AXL/MERTK inhibitor ONO-7475 potently synergizes with venetoclax and overcomes venetoclax resistance to kill FLT3-ITD acute myeloid leukemia

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Received: January 13, 2021.
Accepted: October 28, 2021.
Prepublished: November 4, 2021.
https://doi.org/10.3324 haematol.2021.278369

Supplemental Methods

Validation of cell lines

Cell lines were validated by STR DNA fingerprinting using the AmpF_STR Identifier Kit (Applied Biosystems). The STR profiles were compared to known ATCC fingerprints, and to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (http://bioinformatics.istge.it/clima/). The STR profiles matched known DNA fingerprints or were identified as unique. Authenticated cells are stored under liquid nitrogen and are kept in culture for no more than 3 months. Presence of mycoplasma was tested for using a detection kit from Applied Biological Materials (Richmond, BC, Canada).

Cell lines

Normoxic culture conditions comprised 21% O_2 and 5% CO_2 at 37°C. For experiments involving hypoxic conditions, cells were incubated with 1% O_2 and 5% CO_2 at 37°C.

RPPA

Reverse phase protein analysis (RPPA) was performed by the RPPA Core at the University of Texas MDACC using parental and ABT-199-resistant MV4;11 cells. Cellular proteins were denatured by 1% SDS (with beta-mercaptoethanol) and diluted in five 2-fold serial dilutions in dilution buffer (lysis buffer containing 1% SDS). Serially diluted lysates were arrayed on nitrocellulose-coated slides (Grace Biolab) by Aushon 2470 Arrayer 2 (Aushon BioSystems). The list of antibodies used, including commercial sources and catalog numbers, are available at the RPPA website (Antibody Panel 716 was used; https://www.mdanderson.org/research/research-resources/core-facilities/functional-proteomics-rppa-core/antibody-information-and-protocols.html). A total of 5808 array spots were arranged on each slide

including the spots corresponding to positive and negative controls prepared from mixed cell lysates or dilution buffer, respectively. Each slide was probed with a validated primary antibody plus a biotin conjugated secondary antibody. Only antibodies with a Pearson correlation coefficient between RPPA and western blotting of greater than 0.7 were used in reverse phase protein array study. Antibodies with a single or dominant band on western blotting were further assessed by direct comparison to RPPA using cell lines with differential protein expression or modulated with ligands/inhibitors or siRNA for phospho- or structural proteins, respectively. The signal obtained was amplified using a Dako Cytomation–catalyzed system (Dako) and visualized by DAB colorimetric reaction. The slides were scanned, analyzed, and quantified using a customized software Microvigene (VigeneTech Inc.) to generate spot intensity. Each dilution curve was fitted with a logistic model ("Supercurve Fitting," developed by the Department of Bioinformatics and Computational Biology in MD Anderson Cancer Center, (http://bioinformatics.mdanderson.org/OOMPA)). This fits a single curve using all the samples (i.e. dilution series) on a slide with the signal intensity as the response variable and the dilution steps are independent variables. The fitted curve is plotted with the signal intensities – both observed and fitted - on the y-axis and the log2-concentration of proteins on the x-axis for diagnostic purposes. The protein concentrations of each set of slides were then normalized by median polish, which was corrected across samples by the linear expression values using the median expression levels of all antibody experiments to calculate a loading correction factor for each sample.

qRT-PCR

RNA was extracted and purified from cell pellets using Zymo Quick-RNA columns per manufacturer's instructions (Zymo Research, Irvine, CA) and reverse transcribed using iScript (BioRad, Hercules, CA). qRT-PCR was performed with an ABI StepOnePlus Real Time PCR System using the following Taqman gene expression assays per manufacturer's instructions (AXL Hs01064444_m1, ABL1 Hs01104728_m1,

BCL2 Hs00608023_m1; Applied Biosystems, Foster City, CA). Individual samples were assayed in triplicate. Calculations were performed using the Pfaffl method comparing expression changes between target genes and housekeeping control (ABL1).

Immunoblot analysis

Cells were incubated with vehicle, ONO-7475, and/or ABT-199 and then lysed. Total protein was fractionated by SDS/PAGE. Immunoblot analysis was imaged with ImageJ. Tubulin and β–actin were used as loading controls.

IVIS (Luciferase) Imaging

Imaging was done as in Ruvolo et. al. [1]

Histology and Immunohistochemistry

Tissues were harvested from transplanted mice and fixed in 10% neutral phosphate-buffered formalin (Sigma, St Louis, MO) followed by paraffin embedding. Five-micron sections of formalin-fixed paraffin-embedded tissue samples were either stained with hematoxylin and eosin or subjected to immunohistochemistry. For immunohistochemistry, sections were deparaffinized and antigens were retrieved using citric acid and heat. Evaluation of human cells was determined using a human-specific Ku-80 primary antibody (rabbit α-Ku-80, Cell Signaling, Dancers, MA) and visualized using ABC and DAB kits (Vector Laboratories, Burlingame, CA). Slides were counterstained with Nuclear Fast Red (Sigma Aldrich, St. Louis, MO).

Supplemental Table 1. List of antibodies used in this study.

Antibody	Company	Catalog Number
MCL-1	Cell Signaling	94296S
ERK	Cell Signaling	4696S
p-ERK	Cell Signaling	4370S
AXL	Cell Signaling	8661S
BCL2	Cell Signaling	15071
MCL1	Cell Signaling	94296S
B-RAF	Cell Signaling	14814S
pS6	Cell Signaling	5364S
S6	Cell Signaling	2217S

Supplemental Table 2. Characteristics of AML patient samples used in the *in vitro* studies

Patient #	28	32
Source	PB	PB
Blast	34%	89%
Cytogenetics	45~46,XX,del(7)(q22)[cp2]	46,XY,t(6;22)(q25;q11.2)[8]/47,idem,+8[2]
FLT3 WT/ITD	ITD and D835	ITD
p53	WT	WT
Mutations	*FLT3*, *KRAS*, *NRAS*, *PTPN11*, *RUNX1*, *WT1*	*RUNX1*, *IDH2*, *FLT3*, *DNMT3A*, *ZRSR2*

Supplemental Figure Legends

Supplemental Figure 1. *ONO-7475 combination with ABT-199 induces apoptosis in AML cells.* MV4;11 and MOLM13 cells were treated with vehicle (0.2% DMSO), 10 nM or 50 nM ONO- 7475, 10 nM or 30 nM ABT-199, or combinations of the two agents for 72 hours (MV4;11) or 48 hours (MOLM13). Percent apoptotic cells (A for MV4;11; B for MOLM13) were determined by flow cytometry using Annexin V, and DAPI. Apoptotic cells are Annexin V +/DAPI -. One-way ANOVA with Dunnett's post-test was performed to determine significance (*, $p < 0.033$; **, $p < 0.002$; ***, $p < 0.001$).

Supplemental Figure 2. *ONO-7475 combination with ABT-199 has limited effect on FLT3 WT AML cells*. OCI-AML3 and THP-1 cells were treated with vehicle (0.2% DMSO), 10 nM or 50 nM ONO-7475, 10 nM or 30 nM ABT-199, or combinations of the two agents for 72 hours. Total viable cells (A for OCI-AML3; C for THP-1) and percent apoptotic cells (B for OCI-AML3; D for THP-1) were determined by flow cytometry using Annexin V, and DAPI. Apoptotic cells are Annexin V +/DAPI -. One-way ANOVA with Dunnett's post-test was performed to determine significance (*, $p < 0.033$; **, $p < 0.002$; ***, $p < 0.001$).

Supplemental Figure 3. *ONO-7475 synergizes with ABT-199 to kill MV4;11 cells and MOLM13 cells in hypoxic conditions.* MV4;11 and MOLM13 cells were treated with vehicle (0.2% DMSO), 10 nM, 25 nM, or 50 nM ONO-7475, 10 nM, 25, or 50 nM ABT-199, or combinations of the two agents for 72 hours in 1% O_2. Each graph represents cell viability for MV4;11(A) and MOLM13 (B) cells. One-way ANOVA with Dunnett's post-test was performed to determine significance (*, $p < 0.033$; **, $p < 0.002$; ***, $p < 0.001$).

Supplemental Figure 4. *Combination of ONO-7475 with apoptotic agents is efficacious in ABT-199-resistant cells.* (A) ONO-7475 combination with ABT-199 reduces p-ERK in *FLT3-ITD* AML cells. MOLM13 and MV4;11 cells were treated for 24 hours with vehicle, 10 nM ONO- 7475, 30 nM ABT-199, or a combination of both. Expression of ERK phosphorylated at T202/Y204 (p-ERK T202/Y204), total ERK, MCL-1, CDK1, and Tubulin were determined by western blotting. (B) MV4;11 ABT-199-resistant cells were treated with vehicle (0.1% DMSO), 30 nM ABT-199, 25 μM UMI-77 (an MCL-1 inhibitor), 100 nM ONO-7475, or a combination for 72 hours. Viable cells/ml was determined via Vi-Cell Cell Viability Analyzer (Beckman Coulter).

Supplemental Figure 5. *RPPA profiles for MV4;11 parental and ABT-199-resistant cells*. (A) Altered expression levels in MV4;11 ABT-199 resistant cells compared to parental cells and ONO-7475 treated cells. MV4;11 parental and MV4;11 ABT-199-resistant cells were treated for 24 hours with vehicle (0.1% DMSO) or 100 nM ONO-7475 for 24 hours. Cells (5 million) were collected and sent to the MDACC RPPA Core for analysis. Comparison of proteins expressed in vehicle treated parental cells and ABT-199-resistant cells was performed and proteins displaying a >2-fold change in expression were considered for further study. (B) Comparison of proteins expressed in vehicle treated and 100 nM ONO-7475 treated parental cells and ABT-199-resistant cells was performed and proteins displaying a >2-fold change in expression were considered for further study.

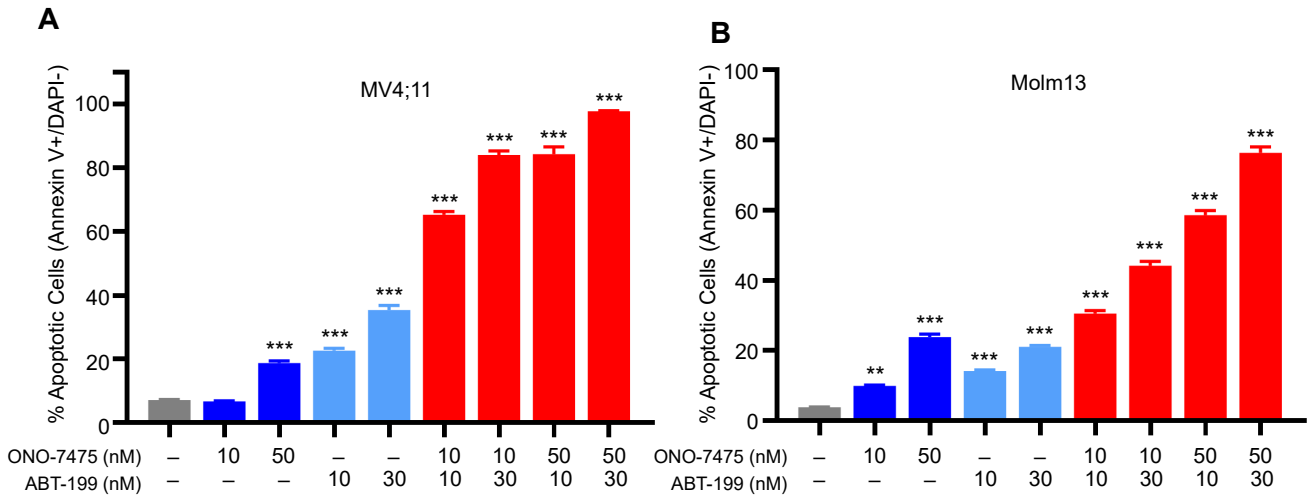
Supplemental Figure 6. *Representative IVIS image of leukemia burden in cell line model treated with ONO-7475 +/- ABT-199.* IVIS image showing radiance in a MOLM1-13 xenograft model.

Supplemental Figure 7. *ONO-7475 reduces leukemic burden in spleens of mice transplanted with a patient AML xenograft.* (A) Analysis of H&E stains of spleens from mice transplanted with the AML PDX model 3028566 was done to determine AML cell engraftment. For the control group, mitotic figures are identified by red arrowheads. For the ABT-199 group, mitotic figures are depicted by red arrowheads and apoptotic cells by green arrowheads. For the ONO-7475 and combination groups, megakaryocytes are depicted by yellow arrowheads and admixed granulocytic and erythroid precursors by green arrowheads. (B) Representative IHC staining with human specific Ku80 antibody using spleens from control and ONO-7475 treated mice engrafted with the AML PDX model 3028566.

Supplemental Reference

1. Ruvolo PP, Ma H, Ruvolo VR, et al. Anexelekto/MER tyrosine kinase inhibitor ONO-7475 arrests growth and kills FMS-like tyrosine kinase 3-internal tandem duplication mutant acute myeloid leukemia cells by diverse mechanisms. Haematologica. 2017; 102 (12): 2048-2057.

Ruvolo Supplemental Figure 1



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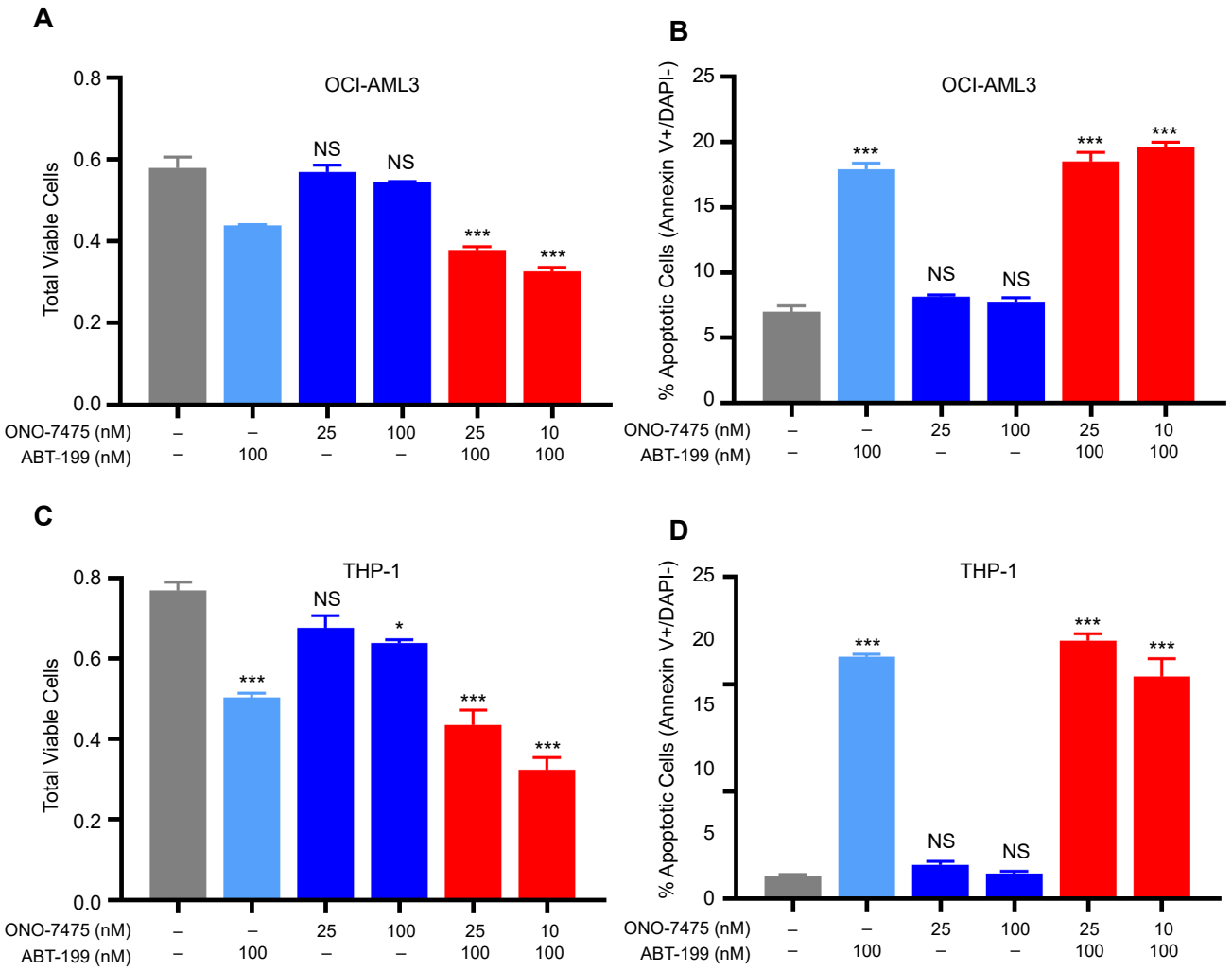

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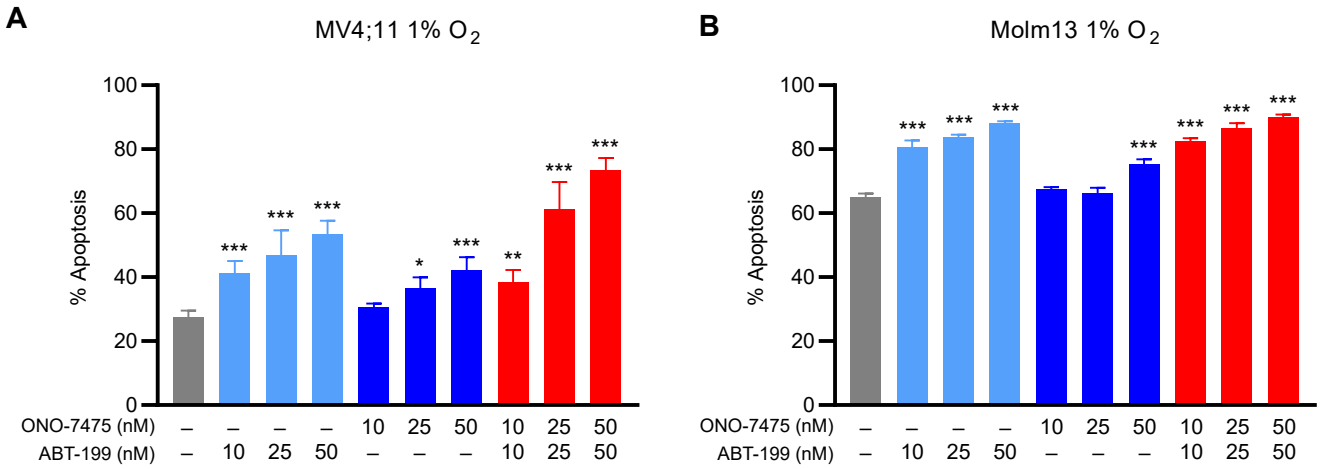

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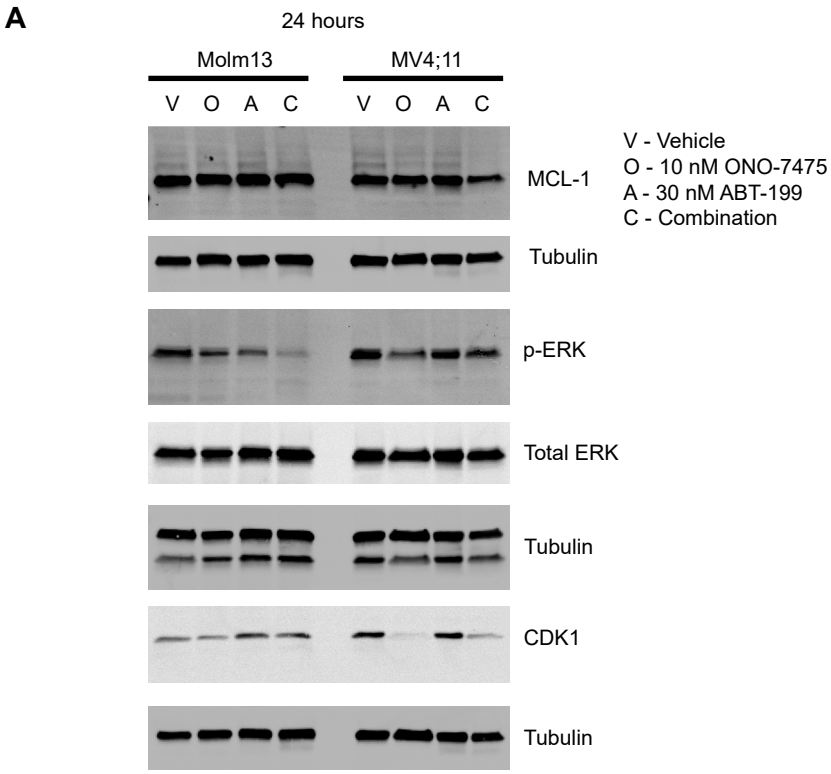
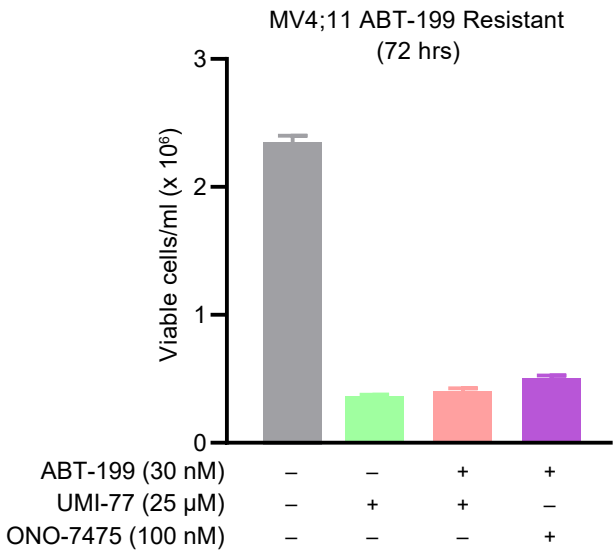
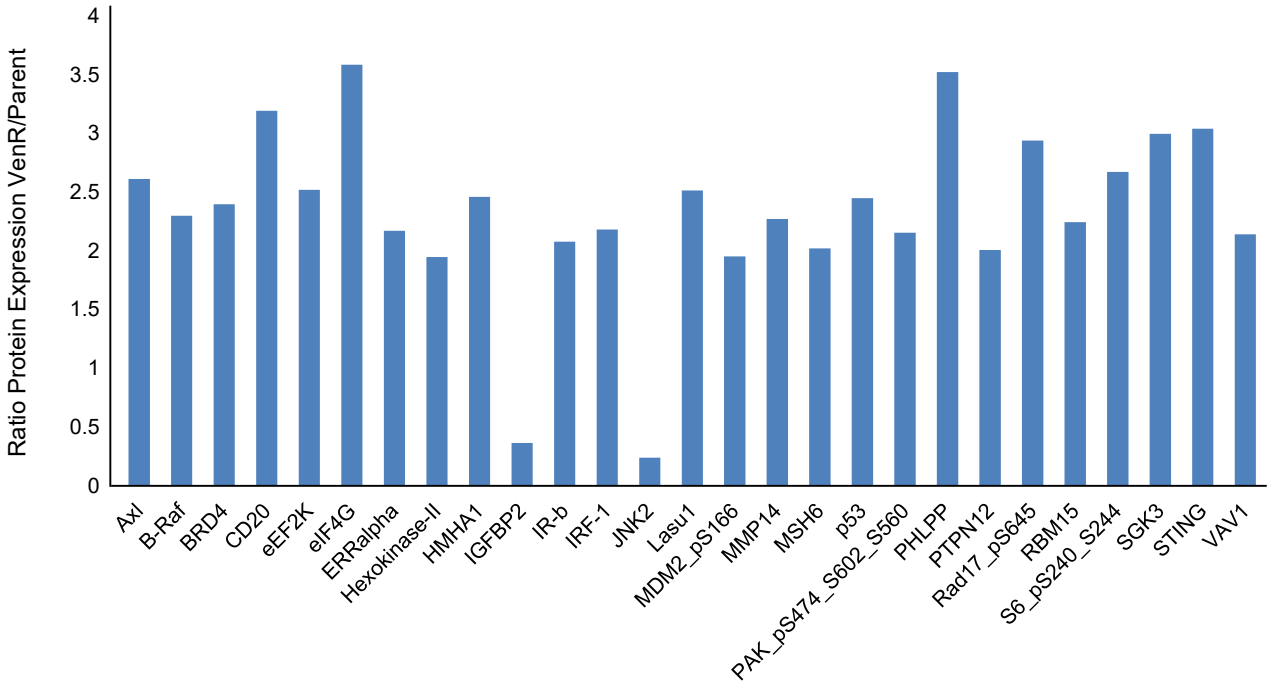




Ruvolo Supplemental Figure 5

A



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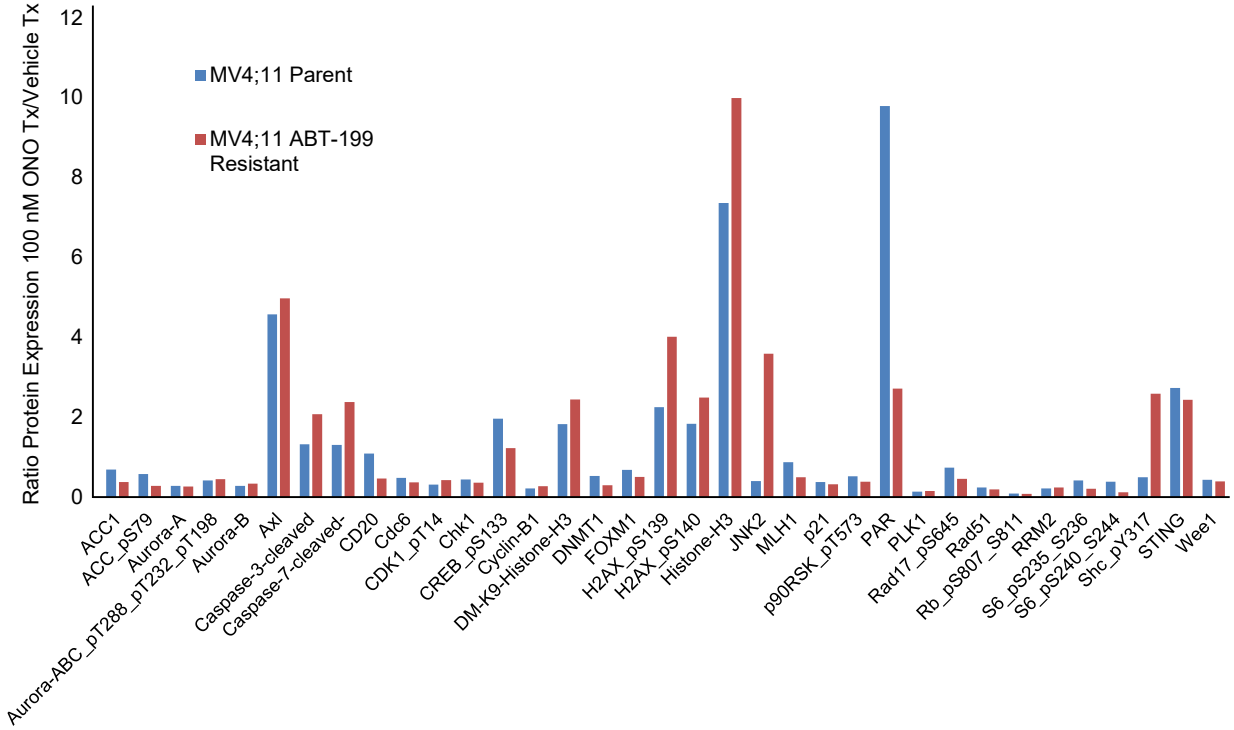

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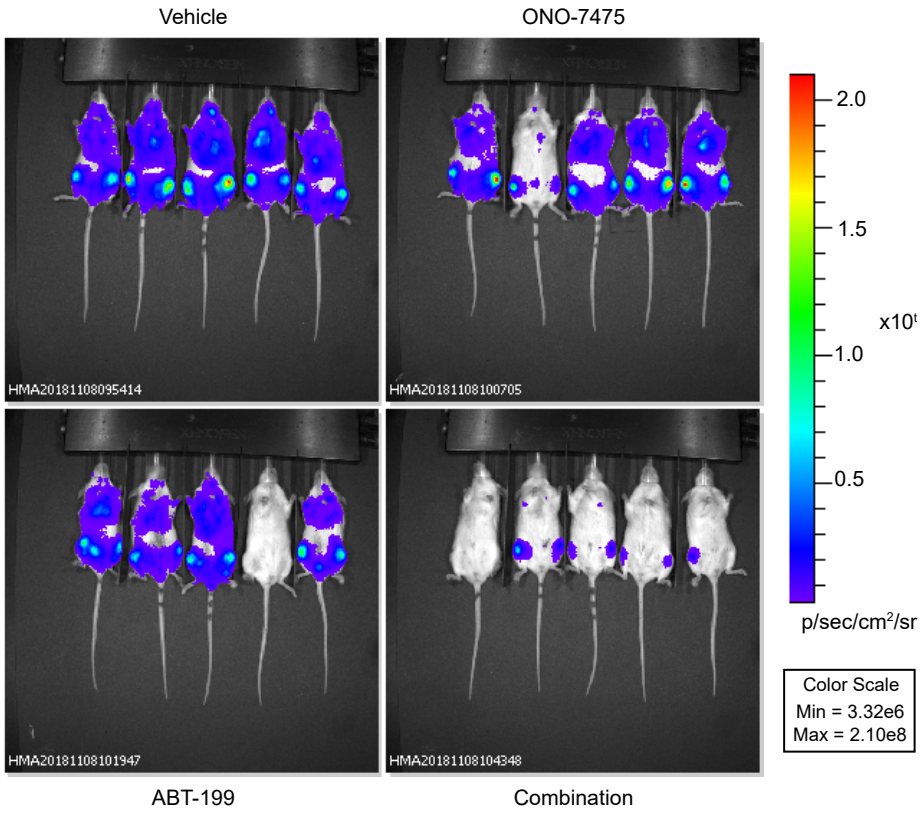

Ruvolo Supplemental Figure 7