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Mcl-1 Dependence Predicts Response to Vorinostat and Gemtuzumab Ozogamicin in Acute Myeloid Leukemia

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Abstract

Older adults with acute myeloid leukemia (AML) are commonly considered for investigational therapies, which often only benefit subsets of patients. In this exploratory, we assessed whether BH3 profiling of apoptotic functionality could predict outcomes following treatment with vorinostat (histone deacetylase inhibitor) and gemtuzumab ozogamicin (GO; CD33-targeted immunoconjugate.) Flow cytometry of BH3 peptide priming with Noxa (anti-apoptotic protein Mcl-1 modulator) correlated with remission induction (p=.026; AUC=0.83 [CI: 0.65–1.00; p=.00042]: AUC=0.88 [CI:0.75–1.00] with age adjustment) and overall survival (p=.027 logistic regression; AUC = 0.87 [0.64–1.00; p=.0017]). This Mcl-1-dependence suggests a pivotal role of Bcl-2 family protein-mediated apoptosis to vorinostat/GO in AML patients.

Keywords

AML; biomarker; personalized medicine; HDAC inhibitors; gemtuzumab ozogamicin

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INTRODUCTION

The outcome of older adults with acute myeloid leukemia (AML) with standard curative-intent chemotherapeutics remains dismal because of increased risks of both treatment-related mortality as well as therapeutic resistance associated with advancing patient age, accumulating medical comorbidities, and changing disease biology [1–3]. As a result, current expert guidelines, e.g. by the National Comprehensive Cancer Network (NCCN), recommend that these patients should receive investigational therapies whenever possible [4].

Considerable effort has focused on the integration of antibody-based therapeutics, most notably the CD33-targeted immunoconjugate, gemtuzumab ozogamicin (GO), alone or in combination with other agents, into the treatment scheme of older adults with AML [5, 6]. One such approach has explored whether histone deacetylase (HDAC) inhibitors such as vorinostat could augment the anti-AML efficacy of GO. Initial *in vitro* studies suggested this possibility by demonstrating that HDAC inhibitors lead to chromatin remodeling that facilitates DNA intercalation of the toxic moiety of GO, a calicheamicin-γ_1 derivative, and enhance GO-induced DNA degradation and cellular apoptosis [7, 8]. As a result of these findings, we conducted a phase 2 trial and studied vorinostat as chemosensitizer with GO in 31 older adults with untreated AML; however, while the treatment regimen was well tolerated, only 7 patients achieved either a complete remission (CR) or CR with incomplete platelet recovery (CRp) [9].

Undoubtedly, pre-treatment biomarkers that accurately predict response and eventual outcome of a treatment regimen would greatly facilitate personalized decision-making . Herein, we investigated whether BH3 profiling, a method for assessing mitochondrial functionality in apoptosis signaling [10–12], could serve as such a biomarker for patients receiving vorinostat/GO for untreated AML. The underlying principle of BH3 profiling is that mitochondrial depolarization following exposure to BH3 domain containing peptides serves as a functional biomarker for a cell's ability to respond to pro-apoptotic cues. As a result of aberrant phenotypes, cancer cells may develop blocks in cell death/apoptosis pathways.[13] BH3 profiling determines if such a dependence on certain apoptosis-regulating proteins occurs in any given cancer cell, and identifies the dependent protein.[14] In turn, this understanding then provides insight into the likelihood of a cancer cell to respond to treatment. The scientific rationale for our study was provided by the fact that members from the calicheamicin family of cytotoxins involve mitochondrial pathways of apoptosis [15], and that HDAC inhibitors have been suggested to exert anti-leukemic cytotoxic effects largely through Bcl-2 family proteins, most notably Mcl-1 [16, 17].

MATERIALS AND METHODS

Study Population and Treatment

Details of the phase 2 trial investigating vorinostat/GO (NCT00673153) have been described previously [9]. Patients aged ≥60 years were eligible if they had untreated primary or secondary AML (other than acute promyelocytic leukemia) according to the 2008 World Health Organization classification, provided they had an Eastern Cooperative Oncology

Group (ECOG) performance status (PS) of 0–3 and adequate organ function. Subjects were ineligible if they were previously diagnosed with another malignancy (unless they were disease-free for >6 months), received prior AML-like systemic therapy, GO or HDAC inhibitors, had central nervous system disease involvement, had a known HIV infection, or had an uncontrolled systemic infection. Patients received vorinostat 400 mg orally once daily on Days 1–9 and GO 3 mg/m^2 on Day 8; hydroxyurea was given to reduce the WBC to less than 10×10^9/L before beginning vorinostat. Those achieving either CR or CRp after 2–3 cycles of therapy (the protocol was amended after 8 enrolled patients to allow a third induction course before response assessment) were eligible to receive one cycle of consolidation treatment with vorinostat/GO at the same doses. Patients could then proceed with vorinostat maintenance therapy as long as CR/CRp was maintained or were removed from study treatment to receive more intensive consolidation therapy including hematopoietic cell transplantation (HCT). Cytogenetic risk-group assignment was according to the Southwest Oncology Group (SWOG)/ECOG criteria. Treatment responses were according to standard criteria by international working groups [3, 18]. The study was approved by the institutional review board of participating institutions, and patients gave informed consent for the clinical trial and associated correlative laboratory studies in accordance with the Declaration of Helsinki.

BH3 Profiling

Thawed aliquots of pretreatment peripheral blood- and bone marrow aspirate-derived mononuclear cells containing leukemic blasts were stained with the antibodies CD45-V450, CD3-Biotin (BD Bioscience, San Jose, CA), and CD20-Biotin (eBiosciences, San Diego, CA) followed by incubation with Streptavidin-APC. Specimens were permeabilized with digitonin and incubated with JC-1 mitochondrial dye and 100 μM BH3 peptides (Bim, Puma, Noxa, Bad, Hrk; Bim and Puma. were also assayed at 0.1 μM and 10 μM, respectively); these peptide sequences have been described previously [14] and were synthesized by New England Peptide (Gardner, MA). Specimens were also incubated individually with dimethyl sulfoxide (DMSO [(1%]) or Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP [10 μM]); the latter serves as an uncoupling reagent control and induces mitochondrial depolarization to 100% completion. The JC-1 signal, proportional to mitochondrial charge, is lost during mitochondrial outer membrane permeabilization; i.e. the full signal is retained with DMSO treatment but lost with CCCP. Peptide induced depolarization is then calculated as a percent relative to the CCCP control which is normalized at 100% priming. Samples were run in duplicate, except in cases where insufficient viable cells were available, on a BD FACS Canto II cytometer (BD Bioscience), and data were analyzed using FACS Diva software. The blast population was identified as CD45 dim, CD3$^-$ and CD20$^-$ [10–12]. The quantifiable propensity of a pro-apoptotic peptide to induce mitochondrial depolarization relative to an uncoupling reagent control was calculated using the median signal intensity of the phycoerythrin channel normalized for DMSO as background (negative control) and CCCP served as 100% priming (positive control) [12]:

$$\%\text{priming}=(1-(\frac{Peptide-CCCP}{DMSO-CCCP}))\times 100$$

Statistical Analysis

Univariate testing association between biomarker status (% priming) and responder or non-responder classification was by logistic regression analysis. We pre-determined a statistical analysis plan with significance set at $p<0.05$. Marker predictive ability was assessed using the area under the receiver operator characteristic curve (AUC). Multivariate analyses were performed using logistic regression and significant adjustment variables from patient clinicopathologic data. Overall survival (OS) was tested for correlation with % priming by logistic regression and AUC. Missing values were treated as imputed data for statistical analyses. Analyses utilized SAS software, version 9.2 (Cary, NC), R version 2.14.2 (Vienna, Austria), and/or Graphpad Prism version 5.04 (La Jolla, CA).

RESULTS

Characteristics of Study Cohort

The original phase 2 study of vorinostat and GO in older adults with untreated AML enrolled 31 patients [9]. Of these, pre-treatment bone marrow (BM) or peripheral blood (PB) specimens were available from 26 patients (83.9%); their pertinent baseline characteristics, stratified by response to induction therapy (i.e. achievement of CR/CRp vs. not) are summarized in Table 1. Patients achieving a CR/CRp were younger than those who failed therapy with vorinostat/GO (p=.022); age was therefore included as covariate in our multivariate analyses of BH3 profiling data. No statistically significant differences were found for any of the other variables in this small patient cohort, although there was a trend towards higher incidence of *NPM1* mutations in responders (p=.084).

BH3 Profiling of Pre-Treatment Patient Specimens

From 26 study participants (median age of 73.8 years [range: 61.1–80.7 years], aliquots of pre-treatment specimens were thawed for the purpose of BH3 profiling. Upon thawing, these specimens yielded cells with excellent viability (median of 82.1% [range: 62.2–97.9%] live cells). They were then subjected to *in vitro* exposure to individual BH3 peptides, including an activator (Bim) and several sensitizers (Noxa, Puma, Bad, Hrk) as surrogates for the function of Bcl-2 family proteins. Twenty-three of 26 tested specimens (n=8 and n=15 from BM and PB, respectively) provided analyzable data, for an overall technical success rate of 88.5%. Three samples were eliminated from statistical analysis due to insufficient cell numbers. Of note, 13 specimens were analyzed in duplicate, with an overall Coefficient of Variation (CV) for repeat samples from individual patients being generally between 3–5%, indicative of a technically robust assay (data not shown).

Association between Priming to BH3 Peptides and Response to Induction Therapy with Vorinostat/GO

The percent priming, i.e. quantifiable propensity of a given BH3 peptide to induce mitochondrial depolarization relative to an uncoupling control agent, for each peptide is summarized in Table 2 separately for patients who responded to study therapy (i.e. achieved either CR/CRp) and those who failed treatment. Among the peptides assayed, only Noxa elicited a statistically significantly different priming between responders (54.1 ± 29.0%

[mean±SD]) and non-responders (23.8 ± 14.9%; p= .027); the percent priming with Noxa for individual patients is depicted in Figure 1A. To test the ability of Noxa to serve as predictive biomarker, we employed the area under the receiver operator characteristic curve (AUC) to analyze the sensitivity and specificity of this biomarker, which yielded an AUC of 0.83 (95%CI: 0.65–1.00; p=0.00042; Figure 1B). Because we found responders to be significantly younger than non-responders (see Table 1), we performed adjusted analyses of Noxa priming in which we accounted for age as second covariate. As shown in Figure 1B, adjustment for age (as a continuous variable) improved the AUC to 0.88 (95% CI: 0.75–1.00). In contrast to Noxa, no statistical association was established between response and priming readout with Bim, Puma, Bad, or Hrk (Table 2).

Association between Priming to BH3 Peptides and Survival

Having established that results from BH3 profiling can predict response to vorinostat/GO, we were interested in investigating whether this approach could also serve to predict survival. As summarized in Table 3, we found a statistically significant association between the extent of priming with Noxa and OS (p=0.026 logistic regression; p=0.037 Cox regression) with an AUC of 0.87 (95% CI: 0.64–1.00; p=.0017). In contrast, there was no statistically significant association between priming with any of the other peptides and OS.

DISCUSSION

It is a recurrent clinical observation that any given chemotherapeutic regimen, be it standard or investigational, will only be successful in a subset of AML patients. Although there has been a long-standing interest in reliable outcome predictors, currently available pretreatment factors such as age or cytogenetics are insufficient to predict treatment success with high accuracy [19].

A biomarker is likely most informative if its metric is in line with the presumed biological mechanism through which a given chemotherapeutic agent exerts its cytotoxic effect [13, 20]. For instance, a Bcl-2 inhibitor will likely show a correlation with priming readout from the Bad peptide, whereas inhibitors reliant on Mcl-1 are likely to show correlation with readout from the Noxa peptide (the respective pro-apoptotic binding partners of these target proteins) [21, 22]. To date, calicheamicin-containing compounds are not known to modulate specific pro-apoptotic cues from either activators or sensitizer classes of BH3-only proteins. However, the intrinsic (mitochondrial) pathway of apoptosis appears to be predominantly utilized during calicheamicin-induced cell death, which may be triggered in a p53-independent and death receptor/FADD-independent manner via activation of mitochondrial permeability transition, cytochrome c release, and engagement of pro-apoptotic Bcl-2 family proteins (e.g. Bax and Bak), and caspase activation [15, 23]. In fact, an inability to activate Bax/Bak was found to be associated with resistance to GO-induced cytotoxicity in AML cells, and knockout of Bax abrogated cell death after calicheamicin treatment [15, 23]. Additionally, administration of HDAC inhibitors, including vorinostat, has been shown to result in down-regulation of Mcl-1 and potentially other anti-apoptotic Bcl-2 family members [16, 24]. Mechanistically, this is consistent with data from preclinical studies of

solid tumors whereby up-regulation of Noxa following vorinostat treatment is correlated with therapeutic efficacy and the ability to overcome acquired multi-drug resistance [25].

Given these preclinical studies implicating pro-apoptotic Bcl-2 family members in the cytotoxic activity of both GO and vorinostat, it is perhaps not surprising that our studies identified the extent of Noxa-induced priming to correlate with the induction response to, and survival after, vorinostat/GO. One question that arises relative to Noxa priming and correlation to clinical outcomes is whether the marker is predictive of response to HDAC inhibitor plus calicheamicin-derived therapy or merely prognostic for patients that are likely to respond to chemotherapy in general. Closer examination of previously published data alongside the data presented herein suggests the former. Specifically, Vo *et al.* found an association between Bim priming and response to topoisomerase inhibitors in $CD34^+$ cells [11]. Likewise, Pierceall *et al.* described a pronounced association between Bim priming and both clinical response and survival among AML patients treated with cytarabine-based regimens [12]. However, in neither of these 2 studies was Noxa significantly associated with clinical outcomes. On the other hand, in the current study, Noxa was the only biomarker found to be significant, whereas there was no such association with Bim. Together, these data are consistent with specific biomarkers from panels of BH3 peptides being predictive of individual treatments (or regimens) and not merely being prognostic for broad chemotherapeutic response.

Mcl-1, Bcl-2 and Bcl-xL protein levels were previously examined in 123 AML patient pretreatment specimens versus chemotherapeutic outcomes with no observable association [26]. Changes in these expression levels in specimens from recurrent patients suggested a role for anti-apoptotic Bcl-2 family members in acquired chemoresistance. Our previous results of pretreatment AML patient s indicated pronounced association of BH3 profiling readout and response while protein expression was not significant [12], indicating definitive divergence in steady state protein levels and BH3 profiling metrics (R^2=0.04).

As strengths of our study, all patients had previously untreated AML and received identical chemotherapy under the auspices of a clinical trial. As a further strength, BH3 profiling experiments and data analyses were performed in a blinded fashion without knowledge of the clinical outcome. Alternatively, acknowledged limitations of the present study are its retrospective nature and the relatively modest number of patients investigated. As patients received a combination therapy of vorinostat and GO, we are unable to discern whether the association between clinical response/survival and Noxa-induced priming is primarily driven by clinical response to vorinostat, GO, or both. As another limitation, most of the responders obtained further non-vorinostat/GO-based consolidation therapy off study, which may impact the survival analyses in our dataset. Finally, for direct clinical applicability, the findings may be somewhat limited as GO is currently not available commercial in many countries (although it has remained fully approved in Japan), and the role of HDAC inhibitors for the treatment of AML remains unclear. In addition to vorinostat, other HDAC inhibitors (e.g. entinostat, panobinostat, ACY-1125) have entered clinical use and/or trial, as have other calicheamicin-containing antibody drug conjugates (e.g. the CD22-targeting immunoconjugate, inotuzumab ozogamicin) [27, 28]; while specific correlative studies will be required, the similarities between these drugs and vorinostat/GO suggest that BH3

profiling, and perhaps in particular response to NOXA-induced priming, could be useful as predictive biomarker(s) to such therapeutics. Indeed, this strategy is consistent with reports in highlighting the biological mechanistic importance of the Noxa/Mcl-1 node in AML-directed therapies [29, 30].

In the current cohort, 7 of the 23 analyzed patients experienced a clinical response to vorinostat/GO, yielding a response rate of 30.4%. In our analyses, the combination of Noxa + age yielded an AUC of 0.88. By closer examination of the ROC curves, all responder patients could be identified (100% sensitivity), while 68.8% of likely non-responder would be identified as such. If the predictive value of such an applied biomarker may be to triage the likely non-responder patients, then the response rate almost doubles from 30.4% to 58.3% (an overall improvement of response rate of 91.7%). While larger cohorts will be required to fully define the extent of medical utility for such a diagnostic test, this early observation may hold promise for the use in experimental regimens such as that studied herein.

In conclusion, our BH3 profiling data in this exploratory study identify Mcl-1 dependence to be associated with response and outcome of vorinostat/GO in older adults with AML. This finding would be compatible with a pivotal role of Bcl-2 family protein-mediated mitochondrial depolarization for the clinical efficacy of GO and vorinostat, consistent with previous *in vitro* studies

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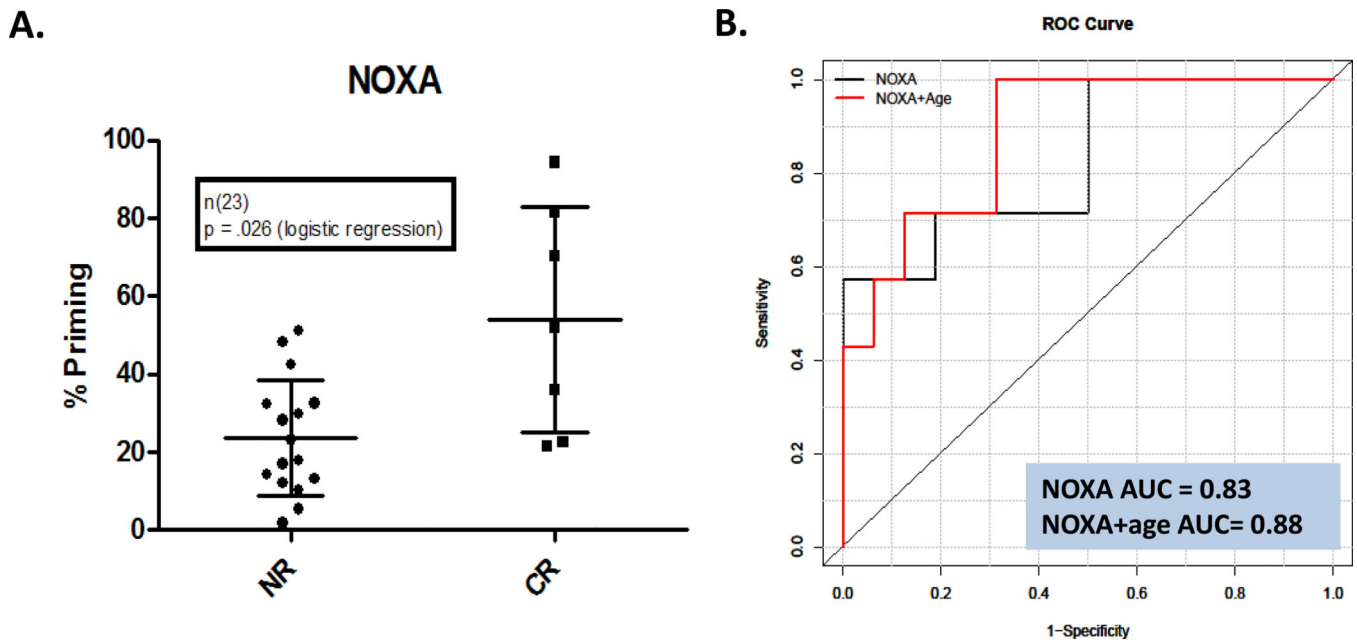

Figure 1. Relationship between Noxa-induced priming and therapeutic response

(A) Dot-plot for the mean %priming (± S.D.) induced by Noxa comparing the 16 non-responder patients ("NR") with those 7 responder patients who achieved a CR or CRp ("CR") with vorinostat/GO. **(B)** ROC-plot of the sensitivity and specificity of Noxa as a predictor of therapeutic response for our study cohort, either when used alone or, in multivariate analysis, combined with age as additional predictive factor.

TABLE I

Baseline Characteristics of Study Population

	Responders (N=7)	Non-Responders (N=16)	All Patients (N=23)	p- value
Median Age (range), years	68.7 (61.1–74.4)	76.3 (64.7–80.7)	73.8 (61.1–80.7)	0.022
Male Gender, n (%)	4 (57.1%)	10 (62.5%)	14 (60.9%)	0.824
Cytogenetic Risk Group, n(%)				0.378
Favorable	0 (0%)	1 (6.2%)	1 (4.3%)	
Intermediate	6 (85.7%)	9 (56.2%)	15 (65.2%)	
Unfavorable	1 (14.3%)	6 (37.5%)	7 (30.4%)	
NPM1 Mutation, n (%)				0.084
Negative	4 (57.1%)	11 (68.8%)	15 (65.2%)	
Positive	3 (42.9%)	0 (0%)	3 (13%)	
FLT3/ITD, n (%)				0.497
Negative	7	9	16	
Positive	0	2	2	
ND	0	5	5	
Antecedent Hematologic Disorder, n (%)	3 (42.9%)	9 (56.2%)	12 (52.2%)	0.890

TABLE II

Relationship Between BH3-Only Peptide Priming and Response to Study Therapy

	Mean %Priming ± SD		p-value (logistic regression)	AUC [95% CI]	p-value (AUC)
	Non-Responders	Responders			
Bad (100 uM)	51.3 ± 24.4	53.2 ± 38.8	0.83	0.51 [0.19, 0.84]	0.93
Bim (0.1 uM)	60.0 ± 21.6	56.4 ± 37.0	0.76	0.50 [0.19, 0.81]	1.00
Hrk (100 uM)	49.6 ± 25.0	54.9 ± 36.7	0.48	0.61 [0.31, 0.91]	0.48
Noxa (100 uM)	23.8 ± 14.9	54.1 ± 29.0	0.026	0.83 [0.65, 1.00]	0.00042
Puma (100 uM)	65.8 ± 28.3	42.3 ± 44.0	0.15	0.63 [0.32, 0.94]	1.00
Puma (10 uM)	51.4 ± 22.2	52.6 ± 35.4	0.91	0.51 [0.21, 0.81]	0.93

TABLE III

Relationship Between BH3-Peptide Priming and Overall Survival

	p-value (logistic regression)	p-value (Cox regression)	AUC [95% CI]	p-value (AUC)
Bad (100 mM)	0.52	0.82	0.60 [0.23,0.97]	0.60
Bim (0.1 mM)	0.71	0.63	0.58 [0.23,0.93]	1.00
Hrk (100 mM)	0.57	0.87	0.61 [0.21,1.00]	0.60
Noxa (100 mM)	0.027	0.037	0.87 [0.64,1.00]	0.0017
Puma (100 mM)	0.55	0.22	0.57 [0.24,0.90]	1.00
Puma (10 mM)	0.94	0.97	0.57 [0.24,0.89]	1.00