SUPPLEMENTAL MATERIALS, METHODS and FIGURES

Reagents

The selective MEK1/2 inhibitor Mirdametinib[1] [PD0325901; (N-((R)-2,3-dihydroxy-propoxy)-3,4-difluoro-2-(2-fluoro-4-iodo-phenylamino)benzamide)] kindly provided to us by Dr J. S. Sebolt-Leopold (Cancer Molecular Sciences, Pfizer Global Research & Development, Ann Arbor, MI) was dissolved in dimethyl sulfoxide (DMSO) at the stock concentration of 100mM and then diluted for the *in vitro* experiments in culture medium at the concentration of 0.5 µM.

Solid ultrapure arsenic trioxide (As_2O_3; ATO; Sigma-Merck Saint-Louis, MO, USA) was solubilized in a solution of 1 M sodium hydroxide (NaOH) and then diluted in PBS. The ATO solution was back titrated with HCl to pH 7.4 and subsequently filtered through a 0.22 µm filter.

Imatinib (Sigma-Merck) was prepared as a 10 mM stock solution in sterile phosphate-buffered saline (PBS) and kept at -20°C.

The ABL1 allosteric activator 5-[3-(4-fluorophenyl)-1-phenylpyrazol-4-yl] imidazolidine-2,4-dione (DPH) from Sigma-Merck was dissolved in DMSO at the stock concentration of 20mM and used at the concentration of 10 µM for the *in vitro* experiments.

Colony Forming Assays

Colony forming assays have been carried out by standard methods by plating either CML cell lines (10^3 cells/plate) in MethoCult™ H4230 or purified CD34+ leukemia cells (10^3 cells/plate) in Methocult™ GF H84444 in presence of Imatinib (1 µM), PD0325901 (0.5 µM), ATO (1 µM for CML cell lines, 0.5 µM for primary samples), DPH (10 µM) or the combination PD+ATO or PD+DPH. Live colonies were detected after 10-14 days of culture by MTT (1 mg/ml) staining and counted using ImageJ quantification software.

Co-culture of leukemic cells with bone marrow stromal cells

Co-culture system between CML cells and adherent HS-5 (human) or MS-5 (murine) cells expressing GFP was performed in RPMI-1640 medium at 10% FBS. Before the experiments stromal cells were plated to confluence and incubated overnight in 6-well or 24-well plates. CML cells (1×10^5 cells/mL) were incubated with PD0325901 for 3 hours in presence or absence of stromal cell line in a 1:1 ratio[2], and then were treated with ATO.

CML cell lines and primary CML cells were seeded in direct contact to HS-5/GFP or MS-5/GFP cells monolayer and after the treatments CML cells were detached by carefully wash off since they did

not firmly adhere to stromal cells and flow cytometry analysis of GFP demonstrated that the purity of CML cell population (GFP-negative) after co-culture was over 98%. Cell death of CML cells was then monitored by flow cytometry analysis of Annexin V-PE/7-AAD staining.

Apoptosis assays

Cytofluorimetric analysis was performed to evaluate the percentage of apoptotic cells by Sub-G1 DNA content and Annexin V-FITC/PI or Annexin V-PE/7-AAD staining. Flow cytometry assay to evaluate Mitochondrial Transmembrane Potential (ΔΨm) was performed according to the manufacturer's protocols (MitoLight®, Chemicon®).

siRNA transfections

Prior to electroporation, CML cells were washed twice with serum-free RPMI 1640 medium and resuspended in Opti-MEM (GibcoBRL, Grand Island, NY) to a final concentration of $24x10^6$ cells/mL. Subsequently, 0.4 mL of cells suspension was mixed with 1 nmol of a smart pool small interfering double-stranded RNAs (siRNA) against TAp73, ΔNp73, MEK1/2 (manuscript, ref. 13-16) or non-specific control siRNA obtained from Dharmacon Tech (Lafayette, Co) and electroporated in a 0.4-cm cuvette using the Gene Pulser electroporation apparatus (Bio-Rad Laboratories Inc, Herculex, California) using a single-pulse protocol (voltage 250 mV/capacitance 1050 µF). 24 and/or 48 hours after transfection CML cells were harvested for Immunoblotting to monitor knockdown protein levels by siRNA.

Electroporation of plasmids

Bone marrow stromal cells HS-5 (human) or MS-5 (murine) were electroporated with 10 µg of pLenti-CMV-GFP-2A-Puro-Blank Lentiviral Control Vector (Applied Biological Materials Inc, Vancouver, BC, Canada) expressing GFP. After electroporation cells were expanded in complete medium for three days after which the medium was replaced with selection medium containing 0.6 µg/mL puromycin for selection of stable transfected pools. Up to 95% of GFP-positive cells were obtained. Stromal cells stably transfected were continuously cultured under puromycin selection. After growth stabilization, the cells were used in the experiments described.

K562 cells were electroporated with 3 µg of pUSE MEK1 (Activated) [S218D/S222D] plasmid (Upstate Biotecnology) expressing Activated MEK1 or empty vector control. After electroporation cells were expanded in complete medium for three days after which the medium was replaced with selection medium containing 0.6 mg/mL G418 for selection of stable transfected pools. K562 stably

transfected [K562-(ca)MEK] were continuously cultured under G418 selection. After growth stabilization, the cells were used in the experiments described.

Western blot

Proteins from whole cell lysates or cytoplasmic and nuclear fractions prepared using the Active Motif's Nuclear Extract Kit (Active Motif, Rixensart, Belgium) were quantified by Bradford assay, separated by SDS-PAGE and analyzed by Western blotting with the antibodies reported in Supplemental Table 3. TINA 2 software was used for densitometric analysis of western blot results. The interaction between ABL1 and BCR with MEK1/2 was evaluated by co-immunoprecipitation analysis by using ExactaCruz kits (Santa Cruz Biotechnology, Santa Cruz, CA) as per manufacturer's instructions. For immunoprecipitation, the antibodies reported in Supplemental Table 3.

Animal studies

Five-week-old non-obese diabetic (NOD)/severe combined immunodeficient (SCID) CB17/Prkdcscid/J (NOD/SCID) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained under the same specific pathogen-free conditions. All procedures involving animal models were performed in accordance with national and international laws and policies. The experimental protocol was approved by the University of Parma Ethics Committee and by the Italian Ministry of Health (art. 5, DL 116/92). NOD/SCID mice were injected intravenously with $4x10^6$ of Ba/F3p210^{T315I} cells. After 5 days, mice with BCR::ABL1^{T315I}-induced leukemia were randomly assigned (n=8 per group) to receive vehicle alone (PBS), PD0325901 (10mg/kg orally), ATO (3,75 mg/kg intraperitoneally), or Imatinib (50mg/kg, twice daily, intraperitoneally) or the combination PD+ATO for three weeks. In this study, no blinding was done.

For our in vivo experiments we utilized the MEK1/2 inhibitor PD0325901 (Mirdametinib) formulated in 0.5% hydroxypropyl methylcellulose (viscosity 4000 centipoise) plus 0.2% Tween 80, and administered by oral gavage.

Solid ultrapure arsenic trioxide (As_2O_3; ATO; Sigma-Merck) was solubilized in a solution of 1 M sodium hydroxide (NaOH) and then diluted in PBS. The ATO solution was back titrated with HCl to pH 7.4 and subsequently filtered through a 0.22 µm filter Costar (Santa Clara, CA). The ATO solution was injected intraperitoneally with 3.75mg/Kg body weight; controls received a daily intraperitoneal injection of PBS (volume weight-determined). In PD plus ATO-treated mice PD was administrated 6 hours before ATO on a five days-a-week schedule for 3 consecutive weeks.

Imatinib was dissolved in sterile water and administered by intraperitoneal injection at 50 mg/kg twice daily for 3 consecutive weeks.

Mice were evaluated for weight loss and overall appearance everyday and the combination at the doses of PD 10mg/kg plus ATO 3.75mg/kg was well tolerated in vivo.

After twenty days of treatment or when mice were moribund, three mice from each group were killed by cervical dislocation and spleens and livers from 20-days treated mice were rapidly harvested and processes for histopathological, immunohistochemical and molecular analysis. Kaplan-Meier survival curve was evaluated from first day of treatment until death or sacrifice (mice were killed when moribund).

Histology and Immunohistochemistry

Necropsies were performed on all subjects and specimens of neoplasia, splanchnic organs and femur bone marrow were collected and formalin-fixed/paraffin embedded. 5 µm thick sections were obtained from samples and hematoxylin and eosin (H&E) stained to evaluate the morphology of tissues and neoplastic cells; the expression of Ki-67 and Cleaved Caspase-3 was evaluated with avidin-biotin-complex (ABC method) according to manufacturer's protocol.

During the treatment with PD and/or ATO, blood samples were collected from the tail vein of mice for differential leukocyte counts once a week. White Blood Cells (WBC) differential counts were performed by microscopy examination on May-Grünwald-Giemsa stained blood films[3].

Slides were examined with Nikon Eclipse E800 microscope (Nikon Corporation, Japan) using Nikon PLAN APO lenses. Sections were photographed at 2x, 10x and 20x (Nikon PLAN APO lenses) with Camera DIGITAL SIGHT DS-Fi1 (Nikon Corporation); pictures were acquired with DS Camera Control Unit DS-L2 (Nikon Corporation) and stored in USB device.

Immunocytochemistry

Immunofluorescence microscopy analysis of ABL1 was performed on K562-R plated on a glass poly-L-lysinate slide by centrifugation with Shandon Cytospin 2 and fixed with 4% paraformaldehyde in PBS for 10 minutes. Cells were then permeabilized in a solution of 0.01% Triton X-100 in PBS for 3 minutes and washed in three changes of PBS. Cells were treated for 30 minutes with 3% BSA in PBS to block unspecific binding sites and incubated with ABL1 antibody (1:200) overnight at 4°C in a dark chamber, followed by Alexa Fluor 594 goat anti-Mouse IgG antibody (2µg/ml), diluted with BSA 3% in PBS for 60 minutes at room temperature in a dark chamber. Nuclei were counterstained with DAPI; the slides were mounted with an aqueous mounting medium. Between each step the slides

were washed with BSA 1% in PBS (three changes for 5 minutes each). The 100x images were captured using the Nikon Eclipse E600 Fluorescence Microscope (Nikon Corporation, Japan) with the Nikon Microscope 100x CFI Plan Fluor Oil Objective and were photographed with DS-Fi2 camera (Nikon); pictures were then acquired and analyzed with the NIS-Elements Microscope Imaging Software (Nikon).

Statistical analysis

The Chou-Talalay method and Calcusyn software (Biosoft, Ferguson, MO) were used to assess synergistic or additive or antagonist effects of combined therapies.

For multiple comparisons a statistical analysis was performed using analysis of variance for repeated measurements followed by a Tukey-Kramer or Dunnet post-tests using JMP version 7.0 statistical software (SAS Institute, Cary, NC).

Survival curves were derived by the Kaplan-Meier method and compared using the log-rank test, and P values for the log rank statistic were adjusted for multiple testing by the Bonferroni method (5 groups, 10 pairwise comparisons) using JMP software.

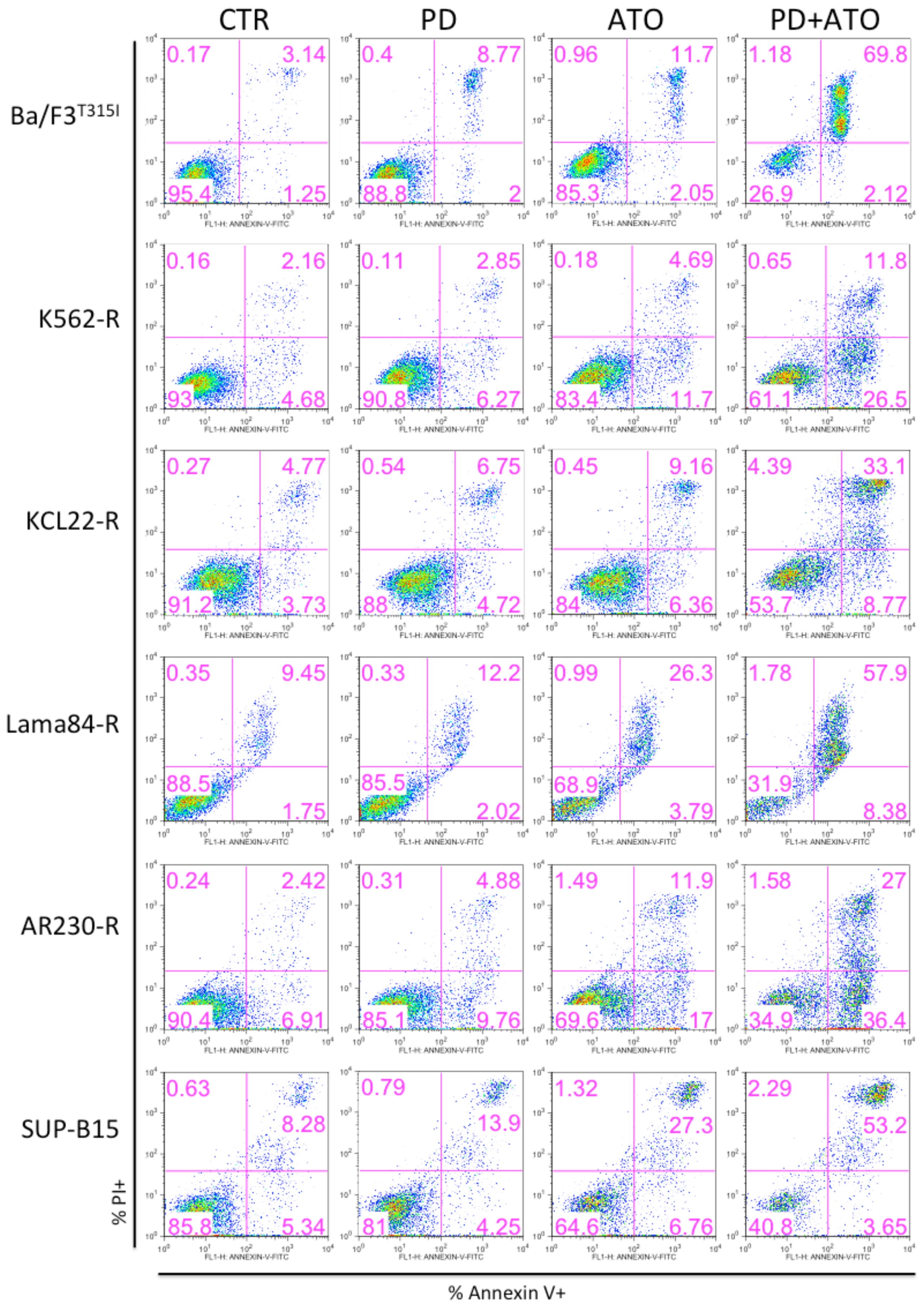
Supplemental References

1. Bain J, Plater L, Elliott M, et al. The selectivity of protein kinase inhibitors: a further update. Biochem J. 2007;408(3):297-315. doi: 10.1042/BJ20070797.

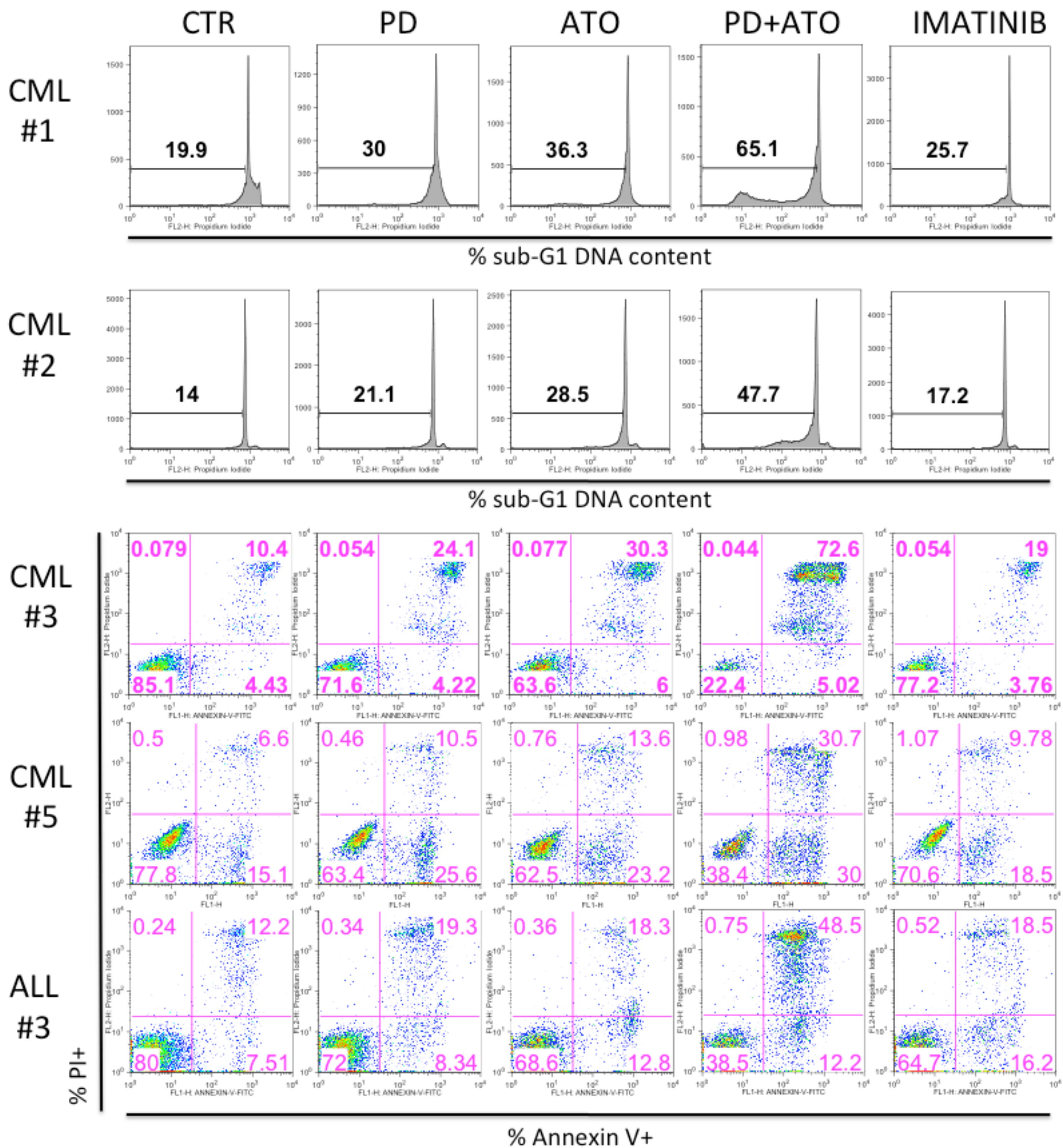
2. Podszywalow-Bartnicka P, Kominek A, Wolczyk M, Kolba MD, Swatler J, Piwocka K. Characteristics of Live Parameters of the HS-5 Human Bone Marrow Stromal Cell Line Cocultured with the Leukemia Cells in Hypoxia, for the Studies of Leukemia–Stroma Cross-Talk. Cytometry A. 2018;93(9):929-940

3. Camacho RE, Wnek R, Fischer P, et al. Characterization of the NOD/scid-[Tg]DR1 mouse expressing HLA-DRB1*01 transgene: a model of SCID-hu mouse for vaccine development. Exp Hematol. 2007;35(8):1219-30.

Supplemental Figure 1A

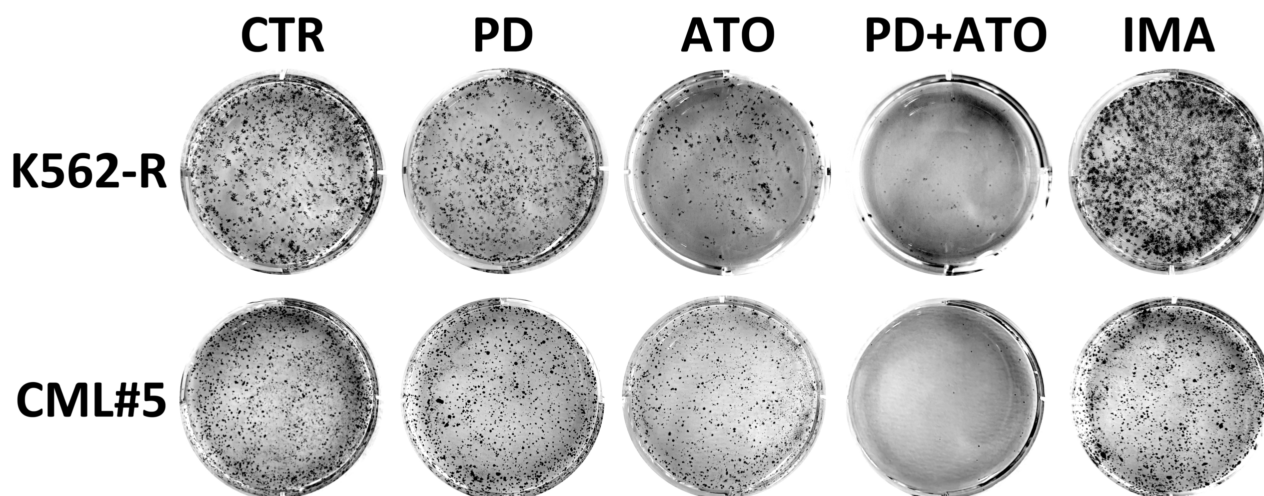


Supplemental Figure 1B



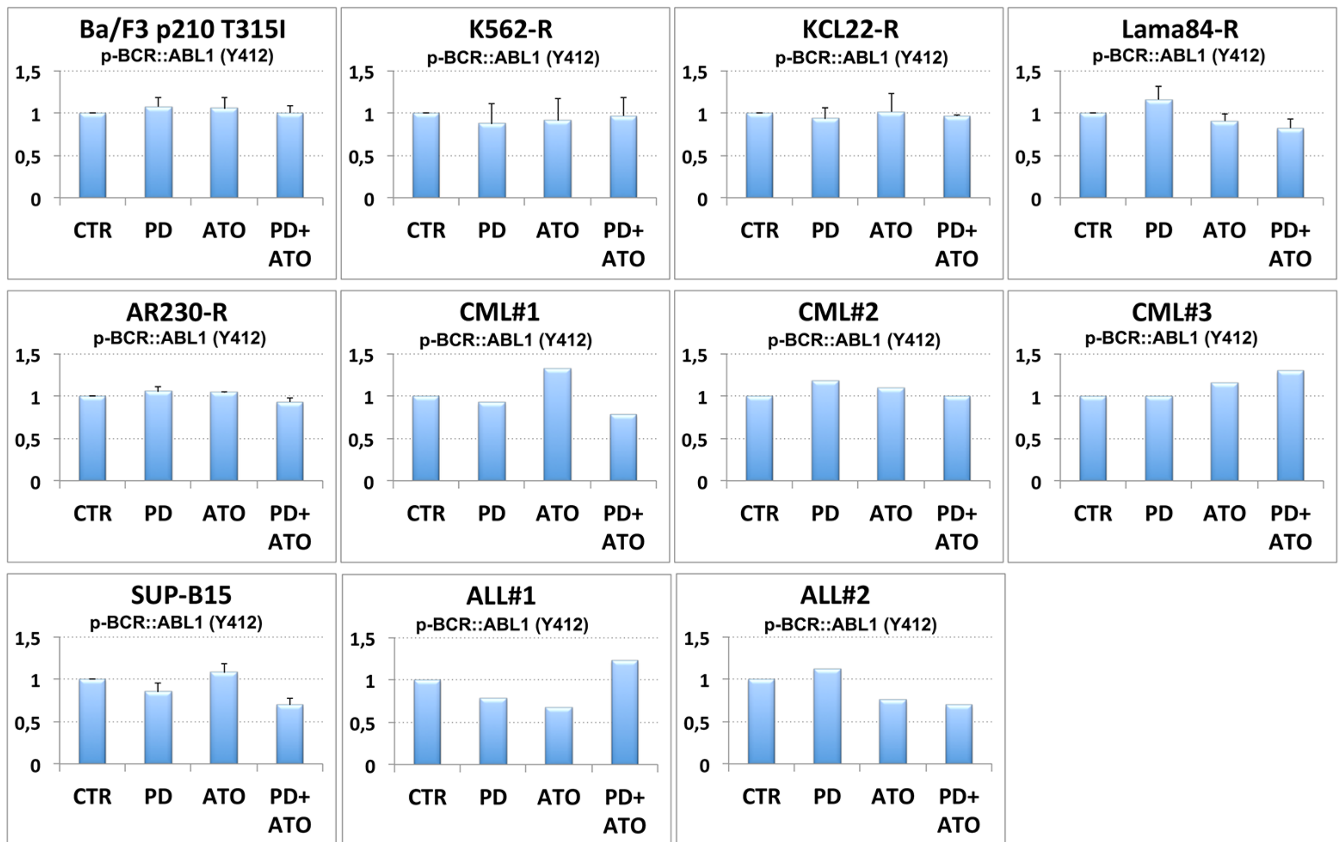
Relative levels of cell death measured by Annexin V-FITC/PI staining or by sub-G1 DNA content in Imatinib-resistant CML and Ph+ ALL cell lines **(A)** and primary leukemic cells from patients with Ph+ CML and ALL (CML#1, CML#2, CML#3, CML5; ALL#3 **(B)** following sequential treatment with PD0325901 (0.5 µM) and ATO (2 µM). To confirm their Imatinib-resistance ex vivo, patient-derived leukemic cells were separately treated with Imatinib (1 µM).

Supplemental Figure 2



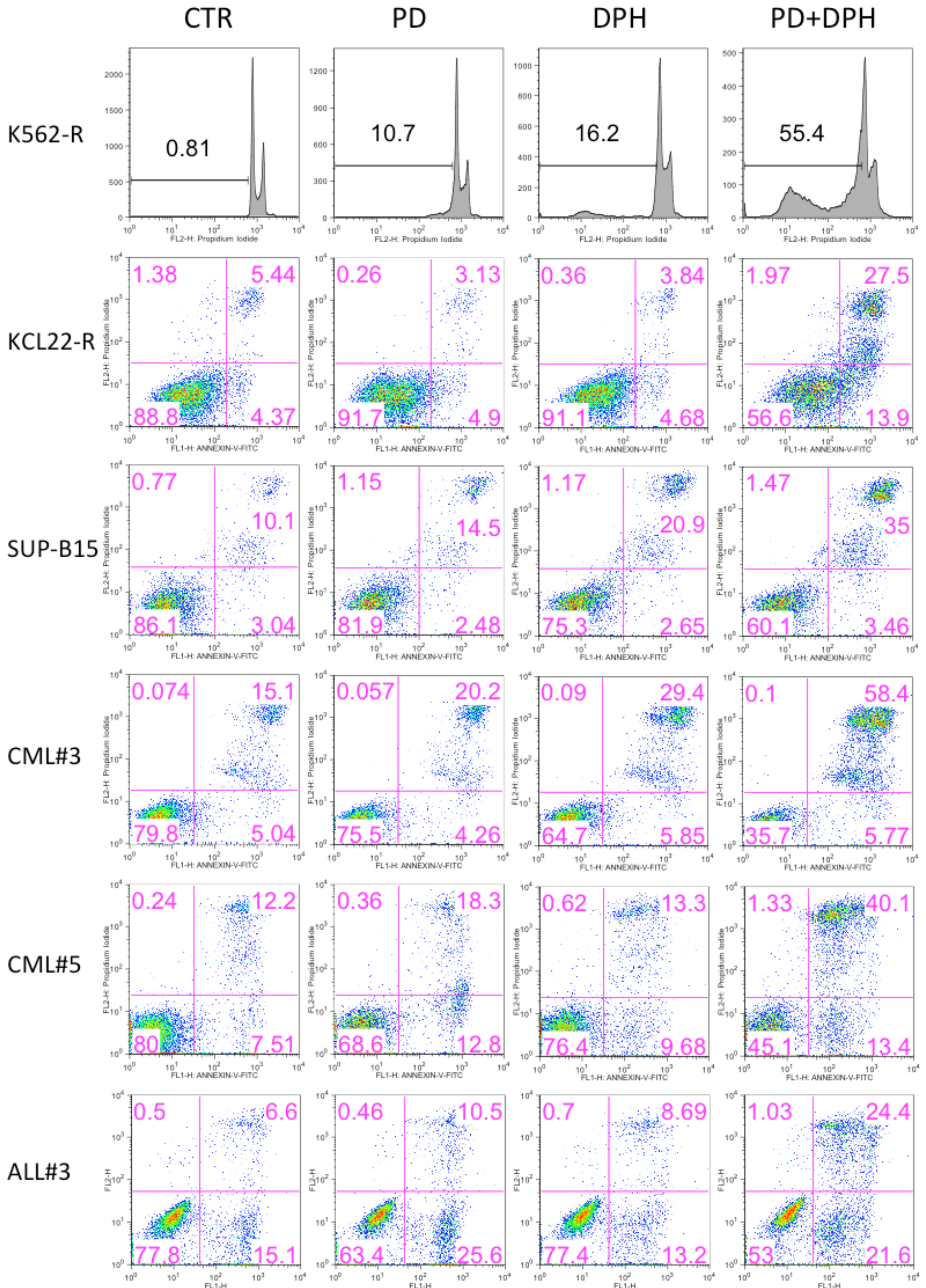
Representative images of cell colony formation assay on K562-R cells and sample CML#5 grown in semi-solid methylcellulose medium (10^3 cells/ml) in presence of PD0325901 (0.5 µM), ATO (1 µM for CML cell line, 0.5 µM for primary sample) or the combination PD+ATO. Live colonies were detected after 10 days (CML cell line) or 14 days (primary sample) of culture by MTT (1 mg/ml) staining.

Supplemental Figure 3



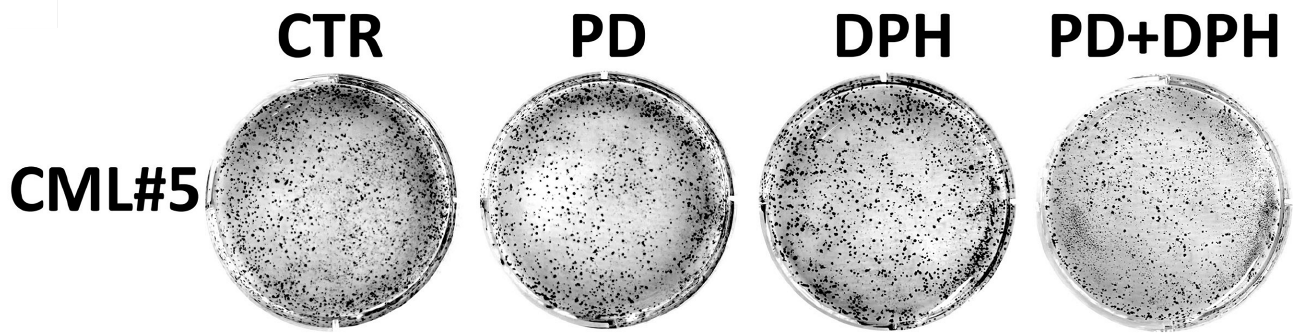
Histogram represents the densitometric analysis of phospho-BCR::ABL1 (Tyr412) western results showed in Figure 2A of the manuscript. The levels of phosphorylated BCR::ABL1 were normalized to total BCR::ABL1. The relative fold change of protein levels was normalized with respect to the level of the untreated control, which was taken as 1.

Supplemental Figure 4



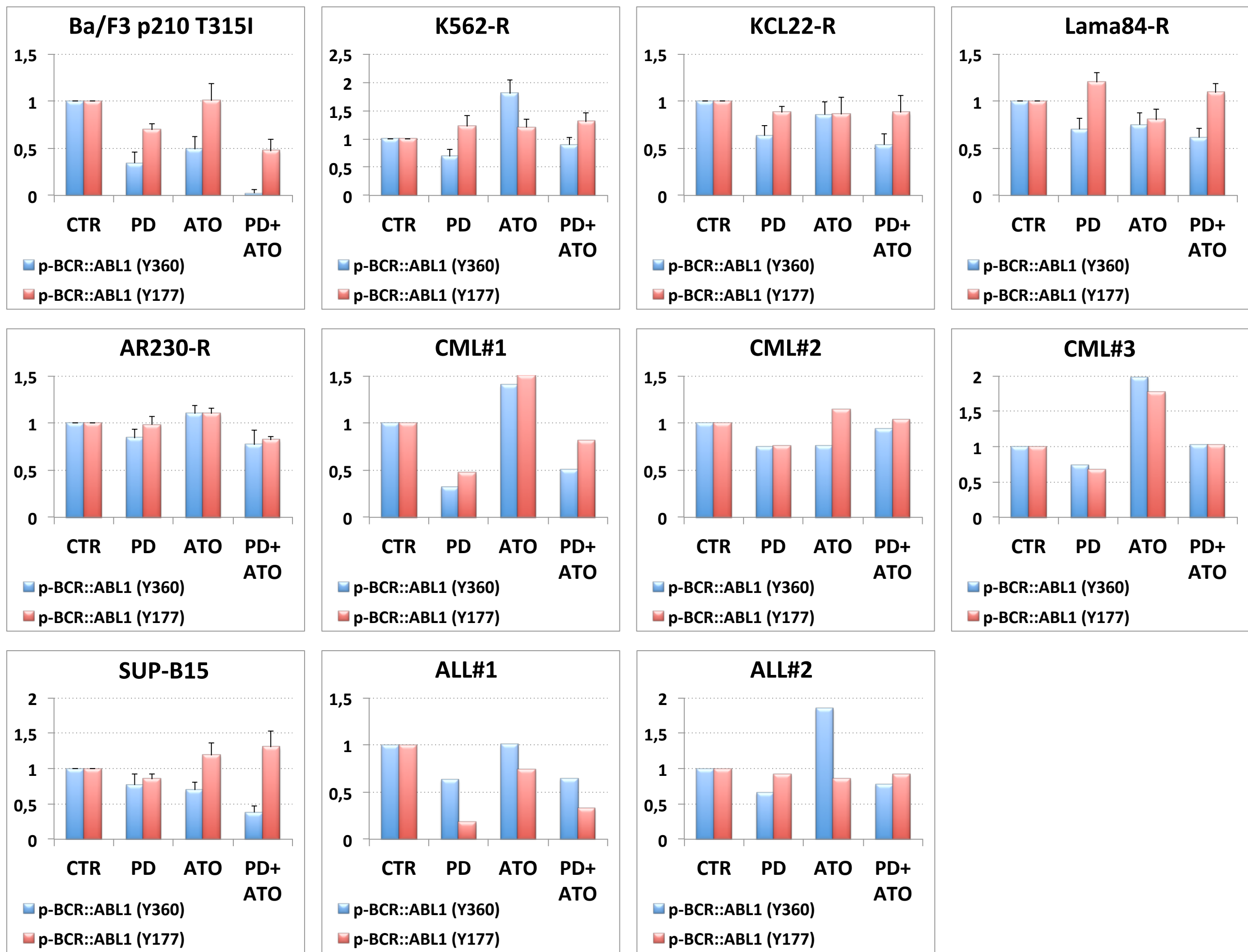
Relative cell death established by sub-G1 DNA content or Annexin V-FITC/PI staining in K562-R, KCL22-R and SUP-B15 cell lines and primary leukemic cells from patients CML#3, CML#5 and ALL#3 after 24h (for SUP-B15 and primary cells) or 48h (for K562-R and KCL22-R) of sequential treatment with PD0325901 (0.5 µM) and the ABL1 activator DPH (10 µM).

Supplemental Figure 5



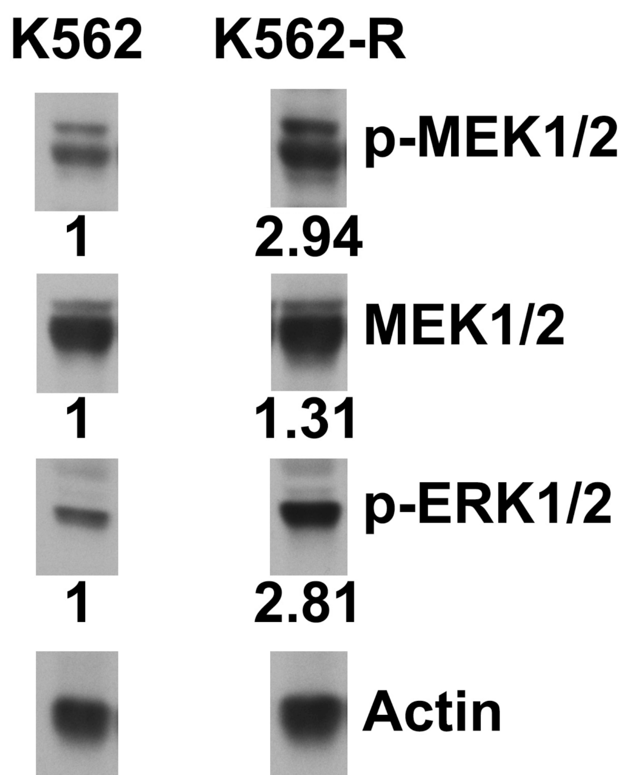
Primary leukemic cells from CML#5 (representative images) were seeded in semi-solid methylcellulose medium (10^3 cells/ml) in presence of PD0325901 (0.5 µM), DPH (10 µM) or the combination PD+DPH. Live colonies were detected after 14 days of culture by MTT (1 mg/ml) staining and counted using ImageJ quantification software.

Supplemental Figure 6



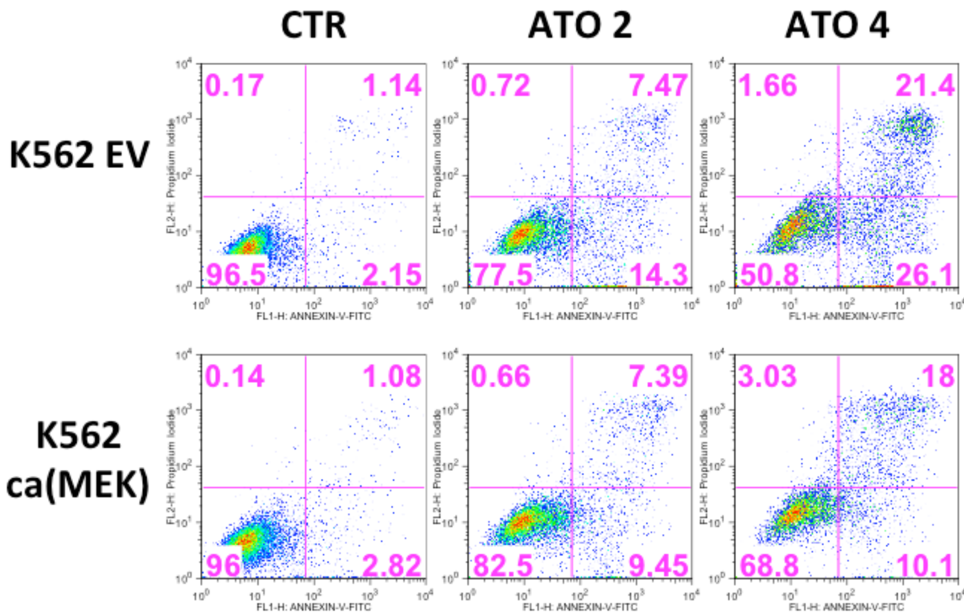
Histogram represents the densitometric analysis of phospho-BCR::ABL1 (Tyr360) and phospho-BCR::ABL1 (Tyr177) western results showed in Figure 4A of the manuscript. The levels of phosphorylated BCR::ABL1 were normalized to total BCR::ABL1. The relative fold change of protein levels was normalized with respect to the level of the untreated control, which was taken as 1.

Supplemental Figure 7

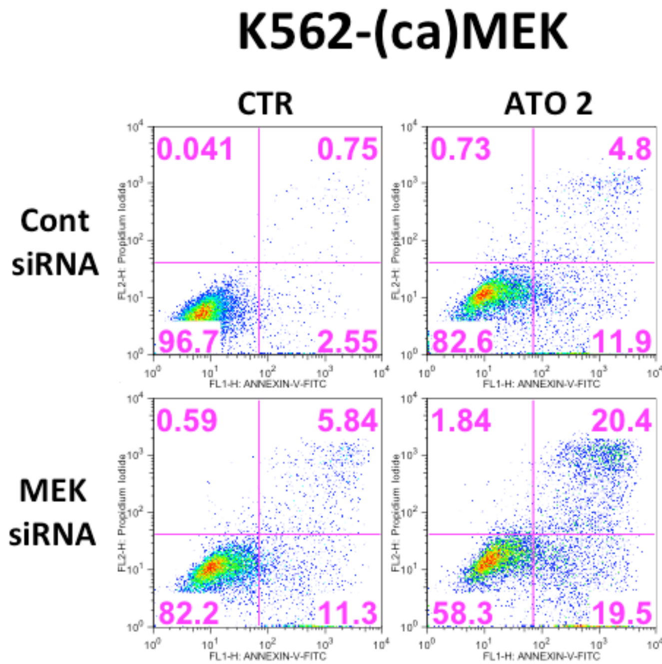


Immunoblotting for phospho-MEK1/2 (Ser217/221), MEK1/2, phospho-ERK1/2 (Thr202/Tyr204) and Actin on Lysates of Imatinib-sensitive K562 and -resistant K562-R cells. Nonadjacent bands from the same blot with an identical exposure were juxtaposed to facilitate comparisons. Bands were then subjected to densitometric scanning and the relative fold change of protein levels was normalized with respect to the level of K562 lysates, which was set as 1.

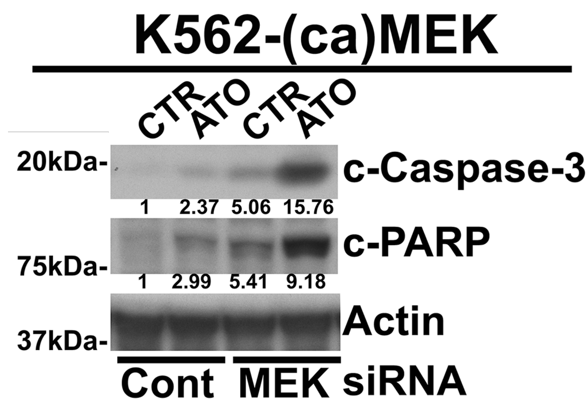
Supplemental Figure 8A



Supplemental Figure 8B

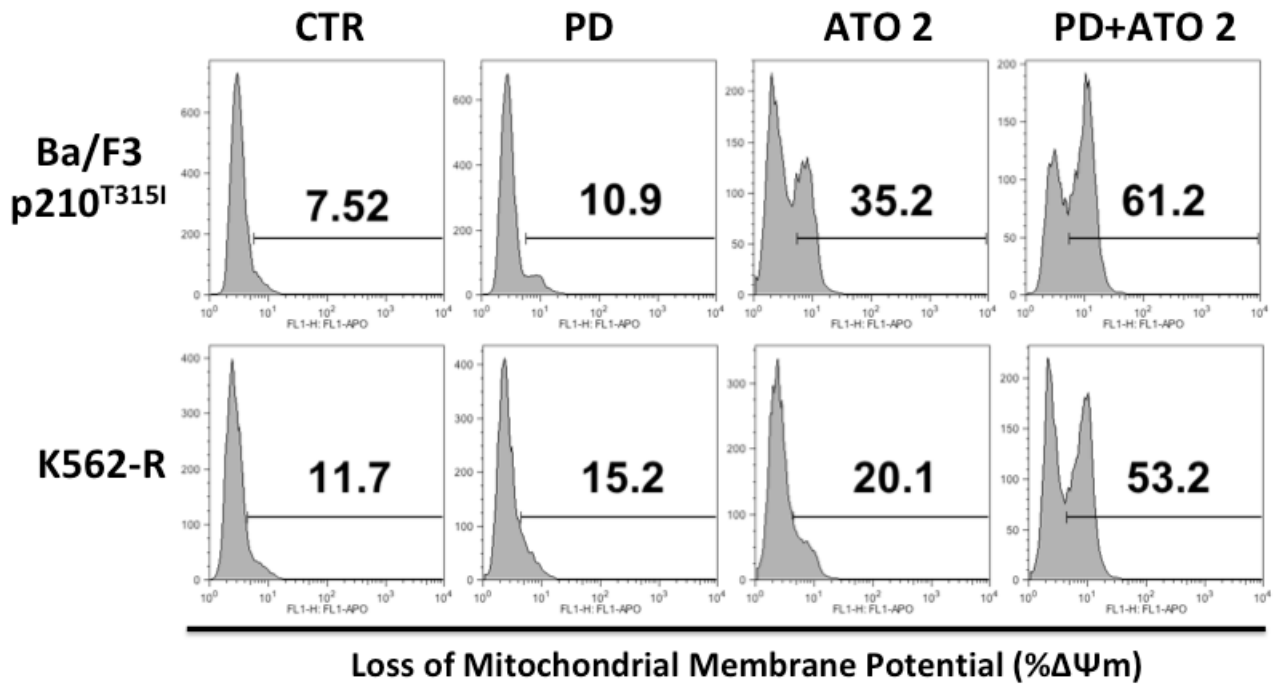


Supplemental Figure 8C



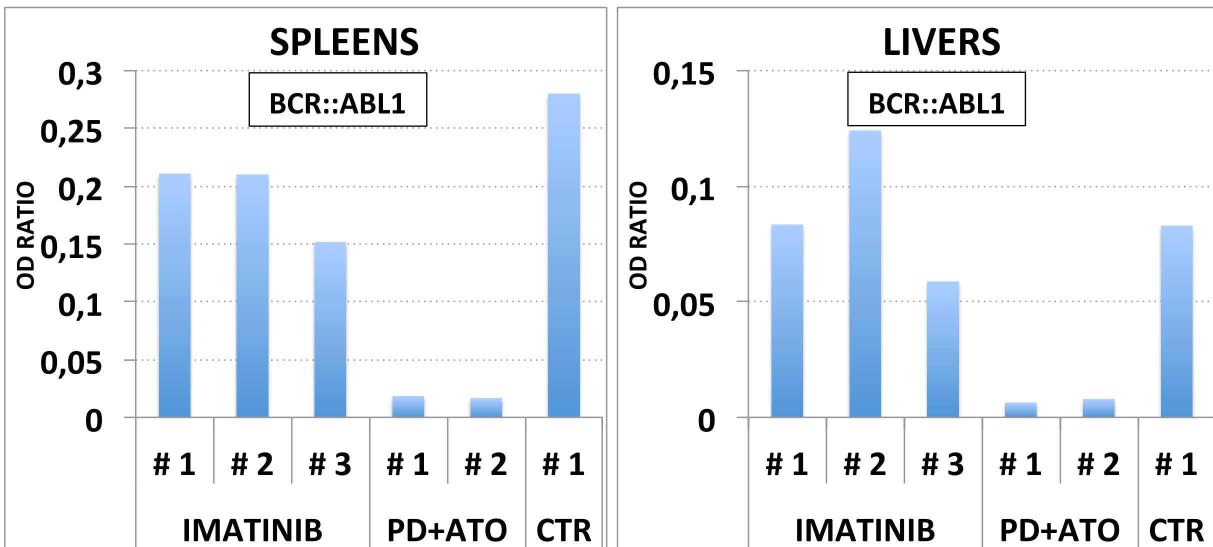
(A) Relative levels of apoptosis revealed by Annexin V-FITC/PI staining in stable clones of K562 cells transfected with empty vector (EV) or with plasmid expressing Activated MEK1 [(ca)MEK] after 72h of ATO treatment (2 and 4 μM). **(B)** K562-(ca)MEK cells were then electroporated with control siRNA or with MEK1/2 siRNA and treated with ATO (2 μM). After 48h the relative levels of apoptotic cell death was revealed by Annexin V-FITC/PI staining. **(C)** Western blot analysis of Cleaved caspase-3, cleaved PARP and Actin from K562-(ca)MEK cells electroporated with control siRNA or with MEK1/2 siRNA and treated with ATO (2μM) for 24h; Cleaved caspase-3, cleaved PARP and Actin are from the same blot.

Supplemental Figure 9

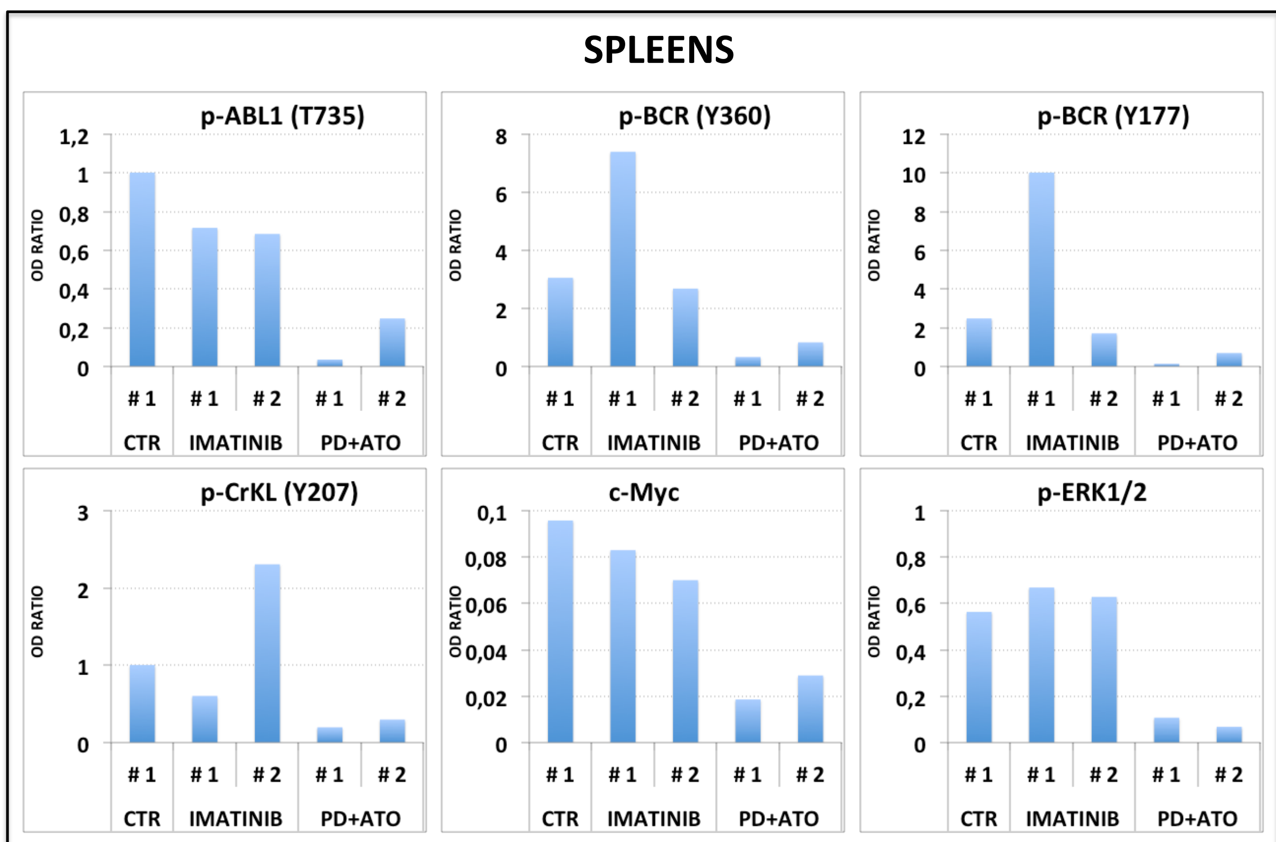


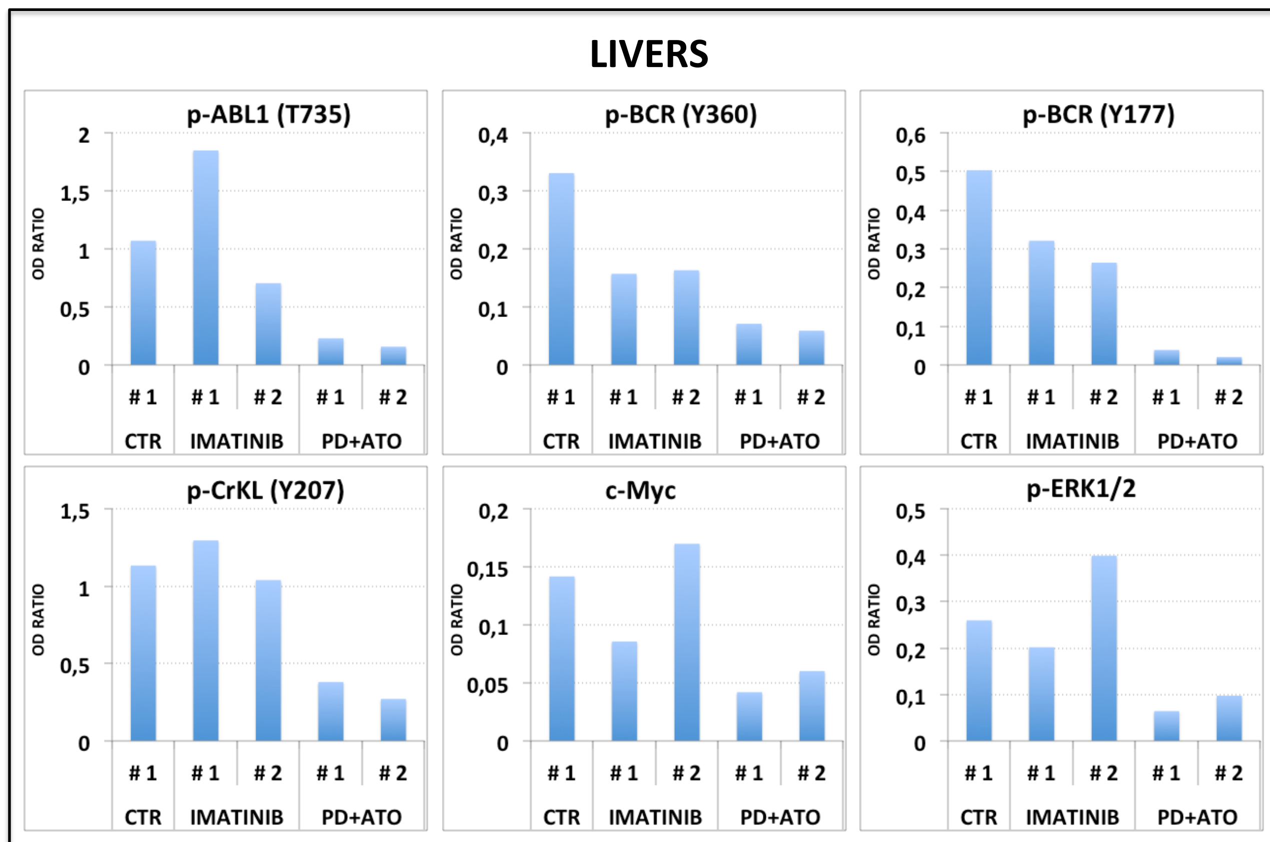
Ba/F3p210^{T315I} cells and K562-R were treated with PD0325901 (0.5 µM) and ATO (2 µM) alone or in combination and after 48h of treatment the percentage of cells displaying loss of mitochondrial membrane potential (ΔΨm) was determined by flow cytometry.

Supplemental Figure 10A



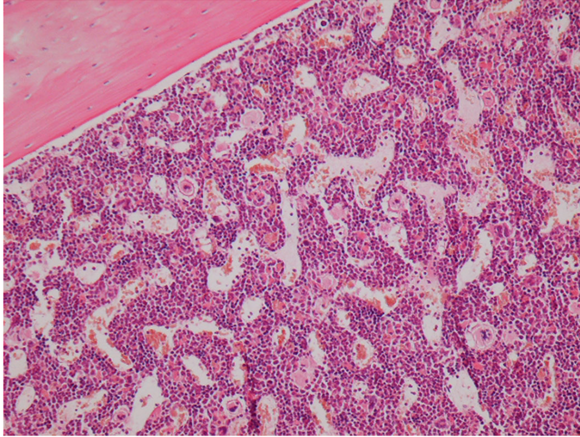
Supplemental Figure 10B

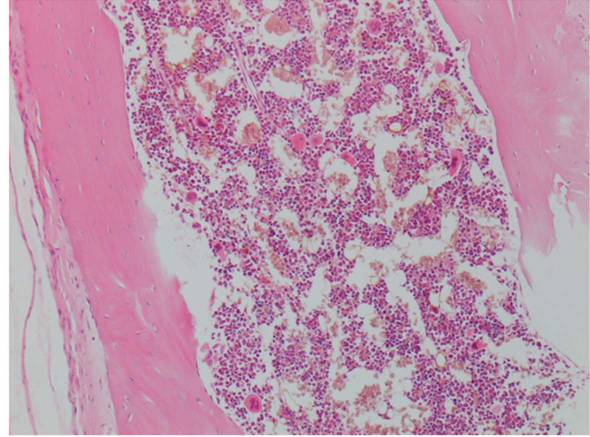




(A) Histograms show densitometry results from western blot analysis of BCR::ABL1 in spleens and livers from mice receiving vehicle, PD+ATO or Imatinib treatments shown in Figure 7A of the manuscript. **(B)** Histograms show densitometry results from western blot analysis of phospho-ABL1 (Thr735), phospho-BCR (Tyr360), phospho-BCR (Tyr177), phospho-CrkL (Tyr207), c-Myc and phospho-ERK1/2 (Thr202/Tyr204) in spleens and livers from mice receiving vehicle, Imatinib or PD+ATO treatments shown in Figure 7B of the manuscript.

Supplemental Figure 11





Bone Marrow from representative untreated or PD+ATO-treated mice were analyzed by hematoxylin and eosin staining (original magnification x10).