Supplemental Information

The novel autophagy inhibitor ROC-325 augments the anti-leukemic activity of azacitidine

Steffan T. Nawrocki[1], Yingchun Han[2], Valeria Visconte[2], Bartlomiej Pryzchodzen[2], Claudia M. Espitia[1], James Phillips[2], Faiz Anwer[1,2], Anjali Advani[2], Hetty E. Carraway[2], Kevin R. Kelly[3], Mikkael A. Sekeres[2], Jaroslaw P. Maciejewski[2] and Jennifer S. Carew[1]

[1]Department of Medicine and The University of Arizona Comprehensive Cancer Center, Tucson, AZ; [2]Taussig Cancer Institute, Cleveland Clinic, Cleveland, OH; [3]Jane Anne Nohl Division of Hematology and Center for the Study of Blood Diseases, University of Southern California Norris Comprehensive Cancer Center, Los Angeles, CA

Materials and Methods

Drugs. The synthesis of ROC-325 was performed as previously described (1). 5-azacytidine was purchased from the cancer center pharmacy.

Cells and cell culture. HL-60, and KG-1 cells were obtained from ATCC (Manassas, VA, USA). MV4-11, MOLM-13, NOMO-1, and PL-21 cells were obtained from the Deutsche Sammlung von Mikororganismen und Zellkulturen (DSMZ, Leibniz, Germany). Human normal CD34+ bone marrow cells were purchased from Stem Cell Technologies (Vancouver, Canada). Cells were cultured with medium supplemented with 10% FBS at 37°C with 5% CO_2 as previously described (2) and were authenticated using short tandem repeat (STR) profiling. Primary AML cells were obtained from patients following informed consent in accordance with an approved IRB protocol and the Declaration of Helsinki.

Chemicals and reagents. Reagents were obtained from the following sources: propidium iodide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and anti-β tubulin antibody (Sigma, St. Louis, MO, USA), anti-active caspase-3 (Cell Signaling, Beverly, MA USA), anti-LC3B and anti-p62 (Abcam, Cambridge, MA, USA), goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Laboratories, West Grove, PA, USA), sheep anti-mouse-HRP and donkey anti-rabbit-HRP (Amersham, Pittsburgh, PA, USA).

Transmission electron microscopy. Transmission electron microscopy of cells was performed as previously described (3). AML cells were treated with ROC-325 for 24 h and harvested for imaging. Briefly, sections were cut in an LKB Ultracut microtome (Leica, Deerfield, IL), stained with uranyl acetate and lead citrate, and examined in a JEM 1230 transmission electron microscope (JEOL, USA, Inc., Peabody, MA, USA). Images were captured using the AMT Imaging System (Advanced Microscopy Techniques Corp, Danvers, MA, USA).

Quantification of lysosomal acidity. Cells were treated with ROC-325 for 6 h. Following drug treatment, cells were stained with 1 μM LysoSensor Green (Thermo Fisher, San Diego, CA, USA) for 2 h. For confocal microscopy imaging, cells were counterstained with PI and imaged using a Leica TCS-SP5II upright confocal/multi-photon microscope (equipped with a Q-Imaging Retiga EXi cooled CCD camera Leica Microsystems, GmbH, Wetzlar, Germany). For FACS-based quantification,

LysoSensor Green fluorescence was measured using a BD FACS Canto II cytometer (BD Biosciences, San Jose, CA. USA).

Immunoblotting. Cells were incubated with ROC-325 alone or in combination with azacitidine. Cells were harvested and were then lysed as previously described (4). Approximately 50 μg of total cellular protein from each sample were subjected to SDS-PAGE, proteins were transferred to nitrocellulose membranes, and the membranes were blocked with 5% nonfat milk in a Tris-buffered saline solution containing 0.1% Tween-20 for 1 h. The blots were then probed overnight at 4 °C with primary antibodies, washed, and probed with species-specific secondary antibodies coupled to horseradish peroxidase. Immunoreactive material was detected by enhanced chemiluminescence (West Pico, Pierce, Inc., Rockville, IL, USA).

Quantification of drug-induced cytotoxicity. Cell viability was assessed by MTT assay. Cells were seeded into 96-well microculture plates and treated with ROC-325 or azacitidine for 72 h. Following drug treatment, MTT was added and cell viability was quantified using a BioTek (Winooski, VT, USA) microplate reader. Pro-apoptotic effects following *in vitro* drug exposure were quantified by propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analysis of sub-G_0/G_1 DNA content as previously described (5) and by measurement of active caspase-3 and Annexin-V FITC by flow cytometry using commercial kits (BD Biosciences, San Jose, CA. USA).

RNA sequencing analysis.

Total RNA was isolated from MV4-11 cells following treatment with control or ROC-325. RNA (2 µg) was subjected to RNA sequencing using an Illumina HiSeq2000 (Otogenetics, Norcross, GA, USA). Analysis was conducted as previously described (6). Twenty-million sequencing reads were generated per sample. 100-basepair paired-end RNA sequencing reads were mapped to the hg19 RefSeq human transcriptome and spliceome by DNAnexus (http://dnanexus.com) using a Bayesian method 17 where a read was mapped when its posterior probability of mapping exceeded 0.9. TMM 18 normalization and the voom-limma approach 19 from the R package limma version 3.18 with R version 3.0.2 were used to perform differential gene expression analysis.

Quantitative real-time polymerase chain reaction. cDNA from ROC-325 treated cells were used for relative quantification by RT–PCR analyses. First-strand cDNA synthesis was performed from 1 µg RNA in a 20 µl reaction mixture using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). *DDX1*, *GADD34* and *GAPDH* transcripts were amplified using commercially available TaqMan® Gene expression assays (Applied Biosystems, Foster City, CA, USA). Relative gene expression was calculated with the $2^{-\Delta\Delta^{Ct}}$ method using *GAPDH* as a housekeeping gene (7).

Immunocytochemistry. Cells were treated with ROC-325 for 24 h. Following drug treatment, cells were fixed with 4% paraformaldehyde, permeabilized using 0.2% triton-X-100, and incubated overnight with anti-LC3B antibody. Alexa Fluor 488 conjugated fluorescent secondary antibody was used to visualize protein localization. DAPI was

utilized to stain the nucleus. Images were captured using an Olympus fluorescent microscope (Center Valley, PA) with a DP71 camera and a 60X objective. Image-Pro Plus software Version 6.2.1 (MediaCybernetics, Bethesda, MD) was used for image acquisition (8).

***In vivo* evaluation of ROC-325 and azacitidine.** MV4-11 cells (1 x 10^6) were suspended in HBSS and injected into the tail veins of NOD/SCID mice. Leukemic mice were randomized into treatment groups and treated with vehicle (water), ROC-325 (50 mg/kg PO), azacitidine (5 mg/kg IV) or both drugs as indicated. Mice were monitored daily and overall survival was quantified. At study completion, bone marrow and spleen specimens from representative animals were excised from each group, formalin-fixed, and paraffin-embedded for immunohistochemical analysis.

Immunohistochemistry. Paraffin-embedded bone marrow and spleen sections were deparaffinized in xylene, exposed to a graded series of alcohol, and rehydrated in PBS (pH 7.5). Heat-induced epitope retrieval on paraffin-embedded sections and probing with specific antibodies was conducted as previously described (9). Positive reactions were visualized using 3,3'-diaminobenzidine (Dako, Glostrup, Denmark). CD45+ cells in bone marrow specimens were visualized by staining with a human-specific anti-CD45 antibody (Cell Signaling Technology, Danvers, MA, USA). Images were captured using an Olympus fluorescent microscope (Center Valley, PA) with a DP71 camera and a 20X objective. Image-Pro Plus software Version 6.2.1 (MediaCybernetics, Bethesda, MD) was used for image acquisition. ImageJ software was used for quantification of LC3B

and p62 levels by densitometric analysis of five random fields containing viable tumor cells as previously described (10). CD45+ cells were scored in five random fields containing viable tumor cells.

Statistical analyses. Statistical significance of differences observed between samples was determined using the Student's *t* test. Kaplan-Meier survival analysis was used to assess the benefit of each treatment *in vivo*. Differences were considered significant in all experiments at $p < 0.05$ with two-sided comparisons.

REFERENCES

1. Carew JS, Espitia CM, Zhao W, Han Y, Visconte V, Phillips J *et al*. Disruption of Autophagic Degradation with ROC-325 Antagonizes Renal Cell Carcinoma Pathogenesis. Clin Cancer Res 2017; 23:2869-79.
2. Swords RT, Kelly KR, Smith PG, Garnsey JJ, Mahalingam D, Medina E *et al*. Inhibition of NEDD8-activating enzyme: a novel approach for the treatment of acute myeloid leukemia. Blood 2010; 115:3796-800.
3. Carew JS, Medina EC, Esquivel JA, 2nd, Mahalingam D, Swords R, Kelly K *et al*. Autophagy inhibition enhances vorinostat-induced apoptosis via ubiquitinated protein accumulation. J Cell Mol Med 2009.
4. Nawrocki ST, Carew JS, Maclean KH, Courage JF, Huang P, Houghton JA *et al.* Myc regulates aggresome formation, the induction of Noxa, and apoptosis in response to the combination of bortezomib and SAHA. Blood 2008; 112:2917-26.

5. Mahalingam D, Medina EC, Esquivel JA, 2nd, Espitia CM, Smith S, Oberheu K *et al*. Vorinostat enhances the activity of temsirolimus in renal cell carcinoma through suppression of survivin levels. Clin Cancer Res 2010; 16:141-53.

6. Visconte V, Avishai N, Mahfouz R, Tabarroki A, Cowen J, Sharghi-Moshtaghin R *et al.* Distinct iron architecture in SF3B1-mutant myelodysplastic syndrome patients is linked to an SLC25A37 splice variant with a retained intron. Leukemia 2015; 29:188-95.

7. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29:e45.

8. Carew JS, Espitia CM, Esquivel JA, 2nd, Mahalingam D, Kelly KR, Reddy G *et al* . Lucanthone is a novel inhibitor of autophagy that induces cathepsin D-mediated apoptosis. J Biol Chem 2011; 286:6602-13.

9. Nawrocki ST, Kelly KR, Smith PG, Espitia CM, Possemato A, Beausoleil SA *et al*. Disrupting protein NEDDylation with MLN4924 is a novel strategy to target cisplatin resistance in ovarian cancer. Clin Cancer Res 2013; 19:3577-90.

10. Mahalingam D, Mita M, Sarantopoulos J, Wood L, Amaravadi RK, Davis LE *et al*. Combined autophagy and HDAC inhibition: a phase I safety, tolerability, pharmacokinetic, and pharmacodynamic analysis of hydroxychloroquine in combination with the HDAC inhibitor vorinostat in patients with advanced solid tumors. Autophagy 2014; 10:1403-14.