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Targets and effectors of the cellular response to aurora kinase inhibitor MK-0457 (VX-680) in imatinib sensitive and resistant chronic myelogenous leukemia

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ABSTRACT

MK-0457 inhibits aurora, BCR-ABL and other kinases and may be clinically active in imatinib resistant leukemia. To define mediators of MK-0457 responsiveness, kinase inhibitory profiles were examined in multiple cell models of imatinib sensitive and resistant disease. Aurora and BCR-ABL kinase inhibition were consistently measured at 20–100 nM and 2–10 μM MK-0457, respectively, but expression of T315I-BCR-ABL and overexpression of Lyn kinase reduced MK-0457 sensitivity. Aurora kinase inhibition was associated with cell cycle restriction and p53 induction and p53-null cells were far less responsive to MK-0457, requiring BCR-ABL inhibitory concentrations for apoptotic activity. In wild-type p53 expressing CML cells MK-0457 sensitivity was modulation by alterations in p53 levels through HDM-2 inhibition and gene silencing. MK-0457 suppressed aurora kinase activity and induced apoptosis in imatinib resistant clinical specimens expressing T315I and other BCR-ABL mutations without effecting BCR-ABL kinase activity. Together, these results suggest that MK-0457 apoptotic activity in CML cells is primarily associated with aurora kinase inhibition but can be altered by multiple molecular changes associated with disease progression or acquisition of imatinib resistance.

1. Introduction

Chronic myelogenous leukemia (CML) is characterized by a specific chromosomal translocation (Philadelphia Chromosome, t9:22) and expression of BCR-ABL, a tyrosine kinase with hematopoetic stem cell transforming activity [1,2]. BCR-ABL serves as a scaffold and kinase to recruit and phosphorylate multiple proteins to bypass cytokine dependence and engage transformation [1,2]. Interruption of BCR-ABL kinase activity results in the induction of apoptosis in CML cells and constitutes the most common form of CML therapy [3–5]. Specific kinase inhibitors such as imatinib and dasatinib prevent BCR-ABL mediated phosphorylation and are active in controlling CML through selective induction of apoptosis in BCR-ABL expressing or "addicted" cells [1–5]. This therapeutic approach is highly effective but can be compromised by multiple mechanisms, including point mutations or amplification of BCR-ABL, overexpression of additional kinases or genes and acquisition of stem cell-like characteristics [5,6].

The most investigated mechanism of kinase inhibitor-based therapeutic failure in CML involves emergence or selection of BCR-ABL mutations at specific sites that disrupt kinase inhibitor binding without incapacitating kinase activity [5]. Of particular importance are specific mutations, such as T315I, which block BCR-ABL inhibitory activity of drugs currently approved for CML therapy (imatinib, dasatinib) [5,6]. In addition, other mutations are reported in various frequencies in a partially predictive tyrosine kinase inhibitor specific fashion, supporting the need for sequential or kinase inhibitor cocktails to prevent emergence of resistant disease through target- or site-specific mutations [7,8].

Until recently, few agents were available to inhibit T315I mutated BCR-ABL [4–7]. Compounds designed to inhibit BCR-ABL in a unique fashion or to overcome the steric hindrance and loss of electrostatic attraction imparted by the T315I mutation were recently reported [4–6]. Another approach to address this need was to examine existing compounds with target-directed activity against other kinases for activity against BCR-ABL and its various mutated forms, including T315I. MK-0457, or VX-680, was originally described as an aurora kinase inhibitor and was subsequently shown to inhibit BCR-ABL and multiple BCR-ABL point mutants, including the T315I mutant [9,10]. Crystallographic and structural analysis predicted binding of MK-0457 to the activated form of BCR-ABL, although other configurations may also exist that are aligned with the conformation of the MK-0457 binding pocket in aurora kinase [9,11]. MK-0457 induces apoptosis in CML cells in vitro but a wide range of IC_{50} concentrations are reported [7,10,12–15]. In addition, clinical activity has been reported in patients that had been previously treated with imatinib but had progressing disease associated with the emergence of the T315I mutation [10]. Although MK-0457 reduced the number of CML cells in these patients, the role of BCR-ABL

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kinase inhibition in this activity is unknown. Given the wide MK-0457 concentration range required to induce apoptosis in CML cells in vitro (5 nM to 5 μM) and the possible broad spectrum of kinase targets for MK-0457 [16], the precise mechanism of action of MK-0457 in imatinib resistant disease needs to be established to conduct and evaluate past and future clinical studies with this and other compounds with similar mechanisms of action.

In this report we assessed the activity of MK-0457 in multiple models of BCR-ABL associated leukemia that are sensitive or resistant to imatinib, with the primary goal of determining the role of BCR-ABL inhibition in its anti-leukemic activity. Using selective and kinase specific substrates, we demonstrate that MK-0457 effectively inhibits aurora A and B kinase activity, and at higher concentrations, affects BCR-ABL kinase activity. However, despite effective aurora kinase inhibition at low MK-0457 concentrations, the apoptotic activity is modulated by additional genes and kinases that are common in CML progressing disease. Understanding the complex target profile and mediators of MK-0457 sensitivity in imatinib resistant disease will increase the potential of agents like MK-0457 to provide effective and safe (concentration-tailored) therapy for tyrosine kinase inhibitor refractory CML patients.

2. Materials and methods

2.1. Cell lines and patient specimens

The isolation, characterization and maintenance of CML cell lines used in this study (K562, K562R, BV-173, BV-173R, WDT-2, WDT-3), were previously described [17–19]. Z-119 cells established from an acute lymphocytic leukemia patient [20] were provided by Dr. Zeev Estrov (Department of Leukemia, University of Texas, M.D. Anderson Cancer Center, Houston, TX). IL-3 dependent BaF3 cells were provided by Dr. Charles Sawyers (University of California, Los Angeles, CA) and were transformed to cytokine independence with unmutated and T315I mutated BCR-ABL as previously described and characterized [18]. Patient specimens were from leukemia patients that had signed an informed consent document approved by the University of Michigan IRB for collection and use of their specimen for analysis. Samples were derived from patients where imatinib therapy failed to continually control their disease. Mononuclear cells were isolated from blood samples by density centrifugation (Ficoll-Hypaque), washed with PBS and aliquots were lysed immediately (for RNA extraction and BCR-ABL mutational analysis) or resuspended in cell culture media (RPMI-1640, 10% fetal bovine serum) and incubated overnight at 37 °C in a 5% CO_2 incubator before treatment with kinase inhibitors [21]. All cell lines were cultured in RPMI 1640 medium (Hyclone, Logan, UT) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY). Medium for IL3-dependent BaF3 cells was supplemented with 5 ng/ml recombinant IL-3 (Peprotech, Rocky Hill, NJ). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Compounds

Imatinib was synthesized, purified and kindly provided by Dr. William Bornmann (Dept. of Experimental Therapeutics, M.D. Anderson Cancer Center). Dasatinib was kindly provided by Dr. Francis Lee (Bristol-Myers Squibb, Princeton, NJ). MK-0457 was provided by Dr. Caroline Buser (Merck & Co. Inc., North Wales, PA). Nutlin-3 was purchased from Cayman Chemical Co. (Ann Arbor, MI).

2.3. Antibodies

Antibodies against pY-CrkL, pS10-HistoneH3, HistoneH3, p-aurora A/B, and aurora A were purchased from Cell Signaling Technology (Danvers, MA). Antibodies specific for CrkL, Lyn and tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pY-Lyn (Epitomics, Burlingame, CA), anti-p53 (CalBiochem, San Diego, CA) anti-PARP (Millipore, Billerica, MA) and anti-actin (Sigma–Aldrich Co., St. Louis, MO) were also purchased for use in this study.

2.4. Preparation of cell lysates

Cell lysates were prepared from PBS-washed cells as previously described [17,21]. Washed cell pellets were solubilized on ice in lysis buffer consisting of 50 mM Hepes, pH 7.6, 250 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 10 μg/ml leupeptin and 10 μg/ml aprotinin. Lysates from clinical samples contained additional phosphatase and protease inhibitor cocktails (Sigma Cat. # P5726 and P8340, respectively). Crude lysates were subjected to centrifugation at 14,000 × *g* at 4 °C for 30 min and the supernatant fraction was used as a source of protein for immunoblotting.

For clinical specimens, mononuclear cells were isolated from blood samples by density centrifugation (Ficoll-Hypaque), washed with PBS and aliquots were lysed immediately (for RNA extraction) or resuspended in cell culture media (RPMI-1640, 10% fetal bovine serum) and incubated overnight at 37 °C in a 5% CO_2 incubator before treatment with kinase inhibitors and preparation of cell lysates.

2.5. Analysis of BCR-ABL mutations

BCR-ABL mutations were analyzed in CML patient specimens as previously described [21]. In brief, total RNA was extracted (RNeasy Protect Mini Kit; QIAGEN, Valencia, CA) and used to prime a one-step RT-PCR reaction (Invitrogen, Carlsbad, CA) using the following primers: CM10 (5′-GAAGCTTCTCCCTGACATCCGT-3′, BCR: 2609-2630) and 3′ *ABL* KD (5′GCCAGGCTCTCGGGTGCAGTCC-3′, ABL: 1292–1271), resulting in a 1.3 kb fragment. The 1.3 kb gel-purified fragment was used as a template to prime a second PCR reaction [5′ *ABL* KD (5′GCGCAACAAGCCCACTGTCTATGG-3′) and 3′ KD] resulting in amplification of the ABL kinase domain.

Second PCR reactions were performed using PCR SuperMix High Fidelity (Invitrogen, Cat. #: 10790-020). The resultant 0.6 kb fragment was isolated by QIAquick Gel Extraction Kit (Cat. #: 28704) and subcloned into the pGEM-T vector (Promega, Cat #: A3600). At least 10 clones containing the ABL kinase domain were directly sequenced using an ABI377 automated sequencer.

2.6. p53 silencing

BV-173R cells were electroporated with 100 nM siRNA against p53 or a scrambled control sequence using the AMAXA nucleoporation system (Gaithersburg, MD). All siRNA was purchased from DHARMACON (Lafayette, CO). Electroporation was performed with freshly washed 2.5×10^6 cells in solution T on a setting of 017, according to the manufacturer's (AMAXA, Gaithersburg, MD) instructions; ∼50% of cells are electroporated under these conditions. Forty-eight hours after electroporation lysates were prepared and analyzed for effects on protein expression and sensitivity to kinase inhibitors (MTT assay).

2.7. Assessment of BCR-ABL kinase inhibition

Equal protein cell lysates from control and kinase inhibitor treated cells were resolved by SDS-PAGE, transferred to nitrocellulose membranes and subjected to immunoblotting with pY-CrkL. Anti-CrkL immunoblotted was subsequently performed on the

stripped blot to demonstrate equal protein loading. Primary antibodies were detected with anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) conjugated with horseradish peroxidase (HRP). Antigens were detected with enzyme-catalyzed fluorescence reagent as described by the manufacturer (G.E. Healthcare, Piscataway, NJ).

2.8. Assessment of aurora kinase inhibition

Four techniques were utilized to detect the state of aurora kinase activation.

2.9. Detection of phosphorylated (activated) aurora kinase

Equal protein lysates from treated and control cells (5×10^6) were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an antibody that recognizes the activated form of aurora A (pT288) and B (pT232). Antigen was detected as described above. The membrane was stripped and reblotted for aurora A to demonstrate equal protein content in each lane.

2.10. Detection of phosphorylated HistoneH3 in acid-soluble protein extracts

Kinase inhibitor treated and control cells (1.5×10^7) were harvested at the interval indicated by centrifugation and washed twice with PBS. Acid-soluble protein was enriched by the procedure recently reported by He et al. [22]. Briefly, PBS-washed cells were resuspended with 10 volumes of lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) and then sulfuric acid (H_2SO_4) was added to a final concentration of 0.2 M and extracts were left on ice for 30 min. Extracts were centrifuged at 12,000 × *g* for 10 min at 4 °C, and the supernatant fractions were transferred to fresh tubes and precipitated on ice for 45 min with trichloroacetic acid (20% final concentration). Samples were then centrifuged at 12,000 × *g* for 10 min at 4 °C, the pellets were washed once with 0.1% acidic acetone and once with pure acetone. Acid-soluble proteins were dissolved in 0.1 N NaOH and stored at −20 °C. To measure phosphorylated HistoneH3 at serine 10 (pS-H3) as a surrogate marker of aurora kinase activity, acid-soluble proteins were rapidly thawed and dissolved in SDS sample buffer and separated by 15% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes (BioRad, Hercules, CA). Membranes were blocked with 5% nonfat dry milk in PBS for 1 h at room temperature and incubated overnight at 4 °C with antibody against pS-HistoneH3 or total HistoneH3. Antigen was detected by incubation with HRP-conjugated secondary antibody followed by enzyme-catalyzed fluorescence according to the manufacturer's instructions (Amersham Biosciences).

2.11. ELISA-based quantitation of pS10-HistoneH3

Due to sample size limitations an ELISA-based analysis of pS-H3 in patient samples was utilized. An immunoassay kit (Cat. #KHO0671) was used according to the manufacturers' instruction (Invitrogen) with slight modification. Mononuclear cells from patients or leukemic cells were washed in PBS, pelleted by centrifugation and subjected to extraction in cell extraction buffer recommended by the kit manufacturer [10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_20_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS. 0.5% deoxycholate, 1 mM PMSF, and protease inhibitor cocktail (Sigma Cat. # P-2714)]. Cells were resuspended on ice at a density of 10^6 cells/50 μl of extraction buffer and extracted for 30 min with intermittent vortexing (every 10 min). The extracts were clarified by centrifugation (18,000 × *g*, 10 min, 4 °C) and 10–20 μl aliquots of supernatant were assessed in a total volume of 100 μl in triplicate assays. A standard curve was provided and run with each assay to allow conversion of absorbance values into the average ± the standard deviation of units/ml of pS-HistoneH3 in each extract.

2.12. Cell staining for pS-HistoneH3

Freshly washed leukemic cells ($1–2 \times 10^5$) were fixed in 4% formaldehyde for 15 min at room temperature and cytospun onto glass slides (700 rpm, medium acceleration, 5 min, Cytopro 7620, Wescor, Logan, UT). After permeabilization with 0.5% Triton X-100 in PBS for 5 min, cells are washed with 5%-BSA in PBS and blocked with 5% normal rabbit serum at room temperature for 30 min. Cytospun and permeabilized cells were incubated with a 1:100 dilution of primary antibody (pS-H3) in 5% BSA dissolved in PBS containing 0.5% Triton X-100 for 2 h at room temperature. After washing with 5% BSA/PBS three times, 1:500 diluted goat anti-rabbit Alexa 488 conjugated secondary antibody (Invitrogen) was incubated with the slide for 1 h at room temperature protected from light. After washing the slide with 5% BSA in PBS three times, nuclei were stained with the addition of Hoechst/DAPI stain (Invitrogen, 1 μg/ml) for 10 min. After one additional PBS wash, 1 drop of fluoromount/prolong gold anti-fading agent (Invitrogen) was added and a coverslip was applied without sealing. The slides were stored in the dark overnight at room temperature and imaged on a fluorescence microscope (Olympus Model IX71, Center Valley, PA) fitted with a digital camera (Olympus DP71). Individual Images were captured at 200× magnification and merged using Olympus DP controller software.

2.13. Cell cycle and apoptosis analysis

Leukemic cells cultured in 6 well plates were treated with vehicle alone or kinase inhibitor as indicated for 60 to 72 h. Cells were harvested, washed in PBS, fixed in 1% paraformaldehyde, rewashed in PBS, and resuspended in 70% ethanol at −20 °C overnight. Cells were washed twice with PBS and stained with 20 μg/mL propidium iodide. DNA content was analyzed on a cytofluorimeter by fluorescence-activated cell sorting analysis (FACScan; Becton Dickinson Biosciences, San Jose, CA) using ModFit software (Verity Software House, Topsham, ME).

Apoptosis in cell lines was determined by immunoblotting lysates from control and treated cells for PARP as previously described [17,21]. For patient derived cell samples apoptosis was estimated by staining with Annexin V fluorescein conjugate according to the manufacturer's instructions (Southern Biotech, Birmingham, AL). Briefly, PBS-washed cells (1×10^5) were incubated in binding buffer with labeled annexin V for 15 min before propidium iodide was added. Cells were analyzed by flow cytometry as described above.

2.14. Cell viability assessment

Viability in inhibitor treated and control cells was estimated by MTT assays as previously described [17,21]. Briefly, 2×10^4 cells were plated into individual wells of a 96-well plate in 100 μl of growth media and incubated at 37 °C overnight. Kinase inhibitors were added in 100 μl of growth media at the indicated final concentration and incubated at 37 °C for the interval noted. All analyses were performed in triplicate. Viable cells were assayed for their ability to transform 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into purple formazan by monitoring absorbance at 630 nm. The concentration of drug that resulted in 50% growth inhibition or loss of viability when compared to vehicle treated control cells was calculated and reported as the IC_{50} value. GraphPad InStat 3 (San Diego, CA) was used to calculate the

average, standard deviation and significance of the concentration-dependent viability change. Differences between treated and control cells were tested using two-tailed unpaired student's *t*-test and *p*-values of <0.05 were considered significant.

3. Results

3.1. Differential sensitivity of imatinib sensitive and resistant CML cells to MK-0457

To understand the effectors and mediators of MK-0457 activity in imatinib sensitive and resistant CML, iso-genetic variants representing imatinib sensitive and resistance CML populations were assessed for drug sensitivity [17,18]. BV-173 and K562 cells express near equal sensitivity to imatinib whereas BV-173R and K562R cells resist imatinib killing through expression of T315I mutant BCR-ABL [18] and Lyn overexpression [17,19], respectively (Fig. 1A, left). MK-0457 treatment reduced the survival of all cell lines but with a wide range (~100-fold) of sensitivities (Fig. 1A, right). MK-0457 IC_{50} values for BV-173 and BV-173R were 10–20 nM while K562 and K562R required 2 and 10 μM MK-0457, respectively to reduce survival by 50%. Analysis of cell cycle changes after treatment for 60 h demonstrated an increase in the subG fraction (suggesting the onset of apoptosis), reduction in G1

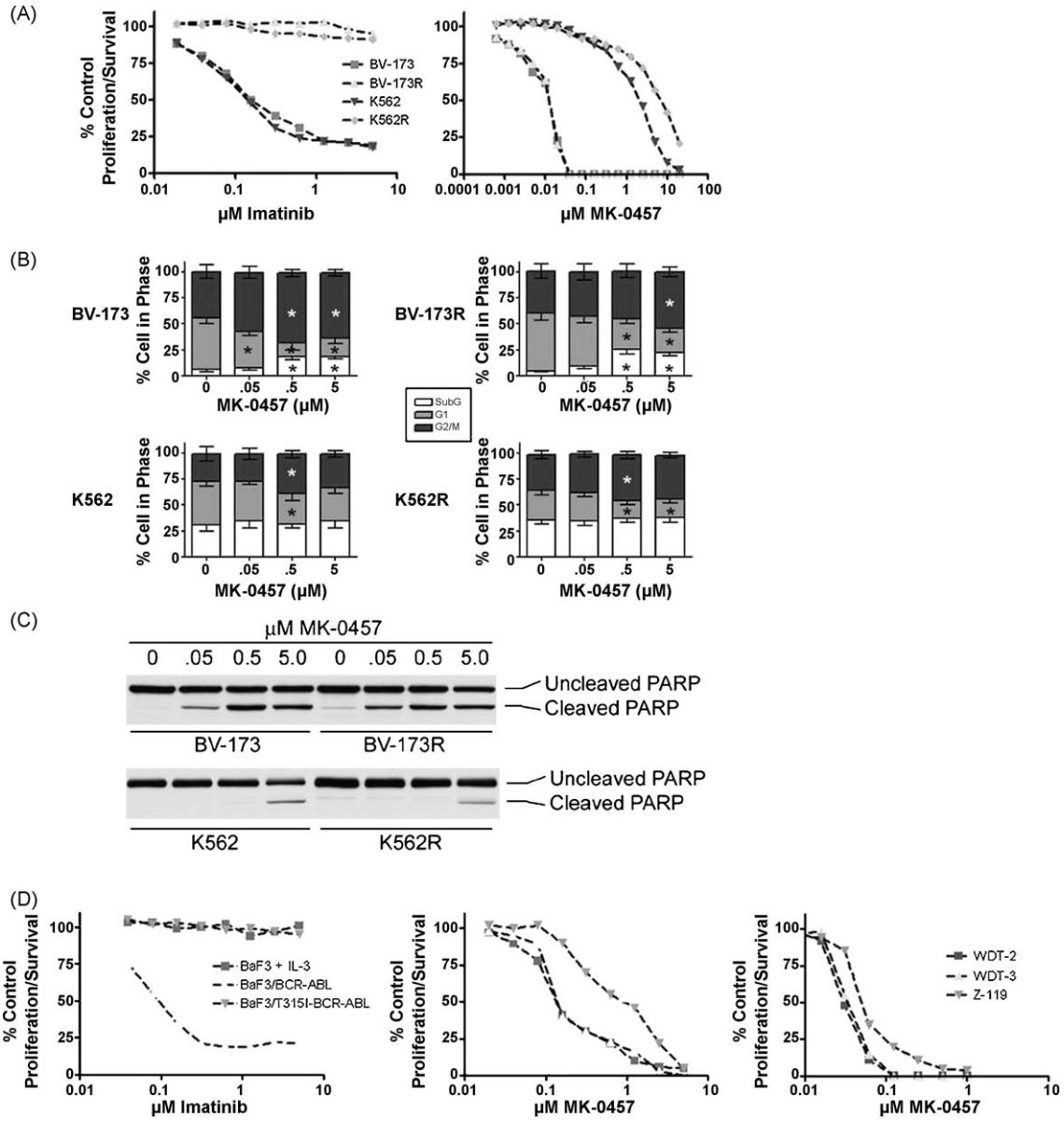

Fig. 1. MK-0457 apoptotic activity in imatinib sensitive and resistant CML. (A) K562, K562R, BV-173 and BV-173R cells were incubated with the indicated concentration of imatinib (left) or MK-0457 (right) for 72 h before analysis of cell proliferation and survival as described in Section 2. The results represent the average of triplicate samples with no more than 5% variance in any data point. The results are representative of 3 independent experiments. (B) CML cells were incubated with the indicated concentration of MK-0457 for 60 h before analysis of cell cycle changes as described in Section 2. The results represent averages derived from triplicate samples ± the calculated S.D. The results shown are representative of two additional independent experiments. *Two-tailed unpaired students *t*-test *p*-value of <0.05 when compared to control. (C) Caspase activation was determined by monitoring PARP cleavage in CML cells after MK-0457 incubation at the indicated concentration for 48 h. These results were similar to those obtained in an additional independent experiment and those obtained after MK-0457 incubated at the same concentrations for 60 h. (D) IL-3 dependent and unmutated or T315I mutant BCR-ABL transformed BaF3 cells were incubated with the indicated concentration of imatinib (left) or MK-0457 (center) for 72 h before analysis of cell proliferation and survival as described in Section 2. Two additional CML derived cell lines and one derived from an ALL patient (Z-119 cells express the p190 form of BCR-ABL) were incubated with MK-0457 at the indicated concentration for 72 h before analysis of proliferation and survival. The results represent the average of triplicate samples with no more than 5% variance in any data point. The results are representative of 2 additional independent experiments.

with an increase in cells at the G2/M phase at 50 and 500 nM MK-0457 in both imatinib sensitive and resistant BV-173 and BV-173R cells (Fig. 1B, top). Treatment with 10-fold higher MK-0457 concentrations did not result in a further increased percentage of cells in the subG fraction. In K562 and K562R cells, MK-0457 did not increase the subG fraction but at intermediate concentrations reduced the percentage of cells in the G1 fraction, while an increase in the percentage of G2/M cells was noted (Fig. 1B, bottom). In

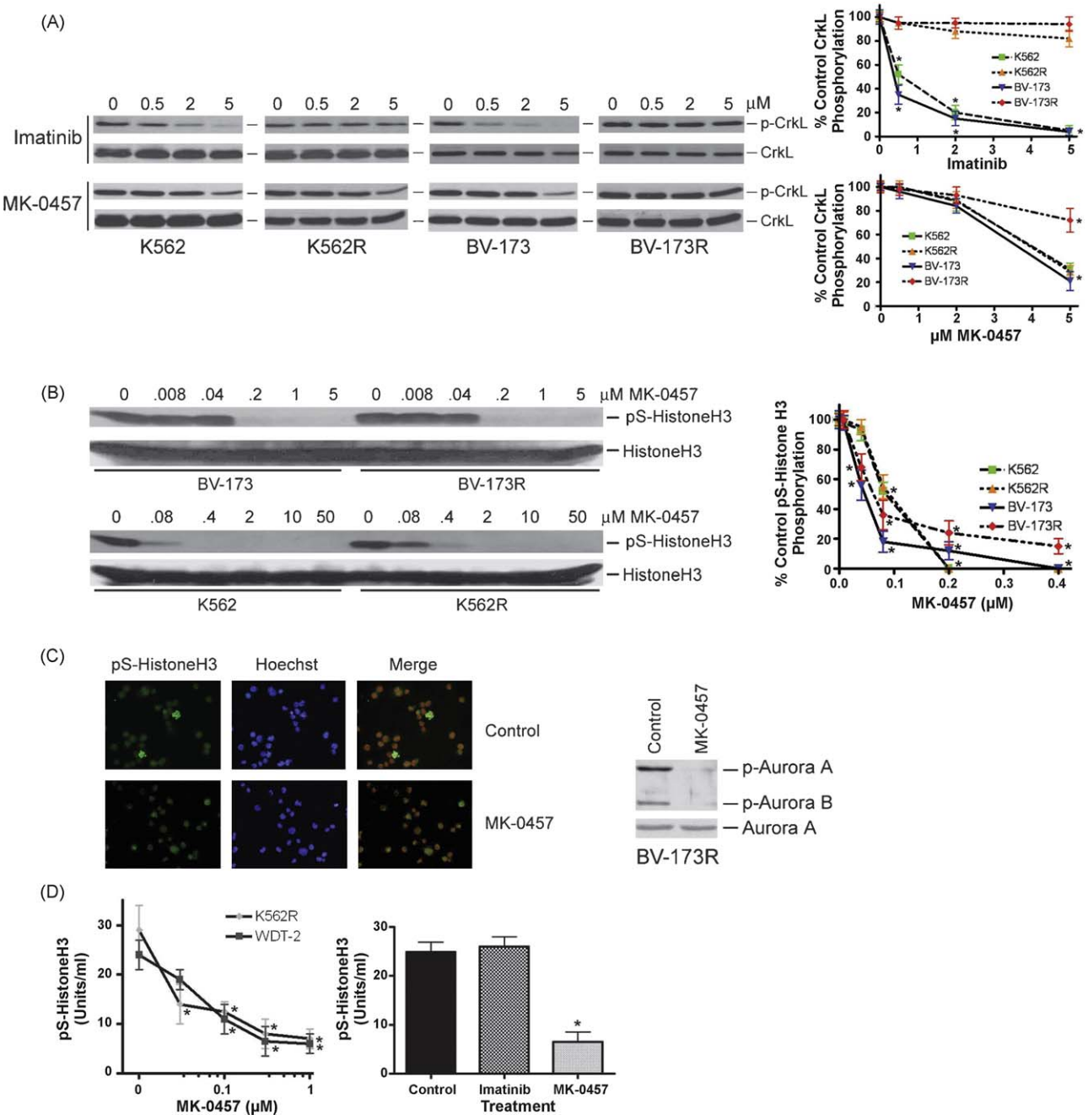

Fig. 2. MK-0457 inhibits BCR-ABL and aurora kinase activity in imatinib sensitive and resistant CML cells. (A) Left – K562, K562R, BV-173 and BV-173R cells were incubated with the indicated concentration of imatinib (top) or MK-0457 (bottom) for 4 h before equal protein cell lysates were prepared and analyzed for BCR-ABL inhibition by immunoblot detection of pY-CrkL. Equal protein loading was determined by reblotting for CrkL. Right – CrkL phosphorylation was quantitated and compared to control levels in immunoblots representative of imatinib (top) and MK-0457 (bottom) treated cells (as shown on the left). The results represent the mean ± S.D. of 3 independent experiments. *Two-tailed unpaired students *t*-test *p*-value of <0.05 when compared to control. (B) Left – CML cells were incubated with the indicated concentration of MK-0457 for 4 h before acid-soluble protein extracts were probed for aurora kinase inhibition by detection of its substrate, HistoneH3. The levels of pS-HistoneH3 in treated and control cells are shown and HistoneH3 was reblotted to demonstrate equal protein load per lane. Right – pS-HistoneH3 phosphorylation was quantitated in immunoblots from control and MK-0457 treated cells (as shown on the left). The results represent the mean ± S.D. of 3 independent experiments. *Two-tailed unpaired students *t*-test *p*-value of <0.05 when compared to control. C. Left – K562 cells incubated with 1 µM MK-0457 or vehicle alone for 4 h were cytospun onto glass slides and stained for pS-HistoneH3 as described in Section 2. Individual cells were detected by Hoechst staining. Digital images were captured and merged to assess pS-HistoneH3 content per cell. Right – BV-173R cells were treated with 1 µM MK-0457 for 4 h before equal protein cell lysates were prepared and assessed for p-aurora A/B levels by immunoblotting (top). To demonstrate equal protein loading, the blot was reblotted for aurora A (bottom). (D) K562R or WDT-2 CML cells (left) were treated with the indicated concentration of MK-0457 for 4 h before equal protein cell lysates were prepared (described in Section 2) and pS-HistoneH3 levels measured by ELISA. Absorbance values were converted to units/ml using a standard curve provided by the manufacturer and run in parallel with unknowns. The results show the average ± S.D. of triplicate assays. Similar results were obtained in two additional independent experiments. Right–K562 cells were treated with 0.5 µM MK-0457 or 5 µM imatinib for 4 h before pS-HistoneH3 levels were assessed in equal protein cell lysates by ELISA. The results represent the average of triplicate assays ±S.D. *Two-tailed unpaired students *t*-test *p*-value of <0.05.

K562 and K562R cells, 5 μM MK-0457 did not lead to greater cell cycle impact than lower concentrations. These results suggested a complex mechanism of apoptosis and cell cycle regulation induced by MK-0457 in CML cells.

To further assess apoptosis in these populations PARP cleavage was monitored 48 h after incubation with the indicated concentration of MK-0457 (Fig. 1C). Low and intermediate concentrations of MK-0457 increased PARP cleavage in BV-173 and BV-173R cells while higher concentrations did not further increase PARP cleavage in either cell type. In K562 and K562R cells, only 5 μM MK-0457 induced PARP cleavage. These results suggest a correlation between MK-0457 sensitivity and induction of apoptosis.

Since previous studies focused on defining the activity of MK-0457 in cells transformed by unmutated and T315I mutant BCR-ABL [9–12,14,15], IL-3 dependent BaF3 and those transformed to cytokine independence with BCR-ABL were screened for sensitivity to imatinib and MK-0457. As shown in Fig. 1D (left), both cytokine-dependent and T315I-BCR-ABL transformed BaF3 cells were unaffected by imatinib and only unmutated BCR-ABL transformants expressed concentration-dependent imatinib sensitivity. Incubation with MK-0457 reduced the viability of both IL-3 dependent and BCR-ABL transformed cells (Fig. 1D, center). However, T315I-transformed BaF3 cells were ~10-fold less sensitive to MK-0457. These results suggest that BCR-ABL is one of several target kinases affected by MK-0457 and expression of T315I-BCR-ABL reduces, but does not eliminate, MK-0457 sensitivity in BCR-ABL transformed BaF3 cells.

To determine whether MK-0457 activity was distinct in other BCR-ABL expressing cells, freshly established WDT-2 and WDT-3 myeloid blast crisis derived CML cell lines and an acute lymphocytic leukemia cell line expressing p190-BCR-ABL (Z-119) were incubated with the indicated MK-0457 concentration and viability was determined after 72 h. All 3 cell lines were sensitive to nM MK-0457 concentrations with only a 2-fold distinction in activity between p210 (WDT-2, WDT-3; IC50 ~30 nM) and p190 (Z-119; IC50 ~60 nM) expressing cells.

3.2. Kinase inhibitory activity of MK-0457 in imatinib sensitive and resistant cells

Kinase inhibition was measured in MK-0457 treated cells by monitoring the extent of BCR-ABL (pY-CrkL) and aurora (pS-HistoneH3) kinase substrate phosphorylation [17,21]. CrkL tyrosine phosphorylation was compared in imatinib sensitive and resistant cell lines treated with the indicated concentration of imatinib or MK-0457. As shown in Fig. 2A, imatinib treatment resulted in a concentration-dependent suppression of CrkL phosphorylation in imatinib sensitive cells with an EC_{50} <500 nM. In both imatinib resistant cell lines, CrkL phosphorylation was not suppressed by 5 μM imatinib. MK-0457 concentrations of 5 μM or greater (BV-173R cells) were required to inhibit CrkL phosphorylation, suggesting that apoptotic and cellular sensitivity to MK-0457 were not directly associated with BCR-ABL kinase inhibition.

Aurora kinase inhibition was also examined in CML cells by measuring the extent of HistoneH3 serine-10 phosphorylation. As shown in Fig. 2B, pS-HistoneH3 levels were highly sensitive to MK-0457 (imatinib was inactive at 5 μM; data not shown) with EC_{50} <80 nM in both imatinib sensitive and resistant cell lines. Aurora kinase and pS-HistoneH3 inhibition were also measured in control and MK-0457 treated cells by immunofluorescence of pS-HistoneH3 in permeabilized cells and by immunoblotting cell lysates with an antibody recognizing the activation site-specific phosphorylation of aurora kinase (A and B). As shown in Fig. 2C, basal staining of pS-HistoneH3 in untreated K562 cells was low but detectable in metaphase cells and 4 h of MK-0457 (1 μM) incubation fully suppressed pS-HistoneH3 detection. Immunoblotting of phosphorylated aurora kinase in control and MK-0457 treated BV-173R cells (1 μM, 4 h) also demonstrated suppression of both aurora A and B activation in inhibitor treated cells. These results confirm direct inhibition of both aurora A and B kinase and the downstream substrate, HistoneH3, by MK-0457.

To measure and quantify aurora kinase activity and its inhibition in clinical specimens ELISA analysis was tested as an alternate to procedures requiring high numbers of cells or extensive handling of samples. Two CML cell lines were treated as indicated in Fig. 2D (left) and analyzed for pS-HistoneH3 levels by ELISA. Concentration-dependent inhibition was detected in both cell lines and the EC_{50} (~40 nM) was similar to that determined by other methods (Fig. 2B and C). Further, imatinib treatment did not suppress pS-HistoneH3 levels in CML cells under these conditions (Fig. 2D, right).

3.3. Concentration-dependent modulation of p53 in MK-0457 treated CML cells

MK-0457 is reported to induce or stabilize p53 in multiple cell types and may play a role in the apoptotic and cell cycle changes associated with aurora kinase inhibition [23–25]. Differential aurora kinase inhibition did not appear to underlie the wide variance in MK-0457 sensitivity in CML cells since low concentrations (<80 nM) of MK-0457 were able to block aurora kinase activity in CML cells with 40 nM and 10 μM apoptotic sensitivity (Figs. 1 and 2B). Since both K562 and K562R cells are p53 protein deficient and insensitive to MK-0457-mediated apoptosis at aurora kinase inhibitory concentrations, p53 may play a role in the apoptotic sensitivity and caspase activation following aurora kinase inhibition. Treatment of wild-type p53 expressing BV-173 or BV-173R cells with low and intermediate levels of MK-0457 increased p53 levels (Fig. 3A). However, 5 μM MK-0457 failed to induce (BV-173) or suppressed (BV-173R) p53 levels when compared to untreated cells. The loss of p53 response at high dose MK-0457 does not appear to be associated with the rapid onset of apoptosis as apoptosis and loss of cell viability are detected only after a 48 h or longer incubation interval. These results suggest that MK-0457 induces a complex concentration-dependent effect on cell survival associated with aurora kinase inhibition, p53 induction and possibly other target kinases.

To determine whether p53 induction is an important regulator of MK-0457 sensitivity alternate mechanisms of p53 induction and suppression were employed in CML cells. Treatment of BV-173R cells with nutlin-3 [26] consistently increased p53 levels while siRNA reduced p53 content (Fig. 3B, top). HDM2 inhibition by nutlin-3 increased p53 levels to a level similar to those achieved by low dose MK-0457 (Fig. 3A and B). Cells pretreated for 24 h with siRNA or nutlin-3 were treated with a range of MK-0457 concentrations to determine the impact of p53 modulation on drug sensitivity. Suppression of p53 by siRNA had no effect on BV-173R cell growth or survival while nutlin-3 (at 0.2 μM) reduced cell growth by ~20% and was used as a baseline to calculate impact on MK-0457 sensitivity. Nutlin-3 increased sensitivity to MK-0457 (~2-fold) while p53 silencing reduced drug sensitivity by 2.4-fold (Fig. 3B, bottom). These results suggest that p53 induction increases the sensitivity of some CML cells to MK-0457. In p53-null CML cells (i.e. K562) MK-0457 activity may be markedly impaired.

3.4. Absence of p53 and Lyn overexpression are associated with MK-0457 resistance

K562R cells are resistant to imatinib due to overexpression of Lyn, a kinase that is not inhibited by clinically achievable

concentrations of imatinib [17,19,21]. Lyn is also associated with CML cell survival and imatinib resistance in some clinical settings [21]. The limited sensitivity of K562R cells to MK-0457 ($IC_{50} \sim 10\ \mu M$) may be related to the inability of this drug to inhibit Lyn kinase activity. To examine this possibility, K562 and K562R cells were treated with imatinib or MK-0457 and Lyn activation was assessed by immunoblotting with an antibody that detects activated Lyn (pY396). Dasatinib, a multi-kinase inhibitor affecting BCR-ABL and Lyn, was also examined for affects on Lyn phosphorylation. As shown in Fig. 3C, neither MK-0457 nor

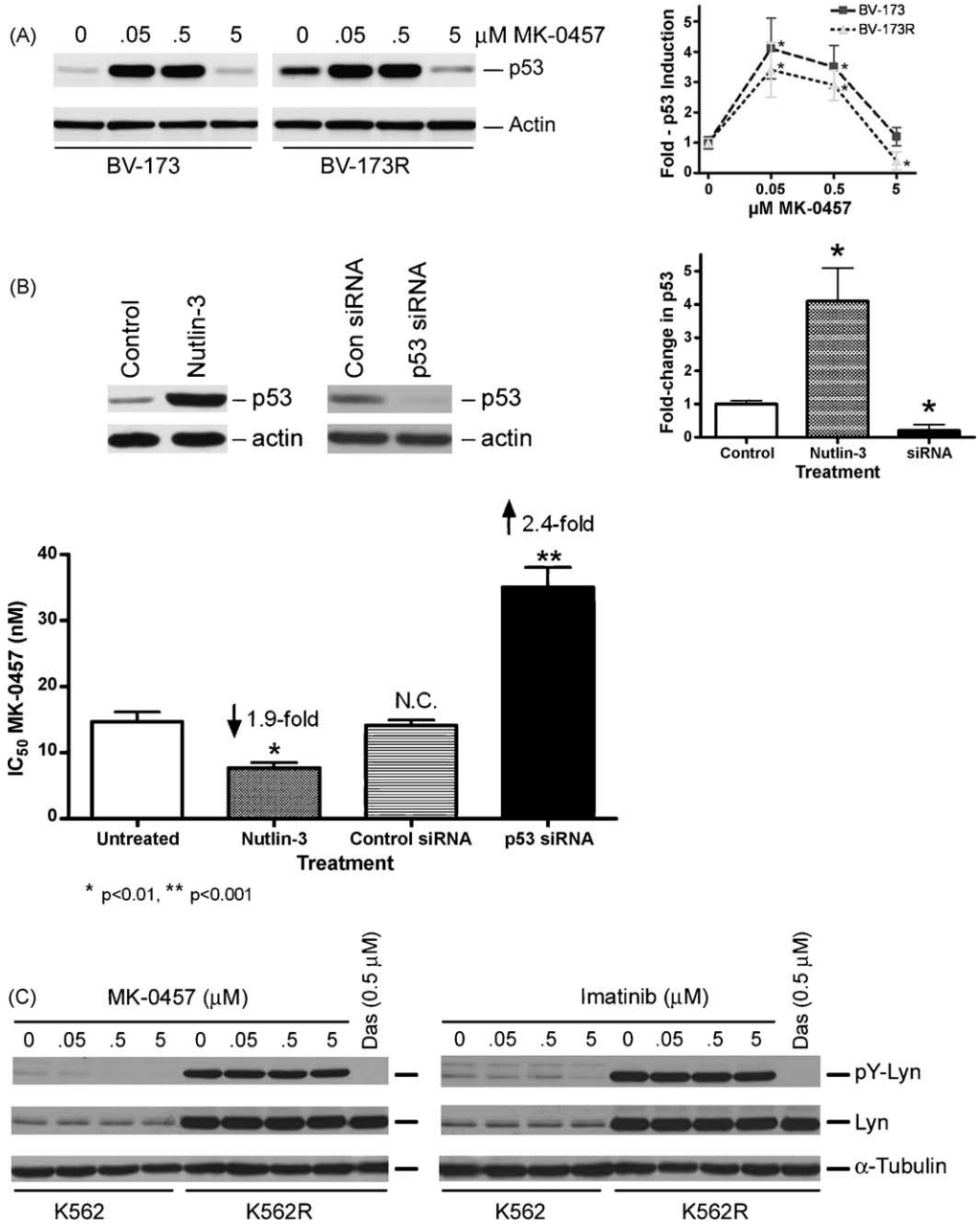

Fig. 3. . Effectors of MK-0457 sensitivity in CML cells. (A) Left – BV-173 or BV-173R cells were treated with the indicated concentration of MK-0457 for 24 h before equal protein cell lysates were immunoblotted for p53. Equal protein loading per lane was determined by reblotting for actin. Representative results from a single experiment are shown. Right – p53 levels in control and MK-0457 treated cells (as indicated) were quantitated in resulting blots (as shown on the left). The results represent the mean ± S.D. of 3 independent experiments. *Two-tailed unpaired students *t*-test *p*-value of <0.05 when compared to control. (B) Top panel, left – BV-173R cells were treated with 1 μM Nutlin-3 or vehicle alone for 24 h before analysis of equal protein cell lysates for p53 levels by immunoblotting. Top panel, right – BV-173R cells were electroporated with control (Con) or p53 specific siRNA and after 48 h cell lysates were probed for p53 levels by immunoblotting. Actin blotting was used to demonstrate equal protein loading per lane. Representative results from a single experiment are shown. Top panel, right – p53 levels in control, Nutlin-3 and siRNA treated BV-173R cells (as indicated) were quantitated in resulting blots (as shown on the left). The results represent the mean ± S.D. of 3 independent experiments. *Two-tailed unpaired students *t*-test *p*-value of <0.05 when compared to control. Bottom panel – BV-173R cells were treated with 0.2 μM Nutlin-3 or vehicle alone for 24 h before incubation with a range of MK-0457 concentrations for an additional 72 h. IC_{50} values were compared between each treatment group and Nutlin-3 only treated cells. In cells treated with Nutlin-3 and MK-0457 combined, Nutlin-3 alone served as a control to calculate the impact on MK-0457 sensitivity. BV-173R cells electroporated with siRNA (as indicated) and after 24 h cells were incubated with a range of MK-0457 concentrations for 72 h to determine the IC_{50}. The IC_{50} values represent the average ± S.D. of three dose–response measurements. *p*-values between treatment groups were calculated as described in Section 2. *Two-tailed unpaired students *t*-test *p*-value of <0.05. Both Nutlin-3 (increased sensitivity by 1.9-fold) and p53 siRNA (decreased sensitivity by 2.4-fold) had significant impact on MK-0457 sensitivity. (C) K562 and Lyn-overexpressing K562R cells were incubated with the indicated concentration of MK-0457 (left) or imatinib (right) for 4 h before equal protein extracts were prepared and immunoblotted for pY-Lyn and reprobed for Lyn and α-tubulin as protein loading controls. Lysates derived from 4 h dasatinib (0.5 μM) treated K562R cells were also included as a positive control for Lyn inhibition. The results are representative of two additional independent experiments.

imatinib at any concentration tested reduced Lyn activation in K562R cells, whereas dasatinib fully reduced Lyn activation at clinically achievable concentrations [27]. These results suggest that MK-0457 exhibits a concentration-dependent range of activities against some but not all kinases relevant to CML cell survival. MK-0457 effectively inhibits aurora kinase activity at low concentrations (Fig. 2B) and induces apoptosis in the majority of CML cells at aurora kinase inhibitory doses (Fig. 1A and D). Unmutated BCR-ABL kinase activity is suppressed at 50- to 100-fold higher MK-0457 concentrations, while the T315I-BCR-ABL mutant requires slightly higher MK-0457 doses to achieve similar levels of BCR-ABL kinase inhibition (Fig. 2A). Finally, MK-0457 is unable to suppress Lyn activation (Fig. 3C). Together, these results suggest that clinically achievable concentrations of MK-0457 may vary widely in their clinical impact on CML patients due to acquisition of molecular changes associated with response to prior therapies and/or disease progression (BCR-ABL mutations, p53 status, Lyn expression).

3.5. MK-0457 induces apoptosis in imatinib resistant CML patient specimens without affecting BCR-ABL kinase activity

Previous reports of clinical MK-0457 activity in an imatinib/nilotinib resistant T315I-BCR-ABL mutant leukemia patient suggested that white blood cell counts were suppressed at MK-0457 doses that did not induce inhibition of CrkL phosphorylation [10]. We obtained mononuclear cell preparations from two patients that progressed on imatinib therapy associated with BCR-ABL mutations. In one patient only T315I-BCR-ABL expression was detected while a second patient had a more complex mutational phenotype (M244V, G250E, M351T). These cells were incubated with imatinib (5 μM), dasatinib (0.5 μM) or MK-0457 at the concentration indicated for 48 h to measure changes in kinase activity and apoptosis. As shown in Fig. 4, imatinib and dasatinib did not inhibit CrkL phosphorylation in patient 1 but reduced pY-CrkL levels in patient 2. MK-0457 at the highest concentration (5 μM) inhibited CrkL phosphorylation but 10- and 100-fold lower doses that limited affects on BCR-ABL substrate phosphorylation.

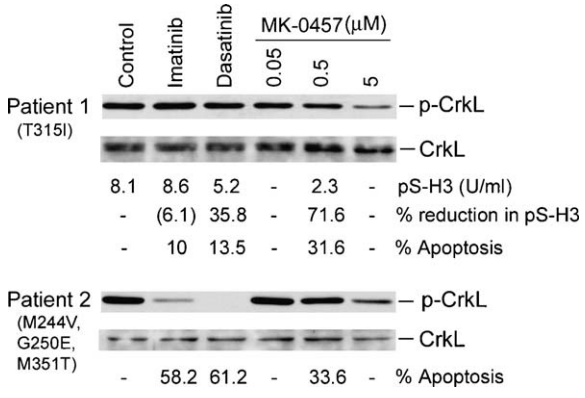

Fig. 4. MK-0457 target kinase inhibition in imatinib resistant CML patient specimens. Mononuclear cells obtained from 2 CML patients with imatinib uncontrolled disease were obtained and RNA was used to analyzed BCR-ABL mutations as described in Section 2 and confirmed by an independent hematopathology lab. Mutations detected in each specimen are illustrated in parenthesis. Freshly prepared cells (not cryo-preserved and recovered cell pellets) were incubated with the indicated concentration of imatinib, dasatinib, MK-0457 or vehicle alone for 48 h. Cell lysates were prepared and equal protein extracts were immunoblotted for pY-CrkL and CrkL. In some samples, apoptosis was measured by flow cytometry as described in Section 2. The % apoptosis, compared to vehicle only treated cells, is tabulated below each sample. For patient 1, sufficient sample was available and pS-HistoneH3 levels in cell lysates were measured by ELISA and units/ml were calculated by comparison with a standard curve run in parallel. The % reduction in pS-HistoneH3 is also tabulated and compared to control, vehicle only treated cells.

In patient 1, sufficient sample was available to measure pS-HistoneH3 levels at the intermediate MK-0457 concentration which demonstrated >71% reduction of pS-HistoneH3. Imatinib had little affect on pS-HistoneH3 levels but dasatinib reduced pS-HistoneH3 by >35%, possibly through indirect effects on DNA synthesis and cytokinesis [28]. MK-0457 at 0.5 μM increased apoptosis by > 31% in either patient sample. In patient 1, neither imatinib nor dasatinib incubation resulted in marked induction of apoptosis while a similar regimen increased apoptosis in patient 2 by >58%. These results suggest that MK-0457 induces apoptosis in the absence of marked changes in BCR-ABL substrate phosphorylation and may be associated with inhibition of aurora or other kinases.

4. Discussion

MK-0457 (or VX-680) was initially described as an aurora kinase inhibitor and was subsequently shown to interfere with BCR-ABL kinase activity [9,10,15,16]. Of particular clinical interest was the observation of MK-0457 inhibitory activity against both the unmutated and T315I mutant BCR-ABL kinase. These properties supported a clinical investigation in imatinib and nilotinib resistant disease [10]. Early reports suggested that white cell counts in some patients were reduced by MK-0457 at doses that did not effect CrkL phosphorylation [10], suggesting clinical activity without BCR-ABL kinase inhibition. To further assess the role of BCR-ABL as a target for MK-0457 based CML therapy we examined the affects of MK-0457 on specific target kinases in imatinib sensitive and resistant iso-genetic variant CML cells. Cells were treated with MK-0457 and apoptotic responses were compared to the profile of kinase inhibitory activities. The prime goal was to more clearly define mediators of MK-0457 anti-leukemic activity so that target-specific impact could be achieved through appropriate dosing. The results suggest that MK-0457 anti-CML activity is controlled by multiple molecular changes associated with prior therapies or disease progression [29].

Although no concentration-dependent distinction in aurora kinase inhibition was measurable in imatinib sensitive or resistant cells (Fig. 2B), MK-0457–mediated BCR-ABL kinase inhibition was reduced in cells expressing the T315I mutant kinase (Fig. 2A). 5 μM MK-0457 suppressed CrkL phosphorylation in all cells except those with endogenous expression of T315I-BCR-ABL (BV-173R). Further, T315I-BCR-ABL transformed BaF3 cells were less sensitive to MK-0457 than those expressing unmutated kinase, as previously reported [7,9,15]. However, it is clear that the broader spectrum of MK-0457 sensitive target kinases in these cells (i.e. Jak-2) limits the capacity of MK-0457 to distinction between BCR-ABL transformation and IL-3 dependent cell survival [30]. Our observations suggest that MK-0457-mediated inhibition of T315I mutant BCR-ABL kinase activity at clinically achievable concentrations plays a minor role in the activity of this drug against T315I associated imatinib resistant leukemia.

MK-0457 induced equivalent dose-dependent apoptosis in CML cells expressing unmutated (BV-173) or T315I (BV-173R) mutant kinase and lethal doses did not interfere with BCR-ABL kinase activity (Fig. 2A and B). Aurora kinase inhibition was measurable in both imatinib sensitive and resistant cells at nM MK-0457 concentrations and was independent of the mechanism of imatinib resistance (Fig. 2B). In all CML cell lines and patient specimens μM dosing was required to inhibit BCR-ABL signaling (Figs. 2A and 4). Similar EC50s against BCR-ABL kinase activity in intact cells from patients and CML cell lines were previously reported by other investigators [10,12–15]. However, BCR-ABL inhibitory MK-0457 concentrations do not appear to be necessary to induce anti-CML activity. Dosing to BCR-ABL inhibitory concentrations may be necessary when aurora kinase inhibition is not coupled to

induction of apoptosis as demonstrated in K562 and K562R cells. In fact, in some CML cells, BCR-ABL kinase inhibitory doses may induce sub-optimal apoptosis as μM concentrations failed to result in greater induction of apoptosis than that achieved at log-fold lower concentrations (Figs. 1C and 3A). In cells with wild-type p53 expression, only low and intermediate concentrations of MK-0457 increased p53 levels while BCR-ABL inhibitory doses had no p53 effect. This concentration-dependent p53 effect of MK-0457 may be related to suppression of aurora kinase mediated p53 phosphorylation as previously described [31,32]. However, higher MK-0457 concentrations fail to engage p53 induction and is possibly related to inhibition of additional stress-sensitive kinases at higher MK-0457 concentrations [30]. In this case, MK-0457 stimulates most effective apoptosis at doses that do not result in BCR-ABL kinase inhibition. This unusual dose–response relationship may be related to broader target kinase inhibition at high MK-0457 doses which blunts the coupling between chromosomal unorganization (through aurora kinase inhibition) and cell cycle regulation (i.e. c-Abl). The absence or presence of functional sensors of chromosomal stress or unorganization (p53, p63. p73) in CML cells may play a major role in determining their apoptotic threshold [23,24,31–34]. The complexities of the cellular response to chromosomal re-organization could not have been predicted or mimicked by in vitro kinase inhibition studies alone and additional tumor cell specific studies are necessary to provide optimal kinase inhibitory conditions that link tumor-specific kinase inhibition to tumor-selective apoptosis.

The presence and function of stress-sensing transcription factors such as p53 may play a key role in aurora kinase anti-leukemic activity. K562 cells are derived from a blast crisis patient and are p53 deficient due to loss of one allele and nucleotide insertion resulting in early translational termination in the other allele [35]. However, these cells retain apoptotic sensitivity to low concentrations of imatinib (EC50 ~200 nM). Both K562 and K562R cells are insensitive to aurora kinase inhibitory MK-0457 concentrations (~40 nM) and MK-0457 apoptosis occurs only at concentrations that aligned with BCR-ABL kinase inhibition. Without the induction and participation of p53 in aurora kinase inhibited cells, apoptosis may be engaged solely through other mechanisms (i.e. BCR-ABL kinase inhibition). The biphasic MK-0457 concentration-dependent effect on two CML kinase targets may be unique to CML. Solid tumor cells with mutations in p53 are reported to be more sensitive to aurora kinase inhibition than wild-type p53 expressing cells through loss of chromosomal disruption-mediated cell cycle check point control resulting in endoduplication and apoptosis [24]. In CML cells, wild-type p53 induction is associated with apoptosis and modulation of p53 levels alters apoptotic sensitivity to MK-0457 (Fig. 3A and B). It will be necessary to determine whether the absence or presence of a mutant p53 regulates MK-0457 activity through distinct pathways in pre-clinical and clinical studies of CML. It should be noted that changes in chromosome 17 are common in CML blast crisis and may be associated with limited chemotherapeutic activity and disease progression [36]. However, p53 mutations are less common in advanced CML than that reported for solid tumors or other hematological malignancies [29]. Detailed and careful CML cell line studies may be essential to fully address the role of aurora kinase, p53 function and BCR-ABL inhibition in the anti-CML activity of MK-0457.

Lyn kinase overexpression and BCR-ABL kinase independent Lyn activation are common in imatinib and nilotinib resistant, BCR-ABL mutation-negative CML patients [17,21,37]. Inhibition of Lyn and other src-family kinases may be essential to re-engage apoptotic responses in that subset of CML patients and may underlie the clinical activity of drugs such as dasatinib which suppresses BCR-ABL and Lyn as well as other kinases [17,21]. MK-0457, like imatinib, suppressed BCR-ABL kinase activity (albeit at different dose-levels) but did not suppress Lyn kinase activation (Fig. 3C). Limited activity against Lyn kinase may also underlie the very limited sensitivity of K562R cells to MK-0457 (Fig. 1). Given the multiple mechanisms of imatinib resistance and mediators of MK-0457 apoptotic activity, it is attractive to propose sequential treatment of CML patients with broader spectrum kinase inhibitory molecules in imatinib resistant disease. Use of dasatinib in combination or sequence with MK-0457 may be most effective in treating imatinib refractory CML patients [7]. However, treatment with a single agent with aurora, BCR-ABL and Lyn kinase inhibitory activity as previously described, may be equally or more effective and ideally suited for these patients [38,39].

The results reported here suggest a complex mechanism and spectrum of kinase inhibition underlies apoptotic sensitivity to MK-0457 in CML. In most cases, BCR-ABL kinase inhibition does not appear to be essential to MK-0457 activity. Additional clinical studies coupled with thorough evaluation of mediators of MK-0457 activity described here (and others) may result in more effective therapy with limited toxicity in imatinib resistant disease.

Contributions by authors

N. Donato wrote the manuscript and analyzed data. D. Fang, H. Sun, D. Giannola and L. Peterson performed experiments and analyzed data. M. Talpaz provided clinical specimens and analyzed data.

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