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ENHANCED AND SELECTIVE KILLING OF CHRONIC MYELOGENOUS LEUKEMIA CELLS WITH AN ENGINEERED BCR-ABL BINDING PROTEIN AND IMATINIB

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Abstract

The oncoprotein Bcr-Abl stimulates pro-survival pathways and suppresses apoptosis from its exclusively cytoplasmic locale, but when targeted to the mitochondrial compartment of leukemia cells, Bcr-Abl was potentially cytotoxic. Therefore, we designed a protein construct to act as a mitochondrial chaperone to move Bcr-Abl to the mitochondria. The chaperone (i.e., the 43.6 kDa intracellular cryptic escort (iCE)) contains an EGFP tag and two previously characterized motifs: 1) An optimized Bcr-Abl binding motif that interacts with the coiled-coil domain of Bcr (ccmut3; 72 residues), and 2) A cryptic mitochondrial targeting signal (cMTS; 51 residues) that selectively targets the mitochondria in oxidatively stressed cells (i.e., Bcr-Abl positive leukemic cells) via phosphorylation at a key residue (T193) by protein kinase C. While the iCE colocalized with Bcr-Abl it did not re-localize to the mitochondria. However, the iCE was selectively toxic to Bcr-Abl positive K562 cells as compared to Bcr-Abl negative Cos-7 fibroblasts and 1471.1 murine breast cancer cells. The toxicity of the iCE to leukemic cells was equivalent to 10 μ M imatinib at 48 hours and the iCE combined with imatinib potentiated cell death beyond imatinib or the iCE alone. Substitution of either the ccmut3 or the cMTS with another Bcr-Abl binding domain (derived from Ras/Rab interaction protein 1 (RIN1; 295 residues)) or MTS (i.e., the canonical IMS derived from Smac/Diablo; 49 residues) did not match the cytotoxicity of the iCE. Additionally, a phosphorylation null mutant of the iCE also abolished the killing effect. The mitochondrial toxicity of Bcr-Abl and the iCE in Bcr-Abl positive K562 leukemia cells was confirmed by flow cytometric analysis of 7-AAD, TUNEL, and annexin-V staining. DNA segmentation and cell viability were assessed by microscopy. Subcellular localization of constructs was determined using confocal microscopy (including statistical colocalization analysis). Overall, the iCE was highly active against K562 leukemia cells and the killing effect was dependent upon both the ccmut3 and functional cMTS domains.

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Disclosure

The authors declare that they have no competing interests.

Supporting Information Available

This information is available free of charge via the Internet at <http://pubs.acs.org/>.

Keywords

cryptic MTS; Bcr-Abl; Bcr-Abl binding; coiled-coil; molecular chaperone

Introduction

The fusion oncoprotein, and constitutively active tyrosine kinase, Bcr-Abl (autoinhibition of c-Abl kinase activity is lost in Bcr-Abl fusion)¹ is the central etiologic agent of chronic myelogenous leukemia (CML) and is exclusively localized in the cytoplasmic space at the plasma membrane primarily through interactions with cytoskeletal actin.¹ Directing a change in Bcr-Abl's subcellular location can change Bcr-Abl from an oncogenic agent into a pro-apoptotic factor.^{2, 3}

Normal c-Abl fulfills a terminal role as a pro-apoptotic factor when targeted to the mitochondria under a variety of cellular insults.^{4, 5} We previously demonstrated that direct targeting of c-Abl to the mitochondria is toxic to leukemia cells.⁶ Therefore, relocating Bcr-Abl to the mitochondria would mimic death-directed mitochondrial c-Abl function which is largely defunct in CML cells.^{7, 8} In this paper, as with c-Abl,⁶ the direct mitochondrial targeting of Bcr-Abl (by fusion to canonical MTSs targeting the mitochondrial matrix,⁹ inner mitochondrial membrane,¹⁰ and the intermitochondrial membrane space¹¹) was cytotoxic.

In light of this, we designed a small protein, the intracellular cryptic escort (iCE), for the purpose of capturing and translocating Bcr-Abl to the mitochondria. The Bcr-Abl capture motif employed for the iCE, was a previously optimized coiled-coil (i.e., the ccmut3)¹² which demonstrated both the ability to bind and, when fused to four nuclear localization signals (NLS), move endogenous Bcr-Abl to the nucleus.¹³ The ccmut3 oligomerizes with the coiled-coil domain of the Bcr portion of Bcr-Abl while possessing a reduced affinity for homodimer formation.¹² We paired the ccmut3 with a 'cryptic' mitochondrial targeting signal (cMTS) that is activated by phosphorylation (by PKA and/or PKC) in a reactive oxygen species dependent manner.⁶

Despite the lack of iCE/Bcr-Abl mitochondrial localization the iCE alone was selectively toxic to Bcr-Abl positive K562 cells to the same degree as 10 μ M imatinib at 48 hours. The killing capacity of the iCE was ablated by substitution of either the ccmut3 (with the Bcr-Abl binding domain of Ras and Rab interactor 1 (RIN1-BD)¹⁴) or the cMTS (with the canonical intermitochondrial membrane space targeting sequence (IMS) from Smac/Diablo¹¹) in two 'mock' iCEs, the RIN-cMTS or the IMS-ccmut3, respectively. The combination of the iCE with imatinib was the most potent inducer of leukemic cell death. This work demonstrates the selective killing of Bcr-Abl positive cells by a designed Bcr-Abl coiled-coil interacting protein where phosphorylation (via PKC and/or PKA) is coincident with the cell death effect.

Experimental Section

Subcloning and construction of plasmids

pEGFP-Bcr-Abl was constructed as previously described.³ Bcr-Abl DNA was also cloned into pmCherry-C1 (Clontech, Mountain View, CA, USA) and pTagBFP-C (Evrogen, Moscow, Russia) at the *EcoRI* site on both vectors creating pmCherry-Bcr-Abl and pBFP-Bcr-Abl, respectively. The pOTC-EGFP-Bcr-Abl was created using an oligonucleotide encoding the MTS from OTC (incorporating the Kozak sequence), 5'-CCGGTCGCCACCATGCTGTTTAATCTGAGGATCCTGTAAACAATGCAGCTTTTA

GAAATGGTCACAACCTTCATGGTTCGAAATTTTCGGTGTGGACAACCACTACAAA ATAAAGTGCA GCGA-3' which was annealed to its complementary strand and subsequently cloned into the *AgeI* site of EGFP-Bcr-Abl. The pIMS-EGFP-Bcr-Abl, pIMS-EGFP, and pIMS-EGFP-ccmut3 were made by annealing and ligating four oligonucleotides encoding the Kozak sequence and IMS signal (1: (5' phosphorylated) 5'-CCGGTGCCACCATGAGAAGCGTGTGCAGCCTGTTTCAGATACAGACAGAGATTCC CCGTGCTGGCCAACAGCAA-3', 2: 5'-GAAGAGATGCTTCAGCGAGCTGATCAAGCCCTGGCACAAGACCGTGCTGACCG GCTTCG GCATGACCCTGTGCGCCGTGCCCATCGGA-3', 3: 5'-TGCCACCATGAGAAGCGTGTGCAGCCTGTTTCAGATACAGACAGAGATTCCCCGT GCTGGCCAACAGCAAGAAGAG-3', 4: (5' phosphorylated) 5'-ATGCTTCAGCGAGCTGATCAAGCCCTGGCACAAGACCGTGCTGACCGGCTTCGG CATGACCCTGTGCGCCGTGCCCATCAGGACCGG-3') followed by insertion into the *AgeI* site of pEGFP-Bcr-Abl, pEGFP, or pEGFP-ccmut3,¹² respectively. The inner mitochondrial membrane targeting sequence (IMM) was incorporated into the pIMM-EGFP-Bcr-Abl and pIMM-EGFP by annealing the 5' phosphorylated oligonucleotide encoding the Kozak sequence and IMM signal, 5'-CCGGTCGCCACCATGTCCGTCCTGACGCCGCTGCTGCTGCGGGGCTTGACAGGC TCGCCCCGGCGGCTCCCAGTGCCGCGCGCCAAGATCCATTCGTTGA-3' with its reverse complement followed by ligation into the *AgeI* site of pEGFP-Bcr-Abl and pEGFP-C1, respectively. The kinase dead mutant of pIMM-EGFP-Bcr-Abl (i.e., pIMM-EGFP-Bcr-Abl-KD) was made using site directed mutagenesis with the primer 5'-CTGACGGTGGCCGTGGCGACCTTGAAGGAGGAC-3' and its reverse complement. The pRIN-cMTS construct was made by PCR amplifying the binding domain of the human RIN1 gene (NM_004292, OriGene, Rockville, MD, USA) with the primers 5'-GCGCGCGCGATCTATGGAAAGCCCTGGAGAGTCAGGCGCG-3' and 5'-GCGCGCGAATTCCTGACCCCACTGAGCTCTCCCTCCGTAGCAGCTGGC-3' and inserted into pEGFP-cMTS using the *BglII* and *EcoRI* sites. The murine glutathione S-transferase A4-4 [Swiss-Prot:P24472.3] cMTS (N-terminal residues, 172–222)¹⁵ was constructed by annealing four oligonucleotides encoding the cMTS (1: (5' phosphorylated) 5'-AATTCGCCCCCGTGCTGAGCGACTTCCCCCTGCTGCAGGCCTTCAAGACCAGA ATCAGC AACATCCCCACCATCAAGAAGTTCCTGCAGCCC-3', 2: 5'-CTGCCGGGCTGCAGGAACCTTCTTGATGGTGGGGATGTTGCTGATTCTGGTCTTGA AGGCCTGCAGCAGGGGGAAGTCGCTCAGCACGGGGGCGG-3', 3: 5'-GGCAGCCAGAGAAAGCCCCCCCCGACGGCCCCCTACGTGGAGGTGGTGAGAAC CGTGCTGAAGTTCGGCGCCGGCTGCTGCCCCGGCTGCTGCTGA-3', 4: (5' phosphorylated) 5'-AATTTAGCAGCAGCCGGGGCAGCAGCCGGCGCCGAACCTTCAGCACGGTTCTCA CCACCTCCACGTAGGGGGCGTGGGGGGGGCTTTCTCTGG-3') simultaneously and then inserting the annealed product into the multiple cloning site (MCS) of EGFP-C1 vector (Promega Biotech, Madison, WI), with or without the ccmut3 sequence present, at the *EcoRI* site creating pEGFP-cMTS⁶ (cMTS) and pEGFP-ccmut3-cMTS (iCE), respectively. The pEGFP-ccmut3-cMTS null (S189A and T193A) was created using site-directed mutagenesis using primers, 5'-GACCAGAATCGCCAACATCCCCGCCATCAAGAAGTTCCTGCAGCCCGGCAGCCA GAGAA-3' and its reverse complement. All constructs were verified by sequence analysis.

Materials

RPMI-1640 medium, MitoTracker Red CM-H₂XRos (MitoTracker CMXros), Hoechst 33342 (cell permeable nuclear stain), 7-aminoactinomycin D (7-AAD; DNA intercalating dye permeable to dying or dead cells), annexin-APC (annexin-V conjugated to

allophycocyanin), staurosporine, Lipofectamine LTX with Plus Reagent, trypan blue 0.4%, phosphate-buffered saline (PBS), fetal bovine serum (FBS), and gentamycin were purchased from Invitrogen (Carlsbad, CA). Penicillin-streptomycin-L-glutamine (P-S-G; 100U/mL), DMEM media, and trypsin were purchased from Gibco BRL (Grand Island, NY). The poly-L-lysine (0.01% solution) was purchased from Sigma-Aldrich (St. Louis, MO). Imatinib (CT-IM001) was purchased from ChemieTek (Indianapolis, IN). QuikChange II XL Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (Santa Clara, CA). Cell Line Nucleofector Kit V was purchased from Lonza Group (Basel, Switzerland). Restriction enzymes (*EcoRI*, *AgeI*, and *BglII*) were purchased from New England Biolabs (Ipswich, MA).

Cell lines and culture conditions

As previously described,⁶ K562 cells (non-adherent human chronic myelogenous leukemia cell line), gift from Dr. K. Elenitoba-Johnson, University of Michigan, and Cos-7 (monkey kidney fibroblast adherent cell line; ATCC) were cultured in RPMI 1640 supplemented with 10% FBS, 1% P-S-G, and 0.1% gentamycin. Murine mammary adenocarcinoma 1471.1 cells, (gift from Gordon Hager, PhD, NCI, NIH) were grown as monolayers in DMEM supplemented with 10% FBS, 1% P-S-G and 0.1% gentamycin. K562 cells were passaged at a density of 0.5×10^5 /mL every other day, for ten passages. Cos-7 and 1471.1 cells were passaged at 80% confluency and split 1:10 in fresh media and discontinued after passage 15. All cells were maintained in a 5% CO₂ incubator at 37°C.

Expression of constructs in K562 leukemia, Cos-7 fibroblast, and 1471.1 breast cancer cells

Constructs were transiently transfected into K562 cells using the Amaxa Nucleofector II, as described previously.³ Briefly, 1×10^6 K562 cells, (initially seeded at a density of 0.5×10^5 cells/mL) between passages 5 to 10, were pelleted and resuspended in 100µL Amaxa Solution V, combined with 2µg of DNA and transfected in an Amaxa cuvette under program T-013. Transfected cells were immediately transferred to a 25cm² flask with 7mL of pre-warmed complete RPMI. Transient transfection of 1471.1 and Cos-7 were carried out in two-well live-cell chambers (Lab-Tek chamber slide system, Nalge NUNC International, Naperville, IL) or sterile 6-well tissue culture plates (Greiner CellStar, Greiner Bio-one GmbH) using Lipofectamine LTX as per manufacturers' instructions between passages 3 and 15 in antibiotic free media.

Cell proliferation

Trypan blue exclusion was used to determine cell proliferation (cell viability)¹⁶ in K562 cells 48 hours post-transfection of EGFP-C1, cMTS, ccmut3, cMTS, and iCE, with and without the presence of imatinib (10µM).

Western blotting

As previously described,¹⁷ cell lysates were prepared in lysis buffer (62.5mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50mM DTT, 0.01% w/v bromophenol blue) followed by standard blotting using antibodies to detect p-Bcr-Abl, p-Crk-L, p-STAT5, and eIF4E as the protein loading control. Primary antibody labels (anti-pAbl (Y245), anti-pCrk-L (Y207), and anti-eIF4E, Cell Signaling Technology; anti-pSTAT5 (Y694), Abcam) were detected with (#7074, Cell Signaling Technology) secondary antibody prior to the addition of ChemiGlo (AlphaInnotech, Cell Biosciences, Santa Clara, CA, USA) chemiluminescent substrate and detection using a FluorChem FC2 imager (AlphaInnotech).

Mitochondrial staining

As previously described,⁶ aliquots of transfected K562 suspension cells (400 μ L) were plated into poly-L-lysine coated 4-well live-cell chambers at least four hours in advance of microscopy. Cells were incubated with MitoTracker Red CM-H₂XRos (K562; 100nM, Cos-7 and 1471.1; 325nM) for 45 minutes at 37°C and protected from light prior to imaging.

Microscopy

Fluorescent images of K562, Cos-7, and 1471.1 live cells were acquired on an Olympus IX81 FV1000-XY spectral confocal microscope (Imaging Core Facility, University of Utah) equipped with 405 nm diode, 488 nm argon, and 543 nm HeNe lasers using a 60X PlanApo oil immersion objective (NA 1.45) using Olympus FluoView software, as previously described.⁶ Excitation and emission filters were as follows: EGFP, 488 nm excitation, emission filter 500–530 nm; MitoTracker Red CM-H₂XRos, 543 nm excitation, emission filter 555–655 nm. Images were collected in sequential line mode with exposure and gain of laser kept constant and below detected pixel saturation for each group of cells. No channel crosstalk was observed. Pixel resolution was kept at 1024 $\times$ 1024 (0–2.5-fold digital zoom) with a pixel dwell time of 12.5 μ s. Imaging of K562 cells for the analysis of DNA segmentation was acquired on an Olympus IX71F fluorescence microscope (Scientific Instrument Company, Aurora, CO) with a 60X PlanApo oil immersion objective (NA 1.4) on an F-view monochrome CCD camera. K562 cells were stained with the nuclear dye Hoechst 33342 (Invitrogen, Carlsbad, CA) at a concentration of 4 μ M.

Image analysis

Images were analyzed as previously described.⁶ Briefly, original images were converted to 8-bit, then stacked as separate channels, and corrected for background noise using ImageJ plugin 'BG subtraction from background' in default mode (i.e., mean background intensity outside of cells was subtracted).¹⁸ Image and statistical analysis was performed with JACoP plugin in ImageJ.¹⁹ Pearson's correlation coefficient (PCC) was generated using Costes' automatic threshold algorithm.^{20, 21} The PCC is dependent upon both the pixel intensity and overlap of signal and has a range of +1 (complete colocalization) to -1 (anti-correlation) with zero correlating with random distribution between comparators.¹⁹ The PCC threshold for defining colocalization (i.e., colocalization due to co-compartmentalization) is 0.6 as per Bolte and Cordelières.¹⁹ Channels have been false-colored (cyan and magenta) using ImageJ LUT for increased visual clarity. Additionally, spatial representation of intensity correlation was included using the Colocalization Colormap ImageJ plugin. 'Colormap' displays positively correlated pixels in hot colors and negatively correlated pixels in cold colors that can be visually interpreted using the color scale bar.²² Identification of segmented nuclei was performed using the nuclear dye H33342 on K562 cells displaying green fluorescence 24 hours post transfection.

7-AAD assay

Flow cytometric assay of cell death was done as previously described.¹² Briefly, K562, Cos-7, or 1471.1 cells were resuspended in 500 μ L ice cold PBS containing 1 μ M 7-aminoactinomycin D (7-AAD) for 30 minutes prior to analysis. Cells that have compromised membrane integrity (late apoptosis/necrosis) are permeable to 7-AAD.²³ Media from adherent Cos-7 and 1471.1 cells was collected prior to trypsinization of cell monolayer and recombined with the enzymatically released cell population for centrifugation and subsequent resuspension. Analysis and gating was performed on a BD FACSCanto II (Flow Cytometry Core Facility, University of Utah) using BD FACSDiva software (BD Biosciences, Franklin Lakes, NJ). At least three separate experiments (n = 3) in duplicate were performed. Compensation controls were included with each experiment.

TUNEL assay

As previously described,²⁴ detection of DNA strand breaks in the K562 cell line was performed using the In Situ Death Detection Kit, TMR red (Roche, Mannheim, Germany) as per the manufacturers' protocol. Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) detects cells that have extensive DNA degradation in late stage apoptosis/necrosis.²⁵ Samples were run on a Becton-Dickinson FACS Aria-II (BD-BioSciences, University of Utah Core Facility) using the 488 nm (for EGFP) and 563 nm (TMR red) laser lines with FACSDiva software. Analysis was performed on EGFP positive cells using preset gates. The TMR red positive cells were detected in the PE (phycoerythrin) channel. Each construct was assayed in at least triplicate (n = 3).

Annexin-V assay

Annexin-V binding was assessed, as previously described,²⁴ 48 hours post-transfection in K562 cells. Externalized phosphatidylserine in the plasma membrane is indicative of early apoptosis and is bound specifically by annexin V.²⁶ K562 cells were suspended in 100 μ l Annexin binding buffer (Invitrogen) and incubated with 5 μ l Annexin-V conjugated to allophycocyanin (Annexin-APC, Invitrogen) for 10 minutes. The incubated cells were then diluted in 400 μ l Annexin binding buffer and analyzed using the FACSCanto-II (BD-BioSciences, University of Utah Core Facility) with FACSDiva software. EGFP (ex. 488 nm and em. 507 nm) and APC (ex. 635 nm and em. 660 nm) fluorescence was collected. Analysis was conducted on EGFP-positive cells using preset EGFP gates. Each construct was tested in triplicate (n=3).

Statistics

Data are shown as mean $\pm$ S.E.M., with one-way ANOVA using Tukey's post-hoc test or two-way ANOVA using Bonferroni post-test (as indicated in figure legends) to compare measurements between experimental data with an N of 3 or greater. Statistical significance was set at $p < 0.05$ (by convention $p < 0.05$ is represented with *; $p < 0.01$ with **; $p < 0.001$ with ***). GraphPad Prism Graph 4 (GraphPad, La Jolla, CA) software was used for generating statistics.

Results

Targeting Bcr-Abl to the mitochondria induces cell death in K562 leukemia cells

Cell death evaluated by flow cytometric analysis of 7-AAD (DNA accessibility) and annexin-APC (phosphatidylserine externalization) staining (fig. 1) showed the toxic consequences of mitochondrially targeted Bcr-Abl (fig. 1A, 3rd column) at 24 hours post-transfection in K562 cells. DNA segmentation analysis of mitochondrially targeted Bcr-Abl also revealed the same pattern (data not shown). The IMM-Bcr-Abl was created by fusing Bcr-Abl to a canonical mitochondrial targeting signal (MTS; derived from cytochrome c oxidase subunit VIII) that is widely used to direct proteins to the mitochondria and the inner mitochondrial membrane in particular (IMM; see table 1).¹⁰ Interestingly, the kinase dead version (i.e., mutation of a single critical lysine ablates the capacity for kinase activity) of mitochondrially targeted Bcr-Abl (IMM-Bcr-Abl-KD; see table 1) yields no statistical difference in cell death to that of IMM-Bcr-Abl (compare fig. 1, 3rd and 4th columns). Furthermore, mitochondrially targeted Bcr-Abl and Bcr-Abl-KD (fig. 1, 3rd and 4th columns) significantly induced more cell death as compared to the gold standard of current CML therapy, imatinib (IM; 10 μ M) (fig. 1B, 2nd column) at 24 hours.

In addition to the IMM, Bcr-Abl was also targeted to the mitochondrial matrix (OTC-Bcr-Abl) and IMS (IMS-Bcr-Abl) (table 1). Figure 2A shows representative images of E-Bcr-Abl or mitochondrially targeted Bcr-Abl in K562 cells compared to MitoTracker CMXros

(MitoTracker) dye. The E-Bcr-Abl construct was not associated with the mitochondria (fig. 2A, compare 1st column with 2nd 'MitoTracker' column) but each of the mitochondrially targeted Bcr-Abl constructs did localize (fig. 2A, 2nd through 4th rows) to the mitochondria. The corresponding EGFP-only constructs (i.e., IMM-EGFP, IMS-EGFP, and OTC-EGFP) were also tested and exhibited mitochondrial localization and low-toxicity. Therefore, only data on the IMM-EGFP is shown (fig. 1, IMM-EGFP). We further characterized the effects of targeting Bcr-Abl to the mitochondrial matrix with the OTC MTS in Cos-7 and 1471.1 cells (supplementary fig. S1).

In K562 cells, the OTC and the IMM fused Bcr-Abl constructs were significantly more toxic (7-AAD positive) than the non-targeted E-Bcr-Abl (fig. 2B, compare 4th and 5th columns to 2nd column) at 48 hours post-transfection. Yet, there was no difference in toxicity between the individual MTS-Bcr-Abl constructs (fig. 2B, compare 3rd, 4th, and 5th columns).

The iCE exhibits selective toxicity to Bcr-Abl positive K562 cells 48 hours post-transfection

Since Bcr-Abl targeted to the mitochondria is toxic (fig. 1, 3rd column), we designed a bimodal construct (intracellular cryptic escort (iCE)) using a previously characterized cMTS⁶ and our optimized Bcr-Abl binding domain, ccmut3¹² (see fig. 3 for description of constructs). The iCE and its component parts (i.e., EGFP, ccmut3, and cMTS; fig. 3) were transfected into Bcr-Abl positive K562 and Bcr-Abl negative Cos-7 and 1471.1 cell lines. The cell death profiles, as measured by flow cytometric analysis of 7-AAD staining, demonstrated a cell type-dependent response to the constructs. The iCE was toxic only in the K562 cell line (fig. 4A, K562, iCE column) as was imatinib (fig. 4A, K562, IM column). There was no significant difference in Cos-7 or 1471.1 between treatment with imatinib or the constructs individually with the exception of 1471.1 cells with the iCE combined with imatinib (fig. 4C, 1471.1, iCE+IM column). This was not evidenced in Cos-7 where combining imatinib and the iCE were not toxic (fig. 4B, Cos-7, iCE+IM column). In contrast, within K562 cells imatinib and the iCE were not different from one another (fig. 4A, K562, compare iCE to IM columns) but both were different from the EGFP control and the iCE components (fig. 4A, K562, EGFP, ccmut3, and cMTS columns). The iCE when combined with imatinib (fig. 4A, K562, iCE+IM column) had the greatest killing effect.

Figure 5A includes the components comprising the iCE (i.e., EGFP, ccmut3, and cMTS) combined with imatinib. There was no difference between imatinib alone and the individual components of the iCE combined with imatinib (fig. 5A, compare IM with EGFP+IM, ccmut3+IM, and cMTS+IM). The cMTS alone was not different from imatinib, however when compared to the iCE alone (fig. 5A, compare cMTS to iCE) the difference was extremely significant ($P < 0.001$). Overall, the iCE+IM was significantly different ($P < 0.001$) in its killing potential when compared to all constructs regardless of imatinib treatment (fig. 5A, compare iCE+IM to EGFP+IM, ccmut3+IM, and cMTS+IM).

Representative histograms (fig. 5B) from a set of K562 cells transfected and/or treated (imatinib (10 μ M) or positive control staurosporine (1 μ M)) samples at 48 hours demonstrate the difference in dead cells when the iCE and imatinib are combined (fig. 5B, compare staurosporine or imatinib to iCE+imatinib). The vertical line within the plot is the gate for 7-AAD positive cells for which the percent is listed in the shaded box. As expected, a similar cell death pattern was seen using a different cell permeable nuclear stain (H33342) to identify nuclear segmentation by microscopy³ (fig. 5C, compare imatinib only (top right set) to iCE + imatinib (lower right set)). The relative health of the cells was revealed by phase contrast as well, where the cells treated with imatinib and the iCE evidence the sequelae of apoptosis/necrosis (e.g. cell shrinkage/swelling and membrane blebbing)³ in comparison to the untreated control (e.g., round cells with intact membranes) (fig. 5C, compare 'Phase' of

iCE, 'no treatment' and 'imatinib' to control, 'no treatment'). The combined treatment of imatinib and the iCE increases DNA segmentation (fig. 5C, compare the stained nuclei of the iCE, 'no treatment' and 'imatinib' to iCE, with 'imatinib'). The peak time for iCE+IM killing of K562 is 48 hours (fig. 5D) whereas it is later for imatinib alone (10 μ M imatinib kills most K562 cells by approximately 72 to 96 hours, our unpublished observations).

Cell viability decreases and apoptosis increases when the iCE is combined with imatinib

Trypan blue exclusion (cell viability), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and phosphatidylserine (PS) externalization (annexin-V binding) demonstrated the antileukemic activity of the iCE in K562 cells (figs. 6A, 6B, and 6C, respectively).

Cell viability was significantly decreased for the iCE compared to EGFP and ccmut3 but not the cMTS (fig. 6A, compare 'no treatment', iCE to EGFP and ccmut3 data points).

However, the decline in cell viability with imatinib present (fig. 6A, 'IM') was extremely significant for the iCE when compared to the other component constructs. The cell viability assessment was completed 48 hours post-transfection but 24 hours post-addition of imatinib (IM). Reducing the incubation time, for this assay, with imatinib to 24 instead of 48 hours allowed better discernment of viable cells (e.g., fig. 5C, bottom row, third column, 'Phase' for iCE + imatinib demonstrates the lack of cells with intact plasma membranes). The level of apoptosis as determined by TUNEL and annexin-APC staining (in contrast to 7-AAD staining which detects late-stage necrotic/apoptotic cells)²⁸ was extremely significant for the combined iCE+IM when compared to IM treated K562 cells (fig. 6B, iCE+IM vs. IM; or fig. 6C compare iCE+IM to ccmut3+IM and cMTS+IM). Both assays were completed 48 hours post-transfection and/or imatinib (10 μ M) treatment and analyzed by flow cytometry.

The iCE colocalizes with Bcr-Abl

Figure 7A shows the cellular localization of the EGFP-tagged iCE or its components (see fig. 3 for domain arrangement of constructs) co-expressed with exogenous Bcr-Abl (either labeled with blue or mCherry fluorescent proteins) in Cos-7 cells. The iCE, like the ccmut3 alone, colocalizes (defined as a Pearson correlation coefficient (PCC) greater than 0.6 as established by Bolte and Cordelières)¹⁹ with Bcr-Abl (Cos-7: fig. 7A, 4th row; PCC=0.63 $\pm$ 0.04 and 1471.1: fig. 7B; PCC=0.83 $\pm$ 0.05). The colocalized iCE/Bcr-Abl exhibits a punctate pattern in both Cos-7 and 1471.1 cells which was different from the typical diffuse pattern of colocalized ccmut3/Bcr-Abl (fig. 7A, compare 2nd row, 1st column (*Bcr-Abl with ccmut3 present*) to 4th row, 1st column (*Bcr-Abl with iCE present*)). The cMTS and EGFP, as expected, do not colocalize with Bcr-Abl in Cos-7 cells (Cos-7: fig. 7A, 3rd row, PCC=0.3 $\pm$ 0.1, for cMTS and Bcr-Abl and fig. 7A, 1st row, PCC=0.02 $\pm$ 0.03, for EGFP and Bcr-Abl). The cMTS alone localized to the mitochondria (fig. 7C, cMTS in K562 (top) and 1471.1 (bottom) compared to MitoTracker) in K562 and 1471.1 cells with higher oxidative backgrounds but not in the 'low ROS' Cos-7 (fig. 7A, 3rd row from top).⁶

The iCE did not associate with the mitochondria

Unlike the cMTS alone which colocalized with the mitochondria in both K562 and 1471.1 cells⁶ (fig. 7C) the iCE remained cytoplasmic in its distribution across all three cell types (fig. 8, compare 'iCE' column to 'MitoTracker' column, 1st through 3rd rows). In K562 cells the ccmut3 alone colocalized with Bcr-Abl and the cMTS alone colocalized with the mitochondria (fig. 7C, top), but the iCE did not localize to the mitochondria (fig. 8, 1st row).

The difference between the iCE and the iCE Null are the S189A and T193A mutations preventing phosphorylation at these sites in the cMTS domain of the iCE Null (fig. 3). Incorporating S189A and T193A mutations (i.e., iCE-Null, see fig. 3) led to a qualitative

distribution difference between the iCE (punctate looking) and the diffuse iCE Null (fig. 8, compare 'iCE' in the 1st row with 'iCE Null' in the bottom row).

The toxic effect of the iCE on K562 cells is lost upon substitution of the cMTS or the ccmut3 with another canonical MTS or Bcr-Abl binding domain

When the ccmut3 is substituted for another Bcr-Abl binding domain (i.e., RIN-BD)¹⁴ to create a mock iCE (fig. 3, RIN-cMTS), the cytotoxic effect remains equivalent to the cMTS alone, and the concomitant use of imatinib does not potentiate toxicity beyond that of imatinib alone (fig. 9A, RIN-cMTS and RIN-cMTS+IM columns). Furthermore, substituting the cMTS with a canonical IMS MTS,¹¹ (fig. 3, IMS-ccmut3) diminished toxicity to that of the ccmut3 alone, or upon the addition of imatinib, no more toxicity than imatinib alone (fig. 9A, IMS-ccmut3 and IMS-ccmut3+IM columns). The mock iCEs localize to different subcellular compartments (fig. 9B, compare top (IMS-ccmut3) and bottom (RIN-cMTS) rows). The IMS-ccmut3 localizes to the mitochondria (fig. 9B, top row, compare 'IMS-ccmut3' to 'MitoTracker' columns) while the RIN-cMTS remains cytoplasmic (fig. 9B, bottom row, compare 'RIN-cMTS' to 'MitoTracker' column). The cellular distribution of IMS-ccmut3 (mitochondrial) and RIN-cMTS (cytoplasmic) remained the same in the presence of 10 μ M imatinib (data not shown).

The toxic effect of the iCE is dependent upon the key phospho-residues in the cMTS domain

Incorporating S189A and T193A mutations (i.e., iCE-Null, *see* fig. 3) into the cMTS domain of the iCE leads to a significant reduction in cell death (i.e., 7-AAD) and apoptosis (e.g., annexin-APC) in K562 cells at 48 hours (figs. 10A and C, compare iCE to iCE Null columns). However, this effect is more pronounced in combination with imatinib (figs. 10A and C, compare iCE+IM to iCE Null+IM columns). The trend is similar for TUNEL staining but did not reach statistical significance (fig. 10B).

Discussion

The ubiquitously expressed tyrosine kinase c-Abl has a pro-apoptotic function at the mitochondria and we have previously demonstrated that direct targeting of c-Abl to the mitochondria induces K562 leukemia cell death.⁶ Based on this we have targeted the constitutively active and oncogenic form of c-Abl (i.e., Bcr-Abl) to the mitochondria. Bcr-Abl is the causative agent for the vast majority of CML cases, and was directed to the mitochondria as a proof of concept that mitochondrial Bcr-Abl could be effectively be used to destroy diseased cells. Yet, the mitochondrial substrates(s) and submitochondrial localization of the pro-apoptotic c-Abl are not known.^{5, 6} Therefore, in order to investigate the potential antileukemic activity of a 'surrogate death-directed c-Abl,' in the form of mitochondrial Bcr-Abl, we targeted exogenous Bcr-Abl by fusion with different MTSs to three submitochondrial regions (table 1). Similar to mitochondrial c-Abl, Bcr-Abl was toxic when targeted to the mitochondria of CML cells (fig. 1, 3rd column). Unlike c-Abl,²⁹ Bcr-Abl's mitochondrial cytotoxic effect was independent of its kinase activity (fig. 1, 4th column). However, there are instances where the kinase activity of c-Abl is dispensable for the induction of apoptosis (e.g., via p38 MAPK).³⁰ Overall, the tyrosine kinase-independent killing of CML cells is compelling because it suggests that the strategy of targeting Bcr-Abl to the mitochondria would be compatible with and complementary to current tyrosine kinase inhibitor (TKI) therapy.

This led to an attempt to move endogenous Bcr-Abl by means of a designed chaperone protein (fig. 3, schematic of constructs) to exploit the toxicity of mitochondrial Bcr-Abl. The intracellular cryptic escort (iCE) was constructed for the purpose of binding and

translocating endogenous Bcr-Abl to the mitochondria. Both the ccm3 and cMTS domains which comprise the iCE have been previously characterized.^{6, 12}

The activation and mitochondrial targeting of the cMTS is limited to cell types with an elevated ROS phenotype.⁶ K562 cells exhibit high basal levels of ROS which is a common pathophysiological feature of malignancy.^{31, 32} The elevated oxidative stress level leads to an increase in PKA and PKC activity which, in turn, phosphorylate (residues S189 (PKA) and/or T193 (PKC)) and thereby 'activate' the cMTS to induce mitochondrial translocation in a Hsp70 dependent manner.^{6, 15} Moreover, the cMTS demonstrated robust and selective mitochondrial targeting when fused to c-Abl (i.e., Abl-cMTS) in K562 cells.⁶

The ccm3 Bcr-Abl binding protein oligomerizes in an antiparallel orientation with the coiled-coil domain of Bcr-Abl.¹² Dixon et al. demonstrated that the ccm3 efficiently bound to and translocated Bcr-Abl to the nucleus when fused to four strong nuclear localization signals (NLS).¹³ However, cell death from nuclear entrapment or nuclear targeting of Bcr-Abl is less when compared to mitochondrially targeted Bcr-Abl. For instance, K562 cell death assessed by nuclear segmentation analysis 24 hours post-transfection with 4NLS-Bcr-Abl or IMM-Bcr-Abl yielded a mean of 12%³ versus 88%, respectively (data not shown).

Consistent with the cytotoxicity (7-AAD staining) elicited by the direct mitochondrial targeting of Bcr-Abl at 48 hours (fig. 2B, 3rd – 5th columns), the iCE also induced cell death (fig. 4A, iCE column) to a similar level within the same timeframe. However, subcellular compartmental analysis of confocal images comparing between the mitochondria (stained with MitoTracker) and fluorescent protein tagged iCE and/or Bcr-Abl did not indicate any mitochondrial accumulation of the iCE or iCE/Bcr-Abl (fig. 9, compare iCE column to MitoTracker column, K562 cells). Therefore, the cytoplasmically localized iCE and the mitochondrially targeted Bcr-Abl (MTS-Bcr-Abl) are likely inducing cell death by different mechanisms.

Our experience with NLSs (e.g., 1 NLS was insufficient to move Bcr-Abl but four NLSs were)³ suggested that perhaps the 'strength' of the cMTS (within the iCE) was not sufficient to overcome the cytoplasmic interactions of Bcr-Abl for efficient translocation to the mitochondria. Anticipating that this could happen, we employed imatinib to better facilitate iCE-to-mitochondrial targeting for two reasons. First, imatinib treatment increases the level of ROS/PKC activity which in turn stimulates increased mitochondrial localization of the cMTS.⁶ Secondly, imatinib bound Bcr-Abl undergoes a conformational change that decreases its association with actin freeing Bcr-Abl for relocalization.³³ Yet, even with the addition of 10 μ M imatinib the iCE did not localize to the mitochondria in K562 cells.

Moreover, in light of the subcellular distribution of the iCE in Bcr-Abl negative cell types, the iCE was likely not hindered from translocating to the mitochondria due to its interaction with Bcr-Abl. Within the context of the Cos-7 cells (which have low inherent ROS and therefore low mitochondrial accumulation of the cMTS)⁶, the iCE was expected to remain cytoplasmic. However, in the Bcr-Abl negative and high ROS 1471.1 cell line the cMTS alone strongly localizes to the mitochondria.⁶ Accordingly, without Bcr-Abl to 'keep' the iCE cytoplasmic the iCE should localize to the mitochondria in this cell line. This was not the case however, and the iCE did not localize to the mitochondria of 1471.1 cells (fig. 8, 3rd row). This result revealed that the ccm3 fusion to the cMTS may compromise the capacity for iCE mitochondrial translocation.

Nonetheless, the antileukemic activity caused by the iCE appears to be entirely dependent upon the combination of the ccm3 and cMTS. Mock 'iCE's' substituting either the ccm3 with RIN-BD (RIN-cMTS; RIN-BD binds SH3/SH2 domains of Bcr-Abl)¹⁴ or the cMTS

with the canonical IMS MTS (IMS-ccmut3; IMS targets the intermitochondrial membrane space)¹¹ failed to restore cell death beyond control levels (fig. 9A). The RIN-cMTS and IMS-ccmut3 results suggest that the toxicity of the iCE is not propagated by either ccmut3 localization to the mitochondria (fig. 9B, top row) or the cMTS remaining bound to Bcr-Abl (at least to the SH2/SH3 domains via RIN-BD, which would put the cMTS domain of the iCE on Bcr-Abl but at a different location) in the cytoplasm (fig. 9B, bottom row). The cytotoxic effect of the iCE is also dependent upon phosphorylation of the cMTS. Mutating the cMTS domain key phospho-residues S189A and T193A (iCE Null; fig. 3) which are required for mitochondrial translocation of the cMTS alone⁶, ablates the killing activity of the iCE.

The ccmut3 alone and imatinib, as expected,¹² both reduced the level of phospho-Bcr-Abl and Bcr-Abl substrate phosphorylation (i.e., p-Stat5 and p-Crk-L), but the iCE and iCE Null were unchanged from control in K562 cells (western blot; data not shown). Therefore, the iCE is not acting as a dominant-negative for Bcr-Abl kinase activity. Yet since the iCE colocalizes with Bcr-Abl it may be disrupting non-kinase Bcr-Abl survival/antiapoptotic function. For instance, the iCE may hinder (e.g., steric interference) kinase-independent signaling from Bcr-Abl such as Src kinase Hck phosphorylation of the Grb2 binding site on Bcr-Abl or Bcr's RhoGEF activity.^{34, 35}

Alternatively, it may be possible that the ccmut3 (binding in an anti-parallel coiled-coil)¹⁷ positions the cMTS where the phosphorylation of the S189 and/or T193 is central to the mechanism for cell death induction. In K562 cells only phosphorylation of the T193 is critical for mitochondrial translocation function of the cMTS whereas S189 is not.⁶ Perhaps the cytotoxic nature of the iCE is also primarily a function of PKC phosphorylation. Although beyond the scope of this paper, a negative feedback coupling of PKC and Bcr-Abl involving transient calcium influx may play a role in the essential nature of cMTS phospho-residues, and T193 in particular. Increased transient calcium influx can stimulate cellular proliferation while conversely, blocking calcium influx via PKC activation is toxic to Ph⁺ leukemia cells.³⁶ Inhibition of Bcr-Abl activity with imatinib relieves negative regulation of PKC and thereby diminishes intracellular calcium flux.³⁶ Perhaps increased stimulation or altered localization of PKC (due to the presence of the iCE) could further diminish transient calcium influx. A fascinating possibility that connects calcium homeostasis and iCE toxicity is depicted in fig. 11.

In this report, we demonstrate that direct targeting of Bcr-Abl to the mitochondria elicits cell death induction in a cell type specific and kinase-independent manner. Additionally, we attempted to harness Bcr-Abl's mitochondrial death induction activity with the use of a designed protein chaperone (i.e., iCE) in order to restore the defunct apoptotic avenue of 'mitochondrial death-directed c-Abl' in CML cells. Though the iCE bound to Bcr-Abl, the iCE/Bcr-Abl complex did not translocate to the mitochondria. However, the iCE combined with imatinib treatment was potentially antileukemic cells as measured by 7-AAD (late apoptosis/necrosis), TUNEL (apoptosis/necrosis), annexin-V (apoptosis) and trypan blue exclusion assay (cell viability).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Direct targeting of Bcr-Abl to the mitochondria causes leukemic cell death. Flow cytometric analysis of EGFP positive K562 cells 24 hours post-transfection stained with 7-AAD and annexin-APC. Both IMM-Bcr-Abl and the kinase dead version (IMM-Bcr-Abl-KD) displayed higher cell death than imatinib or mitochondrially targeted EGFP (IMM-EGFP). Statistical differences were determined using a one-way ANOVA with Tukey's post-hoc test (error bars are $\pm$ S.E.M., N = 4).

A.

B.

Figure 2.

Submitochondrial targeting of Bcr-Abl in K562 leukemia cells. A) Representative images of E-Bcr-Abl or Bcr-Abl fused to a canonical MTS in K562 cells and compared to MitoTracker CMXros staining. Each of the MTS-Bcr-Abl constructs localize to the mitochondria while the non-targeted E-Bcr-Abl remains cytosolic. Channel one (EGFP) and channel two (MitoTracker) have been false-colored, cyan and magenta. Colocalized cyan and magenta pixels show as white in merged ('composite' column) images. The 'Colormap' (*far right column*) is a visual representation of pixel correlation both in space and intensity with positive correlation shown as hot colors and negative correlation in cooler colors and can be interpreted using the color scale bar (shown at bottom of the 'Colormap' column). Scale bars

are 5 μ m. B) Cell death as determined by 7-AAD staining in K562 cells 48 hours post transfection. Statistical differences were determined using a one-way ANOVA with Tukey's post-hoc test (error bars are S.E.M., N = 3).

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Construct composition	Name	Description
<div>EGFPccmut3cMTS</div>	ICE	Protein designed to bind to and move Bcr-Abl to the mitochondria
<div>EGFPccmut3</div>	ccmut3	Optimized Bcr-Abl coiled-coil binding domain
<div>EGFPcMTS</div>	cMTS	MTS that is activated upon phosphorylation
<div>EGFPccmut3cMTS Null</div>	ICE null	Mutant eliminating the key 'activating' phospho-residues in the cMTS (S189A and T193A)
<div>IMSEGFPccmut3</div>	IMS-ccmut3	Mock iCE where the cMTS is substituted for the canonical IMS MTS
<div>EGFPRIN-BDcMTS</div>	RIN-cMTS	Mock iCE with the ccmut3 substituted for the RIN1 Bcr-Abl binding domain

Figure 3. Domain arrangement of constructs. RIN-BD = Abl binding domain from the Ras and Rab interactor 1 (RIN1; binds the SH3/SH2 domains of Bcr-Abl), IMS = intermitochondrial membrane space, ccmut3 = coiled-coil mutation set 3, cMTS = cryptic mitochondrial translocation sequence, Bcr = breakpoint cluster region, Abl = Abelson proto-oncogene, EGFP = enhanced green fluorescent protein. See supplementary figure S2 for domain residue sequences.

Figure 4.

Cell death in Bcr-Abl positive (K562 leukemia) and Bcr-Abl negative (Cos-7 fibroblast and 1471.1 breast cancer) cells 48 hours post-transfection or treatment with 10 μ M imatinib. Cell death was assessed by flow cytometric analysis of 7-AAD. A) K562 cells. B) Bcr-Abl negative Cos-7 cells. C) 1471.1 breast cancer cells. One-way ANOVA with Tukey's post-test performed on individual cell types (error bars are S.E.M., N = 3).

D. **Figure 5.**

The iCE combined with imatinib is toxic to K562 cells. A) This figure is an expansion of the data set seen in Figure 4A, K562 (cell death in K562 at 48 hours). Box and whisker plot showing percent 7-AAD positive cells 48 hours post-transfection and/or treatment with imatinib. The darker shaded boxes (of the box plot) represent the presence of imatinib (10 μ M). Statistical differences were determined using one-way ANOVA with Tukey's post-hoc test (error bars are S.E.M., N = 4). B) Representative set of flow cytometric histograms displaying cell count on the y-axis and 7-AAD intensity on the x-axis. The percent 7-AAD positive (as gated) for the sample is listed in the shaded box and the mean (fig. 4A, K562) and median values (fig. 5A) are listed below. C) Phase-contrast paired with fluorescent images using the nuclear dye, H333342, at 48 hours post-transfection (iCE) or imatinib treatment. Upper left, control K562 cells; lower left, cells transfected with iCE construct; upper right, cells treated with imatinib only; lower right, cells transfected with the iCE and treated with imatinib. White arrow in bottom, rightmost panel indicates a cell with a segmented nucleus. Scale bar is 20 μ m. D) Evaluation of 7-AAD positive K562 cells at 8, 24, and 48 hours post-transfection and/or imatinib treatment. Statistical difference was determined for the parameters of time and treatment using two-way ANOVA with Bonferroni post test (error bars are S.E.M., N = 3).

A. **B.**

C.

Figure 6.

Assessment of cell viability and apoptotic induction in K562 cells. A) Viability 48 hours post-transfection and 24 hours after addition of imatinib. One-way ANOVA with Tukey's post-hoc test was performed within each treatment type (i.e., 'no treatment and 'IM'). B) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was assessed 48 hours post-transfection and/or with imatinib treatment by flow cytometry. C) Phosphatidylserine externalization detection using annexin-APC and flow cytometric analysis 48 hours post-transfection and/or imatinib treatment. Dark shaded columns indicate imatinib treatment. Statistical differences were determined by using one-way ANOVA with Tukey's post-hoc test (for all assays here, error bars are S.E.M., N = 3).

Figure 7.

Representative images of exogenous Bcr-Abl co-expressed with the iCE or its individual components and cMTS mitochondrial localization. Scale bars are 5 μ m. A) Comparison of subcellular localization and association between Bcr-Abl (first column, cyan) and the iCE or iCE component parts (second column, magenta) in Cos-7 cells. The PCC values in the 'Composite' column represent the degree of colocalization between Bcr-Abl and the given construct. B) Co-expression of Bcr-Abl (first column, cyan) and the iCE (second column, magenta) in 1471.1 cells with PCC value below the 'composite' image. C) cMTS expression in K562 and 1471.1 cells (first column, cyan) with MitoTracker staining (second column, magenta).

Figure 8.

Representative images of the subcellular localization of the iCE (or iCE Null, *see* fig. 3) in K562, Cos-7, and 1471.1 cells and compared to MitoTracker. The iCE did not localize to the mitochondria of K562, Cos-7 or 1471.1 cells. Scale bars are 5 μ m.

A. **B.** **Figure 9.**

Domain substitution of either the ccmu3 or the cMTS in the iCE. A) Flow cytometric analysis for 7-AAD was measured 48 hours post-transfection and/or treatment. IM, iCE, iCE +IM, ccmu3, and cMTS values are also in fig. 4A. Imatinib is 10 μ M. Statistical differences were determined using one-way ANOVA with Tukey's post-hoc test (error bars are S.E.M., N = 3). B) Representative images of the subcellular localization of the mock iCE constructs (1st column), IMS-ccmu3 (top) and RIN-cMTS (bottom), in K562 cells stained with MitoTracker (2nd column). Scale bars are 5 μ m.

A. **B.**

C.

Figure 10.

Effect of phospho-residue substitution in the iCE on cell death and apoptosis using flow cytometric analysis at 48 hours in K562 cells. Imatinib is 10 μ M (indicated by shaded columns). A) 7-AAD staining. IM, EGFP, iCE, and iCE+IM, values are also in fig. 4A. B) Apoptosis as measured by TUNEL staining. IM, EGFP, iCE, and iCE+IM, values are also in fig. 6B. C) Annexin-APC staining. IM, EGFP, iCE, and iCE+IM, values are also in fig. 6C. Statistical difference was determined using one-way ANOVA with Tukey's post-hoc test (for all assays here, error bars are S.E.M., N = 3).

Figure 11.

Possible mechanisms for iCE induced toxicity to K562 leukemia cells. With imatinib (IM) present, Bcr-Abl kinase activity is eliminated¹ and the iCE may be potentiating leukemic cell death by altering calcium status and/or blocking kinase-independent oncogenic functions of Bcr-Abl. Bcr-Abl tyrosine kinase (Y kinase) activity stimulates transient calcium influx (which stimulates proliferation) while PKC activation, at the plasma membrane, can have the opposite effect on calcium transients which has been shown to be toxic to leukemia cells.^{36–38} Another cause of iCE enhanced cell death may be through a more complete blockade of Bcr-Abl signaling by steric restriction of kinase-independent pathways.^{34, 39} Steric effects may be caused by the presence of PKA and/or PKC, a conformational change in the cMTS domain, and/or binding of Hsp70 to the phosphorylated cMTS.¹⁵ iCE toxicity requires interaction with Bcr-Abl via the CC domain and the wild-type cMTS (S/T phospho-residues). The ccmut3 competes with Bcr-Abl for oligomerization at the N-terminal portion of the Bcr to Abl fusion at the CC¹², blocking Bcr-Abl transautophosphorylation.¹⁷

Table 1
Canonical mitochondrial targeting sequences used to target Bcr-Abl to the mitochondria

MTSs targeting different mitochondrial compartments were fused to Bcr-Abl and tested for cellular toxicity. KD represents a kinase dead version of Bcr-Abl where a so-called ‘essential lysine’ residue is mutated at the ATP binding site, abolishing all kinase activity.²⁷

Construct	Target compartment	Kinase	Reference
OTC-Bcr-Abl	mitochondrial matrix	active	⁹
IMS-Bcr-Abl	mitochondrial intermembrane space	active	¹¹
IMM-Bcr-Abl	mitochondrial inner membrane	active	¹⁰
IMM-Bcr-Abl-KD	mitochondrial inner membrane	inactive	²⁷
IMM-EGFP	mitochondrial inner membrane	N/A	²⁴
E-Bcr-Abl	cytoplasmic (non-targeted)	active	³