Molecular targeting of the UDP-glucuronosyltransferase enzymes in high-eukaryotic translation initiation factor 4E refractory/relapsed acute myeloid leukemia patients: a randomized phase II trial of vismodegib, ribavirin with or without decitabine

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Supplementary Appendix

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Supplementary Methods

Patient Selection

Patients with AML of M4 or M5 French-American-British FAB subtypes subtype or high-eIF4E (except AML M3), were eligible to participate in this study. All patients must have failed primary therapy (defined as two induction chemotherapies), must have relapsed, or were not suitable candidates for intensive induction chemotherapy. They must have samples evaluable for ribavirin uptake assays by the central laboratory. Patients must have been at least 18 years of age; and must have had an Eastern Cooperative Oncology Group (ECOG) performance status lower than 2; a life expectancy of at least 4 weeks; adequate hepatic and renal function (hepatic transaminase level lower than 2.5 times the institutional upper limit of normal ULN, total bilirubin level less than 1.5 times the ULN, serum creatinine level below 1.5 times the ULN). No concurrent anti-cancer therapy except adjuvant antihormonal agents for breast cancer or for limited stage prostate cancer. Hydroxyurea was allowed up to the end of cycle 2. Patients with uncontrolled central nervous system (CNS) leukemia, active cardiovascular disease, hemoglobinopathies, intercurrent illness or medical condition precluding safe administration of the study drug and known human immunodeficiency virus infection were not permitted on study. Patients with a history of other active malignancy were also excluded. Patients who were disease-free for 2 years or had a history of completely resected non-melanoma skin cancer or successfully treated *in situ* carcinoma were eligible. Pregnant and breastfeeding patients were excluded.

Evaluation of response

To assess response to therapy, a bone marrow aspirate was performed prior to study start and at the end of every 28-day cycle. Clinical responses were assessed using the Cheson criteria [1]. A complete remission (CR) was defined as absence of leukemic blasts from peripheral blood, fewer than 5% blasts in bone marrow, peripheral level of hemoglobin higher than 90 g/L (higher than 9 g/dL), platelet count greater than 100×10^9/L and absolute neutrophil count greater than 1×10^9/L. A designation of complete remission with incomplete blood count recovery (CRi) required that all criteria for a CR were met, but that there was either a residual neutropenia ($<1 \times 10^9$/L) or thrombocytopenia ($<100 \times 10^9$/L). Partial remission (PR) required the hematologic criteria for CR, and a 50% reduction in bone marrow blasts with a post-treatment blast count between 5 and 25%. A blast response (BR) required a greater than 2-log decrease in absolute peripheral blood blast count and/or at least a 50% decrease in bone marrow blast percentage sustained for a 28-day period in the absence of fulfilling the criteria for a CR, CRi, or PR. Progressive disease (PD) was defined as a 50% increase in the absolute number of blasts in the bone marrow relative to baseline, or an increase in the absolute peripheral blast count of at least 10×10^9/L. Stable disease (SD) was defined as failure to achieve a BR, yet not fulfilling the criteria for PD. The best response for each patient was recorded.

Study design and sample size

Each arm was analyzed separately. The run-in patients were included in the assessment of efficacy. Each arm had a minimum of 3 patients and a maximum of 6 patients for the run-in. These patients were part of the 21 patient sample size planned for each arm.

Simon two-stage design [2] was used. Based on the response rates of ribavirin in AML, ribavirin was estimated to be associated with an overall response rate (ORR) of 15%. Adding vismodegib and/or decitabine was estimated to increase the ORR rate to 40% assuming a potentially synergistic or additive effect of these agents. Therefore, the null hypothesis was the true response rate of 15% was be tested against a one-sided alternative. In the first stage, 9 patients were to be accrued. If there was 1 or fewer responses in these 9 patients, further enrollment in the cohort would be terminated for futility. Otherwise, 12 additional patients would be accrued for a total of 21 patients. The null hypothesis would be rejected if 6 or more responses were observed in 19 evaluable patients. This design yields a type I error rate of 0.05 and power of 80% when the true response rate is 40%. Further, it allowed a 10% dropout rate for non-evaluable patients. The sample size above was estimated separately but was the same for both arms. The per-protocol population was defined as all patients who remained on ribavirin, vismodegib and/or decitabine therapy and completed at least one end-of-cycle post baseline assessment. Any patient who discontinued therapy after Day 15 and before the end-of-cycle 1 post baseline assessment because of clinical progression, was included in this population. Patients evaluable for response were used for the interim and subsequent analyses of efficacy parameters.

Next Generation Sequencing

DNA samples were obtained from patients' blood and/or bone marrow blasts. Libraries were created from these samples. Hybridization from xGen Hybridization Capture of DNA libraries from IDT probes (myeloid panel) were used. Single sample variant calling was performed using NextGENe proprietary bioinformatics pipeline which aligned the sequenced

reads to the human reference genome hg19[3]. Variants that had an allelic frequency below 5% or labeled as VUS, Benign, or Likely Benign in a public database (such as Clivar or COSMIC) were filtered out.

Correlative studies

Drug uptake

To assess efficient drug uptake during screen for eligibility for study, sorted blasts were seeded in 24-wells plates at 100,000 cells/300 µl media/per well. They were then treated with 0.66µM ^{3}H-Ribavirin (Moravek, Brea, CA, USA) and incubated at 37°C for 6-10 hrs. After treatment cells were washed twice with ice-cold PBS and lysed with 2 volumes of NaOH (1N) and then neutralized with equal volume of HCl (1N). Samples were mixed with 5 ml Ultima Gold Scintillation cocktail (Perkin Elmer, Waltham, MA, USA) and radioactivity was measured using a liquid scintillation counter (Tri-Carb 2800 TR, PerkinElmer). As control, AML cell lines, uptake-resistant AML cell lines (2) and normal CD34+ cells were used (Lonza, Hayward, CA, USA or ATCC Manassas, VA, USA).

RNA extraction, reverse transcription and quantitative PCR

RNA from sorted AML blasts was extracted using Trizol (Invitrogen, Waltham, MA, USA) and Direct-zol RNA extraction kit (Zymo Research, Irvine, CA, USA) and was reverse transcribed using Supervilo kit (Invitrogen). Quantitative PCR was performed using with SensiFast SYBR Lo-ROX kit (Meridian Bioscience, Cincinnati, OH, USA) on QuantStudio 7 or ViiA 7 thermal cyclers (Applied Biosystems, Waltham, MA, USA). The primers for the target genes (ENT1, ADK and

Gli1) as well as for the reference genes (Ubc, RPIIa, G6PDH, RPL13a) have been described previously[4].

UGT1A antibody purification

The pan-UGT1A antibody (Antibodies-online.com, Limerick, PA, USA) was found to have extra bands when tested by western blot and thus was further affinity purified using lysates of HEK293T cells overexpressing UGT1A1, UGT1A4, UGT1A6 and UGT1A9 constructs (Genescript, Piscataway, NJ, USA) to provide antigens for purification followed by standard affinity purification[5]. These UGT1A constructs were selected based on previous studies which suggested these were involved in the glucuronidation of ribavirin[6]. However, the UGT1A family consists of 9 members so we cannot rule out that other UGT1A family members also contribute to glucuronidation but were not assessed here.

Immunofluorescence and confocal microscopy

For immunostaining of eIF4E sorted blasts were fixed in methanol (10 minutes at -20°C), incubated for 1h in blocking solution (10% FBS, 0.1% Tween20 in PBS) and overnight with eIF4E-FITC antibody diluted 1:25 (BD Biosciences, Mississauga, ON, Canada), washed 3 times with 1xPBS and mounted with Vectashield with DAPI (Vector Laboratories, Newark, CA, USA). Similar method was used when staining with UGT1A antibody dilution 1:100 (Antibodies-online, further purified in-house), except that following overnight incubation with the primary antibody an anti-rabbit AlexaFuor555 (Cell Signaling, Danvers, MA, USA) was incubated with the slides for 1h at room temperature, followed by washes and mounting. When not enough material was available for single staining, cells were double stained with unconjugated eIF4E antibody 1:25

dilution (BD Biosciences) and UGT1A antibody 1:100 dilution (Antibodies-online.com, further purified in-house), followed by anti-mouse Alexa-Fluor488 and anti-rabbit AlexaFluor555 (Cell Signaling) respectively. Images were acquired on a LSM700 microscope (Zeiss), with a 63x objective. For quantification purposes, samples were stained at the same time with the same aliquot of eIF4E and UGT1A antibodies and images acquired at the same time with the same confocal settings to ensure differences in intensity were attributable to the specimens. eIF4E and UGT1A protein levels in isolated blasts were measured by IFCLM and quantified on a per cell basis using FIJI quantifying more than 35 cells per condition. In some cases, it was necessary to compare BM and PB in different cycles due to specimen availability; however, when PB and BM from the same cycle could be compared, these were generally in agreement. When these were not in agreement or when samples were not available for analysis, samples were determined to be "N/A" for not assessable. Not all patients had specimens available at end of treatment (EOT) and in these cases last available sample (LAS) is reported. Micrograph were displayed using Adobe Photoshop, graphs were generated using GraphPad Prism 7.

Western blotting, RNA interference and Vismodegib treatment

Experiments with RNAi were performed as previously described[6]. Briefly, HepG2 cells were transfected with Lipofectamine 2000 (Invitrogen) and 60 nM RNAi duplexes for 96 hours and analyzed by WB. Control RNAi duplex: si Luc sense: (CACGUACGCGGAAUACUUCGA), si Luc anti-sense: (CAUUUCGAAGUAUUCCGCGUACGUGUU). For siUGT1A three sequences were used such that the total amount of the RNAi mix is equal to 60 nM (Integrated DNA technologies, catalogue number HSC.RNAI.N001072.12.2_10NM; HSC.RNAI.N001072.12.10_NM;

HSC.RNAI.N007120.12.9_NM). Immunoblotting was performed as described previously[6]. Antibodies used were in-house purified UGTA1 (originally from Antibodies online), Gli1 (Cell Signaling) and Actin (Sigma Aldrich, Mississauga, ON, Canada).

Cells were treated with either vismodegib (Selleckchem, Houston, TX, USA) or in parallel, phosphate buffered saline. Specifically, vismodegib was added to a final concentration of 200 nM at 0 and again at 48 hours. Cells were collected at 96 hours for analysis.

Supplementary Table 1. Treatment-emergent AEs of any grade, irrespective of causality, reported in ≥10% of patients

		ALL Patients (n=23)				Decitabine + VR (n=15)				VR (n=8)			
Adverse Events		ALL		Grade≥3		ALL		Grade≥3		ALL		Grade≥3	
		n	%	n	%	n	%	n	%	n	%	n	%
Gastrointestinal	Nausea	14	61	2	9	11	73	2	13	3	38	0	0
	Diarrhea	12	52	1	4	10	67	1	7	2	25	0	0
	Vomiting	11	48	0	0	9	60	0	0	2	25	0	0
	Anorexia	8	35	0	0	4	27	0	0	4	50	0	0
	Dysgeusia	8	35	0	0	6	40	0	0	2	25	0	0
	Constipation	7	30	0	0	4	27	0	0	3	38	0	0
General Disorders	Edema limbs	5	22	0	0	4	27	0	0	1	13	0	0
	Fatigue	10	43	3	13	7	47	2	13	3	38	1	13
Infection related	Febrile Neutropenia	15	65	15	65	12	80	12	80	3	38	3	38
	Infection other	9	39	5	22	6	40	4	27	3	38	1	13
	Pyrexia	8	35	1	4	6	40	1	7	2	25	0	0
	Lung Infection	7	30	7	30	6	40	6	40	1	13	1	13
	Skin infection	4	17	2	9	4	27	2	13	0	0	0	0
	Respiratory infection other than lung	3	13	1	4	2	13	1	7	1	13	0	0
	Bacteremia	3	13	3	13	3	20	3	20	0	0	0	0
Laboratory Tests	Anemia	7	30	7	30	4	27	4	27	3	38	3	38
	Thrombocytopenia	5	22	5	22	3	20	3	20	2	25	2	25
	Neutropenia	4	17	4	17	2	13	2	13	2	25	2	25
	Hypokalemia	9	39	8	35	6	40	6	40	3	38	2	25
	Hypomagnesemia	7	30	1	4	6	40	1	7	1	13	0	0
	Increased leukocytes	7	30	2	9	3	20	1	7	4	50	1	13
	Bilirubin increased	5	22	1	4	3	20	0	0	2	25	1	13
	Hypophosphatemia	5	22	5	22	3	20	3	20	2	25	2	25
	ALT increase	5	22	1	4	5	33	1	7	0	0	0	0
	Hypocalcemia	3	13	1	4	1	7	1	7	2	25	0	0
	AST increase	3	13	0	0	3	20	0	0	0	0	0	0
	Alkaline phosphatase increase	3	13	0	0	2	13	0	0	1	13	0	0
Nervous System Disorders	Dizziness	4	17	1	4	2	13	0	0	2	25	1	13
	Insomnia	4	17	0	0	1	7	0	0	3	38	0	0
Pain	Pain in extemity	4	17	0	0	0	0	0	0	4	50	0	0
	abdominal pain	4	17	0	0	3	20	0	0	1	13	0	0
	Pain other	4	17	1	4	3	20	1	7	1	13	0	0
	Chest pain	3	13	0	0	0	0	0	0	3	38	0	0
	Headache	3	13	1	4	2	13	1	7	1	13	0	0
Respiratory	Cough	9	39	0	0	5	33	0	0	4	50	0	0
	Dyspnea	6	26	3	13	4	27	2	13	2	25	1	13
	Nasal congestion/runny nose	4	17	0	0	3	20	0	0	1	13	0	0
	Epistaxis	5	22	1	4	4	27	1	7	1	13	0	0
	Sore throat	4	17	0	0	3	20	0	0	1	13	0	0
Skin	Skin rash	5	22	1	4	4	27	1	7	1	13	0	0
	Skin lesion	4	17	0	0	2	13	0	0	2	25	0	0
	Ulcer mouth	3	13	0	0	3	20	0	0	0	0	0	0
Vascular Disorders	Hypotension	4	17	1	4	4	27	1	7	0	0	0	0

V: vismodegib R: ribavirin

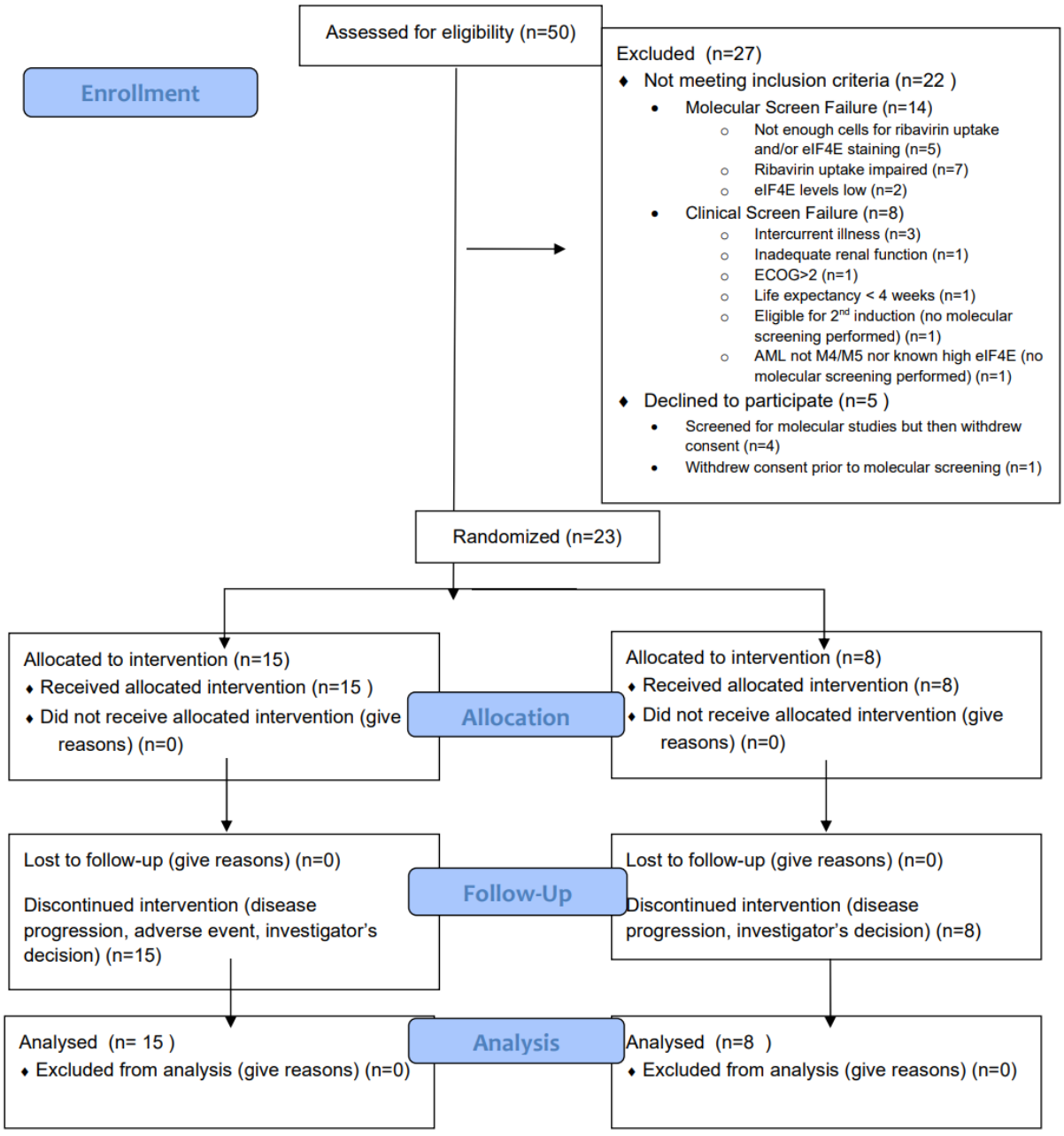
Supplementary Table 2. Molecular response for *GLI1* and *ADK* RNA levels.

Response	Patient ID	*GLI1* RNA**largest change/EOT or LAS	*ADK* RNA**largest change/EOT or LAS
PR	A-011	3CT: 0.886 ± 0.217 *	3CT: 0.753 ± 0.191 *
BR	B-004	1CT: 0.885 ± 0.132 *	10CT: 1.165 ± 0.136
BR	C-003	2CT: 1.562 ± 0.314 *	2CT: 0.658 ± 0.108 *
BR	B-001	1CT: 0.211 ± 0.070 *	1CT: 1.027 ± 0.291 *
SD	C-002	1CT: 0.570 ± 0.112 /4CT: 9.752 ± 2.60 ł *	5CT: 0.706 ± 0.168 ł *
SD	A-008	4CT: 0.700 ± 0.224	4CT: 0.953 ± 0.168
SD	A-001	N/A	N/A
SD	A-002	2CT: 0.503 ± 0.332	1CT: 0.264 ± 0.074 */2CT: 0.872 ± 0.194 *
SD	A-010	2CT: 0.810 ± 0.165	1CT: 0.954 ± 0.293 *
SD	B-002	N/A	1CT: 0.541 ± 0.030
SD	C-004	N/A	1CT: 0.778 ± 0.175
PD	A-004	1CT: 0.758 ± 0.174 *	1CT: 1.433 ± 0.363 *
PD	A-005	1CT: 3.964 ± 1.293 *	1CT: 1.115 ± 0.204 *
PD	A-006	1CT: 0.601 ± 0.159 *	1CT: 0.835 ± 0.124 *
PD	A-009	1CT: 14.372 ± 3.364	1CT: 0.784 ± 0.167
PD	C-001	1CT: 0.287 ± 0.110	1CT: 1.540 ± 0.411

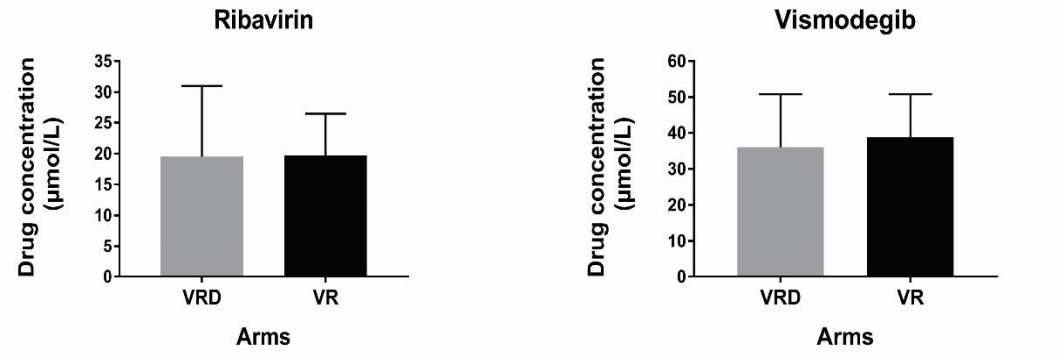
Supplementary Table 2. Molecular response for *GLI1* and *ADK* RNA levels. When available, RNAs were analyzed relative to BT (before treatment) set at 1 using RT-qPCR. For 11 of these patients, both UGT1A protein (Table3) and *GLI1* RNA levels could be evaluated, but not always at the same cycle making it difficult to see if these correlated [4, 6]. *GLI1* RNA levels may not always predict GLI1 protein and Vismodegib targets GLI1 protein localization; unfortunately, we had insufficient material to examine this. GLI1 is sufficient to drive UGT1A protein production (Figure 2B) consistent with previous reports[5, 6]. In some cases, it was necessary to compare BM and PB in the different cycles due to specimen availability; when PB and BM from the same cycle could be compared, these were generally in agreement. When this was not the case or if there were no samples available for analysis samples were determined to be “not assessable” (N/A). Abbreviations are as follows: PR: partial remission, BR: blasts response, SD: stable

disease, PD: progressive disease, EOT: End of treatment, LAS: last available cycle, *: Not EOT, ł: Cycle1Day15 sample used to compare because BT sample was not available.

Supplementary Figure 1. Consort Flow Diagram



Supplementary Figure 2. Ribavirin and vismodegib levels in patient plasma.



Supplementary Figure 2. Ribavirin and vismodegib levels in patient plasma. Average ribavirin and vismodegib plasma concentrations (in micromolar) as measured by mass spectrometry on Cycle1 day 15 are comparable between the two study arms. Error bars indicate standard deviation. The graph was generated using GraphPad Prism 7.

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