed to contour plots with K562 cells to distinguish cell type) from each transfection and treatment group showing only EGFP and mCherry-gated cells. Q1 = 7AAD+/AnnexinV– (necrotic); Q2 = 7AAD+/AnnexinV+ (apoptotic); Q3 = 7AAD–/AnnexinV– (healthy); Q4 = 7AAD–/AnnexinV+ (apoptotic) **G**) Percentage of apoptosis and necrosis induced by each transfection and treatment group (plots A–F), n=3. Values reported as overall mean ± SD; one-way ANOVA with Tukey's post test, *** p < 0.005. Notable significance shown.

Transformative Ability - K562 Cells

Figure 6.

Colony forming assay: transformative ability of K562 cells with different treatment groups. Colony forming units counted seven days after seeding transfected cells into methylcellulose media supplemented with corresponding dose of ponatinib. Data presented as percentage of growth from EGFP control with no ponatinib added (n=3 in duplicate). Values reported as overall mean $\pm$ SEM; one-way ANOVA with Tukey's post test, * $p < 0.05$, *** $p < 0.005$. Notable significance shown.

Transformative Ability - Ba/F3-p210

Figure 7.

Colony forming assay: transformative ability of Ba/F3-p210 cells with different treatment groups. Colony forming units counted seven days after seeding transfected cells into methylcellulose media supplemented with corresponding dose of ponatinib. Data presented as percentage of growth from EGFP control with no ponatinib added (n=3 in duplicate). Values reported as overall mean $\pm$ SEM; one-way ANOVA with Tukey's post test, * $p < 0.05$, ** $p < 0.01$. Notable significance shown.

Figure 8.

Induction of apoptosis and necrosis measured by Annexin V and 7AAD staining in Ba/F3-p210-T315I cells. 72 hours following transfection and treatment with ponatinib, Ba/F3-p210-T315I cells were gated on EGFP and mCherry fluorescence and the percentage apoptotic/necrotic cells was assessed as before. **A–H)** Representative individual scatterplots from each transfection and treatment group showing EGFP and mCherry-gated cells. Q1 = 7AAD+/AnnexinV– (necrotic); Q2 = 7AAD+/AnnexinV+ (apoptotic); Q3 = 7AAD–/AnnexinV– (healthy); Q4 = 7AAD–/AnnexinV+ (apoptotic). **I)** Percentage of apoptosis and necrosis induced by each transfection and treatment group (plots A–H), n=3. Values reported as overall mean ± SD; one-way ANOVA with Tukey's post test, ***p < 0.005.

Transformative Ability - Ba/F3-p210-T315I

Figure 9.

Colony forming assay: transformative ability of Ba/F3-p210 cells harboring T315I mutation (Ba/F3-p210-T315I cells) with different treatment groups. Colony forming units counted seven days after seeding transfected cells into methylcellulose media supplemented with corresponding dose of ponatinib. Data presented as percentage of growth from EGFP control with no ponatinib added (n=3 in duplicate). Values reported as overall mean $\pm$ SEM; one-way ANOVA with Tukey's post test, ** $p < 0.01$, *** $p < 0.005$. Notable significance shown.