The new small tyrosine kinase inhibitor ARQ531 targets acute myeloid leukemia cells by disrupting multiple tumor-addicted programs

Debora Soncini,[1] Stefania Orecchioni,[2] Samantha Ruberti,[1] Paola Minetto,[1,3] Claudia Martinuzzi,[1] Luca Agnelli,[4] Katia Todoerti,[4] Antonia Cagnetta,[1,3] Maurizio Miglino,[1,3] Marino Clavio,[1,3] Paola Contini,[5] Riccardo Varaldo,[6] Micaela Bergamaschi,[1] Fabio Guolo,[1] Mario Passalacqua,[7] Alessio Nencioni,[5] Fiammetta Monacelli,[5] Marco Gobbi,[1,3] Antonino Neri,[4] Giovanni Abbadessa,[8] Sudharshan Eathiraj,[8] Brian Schwartz,[8] Francesco Bertolini,[2] Roberto M. Lemoli[1,3] and Michele Cea[1,3]

[1]Chair of Hematology, Department of Internal Medicine and Specialities (DiMI), University of Genoa, Genoa, Italy; [2]Laboratory of Hematology-Oncology, European Institute of Oncology IRCCS, Milan, Italy; [3]IRCCS Ospedale Policlinico San Martino, Genoa, Italy; [4]Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy; [5]Department of Internal Medicine and Specialities (DiMI), University of Genoa, Genoa, Italy; [6]Division of Hematology and Hematopoietic Stem Cell Transplantation Unit, Ospedale Policlinico San Martino, Genoa, Italy; [7]Department of Experimental Medicine (DIMES), University of Genoa, Genoa, Italy and [8]ArQule, Burlington, MA, USA

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Correspondence: *MICHELE CEA* - michele.cea@unige.it

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell lines

The AML cell lines ML2, MOLM-14, MV4-11, HL60, NOMO-1, OCI-AML2, OCI-AML3 and NB4 were provided by collaborators or purchased from ATCC or DSMZ (Braunschweig, Germany). All cell lines were cultured in RPMI-1640 medium containing 10% FBS (GIBCO, Life Technologies, Carlsbad, CA), 2 μM l−1 glutamine, 100 U ml−1 penicillin, and 100 μg ml−1 streptomycin (GIBCO, Life Technologies, Carlsbad, CA). 293T cell line was purchased from ATCC and cultured in DMEM containing 10% FBS (GIBCO, Life Technologies, Carlsbad, CA), 4mM glutamine, 50 U ml/m1 penicillin, and 50 U/ml streptomycin (GIBCO, Life Technologies, Carlsbad, CA).

Primary cells

All studies involving human samples were performed under Policlinico San Martino Hospital (Genoa, Italy) IRB committee-approved protocols, after informed consent; de-identified samples were utilized. Primary leukemic cells were obtained from peripheral or bone marrow blood of 13 AML patients at diagnosis, before treatment. (Table 1) (1) The percentage of leukemic blasts was always > 90%. Normal $CD34^+$ cells (HSCs) were isolated from the bone marrow (BM) of healthy donors using anti-CD34 magnetic-activated microbeads and MiniMacs high-gradient magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany). This strategy generated highly purified $CD34^+$ cells (> 90%, as confirmed by FACS staining). Cells were either used immediately for viability assays or for mRNA isolation, or stored at −80°C in medium containing 50% FBS and 10% DMSO. Normal or AML MSCs, isolated as previously described, (2) were seeded in 96 well plates 1 × 104/well, AML cells were added to the confluent layer at 10:1 ratio. After overnight incubation, cells were used as indicated. Normal mononuclear cells (MNCs), obtained from the BM of healthy donors were isolated by Ficoll-Hypaque centrifugation (Amersham Bioscience, Piscataway, NJ, USA).

Cell Viability and Apoptosis Assay

Cell viability was assessed by using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA), as previously described. (3) Apoptosis was evaluated by flow cytometric analysis following Annexin V–FITC (BD Biosciences, 556419) and PI (BD Biosciences, 51-6621E) staining, according to manufacturer's instructions. The percentage of cells undergoing apoptosis was defined as the sum of early apoptotic (annexin V+PI−) and late apoptotic (annexin V+PI+) cells.

RNA Seq processing, differentially expressed genes detection and gene set enrichment analysis

OCI-AML3 cells were treated with 1 μM ARQ 531 or vehicle control for 12 hours. RNA was prepared as mentioned earlier. A starting amount of 500 ng of RNA was used to prepare polyadenylate-enriched, single bar–coded libraries using the NEBNext Kit. Quality control of the libraries was evaluated by Bioanalyzer analysis with High Sensitivity chips (Agilent Technologies). Sequencing was performed on a HiSeq 2500 (Illumina) by 2× 50–base pair paired-end reads at the Biopolymers Facility of Harvard Medical School. We used Bcbio-next gen (https://github.com/bcbio/bcbio.nextgen/) to process the RNA-seq data. Briefly, *cutadapt* (https:// github.com/marcelm/cutadapt/) was used to trim adapters; trimmed reads were aligned to human reference genome (GRCh37) by tophat2; and read counts for each gene were calculated by HTSeq under standard parameters. Genes with low expression (fragments per kilobase million, FPKM, <1 across all samples) were filtered out. *Degust* (http://degust.erc.monash.edu/) was used for data visualization and differential analysis using *edgeR.* GSEA (https://software.broadinstitute.org/gsea/) was used on the pre-ranked gene lists, by applying 1000 permutations and using a weighted statistic enrichment. Significant enriched gene sets (16) were selected with a threshold FDR<0.25. Gene sets were downloaded from the Broad Institute's MSigDB (https://software.broadinstitute.org/gsea/msigdb/).

Western blotting

Whole-cell lysates were prepared as previously described. (3) Protein concentrations were determined by Bradford assay (Bio-Rad, CA), and equivalent amounts (40μg) were subjected to SDS-PAGE, transferred to PVDF membranes immunoblotted with following antibodies: anti-GAPDH (#5174, Cell Signaling Technology), -phospho-Histone H2A.X (Ser139) (#05-636, Millipore), -RAD51 (#588-B01P, Novus Biologicals), -Phospho-Btk (Tyr223) (#5082, Cell Signaling Technology), -BTK (#8547, Cell Signaling Technology), -γ tubulin (#MA1-850, ThermoFischer Scientific), -PARP (#9532, Cell Signaling Technology), -Caspase3 (#9662, Cell Signaling Technology), -Bcl2 (#sc-509, Santa Cruz Biotechnology), -Mcl1 (#sc-819, Santa Cruz Biotechnology), -Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#4370, Cell Signaling Technology), -ERK1/2 (#9102, Cell Signaling Technology), Phospho-Akt (Ser473) (#4058, Cell Signaling Technology), AKT (#9272, Cell Signaling Technology), phospho-c-Raf (Ser338) (#9427, Cell Signaling Technology), phospho-MEK1/2 (Ser217/221) (#9154, Cell Signaling Technology), c-MYC (#9402, Cell Signaling Technology), Cyclin D1 (#2978, Cell Signaling Technology), Cyclin D3 (#2936, Cell Signaling Technology), Phospho-eIF4E (Ser209) (#9741, Cell Signaling Technology), Non-phospho-4E-BP1 (Thr46) (#4923, Cell Signaling Technology), Phospho-eIF2α (Ser51) (#3398, Cell Signaling Technology), Phospho-p70 S6 Kinase (Thr389) (#9206, Cell Signaling Technology), ASCT2 (#8057, Cell Signaling Technology), GLUT1 (#12939, Cell Signaling Technology), RAD51 (#sc-8349, Santa Cruz Biotechnology), c-MYB (clone 1-1) (#05-175, Millipore). Band intensities were quantified by Quantity One SW software (Bio-Rad Laboratories, Inc) using standard ECL Western Blotting Detection Reagents (Thermo Fisher Scientific, IL). Densitometric analysis of western blots was carried out using ImageJ software version 1.48 (National Institute of Health).

Lentiviral mediated gene transfer

pLV sh SCRAMBLE and pLV shBTK lentiviral vectors were purchased from Vector Builder (Vector Builder Inc., Santa Clara, USA) . For lentiviral transduction, 1×10^6 293T cells were plated on 60 mm Petri dishes and allowed to adhere for 24 h. Thereafter, cells were transfected with 1μg of lentiviral plasmidic DNA and 700ng of each of three packaging vectors (pRP CMV VSVG; pRP CMV gag:pol RRE; pRP CMV RSV Rev), using TransIT-293 (Mirus Bio, Madison, WI) according to the manufacturer's instructions. 48 and 72h after transfection, the supernatant containing lentiviral particles was harvested, filtered with a 0.45-μm-diameter filter, and used to infect 1.5×10^6 AML cells. AML cells were spinoculated at 750g for 45 min in presence of 8 μg ml−1 polybrene, (Santa Cruz Biotechnologies, CA), incubated with viral supernatant for 6 h and left overnight in normal culturing medium. The day after, a second cycle of infection was performed. Successfully infected cells were selected using a suitable concentration of puromycin (1 μg ml−1). 48 and 72 h after selection, the transduction efficiency was approximated by counting the proportion of cells expressing the fluorescent protein (GFP) using a fluorescence microscope (Nikon Eclipse 80i, Nikon, Melville, NY); and the knockdown efficiency was validated by protein level with WB analysis. Functional studies were performed as described below.

RNA Extraction and RT PCR.

Total RNA was extracted from cells using RNeasy Plus mini kit (Qiagen S.r.l., Milan, Italy) according to the manufacturer's instructions. 1 μg RNA was reverse transcribed in a final volume of 100 μl using High Capacity cDNA Reverse Transcription kit (Life Technologies, Monza, Italy). 5 μl of the resulting cDNA were used for qPCR with a QuantStudio5 Real-Time PCR (Applied Biosystems by Life Technologies, Monza, Italy). Primer sequences were as follows: MYB Fw 5'-CAAGCTCCGTTTTAATGGCAC-3', Rev 5'-ATCTTTCCACAGGATGCAGG -3' ; GAPDH Fw 5'-TCTCCTCTGACTTCAACAGCGAC-3', Rev 5'-CCCTGTTGCTGTAGCCAAATTC-3'. mRNA levels were detected using SYBR Select Master Mix (Applied Biosystems, Italy) according to the

manufacturer's protocol. Gene expression was normalized to housekeeping gene expression (GAPDH). Comparisons in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

Colony forming unit (CFU) assay

Immediately after their purification, $CD34^+$ HSCs were plated in HSC004 methylcellulose medium (R&D) supplemented with EPO 3 IU/ml and IL-6 20ng/ml at a concentration of $1x10^3$ cells per plate and treated (in duplicate) with different concentrations of ARQ531 or DMSO as control. All plates were incubated at 37°C, 5% CO2 for 10-14 days before counting the number of colonies.

BMSC conditioned media

10×10^5 Healthy donor- or Patient derived-BMSC were plated in 6 well plates and left to adhere for 24 hours. The day after, medium was replaced with 2 ml of complete RPMI-1640 and cells were cultured for 5 days. Thereafter, BMSC-conditioned media were collected, filtered with 0,45 µM filters and immediately used for AML cells resuspension. Finally, drugs or vehicle were added at 20X concentration, in order to not dilute conditioned media. After 48 hours cell viability was measured as indicated.

Tumor cell-specific bioluminescence imaging in co-cultures with stromal cells

$7x10^3$ $Luciferase^+$ AML cells (OCI-AML2 Plv sv40 GFP/luc+) were plated in 96-well optical white plates (Corning, Cat.No.3903) in the presence or absence of pre-plated $luciferase^-$ primary stromal cells (20×10^3 cells seeded 24hours before) and treated with drugs or veichle (DMSO), as indicated in each experiment. After 48 h of treatment, AML cell specific viability was assessed with Nano-Glo® Live Cell Assay System (Promega, Cat.No. N2011)

Nucleofection

HL60 cells were transfected by using the 4D-Nucleofector™ System (Lonza), according to manufacturer's instruction. Small interfering RNAs (siRNAs) targeting human BTK (ON-TARGET plus SMART pool, #L-003107-00-0005) and a non-targeting negative control (ON-TARGET plus non-

targeting pool #D-001810-10-05) were purchased from Dharmacon. siRNAs targeting human Myc or Myb were purchased from ThermoFisher Scientific (Myc siRNA#1 Dharmacon J-003282-23; Myb ThermoFisher Scientific #AM16708, pool of siRNA ID 115653 and 10768); #AM16708, pool of siRNA ID 115653 and 107687). For each nucleofection, 2 x 10^6 cells was pulsed with the EN-138 program, using Amaxa SF Cell line 4-D Nucleofector X KitL (Cat.No. V4XC-2024, Lonza). In this procedure, all siRNAs were used at the final concentrations of 500 nM. After 24h from nucleofection, cells were cultured and then treated or collected for further experiments.

Immunofluorescence and focal microscopy

Cells were prepared as previously described, (3) using specific primary and secondary antibodies. The slides were then mounted with ProLong Gold Antifade reagent (Invitrogen, Life Technologies, Carlsbad, CA), and images were taken using a Leica TCS SP confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany), equipped with 476, 488, 543 and 633 excitation lines with a 60 x Plan Apo oil objective.

Human Data Sets.

Expression levels of BTK were obtained from the TCGA cohort among their low or high expression of BTK. (BloodPortal data of BTK probe 205504_at from U133 Plus 2.0 array).

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3. Cagnetta A, Soncini D, Orecchioni S, Talarico G, Minetto P, Guolo F, et al. Depletion of SIRT6 enzymatic activity increases acute myeloid leukemia cells' vulnerability to DNA-damaging agents. Haematologica. 2018 Jan;103(1):80-90.

Supplementary Figure Legends.

Supplementary Figure 1. BTK expression in AML cells. BTK mRNA expression in AML as compared to other cancers, on the basis of data from The Cancer Cell Line Encyclopedia (CCLE) database. Data are presented as mean log2 expression with range; in red are highlighted AML cell lines (n=39).

Supplementary Figure 2. Anti-tumor activity of ARQ531 is dependent of caspase activation. A) HL60, OCI-AML2 and primary AML-004 cells were treated with increasing doses of ARQ 531 (1-10 μM) or DMSO for 48 hours. Apoptotic cell death was measured by Annexin V/PI staining and flow cytometric analysis. The percentage of each group is shown as solid columns. Data are derived from 3 independent experiments. **B)** HL60 cells were pretreated with caspase inhibitors (zVAD-fmk 50 μM), for 2 hours and then incubated with ARQ531 at indicated concentrations for 48 hours. Specific cell death was then measured with MTS assay.

Supplementary Figure 3. ARQ531 and Ibrutinib inhibit AML cell migration in response to SDF-1. HL60 cells were pretreated with ibrutinib (500nM) or ARQ531 (500nM) for 1 h before wash-off and then placed in the upper well of a 8.0μM transwell plate. The lower chamber contained 500ul of serum free media supplemented with SDF1 (100 ng/ml) for 3 hours and then assessed for cell number using trypan blue staining. Data were normalized to DMSO treated cells.*** p=.001; **** p<.0001

Supplementary Figure 4. Molecular perturbation triggered by ARQ531 in OCI-AML3 cells. A) Principal component analysis (PCA) of control and ARQ531 treatment on OCI-AML3 cells. **B)** GSEA analysis was performed on the entire set of signatures available from the Molecular Signatures Database (MSigDB). **C)** mRNA levels of selected genes were analyzed by qPCR in OCI-AML3 cell lines treated with 0.3 and 1 μM of ARQ 531 or ibrutinib for 24 hours. The graph shows the fold change compared to untreated cells.

Supplementary Figure 5. The pro-survival MAPK pathway has a crucial role for anti-AML activity of ARQ 531. A) HL60 cells were treated with increasing concentrations of ARQ531 w/w 20% FBS. After 24 hours, cells were collected for western blot analysis. In parallel viability was measured by MTS assay after 48 hours of drug exposure. **B)** HL60 cells treated with ARQ531 or Ibrutinib in presence or not of 20% FBS for 24h were subjected to blot analysis for phospho-ERK1/2, ERK1/2, c-Myc and tubulin as shown.

Supplementary Figure 6. ARQ531 treatment enhances genomic instability of AML cells. A) 3 x 10^6 HL60 cells were treated with ARQ531 for 24hours, using Doxorubicin as positive control. Thereafter, cells were washed with PBS, fixed and stained. γH2AX foci and nuclei (Q-nuclear) were visualized by confocal microscopy. **B)** 2 x 10^5 HL60 cells were plated in 96 well plate and treated for 48 hours with indicated doses of ARQ531 (0.3 μM) in presence or not of DNA damaging agents including idarubicin and Ara-C at indicated doses. Cell viability was than measured by MTS assay. Combination index was calculated by CalcuSyn software and reported above the columns indicating specific co-treatment. Data are represented as mean +/− SD in all histograms (n = 2). **0.01<p<0.05; ***p < 0.001.

Supplementary Figure 7. ARQ531 targets BTK and Myb in AML cells. A) Treatment of BTK-KD HL60 cells with increased doses of Mebendazole (0.2-1.8 μM) resulted in higher anti-AML activity of this drug. **B)** BTK, MYC/MYB, and especially triple gene knockdown considerably reduced viability of HL60 cells as measured by Typan blue staining. Displayed are the mean of triplicates.

Supplementary Figure 8. MYB and BTK expression highly correlate in AML patients. A) Microarray data from GSE13204 database are expressed as histogram plots (25th-75th percentiles) for BTK (A) and MYB (B) expression in AML patients (n = 542) and ND (n = 73). The data were log2 transformed and median centered (dark lines). C) Microarray gene expression data from GSE13204 data sets was robust multiarray average normalized and the correlation between BTK and MYB expression

in AML patients was assessed by the Spearman rank-order correlation, where $P < .05$ was considered as statistically significant.

Supplementary Figure 9. ARQ531 inhibits tumor growth in AML patient-derived xenograft (PDX) mice model. Tumor engraftment was determined by flow cytometry in bone marrow (BM) and spleen at day 53. Evaluated markers: human CD45. Any significant difference was observed among groups.

Supplementary Figure 10. ARQ531screening analysis on Src-family kinases. Immunoblots for CSK, FGR, HCK, YES, FYN, LCK pSRC and tubulin on OCI-AML3 cells following ARQ531 treatment at 24 hours at indicated doses.

Figure S1

A

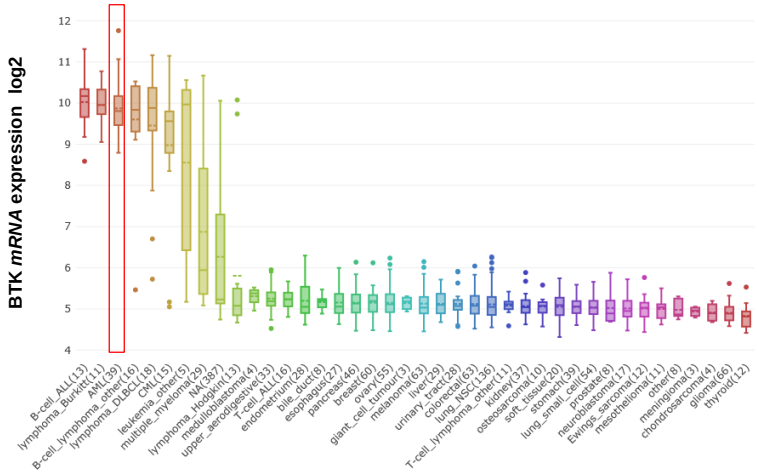
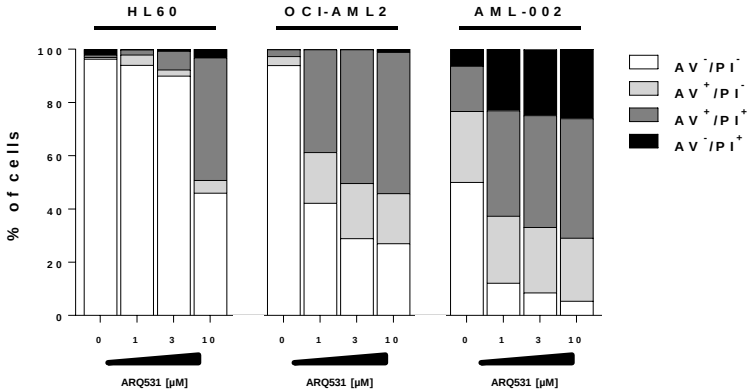

BTK mRNA expression log2
12
11
10
9
8
7
6
5
4
B-cell_ALL(13)
lymphoma_Burkitt(11)
AML(39)
B-cell_lymphoma_other(16)
lymphoma_DLBCL(18)
CML(15)
leukemia_other(5)
multiple_myeloma(29)
lymphoma_Hodgkin(13)
NA(387)
medulloblastoma(4)
upper_aerodigestive(33)
T-cell_ALL(16)
endometrium(28)
bile_duct(8)
esophagus(27)
pancreas(46)
breast(60)
ovary(55)
giant_cell_tumour(3)
melanoma(63)
liver(29)
urinary_tract(28)
colorectal(63)
lung_NSCL(136)
T-cell_lymphoma_other(11)
kidney(37)
osteosarcoma(10)
soft_tissue(20)
stomach(38)
lung_small_cell(54)
prostate(8)
neuroblastoma(17)
Ewings_sarcoma(12)
mesothelioma(11)
other(8)
meningioma(3)
chondrosarcoma(4)
glioma(66)
thyroid(12)

Figure S2

A


HL60
OCI-AML2
AML-002
% of cells
100
80
60
40
20
0
0 1 3 10
ARQ531 [µM]
AV-/PI-
AV+/PI-
AV+/PI+
AV-/PI+

B

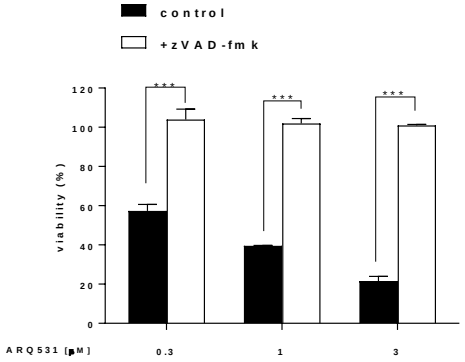
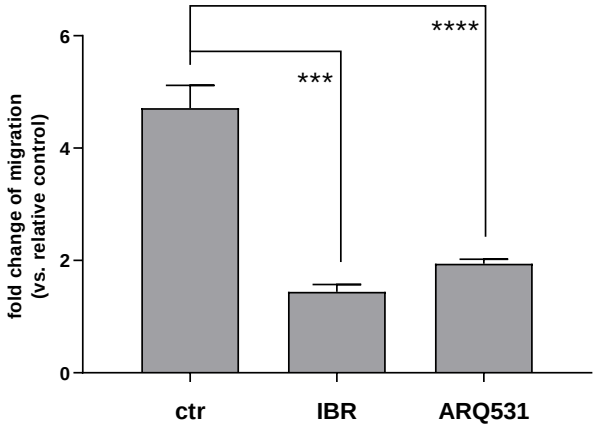
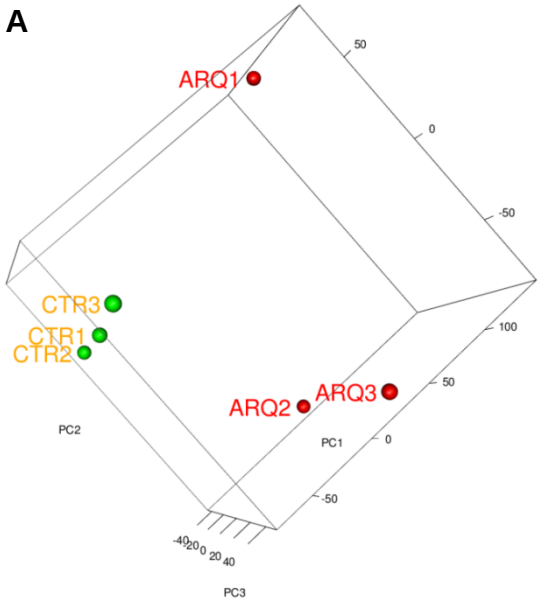

control
+zVAD-fmk
viability (%)
120
100
80
60
40
20
0
***
ARQ531 [µM]
0.3
1
3

Figure S3


fold change of migration
(vs. relative control)
6
4
2
0
***
****
ctr
IBR
ARQ531

A



B

Myc-dependent Gene Set	Size	ES	NES	FDR q-val
SCHUHMACHER_MYC_TARGETS_UP	39	-0,59	-2,97	<0.0001
HALLMARK_MYC_TARGETS_V1	87	-0,48	-3,01	<0.0001
HALLMARK_MYC_TARGETS_V2	33	-0,62	-2,81	<0.0001
MYC_UP.V1_UP	32	-0,62	-2,91	<0.0001
WEI_MYCN_TARGETS_WITH_E_BOX	166	-0,56	-3,99	<0.0001
MENSSEN_MYC_TARGETS	31	-0,61	-2,81	<0.0001
DANG_MYC_TARGETS_UP	38	-0,51	-2,57	<0.0001
KIM_MYC_AMPLIFICATION_TARGETS_UP	34	-0,53	-2,51	<0.0001
SCHLOSSER_MYC_TARGETS_AND_SERUM_RESPONSE_DN	26	-0,56	-2,37	0,001
SCHLOSSER_MYC_TARGETS_REPRESSED_BY_SERUM	43	-0,46	-2,31	0,002

C

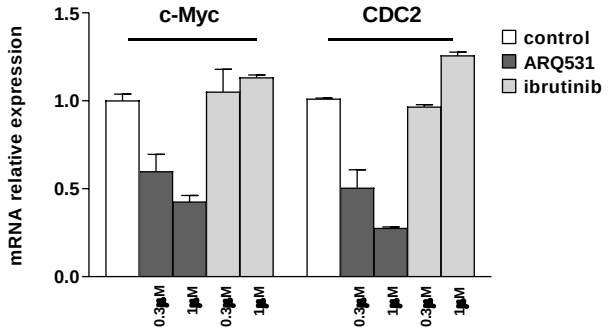


Figure S5

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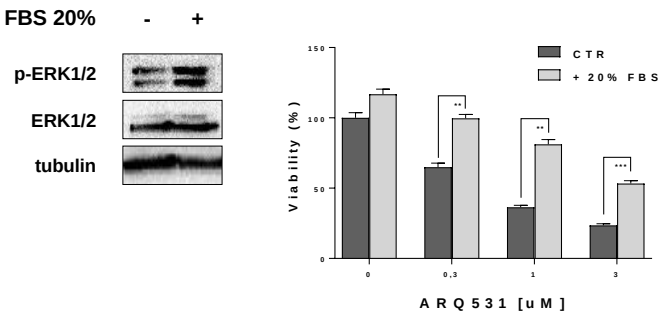

FBS 20%
-
+
p-ERK1/2
ERK1/2
tubulin
CTR
+ 20% FBS
Viability (%)
150
100
50
0
**
**
***
0
0,3
1
3
ARQ531 [uM]

B

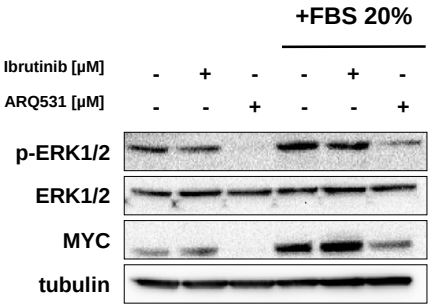
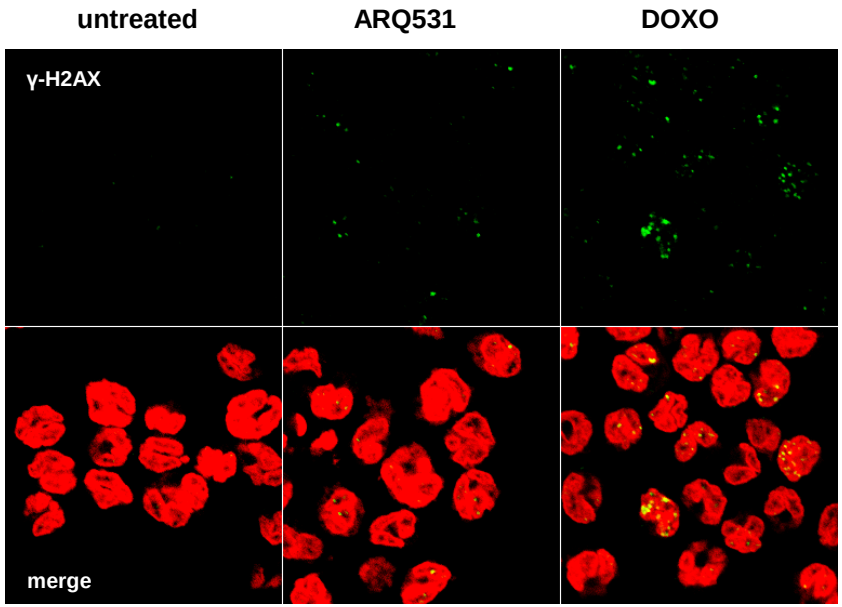

+FBS 20%
Ibrutinib [μM]
ARQ531 [μM]
- + - - + -
- - + - - +
p-ERK1/2
ERK1/2
MYC
tubulin

Figure S6

A


untreated
ARQ531
DOXO
γ-H2AX
merge

B

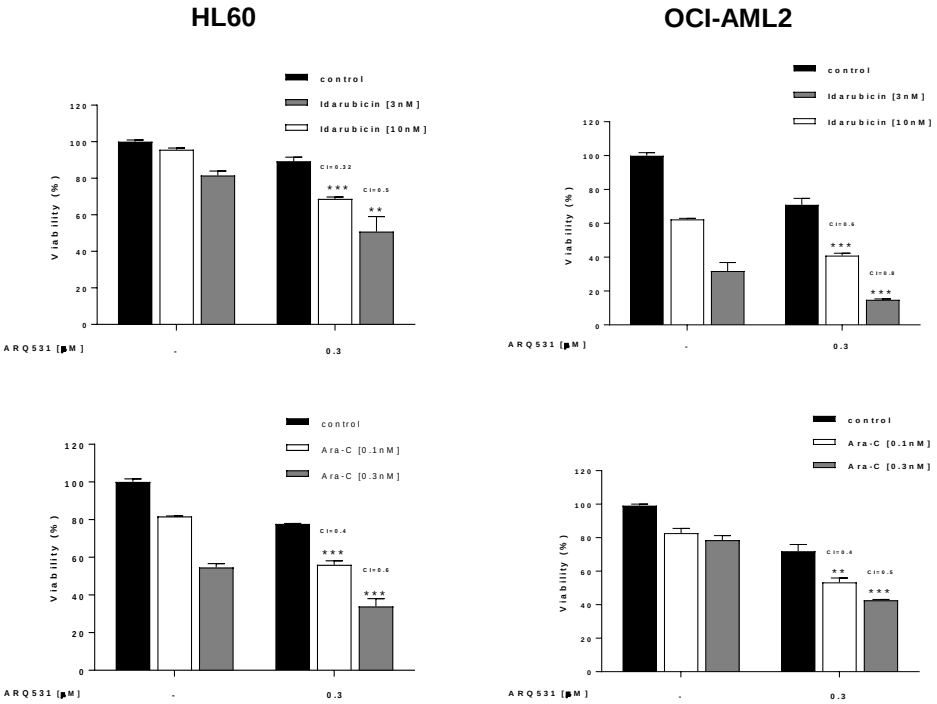
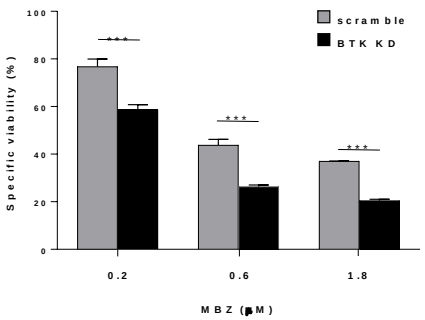

HL60
OCI-AML2
control
Idarubicin [3nM]
Idarubicin [10nM]
Ara-C [0.1nM]
Ara-C [0.3nM]
Viability (%)
ARQ531 [μM]
0.3

Figure S7

A


scramble
BTK KD
Specific viability (%)
100
80
60
40
20
0
***
***
***
0.2
0.6
1.8
MBZ (μM)

B

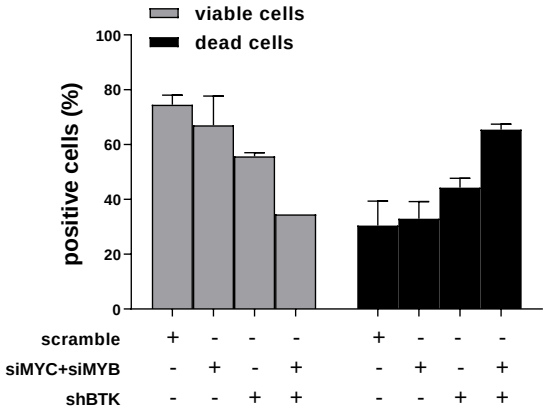
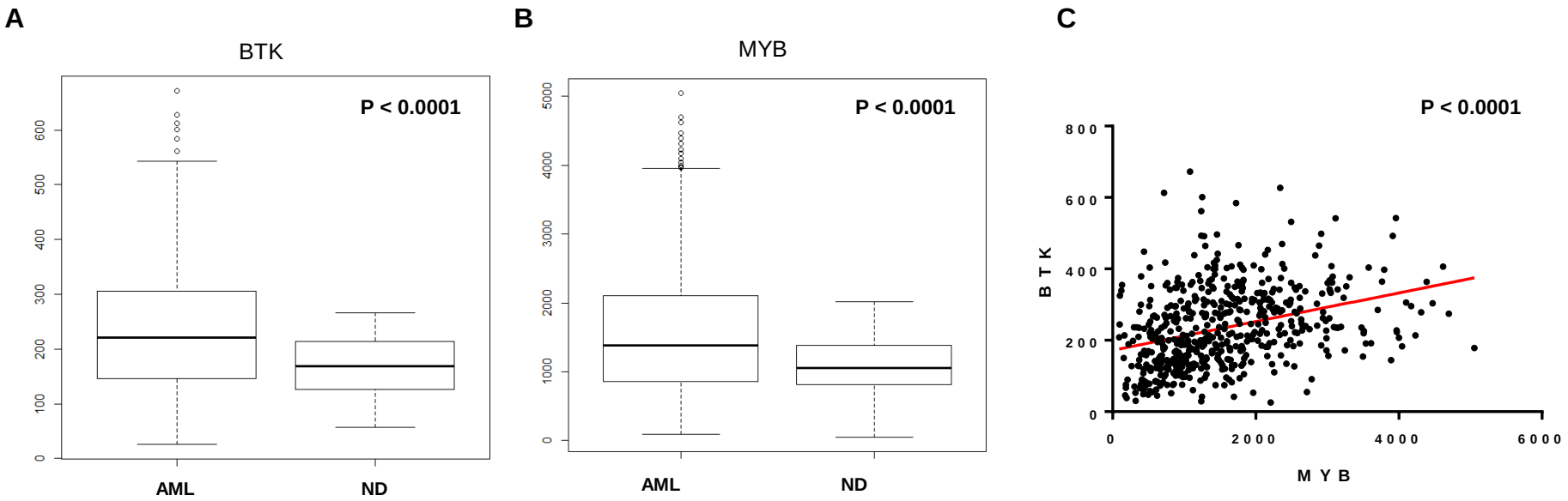

viable cells
dead cells
positive cells (%)
100
80
60
40
20
0
scramble + - - - + - - -
siMYC+siMYB - + - + - + - +
shBTK - - + + - - + +

Figure S8


A
BTK
P < 0.0001
0
100
200
300
400
500
600
AML
ND
B
MYB
P < 0.0001
0
1000
2000
3000
4000
5000
AML
ND
C
P < 0.0001
BTK
0
200
400
600
800
MYB
0
2000
4000
6000

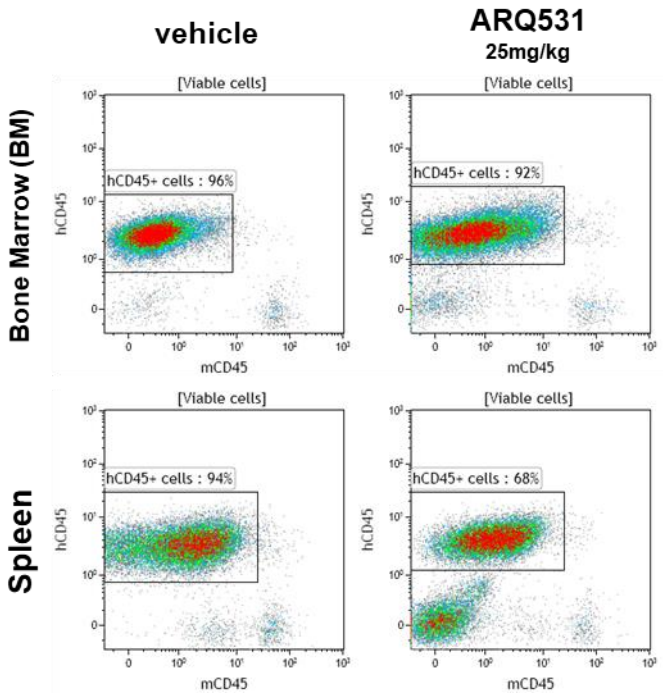

vehicle
ARQ531
25mg/kg
Bone Marrow (BM)
Spleen
[Viable cells]
hCD45+ cells : 96%
hCD45+ cells : 92%
hCD45+ cells : 94%
hCD45+ cells : 68%
hCD45
mCD45

Figure S10