DISRUPTION OF BCR-ABL COILED-COIL OLIGOMERIZATION BY DESIGN*
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Abstract: Oligomerization is an important regulatory mechanism for many proteins, including oncoproteins and other pathogenic proteins. The oncoprotein Bcr-Abl relies on oligomerization via its coiled-coil domain for its kinase activity, suggesting that a designed coiled-coil domain with enhanced binding to Bcr-Abl and reduced self-oligomerization would be therapeutically useful. Key mutations in the coiled-coil domain of Bcr-Abl were identified which reduce homo-oligomerization through intermolecular charge-charge repulsion yet increase interaction with the Bcr-Abl coiled-coil through additional salt bridges, resulting in an enhanced ability to disrupt the oligomeric state of Bcr-Abl. The mutations were modeled computationally to optimize the design. Assays performed in vitro confirmed the validity and functionality of the optimal mutations which were found to exhibit reduced homo-oligomerization and increased binding to the Bcr-Abl coiled-coil domain. Introduction of the mutant coiled-coil into K562 cells resulted in decreased phosphorylation of Bcr-Abl, reduced cell proliferation, and increased caspase-3/7 activity and DNA segmentation. Importantly, the mutant coiled-coil domain was more efficacious than the wild-type in all experiments performed. The improved inhibition of Bcr-Abl through oligomeric disruption resulting from this modified coiled-coil domain represents a viable alternative to small molecule inhibitors for therapeutic intervention.

Coiled-coil domains are ubiquitous protein structural motifs found in approximately 10% of all eukaryotic proteins (1). Characterized by a heptad repeat (sequence of seven amino acids) and association of two or more α-helices, coiled-coils provide oligomerization capabilities (both homo- and hetero-oligomerization) useful for structural scaffolding and protein recognition. Coiled-coil domains are critical for regulating many processes involved in the pathogenesis of various diseases (2). Thus, rationally designed coiled-coils can be used as therapeutics by interfering with the activity of a pathogenic protein through oligomeric disruption. One example is enfuvirtide (Fuzeon®), a fusion inhibitor that disrupts the helix bundle formation necessary for HIV-1 viral entry (3), spawning the next-generation of rationally designed fusion inhibitors (4-8). The coiled-coil reported here was designed to bind to the target (Bcr-Abl) better than the target protein binds to itself while exhibiting minimal homo-oligomerization.

Coiled-coils are very well characterized, and the high correlation between their sequence and structure (9) is advantageous for rational design. Coiled-coil oligomerization involves hydrophobic interactions, salt bridge formation, and helicity. In the heptad repeat, hydrophobic packing at the “a” and “d” positions (see Figure 1, green residues) helps drive association with further stability provided by the residues at positions “g” and “e” which are commonly charged and interact to form salt bridges (see Figure 1, red and blue residues). Since charged residues are routinely found at the “g” and “e” positions, mutating these residues in a rational manner to add salt bridges to favor formation of hetero-oligomers (10-12) and charge-charge repulsions to reduce the formation of homo-oligomers (13,14) can change the affinity and specificity of the coiled-coil dimer.

Bcr-Abl exists primarily as a tetramer (more specifically as a dimer of dimers), facilitating the trans-autophosphorylation necessary to activate the tyrosine kinase domain (15,16). Oligomerization of Bcr-Abl is achieved through a coiled-coil domain at the N-terminus of the protein. Bcr-Abl constructs lacking the N-
terminal coiled-coil fail to induce chronic myelogenous leukemia (CML) in a murine model (15,17) thus setting the stage for oligomeric disruption as a therapy. Proof of concept for using the coiled-coil domain to inhibit Bcr-Abl activity has already been demonstrated through retroviral transfection of the wild-type coiled-coil domain or addition of the purified protein attached to a cytoplasmic transduction peptide (18-22). To further this approach, we designed and tested a mutant coiled-coil (CCmut2) using both computational and in vitro experiments. This CCmut2 disfavors self-oligomerization (CCmut2:CCmut2) yet appears to bind more tightly to the target Bcr-Abl coiled-coil domain (CCmut2:Bcr-Abl) than the target’s native oligomerization partner (Bcr-Abl:Bcr-Abl), and resulted in superior inhibition of Bcr-Abl.

The coiled-coil domain from Bcr-Abl consists of 72 amino acids composed of two α-helices (α1: residues 5-15 and α2: residues 28-67) that form an N-shaped configuration with two parallel helices connected by a short linker (see Figure 1A) (23). Upon dimerization, the resulting coiled-coil has an anti-parallel orientation with α2 at the core and α1 latching onto the backside of the opposing α2 helix (domain swapping) (1,23). The majority of the dimer interface is composed of the classic knobs-in-holes type hydrophobic interactions from residues at the “a” and “d” positions (24). Further stabilization comes from 4 inter-chain salt bridges between residues in the α2 helices (Figure 1A), as well as packing of aromatic residues from the α1 helix and opposing α2 helix. As seen in Figure 1A, there are two uncharged residues (S41 and Q60) that are in the appropriate position for the formation of salt bridges with two charged residues (E32 and E48). Thus, mutation of S41 and Q60 to positively charged amino acids has the potential to provide two additional salt bridges with Bcr-Abl (Figure 1B), and thus enhance binding. However, while this provides more salt bridges in the hetero-oligomer, these mutations alone are undesirable as they allow the formation of a greater number of salt bridges in the homo-oligomer. To reduce homo-oligomerization in the mutant coiled-coil, residues proximal to charged residues on the opposing helix were considered as candidates for mutation to introduce charge-charge repulsion (13,14). L45 and E48 were identified as two such residues (Figure 1C). In addition, previous reports have incorporated a C38A mutation primarily for crystallization purposes (1,23), and this mutation was also studied. Putative mutations were investigated first through molecular modeling and state-of-the-art biomolecular simulation and free energy analysis to ascertain the impact on the coiled-coil structure and stability. Such in silico methods provide a means to efficiently (and inexpensively) assess the influence of mutation. The resulting optimized mutant coiled-coil (CCmut2) contains 5 mutations (C38A, S41R, L45D, E48R, and Q60E) and was further assessed in vitro. Taken together, these results demonstrate the effectiveness of the mutant coiled-coil domain, and importantly, further illustrate the ability to rationally modify an existing coiled-coil domain to improve therapeutic efficacy.

EXPERIMENTAL PROCEDURES

Computational modeling and simulation-
The initial model structure was obtained from the crystal structure (refined to 2.2 Å resolution) of the N-terminal oligomerization domain of Bcr-Abl (PDB ID: 1K1F, chains A and B) (25). Selenomethionine residues were mutated to methionine and position 39 was mutated back to cysteine to maintain consistency with the natural protein. Amino acid side-chain mutations were introduced using Deepview (Swiss PDB Viewer) (26) and by hand with the LEAP module from AMBER 9 (27). Molecular dynamics (MD) and free energy simulations were performed to assess the structure and stability of the model coiled-coil structures. Such methods have proven useful for reproducing and predicting the structure of proteins, including the folding of small proteins and influence of amino acid side chain substitutions (28-34). All simulations were completed using the AMBER modeling suite (27), the AMBER ff03 protein force field (35), with explicit solvent and counter-ions using standard simulation protocols, including minimization and ~40-90 ns of MD sampling with Ewald treatments of the electrostatics (36). For further detail, refer to the supplemental data. Representative plots of the root-mean-squared deviations (RMSd) to the initial structure of the coiled-coil during the MD simulations can be found in Figure S1 of the supplemental data.
effectively the time history of the atomic motions of the model structures at different intervals over the simulation, were analyzed with various tools. Quantification and comparison of the relative helical content was measured by calculating mean residue ellipticities at 222 nm (representative of helix content in CD spectra) of 5 individual 500 ps average structures spanning the final 5 ns of simulation of each coiled-coil dimer using the DichroCalc program (37). Structural helical content, or percent helicity defined as the number of residues in an α-helix divided by the total number of residues, was also calculated based on secondary structure as determined by peptide backbone Ψ and Φ torsions from the final 10 ns of simulation using the DSSP method (38) as implemented in UCSF Chimera (39). Intrahelical hydrogen bonds (i.e. carbonyl oxygen of residue ‘i’ to the amine nitrogen of residue ‘i+4’) were calculated over the final 10 ns of MD with distances less than 3.5 Å indicative of a hydrogen bond. To estimate the relative binding free energies of the coils in the dimers, MM-PBSA as implemented in AMBER (40) and described by Gohlke and Case (41) was applied to independent MD trajectories for the dimer and individual monomers. In addition to the post-processing of MD results, calculations of the relative free energy of binding with respect to the wild-type dimer (ΔΔG_{binding}) were completed using more detailed thermodynamic integration (TI) methods for the CCmut2 dimers (42). On the basis of a thermodynamic cycle (see supplemental data Figure S2), the relative free energy of binding can be calculated by “mutating” the original protein (λ = 0) to incorporate designed amino acid side chain point mutations (λ = 1) in both the dimer and monomeric states over different λ states in silico. Incorporation of the five amino acid mutations considered using this approach was accomplished stepwise (see Figure 1B). Two steps were required to incorporate all five mutations to form the heterodimer mutant (CC-CCmut2) and an additional two steps to perturb the transition dimer into the homodimer (CCmut2-CCmut2). Similarly, two steps were used to incorporate the five mutations in the unbound monomer. Relatively long (6 ns equilibration, > 6 ns accumulation) MD simulations were performed at each λ for the TI. Further technical details are provided in the supplemental data.

Construction of plasmids and mutagenesis- The gene encoding the coiled-coil domain from Bcr-Abl (43) was amplified via PCR with the primers 5’- gtgaactcaggttagttagggacccgggtg-3’ and 5’-atgcctgtggacccgggtcagcttctc-3’ and subcloned into the XhoI site of the plasmid pEGFP-PS, an optimized protein switch (PS) (44,45) for use in the nuclear translocation assay (46), and pEGFP-C1 (Clontech, Mountain View, CA, USA) for other experiments. These plasmids, named pPS-CC and pEGFP-CC respectively, were then used as the template DNA for site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) to generate five amino acid mutations (S41R, L45D, E48R, V49D, and Q60E). The primers used for the mutagenesis were 5’-gggagcgctgcaaggcccgcattcggcgcgacgagcagcgggacaacccaggagctggatctacatgtagtacggtgcctgaccaggg3’ and the reverse complement. Another site-directed mutagenesis was carried out to make C38A and D49V mutations with the primers 5’-caggagctggagcgcgccaaggcccgcattcg-3’ (and reverse complement) and 5’-gggagcgctggagtacagcgggctcctc-3’ (and reverse complement) respectively. The final constructs were termed pEGFP-CCmut2 and pPS-CCmut2. The primers 5’-cgaagggagctcccatccatcatcaactcaatgtagtacagtgtcctgaccaggg3’ and 5’-agcatggatcctcggtgacccggggtctc-3’ were used to PCR amplify wild-type and mutant coiled-coil domains and subcloned into pMAL-c2x (New England Biolabs, Ipswich, MA, USA) at the Sacl and BamHI sites to generate pMAL-H6-PP-CC and pMAL-H6-PP-CCmut2 used for protein expression. The primers 5’-tgtaacctggttagttagggacccgggtg-3’ and 5’-atgcctgtggacccgggtcagcttctc-3’ were used to PCR amplify both coiled-coil genes (wild-type and mutant), and each was subsequently subcloned into the XhoI site of pDsRed2-N1 (Clontech) to make pDsRed-CC and pDsRed-CCmut2. Similarly, the primers 5’-tgtaacctggttagttagggacccgggtg-3’ and 5’-cggggcgcggcgcggcgtatctggctcttctc-3’ were used to insert the genes into the plasmid pEFVP16 (mammalian two-hybrid prey vector containing the VP16 activation domain, obtained from Dr. T.H. Rabbitts, LIMM, Leeds, UK) at the SfiI and NotI sites to generate pEFVP16-CC and
pEFVP16-CCmut2. The primers 5'-tgtaagaattcatggtggacctggtg-3' and 5'-atgctgaattcaccggtcatagctcttc-3' were used to insert the coiled-coils into the vector pM1 (mammalian two-hybrid bait vector containing the Gal4 binding domain, obtained from Dr. T.H. Rabbitts) at the EcoRI site to generate pM1-CC and pM1-CCmut2.

**Cell lines and transient transfection** - Cells were maintained in a 5% CO2 incubator at 37 °C. K562 (a kind gift from Kojo Elenitoba Johnson, Univ. of Michigan) and Cos-7 cells (ATCC) were grown in RPMI (GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT, USA), 1% penicillin-streptomycin (GIBCO), 0.1% gentamicin (Hyclone), and 1% L-glutamine (Hyclone). K562 cells were passaged every two days and maintained between 0.1 - 1 x 10^6 cells/mL. The Amaxa Nucleofector II (Lonza Group Ltd, Basel, Switzerland), was used to transfect 2 x 10^6 cells with 5 - 8 μg DNA in solution V following the manufacturer’s recommended protocol and nucleofection program (T-013). Cos-7 cells were passaged every 2-3 days and transfected 24 hrs after seeding the cells using Lipofectamine LTX (Invitrogen) as recommended by the supplier.

**Protein Purification and Circular Dichroism (CD)** - Fusion proteins consisting of maltose binding protein (MBP), a His-tag, a PreScission Protease site, and CC or CCmut2 were expressed in BL21star DE3 E. coli cells (Invitrogen) from pMAL-H6-PP-CC or pMAL-H6-PP-CCmut2 plasmids. The proteins were purified over amylose resin (New England Biolabs) with gravity flow, cleaved with PreScission Protease (a kind gift from Dr. Chris Hill, Utah), and MBP-H6 removed by purification over HiPur Cobalt resin, (Thermo Scientific, Waltham, MA, USA). Reverse phase HPLC was performed as a final purification before lyophilizing the protein. CC and CCmut2 purified proteins were confirmed through mass spectroscopy (Mass Spectroscopy and Proteomics Core Facilities, U. of Utah). CD experiments were performed on an Aviv 410 CD spectrometer (Biomedical Inc., Lakewood, NJ, USA). Measurements from 190-300 nm (1 nm steps) were taken on 10 μM protein solutions in PBS (50 mM sodium phosphate, 150 mM NaCl, 0.5 mM DTT, pH 7.2) in a 1 mm pathlength cuvette. 3 sec equilibration times were allowed prior to each measurement, and the signal was averaged over 3 sec. The average of 3 scans was used for each solution. Thermal denaturation was monitored in a 1 cm cuvette at 222 nm every 2 °C from 10 °C to 95 °C, and back down to 10 °C in 10 °C steps. The protein concentrations used were 10 μM CC, 10 μM CCmut2, or 5 μM CC + 5 μM CCmut2. In addition, a mixing cell cuvette was used with 2.5 μM CC in one chamber and 7.5 μM CCmut2 in the other chamber with spectra acquired prior to and post mixing. After mixing and before data acquisition, the sample was incubated at 80 °C for 10 min, and then allowed to re-equilibrate at 10 °C.

**Nuclear Translocation Assay** - The nuclear translocation assay (NTA) was performed as previously described (46). Briefly, this assay uses a nuclear-localization inducible protein switch fused to a protein of interest and measures its ability to translocate a second protein into the nucleus. Here, the protein fused to the protein switch was either the coiled-coil domain or mutant coiled-coil domain, and its ability to translocate a cotransfected coiled-coil domain or mutant coiled-coil domain into the nucleus was measured. 24 hrs after transient transfection of Cos-7 cells, 200 nM dexamethasone, or an equal volume of ethanol (carrier) was added to the cells and incubated for 1 hr. 0.5 μL H33342 (nuclear dye) was added and incubated for another 15 min. before imaging the cells with a fluorescence microscope as previously described (43). The percentage of protein localized in the nucleus was quantified with and without ligand to determine the nuclear increase after ligand induction.

**Mammalian Two-Hybrid Assay** - The pM1-CC or pM1-CCmut2 (bait), pEFVP16-CC or pEFVP16-CCmut2 (prey), pG5-Fluc (reporter; obtained from Dr. T.H. Rabbitts), and pRL-CMV (for normalization; obtained from Dr. T.H. Rabbitts) plasmids were co-transfected in RPMI without antibiotics into 7.5x10^4 Cos-7 cells in a white 96-well plate (Cellstar, Greiner Bio-One, Monroe, NC, USA) in a 10:10:10:1 ratio. 24 hrs after transfection the media was replaced with complete RPMI, and 48 hrs after transfection both the Firefly and Renilla luminescence were measured using the Dual-Glo Luciferase Assay (Promega) reagents per the manufacturer’s
recommendations. The mean from duplicate samples were taken from 5 separate experiments. pAD-SV40 and pBD-p53 (Stratagene) plasmids were used for the positive control, and pM1 lacking the coiled-coil gene was used as the negative control. A relative response ratio was calculated by first normalizing the individual Firefly luminescence to the Renilla luminescence. The negative control was subtracted from the mean of the duplicate experimental values, and scaled by dividing by the difference between the positive and negative controls ($\frac{\text{Experiment} - \text{Ctrl}}{\text{Ctrl}^+ - \text{Ctrl}^-}$).

**Cell Proliferation and Western Blotting** - 48 hrs following transfection of K562 cells with pEGFP-C1, pEGFP-CC, or pEGFP-CCmut2, trypan blue exclusion was used to determine cell proliferation (cell viability). 3 x 10$^6$ cells were pelleted and resuspended in 600 μL lysis buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue). Standard western blotting procedures were followed using antibodies to detect the phosphorylated forms of Bcr-Abl, STAT5, CrkL, as well as β-actin as a loading control. The primary antibodies (anti-pAbl(Y245): 73E5, Cell Signaling Technology; anti-pSTAT5(Y694): E208, Abcam; anti-pCrkl(Y207): #3181, Cell Signaling Technology; anti-actin: mAbcam 8226, Abcam) were detected with anti-mouse (Ab6814, Abcam) or anti-rabbit (#7074, Cell Signaling Technology) HRP-conjugated antibodies before the addition of ChemiGlo (AlphaInnotech, Cell Biosciences, Santa Clara, CA, USA) chemiluminescent substrate and detection with a FluorChem FC2 imager (AlphaInnotech). The resulting bands were quantified through densitometry and normalized to the β-actin bands.

**Colony Forming Assay**- 24 hrs following transfection of pEGFP-C1, pEGFP-CC, or pEGFP-CCmut2, K562 cells were resuspended in Iscove’s modified Dulbecco’s media (Stem Cell Technologies, Vancouver, BC, Canada) with 2% FBS, and 1000 cells were seeded in Methocult H4230 methylcellulose medium (Stem Cell Technologies) in the absence of cytokines. Imatinib mesylate (IM; a kind gift from Novartis) was added to 1000 untransfected K562 cells seeded in Methocult at the time of seeding. Each group of treated cells was seeded into two separate plates. Colony formation was assessed 7 days after seeding cells by counting colonies in a 200 μm$^2$ area of the plate and calculating the mean number of colonies per treatment. Experiments were replicated at least 3 times and compared to control (cells transfected with pEGFP-C1).

**Caspase-3/7 Assay**- 48 hrs following transfection of pEGFP-C1, pEGFP-CC, or pEGFP-CCmut2, 3 x 10$^6$ K562 cells were pelleted and resuspended in 50 μL lysis buffer provided in the EnzChek Caspase-3/7 Assay Kit #2 (Invitrogen). Cells were frozen at -80 °C, and then centrifuged at 5,000 x g for 5 min. Lysates were transferred to a black 96-well plate (Cellstar, Greiner Bio-One, Monroe, NC, USA), and 50 μL 2x AMC-DEVD substrate was added and incubated at room temperature for 30 min. Fluorescence was measured with excitation 342 nm and emission 441 nm on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA).

**Fluorescence Microscopy & DNA Segmentation** - 48 hrs following transfection of pEGFP-C1, pEGFP-CC, or pEGFP-CCmut2, K562 cells were transferred to 2-well live cell chambers, 1 μL of the nuclear stain H33342 was added, and the cells were incubated at 37°C for 15 min. Cells were then analyzed with an inverted fluorescence microscope (Olympus IX701F, Scientific Instrument Co., Sunnyvale, CA, USA) with high-quality narrow band GFP filter (excitation HQ480/20 nm, emission HQ510/20 nm, beam splitter Q4951p, Chroma Technology Corp., Brattleboro, VT, USA). Cells were photographed with an F-view Monochrome CCD camera using a 60x objective, and were selected based on EGFP fluorescence. The nuclei of at least 50 transfected cells (EGFP fluorescence) per group were classified as either healthy (round or kidney shaped) or segmented (punctate) (47,48) and the percentage of cells with segmented DNA was calculated.

**RESULTS**

**Computational Modeling**. Rational design, molecular modeling, molecular dynamics (MD), and free energy simulations were used to predict favorable attributes, specificity, and energetics in order to facilitate the choice of coiled-coil modifications that stabilize binding of the mutant coiled-coil domain with Bcr-Abl.
while destabilizing self-oligomerization (CCmut2:CCmut2). Initial simulations monitored increases in $\alpha$-helicity as an indirect correlate for improved free energy of binding of mutant pairs (49,50) and focused on mutations to potentially improve salt bridge interactions, increase stability through formation of a disulfide bond, improve helicity in the backbone though alanine mutations to position “f”, and destabilizing mutations to improve specificity.

Alanine mutations in the peptide backbone of the C-terminal coiled-coil region were designed at residues Gln33, Gln47, Phe54, and Thr61 to increase the $\alpha$-helicity (49-51). Comparisons of the helicity as measured by circular dichroism and secondary structure between the homodimer (CCmut:CCmut) and heterodimer (CCmut:CC) suggested that this design actually decreases the helicity and shows poor specificity for the heterodimer over the homodimer (see table 1, #3, CD and secondary structure) Visualization of the coiled-coil monomer structure suggests that the designed alanine mutations have disrupted local intra-helical hydrogen bonds which may affect secondary structure and protein folding.

Cys38 exists as unbound free thiol in the native protein, and its close proximity to position 52 might allow the formation of a disulfide bond that could further stabilize the structure. An engineered disulfide was modeled by incorporating a cysteine residue at position 52. Visualization of the heterodimer and analysis of the structure helicity (see table 1, #4, CD) suggests that the geometry of the disulfide is not ideal and introduces structural disturbances.

Three point mutations were designed in the wild-type monomer to improve binding to the oncoprotein: S41R, E48R, and Q60E. When these three designed point mutations were modeled, helicity of the homodimer and heterodimer mutants exceeded the wild-type dimer (see table 1, compare #1 vs. #2) suggesting improved binding due to more favorable electrostatic interactions. The improved helicity of the mutant homodimer over the Bcr-Abl wildtype dimer may, however, result in decreased specificity for the heterodimer form. Destabilizing point mutations to improve the heterodimer specificity included designed aspartate mutations in the hydrophobic core (L45D and L49D), and were evaluated as both single and double mutations. Both mutations together decreased the secondary structure (and stability) of the heterodimer (see table 1, #6 and #9), whereas L45D maintained heterodimer specificity (see table 1, #5). The final mutation converted an exposed thiol group (Cys38) to alanine and, in conjunction with the four prior mutations, stabilized the heterodimer over both the mutant homodimer and the wild-type dimer (see table 1, compare #1 vs. #8). Thus, the optimal mutant coiled-coil domain (termed CCmut2) contains C38A, S41R, L45D, E48R, and Q60E mutations. Specificity as measured by helicity suggests that the mutant homodimer (CCmut2:CCmut2) is significantly less stable than the wild-type (CC:CC) and the heterodimer (CC:CCmut2).

MM-PBSA and thermodynamic integration energy calculation methodologies, the latter being more quantitative and accurate, were subsequently used to analyze the two sets of mutations. Both approaches validated the previous indications which suggested CCmut2 exhibits improved heterodimer stability compared to the wild-type oligomerization (see table 2). Further, CCmut2 is shown to have reduced homo-oligomerization. Together, MM-PBSA and thermodynamic integration energy calculations suggest improved binding and specificity of CCmut2.

In vitro Experiments Validate the Design. To substantiate the computational circular dichroism calculations, wavelength scans were performed on protein solutions of CCmut2, CC, and a mixture of CCmut2 and CC. As seen in figure 2A, all three protein solutions produced the typical pattern characteristic of $\alpha$-helices with similar helicity. Given the relatively small number of mutations that distinguish CCmut2 from wild-type CC, and since the mutations are designed to alter oligomerization while retaining helicity, it is reasonable to not expect major differences in their experimentally measured helicity. However, the modifications do clearly destabilize the mutant homodimer as is made apparent in the thermal denaturation of the proteins (figure 2B). While CC demonstrated a melting temperature ($T_m$) consistent with previous reports of 83 °C (23), a large decrease in $T_m$ is found for the CCmut2, $T_m = 63$ °C (figure 2B), confirming the decreased stability of the mutant homo-oligomer. When CCmut2 was mixed with CC in a 1:1 ratio two
distinct melting transitions are evident (figure 2B, black triangles), however a third \( T_m \) was not readily apparent. This suggests the CC-CCmut2 hetero-dimer is either iso-energetic with one of the two homo-dimers or that it simply did not form. To further assess the formation of the hetero-dimer a 3:1 (CCmut2:CC) ratio was studied in a mixing cell cuvette (figure 2C) both before and after mixing. The mixing shifts the curve, most predominantly at lower temperatures and only slightly at higher temperatures, suggesting the formation of a new species (as seen in figure 2C). The shift in the curve at lower temperatures can be accounted for by the decreased CCmut2 concentration due to formation of hetero-dimers. The small difference in the curves at higher temperatures suggests the formation of hetero-oligomers that are nearly iso-energetic or slightly less stable than the CC homo-oligomers. Consistent with the computational modeling, the primary improvement made through these mutations is in the specificity granted by a less stable mutant homo-dimer while retaining the ability to oligomerize with wild-type.

The optimally designed mutant coiled-coil was created through site-directed mutagenesis on a plasmid encoding the Bcr-Abl coiled-coil for cell based in vitro experiments and further validation. First, the nuclear translocation assay (NTA) (46) was used to study the interaction (Figure 3a). This assay measures the ability of a nuclear-inducible protein switch (PS) fused to one form of the coiled-coil domain to alter the nuclear localization of another form of the coiled-coil domain. Essentially, the interaction between the coiled-coil domains is indicated by an increase in fluorescence in the nucleus. The high level of nuclear translocation resulting from the mutant coiled-coil domain (Figure 3a, middle column) is likely to stem from both the reduced homo-oligomerization as well as the improved binding to the wild-type coiled-coil domain. To specifically address whether the designed mutations were limiting the homo-oligomerization, the interaction between two mutant coiled-coil domains (CCmut2:CCmut2) was assayed and found to be indistinguishable from the negative control (not included in graph). These same interactions were also studied in a mammalian two-hybrid assay to further validate the results. Similar to the NTA results, the greatest binding was found between the mutant and wild-type coiled-coil domains (CCmut2:CC, Figure 3B, 2nd column), and the homo-oligomerization between two mutants (CCmut2:CCmut2, Figure 3B, 3rd column) was not statistically distinguishable from the negative control (not included in graph). Together, the NTA and two-hybrid results indicate the proposed mutations have reduced the homo-oligomerization of the mutant coiled-coil domain and improved the binding to the wild-type coiled-coil domain.

Next, the oligomeric disruption of Bcr-Abl was indirectly measured through assaying the activity of Bcr-Abl. As the oligomeric state of Bcr-Abl is correlated to its activity, if the oligomerization is disrupted it will cause a reduction in Bcr-Abl activity (decrease in phosphorylation). After transfecting either the wild-type or mutant coiled-coil domain into K562 cells, western blotting with an antibody that specifically recognizes the phosphorylated form of Bcr-Abl was used to determine the activity. Under identical experimental conditions, the wild-type coiled-coil domain reduced the phosphorylation level to 35% (Figure 4A, last column). Further, the phosphorylation of two proteins known to be phosphorylated by Bcr-Abl, STAT5 and CrkL, was also tested. Again, the wild-type coiled-coil domain had minimal effect while the mutant coiled-coil domain reduced the phosphorylation of both proteins (Figure 4B, 3rd and 4th columns). The decreased phosphorylation of Bcr-Abl suggests that the mutant coiled-coil domain is capable of interacting with the endogenous Bcr-Abl, and not just the isolated coiled-coil domain as used in the previous NTA and two-hybrid experiments. Moreover, the decreased level of phosphorylation provides insight into the oligomeric disruption and inhibitory potential of the mutant coiled-coil domain.

Inhibition of Bcr-Abl through oligomeric disruption, as with inhibition through tyrosine kinase inhibitors, should relieve the upregulation of signaling pathways resulting in mis-regulated cell proliferation. The effect of the coiled-coil domains on cell proliferation was measured through cell counts with trypan blue exclusion. While the wild-type coiled-coil domain demonstrated a slight effect on the number of
proliferating cells, the mutant coiled-coil domain was most effective at decreasing the number of proliferating cells (Figure 5A). Furthermore, the effect on proliferation was measured via a colony forming assay, and again the mutant coiled-coil domain was found to cause the greatest reduction in cell proliferation (Figure 5B, column 2), similar to that seen with imatinib (Figure 5B, column 3).

As CML cells become dependent on the signaling pathways upregulated by Bcr-Abl, the inhibition of Bcr-Abl and reprieve of that signaling should also induce apoptosis. As one indication of the ability of the coiled-coil constructs to induce apoptosis, the activity of caspase-3/7 was measured in a fluorimetric assay. In a trend similar to that found in all previous experiments, the mutant coiled-coil domain again produced the greatest result, and was the only construct able to induce the activation of caspase activity at a statistically significant level (Figure 5C, 3rd column). Similar experiments with CCmut2 in cells that do not express Bcr-Abl (1471.1 and Cos-7), as expected, did not show an increase in caspase activity (data not shown).

Finally, as a measure of late stage apoptosis, DNA segmentation (of K562 nuclei) was measured (47,48). Cells transfected with CCmut2 revealed segmented nuclei, a hallmark of apoptosis as shown in Figure 6 (column 3, arrows). CC and control (EGFP) transfected cells had healthy (round) nuclei. The percentage of CCmut2 transfected cells demonstrating apoptosis was 29.4% compared to 4.29% for CC and 0.75% for EGFP control. Additionally, phase contrast images (Figure 6, column 1) demonstrated morphological changes indicative of apoptosis including zeotic blebbing, cell shrinkage, and cell fragmentation (43,48). Finally, in a control cell line (1471.1 cells) minimal DNA segmentation (less than 2%) was observed after EGFP, CC, or CCmut2 was transfected (data not shown). The inhibition of cell proliferation and the induction of apoptosis illustrate the therapeutic potential of oligomeric disruption through this modified coiled-coil.

DISCUSSION

Rational design, molecular modeling, MD simulation, and free energy analysis identified modifications to the Bcr-Abl coiled-coil to improve interaction with Bcr-Abl, while also reducing mutant homodimer (CCmut2:CCmut2) interactions. The optimal set of mutations served as the lead, reducing the need to test an overwhelming number of possible mutations or combination of mutations in vitro. The in vitro experiments performed with this construct confirmed the computational results and demonstrated that this designed mutant coiled-coil has an enhanced capability to oligomerize with Bcr-Abl. The design incorporated charge-charge repulsions between two mutant coiled-coil domains to reduce the homo-oligomerization, thereby making the mutant more available for interaction with the target, Bcr-Abl. Residues with the potential to form additional favorable electrostatic interactions with Bcr-Abl were also introduced to increase the binding affinity between the mutant and Bcr-Abl. Although further structural characterization could confirm that the hypothesized interactions are indeed occurring, both the computational modeling and in vitro experiments strongly indicate that the modifications to the coiled-coil domain lead to a more specific, better binding coiled-coil partner for Bcr-Abl.

Although an isolated coiled-coil domain from Bcr-Abl should, in principal, oligomerize with Bcr-Abl, the isolated coiled-coil domain also has the ability to form homo-oligomers. Given that an isolated coiled-coil domain is smaller, there is likely less entropic penalty for formation, and therefore it should be less effective as a therapeutic due to the decreased effective concentration of the monomer and need for dissociation of the dimer for activity. Our approach to a more potent therapeutic involved the design of a coiled-coil domain with reduced ability to self-oligomerize while also exhibiting enhanced oligomerization with the target. This goal was confirmed through both computational modeling and in vitro experiments. Alternative treatments for CML are still needed due to the inability to eliminate CML stem cells, resistance to small molecule inhibitors, and ineffective treatment of advanced stages of the disease (52-56). Given the importance the coiled-coil domain has in the regulation of Bcr-Abl activity, it has long been hypothesized that this domain could be used therapeutically. This modified Bcr-Abl coiled-coil domain has a heightened ability to inhibit the
oncogenicity of Bcr-Abl and warrants further exploration as an alternative approach to treat CML.

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Footnotes

5 The abbreviations used are: HIV-1 human immunodeficiency virus type 1; CC, wild-type coiled-coil domain from Bcr-Abl; CCmut1, mutant coiled-coil domain with S41R, L45D, E48R, V49D, and Q60E mutations; CCmut2, mutant coiled-coil domain with C38A, S41R, L45D, E48R, and Q60E mutations; CML, chronic myelogenous leukemia; PDB, protein data bank; AMBER, assisted model building with energy refinement; MD, molecular dynamics; CD, circular dichroism; RMSd, root-mean-squared deviation; MM-PBSA, molecular mechanics Poisson-Boltzmann/surface area; FBS, fetal bovine serum; NTA, nuclear translocation assay; CFA, colony forming assay; IM, imatinib mesylate; AMC-DEVD, 7-amino-4-methylcoumarin-aspartate-glutamate-valine-aspartate; PS, protein switch; * This work was supported in part by National Institutes of Health Grants CA129528 (to C.S.L.), GM079383 (to T.E.C., III), an American Foundation for Pharmaceutical Education (AFPE) Predoctoral fellowship (to A.S.D) and the ALSAM Foundation (to A.A.S). Computer time was supported by National Science Foundation TG-MCA01S027 (to T.E.C., III) and CHPC (University of Utah).

Figure Legends

Table 1. Overview of comparative helicity calculations for various mutations of the Bcr coiled-coil domain. Helicity of simulated coiled-coil dimers using the calculated circular dichroism of the peptide, the percent of the residue which is defined as α-helical according to Ψ and Φ backbone dihedral torsions (using the DSSP method), and the percent of α-helical specific hydrogen bonds (< 3.5 Å) formed between i and i+4 residues at ps intervals over the final 10 ns of MD relative to the total number of potential interactions (the total number of residues minus 4). Row #6 was the only set of mutations tested that indicated a more favorable homodimer than the heterodimer, and was termed CCmut1 and used in subsequent MM-PBSA experiments as a negative control. Row #8 contains the set of mutations found to exhibit the optimal reduced homo-oligomerization paired with improved hetero-oligomerization and was termed CCmut2 for subsequent MM-PBSA and thermodynamic integration studies, as well as the in vitro experiments.

Table 2. MM-PBSA and thermodynamic relative free energy of binding results (in kcal/mol). MM-PBSA results were found by subtracting the absolute free energies of the unbound monomers from the
calculated free energy of coiled-coil dimer using separate MD trajectories. Thermodynamic integration calculations followed the scheme described in supplemental data Figure S2 using intermediate coiled-coil dimers to build a consistent transition from the wild type coiled-coil dimer to the CCmut2 dimers. Results from both calculations were reported relative to the wild-type coiled-coil dimer (CC-CC).

**Figure 1:** Ribbon diagrams, with the corresponding helical wheel diagram below, of the wild-type homodimer (A), wild-type:CCmut2 heterodimer (B), and CCmut2:CCmut2 homodimer (C). Grey ribbon (ribbon diagrams) or dots (helical wheel diagrams) represents the wild-type coiled-coil domain and cyan represents CCmut2. The side chains of key residues (E34, K39, S/R41, L/D45, E46, E/R48, R53, R55, and Q/E60) are shown as red (acidic), blue (basic), green (hydrophobic), yellow (serine), or black (glutamine) spheres (ribbon diagrams) or font (helical wheel diagrams). Dotted lines indicate possible ionic interactions and solid lines indicate charge-charge repulsions. Ribbon diagrams were generated with UCSF Chimera starting with the Bcr coiled-coil domain crystal structure (PDB ID: 1K1F).

**Figure 2:** Circular dichroism wavelength scans and thermal denaturation. A) Alpha-helices exhibit a characteristic double absorption minimum at ≈208 nm and 222 nm. CC: green circles, CCmut2: blue squares, Mix: black triangles. The lines represent the average from 3 scans. B) Thermal denaturation curves in 1cm cuvette. Ratio of CC to CCmut2 used in mix was 1:1. CC: green circles, CCmut2: blue squares, Mix: black triangles. C) Thermal denaturation curves in mixing cell cuvette using 2.5 μM CC and 7.5 μM CCmut2. Separate (pre-mixing): green circles, Mix: blue squares.

**Figure 3:** Binding of the homo- and heterodimers tested through the nuclear translocation assay (NTA) and mammalian two-hybrid assay. A) Figure modified from Dixon and Lim (46). The NTA measures the ability of a nuclear-inducible protein switch (PS) fused to a protein of interest to cause a second interacting protein to translocate into the nucleus, and is monitored through fluorescence microscopy (fused to EGFP and DsRed respectively). The assay was performed in Cos-7 cells that do not contain Bcr-Abl. Each experiment was performed in triplicate with at least eight cells analyzed per experiment. Statistical significance determined using one-way ANOVA with Tukey’s post test. * p<0.01, **p<0.001 compared to control (pDsRed2-N1/EGFP-PS-CC, not included in graph); B) Mammalian two-hybrid assay in Cos-7 cells. Statistical significance determined using one-way ANOVA with Tukey’s post test. * p<0.01 compared to CC:CC interaction (n=5).

**Figure 4:** Representative images of western blots to detect the phosphorylated form of Bcr-Abl (A) and two substrates of Bcr-Abl, STAT5 and CrkL (B). The phosphorylation of Bcr-Abl is indicative of the tyrosine kinase activity, and is shown to be decreased by the addition of CCmut2 (percent p-Bcr-Abl from untreated K562 cells is indicated graphically). The proteins STAT5 and CrkL are also phosphorylated when Bcr-Abl is active, and are secondary indicators of the Bcr-Abl activity. Western blotting followed by densitometry was replicated 3 times on lysates from 3 separate transfections. The level of p-Bcr-Abl, as a percentage of the untreated cells, is shown graphically in A, and the level of p-STAT5 and p-CrkL (±st. dev.) is indicated above the representative images. Statistical significance was determined using one-way ANOVA with Tukey’s post test (n=3). *p<0.05, **p<0.01 compared to cells transfected with EGFP.

**Figure 5:** Inhibition of the Bcr-Abl through expression of CCmut2 results in decreased proliferation of K562 cells and activation of apoptosis. A) Proliferation of K562 cells as determined by cell counts with trypan blue exclusion. B) Proliferation of K562 cells as determined by colony forming assays; IM, Imatinib mesylate. C) Induction of apoptosis as measured through activation of caspase-3/7. For A-C, statistical significance was determined using one-way ANOVA with Tukey’s post test. * p<0.01, **p<0.001 compared to control (cells transfected with pEGFP-C1).

**Figure 6:** Fluorescence microscopy for morphological evaluation of nuclei. Cells transfected with
CCmut2 (bottom row) appear shrunken (column 1, arrows) or zotic (column 1, arrowheads) and exhibit segmented (punctuate) nuclei (column 3, arrows), hallmarks of late apoptotic cells. The far right column summarizes the percentage of transfected cells determined to have segmented DNA. Statistical significance was determined using one-way ANOVA with Tukey’s post test. *p<0.001 compared to control (cells transfected with pEGFP-C1).

Tables

Table 1:

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Table 2:

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Figures

Figure 1:

Figure 2:
Figure 3:

A

Nuclear Translocation Assay

Percent Nuclear Increase (EGFP/DsRed)

B

Two-Hybrid Assay

Relative Response Ratio

Figure 4:

A

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B

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