Mechanisms and potential molecular markers of early response to combination epigenetic therapy in patients with myeloid malignancies

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Abstract. Combination epigenetic treatment (EGT) utilizing DNA methyl transferase inhibitors (DNMTi) and histone deacetylase inhibitors (HDACi) may be more efficacious than single agent treatment in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). The molecular mechanisms behind the potential clinical efficacy of combination EGT treatment are incompletely understood and the frequently lengthy EGT regimes required to determine clinical response have generated a significant demand for early molecular markers of treatment response. Our study aimed to identify the effect of combination azacitidine (AZA) and panobinostat (LBH589) on expression levels of a panel of genes implicated in the pathogenesis of high-risk MDS or AML in HL-60 cells. We also characterized gene expression profiles in peripheral blood mononuclear (PBMCs) from patients in a recently reported phase Ib/II clinical trial using the combination of AZA and LBH589 and correlated these findings with clinical response to treatment. *In vitro* analysis demonstrated increased expression of caspase-3, Nor-1, *NUR77*, $p15^{INK4B}$ and $p21^{WAF1/CIP1}$ and decreased expression of Bcl-xL in HL-60 cells treated with combination EGT. Analysis of patient samples prior to treatment demonstrated a significant reduction in *NUR77* and $p21^{WAF1/CIP1}$ expression compared to healthy controls. *NUR77* and $p21^{WAF1/CIP1}$ levels were similar between treatment non-responders and responders at screening. Early post first cycle treatment (day 25) analysis demonstrated a significant increase in expression of both *NUR77*, and $p21^{WAF1/CIP1}$. A significant increase in *NUR77*, and $p21^{WAF1/CIP1}$ together with a trend to increase in $p15^{INK4B}$ first cycle expression was observed in treatment responders compared to non-responders. In summary, combination AZA and LBH589 epigenetic treatment is associated with *in vitro* and *in vivo* modulation of genes implicated in the pathogenesis of MDS/AML. Early expression of *NUR77* and $p21^{WAF1/CIP1}$ correlated with clinical response to combination EGT suggesting investigation for potential use as molecular markers of early treatment response may be warranted.

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Introduction

The potential for enhanced therapeutic responses to combination DNMTi and HDACi EGT is predicated on evidence from basic molecular studies identifying interaction of cellular methylation and acetylation machinery in the transcriptional regulation of gene expression (1,2). Subsequent *in vitro* and *in vivo* studies, particularly involving hematological malignancies, have identified enhanced regulation of gene expression and responses to combination EGT lending support to basic molecular studies (3-8).

Clinical studies evaluating the efficacy of combination EGT have, in part, contributed to establishing the molecular mechanisms for the observed therapeutic effects of EGT although have failed to consistently demonstrate changes in early global and gene specific methylation or acetylation status correlate with clinical effect (9-12). Given the duration of EGT either alone e.g., AZA or in combination with HDACi frequently requires several cycles to determine evidence of clinical benefit a significant need exists for identification of molecular markers for determination of early response to (combination) EGT in order to facilitate accurate selection of patients for continued epigenetic treatment, avoidance of unnecessary cost and exposure to side effects in patients unlikely to respond, and to avert delays in provision of more appropriate treatments where possible.

Our previous *in vitro* studies and recent clinical report investigating the effects of combination AZA and HDACi EGT identifies modulation of expression of genes implicated in the pathogenesis of MDS and AML (8,13,14). We sought to characterize the *in vitro* expression of genes implicated in

the pathogenesis of MDS/AML and potentially involved in the transcriptional response to the specific combination of AZA and LBH589 EGT in HL-60 cells. In addition we evaluated the temporal expression of genes identified as being regulated from our *in vitro* studies in peripheral blood mononuclear cells (PBMCs) from trial patients with high-risk MDS and AML receiving combination AZA and LBH589 EGT and correlated these findings with subsequent clinical responses, predominately observed after a minimum of 3 cycles of therapy (14), with a view to identification of potential early molecular markers of combination EGT treatment response.

Materials and methods

Cell culture. HL-60 cells were cultured in RPMI-1640 (Gibco BRL) containing 10% heat inactivated fetal calf serum and kept in a 5% CO_2 incubator at 37˚C. Agents: azacitidine (AZA), the kind gift of Celgene, Australia and panobinostat (LBH589), the kind gift of Novartis, Australia, were added to plates for 48 h. AZA was dissolved in H_2O with 0.2% acetic acid and used at a final concentration of 1.0 μM. LBH589 was dissolved in PBS with 1% DMSO and used at a final concentration of 20 nM.

Growth inhibition assay. HL-60 cells in log phase were plated at a density of $0.2x10^6$ in 10 ml of medium. Cells were harvested at 48 h. Cell viability was assessed using 0.4% trypan blue staining immediately after culture. Black staining cells were considered as non-viable cells, and unstained bright cells as viable. All experiments were repeated 3 times with averages displayed graphically.

Normal healthy control and clinical trial patient samples
RNA extraction. TRIzol was used to extract RNA from PBMC fractions from healthy donors (n=7) or day 25 samples from patients in a recently completed phase Ib/II clinical trial (14). The clinical trial consisted of a 5-day schedule of AZA followed by LBH589 in high-risk MDS or AML. AZA 75 mg/m^2 was injected subcutaneously on days 1-5. LBH589 was administered orally 3 times a week (Mon/Wed/Fri) starting on day 5 for 7 doses of each 28-day cycle. Between January 2010 and January 2012, 40 evaluable patients were enrolled. There were 30 patients with AML and 10 patients with high-risk MDS, 23 patients (Table I) had data enabling evaluation of molecular marker expression and correlation with treatment response determined either 1, 3 or 6 months after treatment commencement. Treatment responses were defined according to International Working Group criteria for AML and MDS (15).

PCR
Semi-quantitative reverse transcription-PCR (RT-PCR). RT-PCR was performed on total TRIzol extracted RNA from HL-60 cells untreated or treated for 48 h with AZA or LBH589 or a combination of AZA and LBH589. The primers used for $p21^{WAF/CIP1}$ were: forward, 5'-ATT AGC AGC GGA ACA AGG AGT CAG CAT-3'; and reverse, 5'-CTG TGA AAG ACA CAG AAC AGT ACA GGG T-3'. The primers used for Bcl-xL were: forward, 5'-TTG GAC AAT GGA CTG GTT GA-3'; and reverse, 5'-GTA GAG TGG ATG GTC AGT G-3'. The primers used for caspase-3 were: forward, 5'-GCA GCA AAC CTC AGG GAA AC-3'; and reverse, 5'-TGT CGGCAT ACT GTT TCA GCA-3'. The human β-actin gene was used as an internal control. The forward primer for β-actin was 5'-GAC AGG ATG CAG AAG GAG ATT ACT-3' and the reverse primer was 5'-TGA TCC ACA TCT GCT GGA AGG T-3'.

Real-time PCR. Reaction volumes of 20 μl contained SYBR Green 1 buffer and forward and reverse primers for target genes. The primers used for Bcl-xL were: forward, 5'-GGC TGG GAT ACT TTT GTG GA-3'; and reverse, 5'-GTA GAG TGG ATG GTC AGT G-3'. The primers used for caspase-3 were: forward, 5'-CAG TGG AGG CCG ACT TCT TG-3'; and reverse, 5'-TGT CGG CAT ACT GTT TCA GCA-3'. The primers used for $p15^{INK4B}$ were: forward, 5'-AGT CAA CCG TTT CGG GAG GC-3'; and reverse, 5'-ACC ACC AGC GTG TCC AGG AAG-3'. The primers used for $p21^{WAF/CIP1}$ were: forward, 5'-TGG ACC TGT CAC TGT CTT GT-3'; and reverse, 5'-TCC TGT GGG CGG ATT AG-3'. The primers used for *Nor-1* were: forward, 5'-GTC CTC AGA CTT TCC ATC AGG T-3'; and reverse, 5'-GAT CAG TAA ATC CCG GAA TCC-3'. The primers used for *NUR77* were: forward, 5'-GCT GCA GAA TGA CTC CAC C-3'; and reverse, 5'-ACA GCA GCA CTG GGC TTA-3'. The primers used for β-actin were forward, 5'-GAC AGG ATG CAG AAG GAG ATT ACT-3'; and reverse, 5'-TGA TCC ACA TCT GCT GGA AGG T-3'. Each PCR run also included wells of no template control (NTC). A melting point dissociation curve generated by the instrument was used to confirm that only a single product was present. The fluorescence data were quantitated using the threshold cycle (Ct) value. Data were normalized to β-actin and presented as the mean-fold change. PCR of each patient sample was completed a minimum of two times to ensure consistency (8).

Statistical methods. The effects of AZA and LBH589 alone and in combination on HL-60 cell growth, $p15^{INK4B}$, $p21^{WAF1/CIP1}$, caspase-3, Bcl-xL, *NUR77* and Nor-1 mRNA expression were assessed by Student's t-test and analysis of variance (ANOVA). Data were expressed as means ± SEM and $p<0.05$ was considered statistically significant.

Results

AZA and LBH589 alone and in combination inhibits HL-60 cell growth. We examined the effects of AZA and LBH589 alone or in combination on the growth of the human leukemia cell line HL-60. Cells were exposed to AZA (1.0 μM) and/or LBH589 (20 nM) for ≤48 h. Treatment doses were selected based on inhibition of cell growth concentrations identified in previous *in vitro* studies (3,4,7). AZA or LBH589 treatment alone significantly inhibited HL-60 cell growth *in vitro* with LBH589 being the more potent agent. LBH589 in combination with AZA demonstrated a non-significant increase in inhibition of cell growth over AZA or LBH589 treatment alone (Fig. 1).

Effect of AZA and LBH589 on expression of Bcl-xL, caspase-3, Nor-1, NUR77, $p15^{INK4B}$ and $p21^{WAF1/CIP1}$ mRNA in HL-60 cells. As we have previously identified a combination of other hydroxamate HDACi's and AZA as producing

Table I. Clinical and molecular response correlation.

Patient	Age/sex	Diagnosis	Best response	*NUR77* mRNA induction[a]	p21 mRNA induction[b]
1	68 M	MDS	Marrow CR	Yes	NA
2	70 M	AML	PR	Yes	Yes
3	72 F	MDS	CR	Yes	No
4	61 M	MDS	Resistant	No	NA
6	63 F	MDS	CR	Yes	NA
7	58 M	AML	Resistant	No	No
8	75 M	AML	Resistant	No	No
9	72 M	AML	CR	No	Yes
11	73 M	AML	Resistant	No	NA
12	67 F	MDS	PR	Yes	NA
13	79 M	MDS	SD	Yes	NA
16	72 M	MDS	Resistant	No	No
17	60 F	AML	Resistant	No	Yes
18	67 F	MDS	PR	Yes	Yes
19	80 M	AML	Resistant	No	Yes
22	78 M	AML	CR	Yes	Yes
23	69 M	AML	Resistant	No	No
24	72 M	AML	PR	No	Yes
26	75 M	MDS	PD	Yes	No
28	69 M	AML	PR	No	Yes
31	56 M	AML	Resistant	No	No
33	62 F	MDS	Marrow CR	Yes	No
34	70 F	AML	Resistant	Yes	No

[a]2.5-fold or greater *NUR77* induction over Screen. [b]4-fold or greater $p21^{WAF/CIP1}$ induction over Screen. NA, data not available.

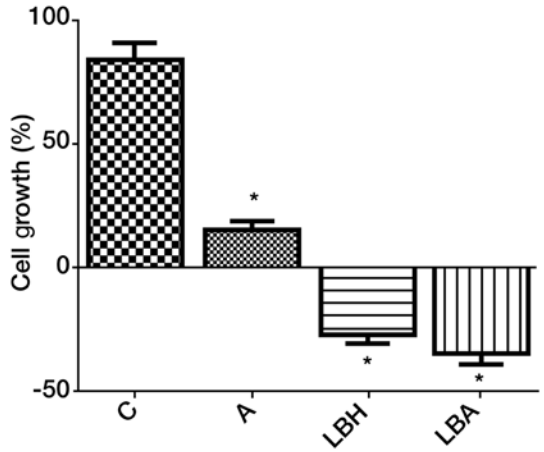

Figure 1. Effects of AZA and/or LBH589 on HL-60 cell growth. HL-60 cells were treated with AZA and/or LBH589 for 48 h. Cell growth (%) = (the total number of viable cells at 48 h - the total number of viable cells at the beginning of the experiment / the total number of viable cells at the beginning of the experiments). C, HL-60 cells without treatment; A, 1.0 μM AZA; LBH, 20 nM LBH589; LBA, 20 nM LBH589 + 1.0 μM AZA. *p<0.001 vs C, n=3.

modulation of several genes implicated in the pathogenesis of MDS including the putative tumor suppressor gene *NUR77* (8,13,16) we were interested in determining the effect of the hydroxamate HDACi LBH589 in combination with AZA on gene expression in HL-60 cells. LBH589 or AZA alone increased $p21^{WAF1/CIP1}$ and caspase-3 expression with LBH589 being the more potent agent (Fig. 2A). The combination of AZA with LBH589 demonstrated a non-significant increase in $p21^{WAF1/CIP}$ expression over single agent treatment (Fig. 2A). LBH589 or AZA alone significantly decreased expression of Bcl-xL expression and the combination of AZA with LBH589 significantly decreased Bcl-xL expression over single agent treatment (Fig. 2A).

Real-time PCR analysis demonstrated AZA, and LBH589 alone significantly increased expression of the novel tumor suppressor genes, *NUR77*, Nor-1 and $p15^{INK4B}$ with LBH589 demonstrating a more potent single agent effect in relation to induction of *NUR77* expression (Fig. 2B). The combination of AZA with LBH589 identified a non-significant increase in $p15^{INK4B}$, *NUR77* and Nor-1 expression over single agent treatment (Fig. 2B).

Effect of AZA and LBH589 on the expression of Bcl-xL, caspase-3, Nor-1, NUR77, $p15^{INK4B}$ and $p21^{WAF1/CIP1}$ mRNA in patient samples. Analysis of gene expression in patient samples prior to treatment (screening) demonstrated a significant reduction in *NUR77* and $p21^{WAF1/CIP1}$ mRNA expression compared to healthy controls (Fig. 3A). *NUR77* and $p21^{WAF1/CIP1}$

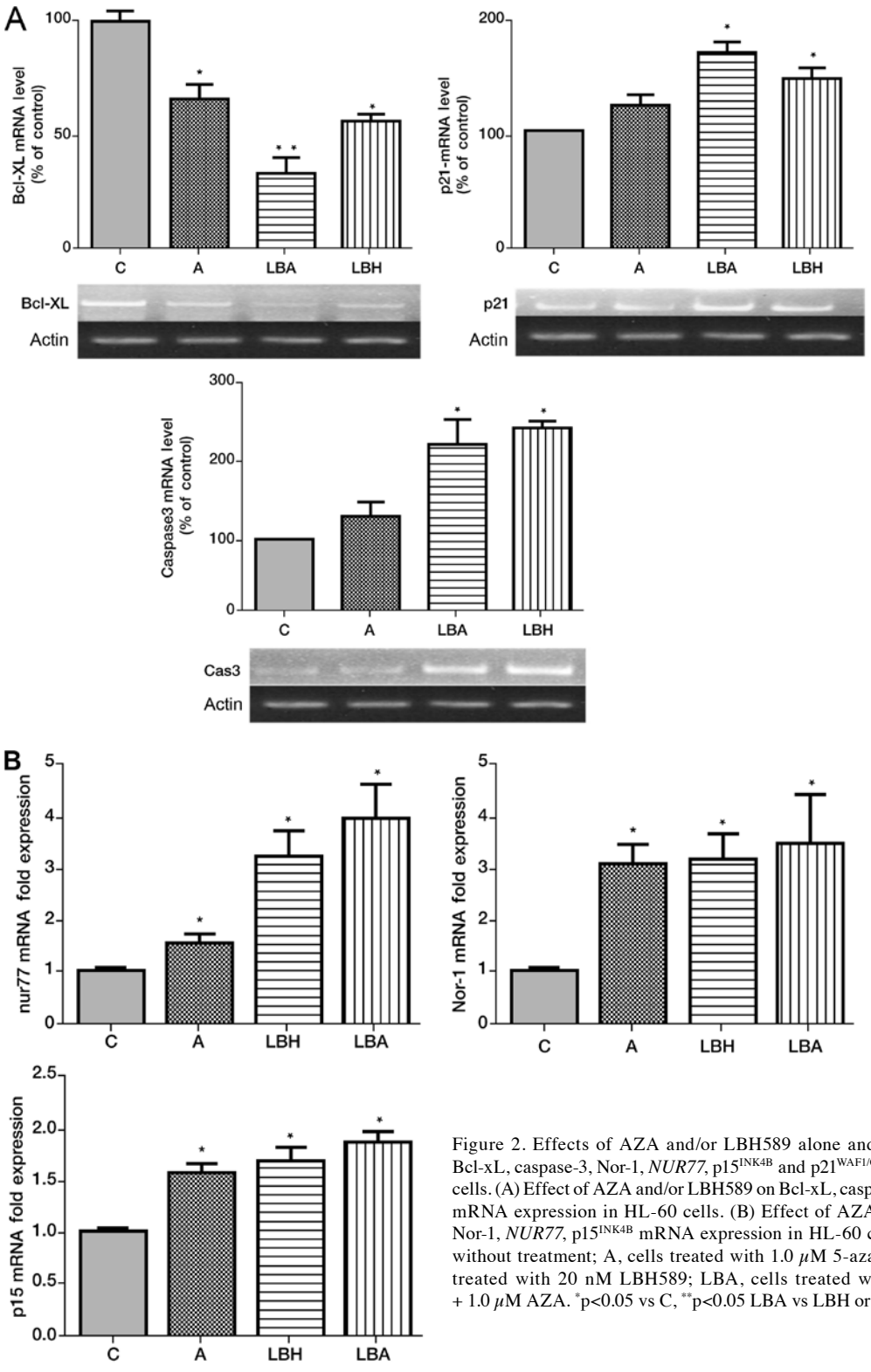

Figure 2. Effects of AZA and/or LBH589 alone and in combination on Bcl-xL, caspase-3, Nor-1, *NUR77*, $p15^{INK4B}$ and $p21^{WAF1/CIP1}$ mRNA in HL-60 cells. (A) Effect of AZA and/or LBH589 on Bcl-xL, caspase-3 and $p21^{WAF1/CIP1}$ mRNA expression in HL-60 cells. (B) Effect of AZA and/or LBH589 on Nor-1, *NUR77*, $p15^{INK4B}$ mRNA expression in HL-60 cells. C, HL-60 cells without treatment; A, cells treated with 1.0 μM 5-azacitidine; LBH, cells treated with 20 nM LBH589; LBA, cells treated with 20 nM LBH589 + 1.0 μM AZA. $^{*}p<0.05$ vs C, $^{**}p<0.05$ LBA vs LBH or AZA, and C, n=3.

mRNA expression levels were similar between treatment non-responders and responders at screening (Fig. 3B). Post treatment (day 25, first cycle) demonstrated a significant increase in expression of both *NUR77*, and $p21^{WAF1/CIP1}$ ranging from 1.5- to 6-fold over screening levels (Fig. 3C). Importantly a significant increase in *NUR77*, and $p21^{WAF1/CIP1}$ (Table I and Fig. 3C) together with a trend to increase in $p15^{INK4B}$ expression was observed in treatment responders compared to non-responders (Fig. 3C).

Discussion

Novel epigenetic treatments for myelodysplastic syndrome (MDS) including DNA methyl transferase inhibitors (DNMTi) and potentially histone deacetylase inhibitors (HDACi) are able to improve survival (17,18). However, not all patients respond and treatment regimens are often lengthy with responses frequently only observed after several cycles of therapy (17,18). In order to reduce unnecessary treatment exposure, associ-

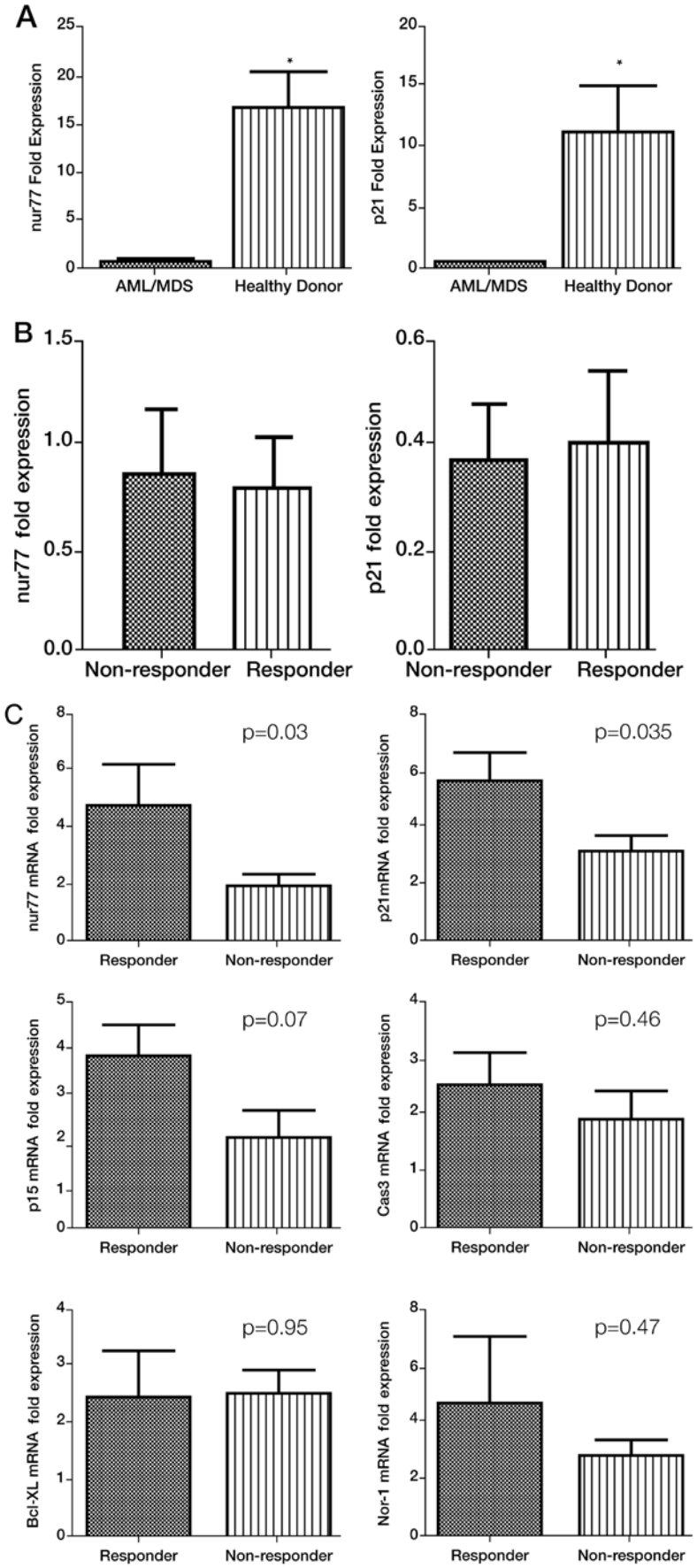

Figure 3. Effects of combination LBH589 and AZA on Bcl-xL, caspase-3, Nor-1, *NUR77*, $p15^{INK4B}$ and $p21^{WAF1/CIP1}$ expression and correlation with clinical response in PBMC's. (A) Pre-treatment patient and healthy control *NUR77* and $p21^{WAF/CIP1}$ mRNA expression levels. (B) Pre-treatment *NUR77* and $p21^{WAF/CIP1}$ mRNA expression levels for responders and non-responders. (C) Correlation of gene expression with clinical response to epigenetic treatment, day 25 mRNA expression levels for *NUR77*, $p21^{WAF/CIP1}$, $p15^{INK4B}$, caspase-3, Bcl-xL and *NOR-1* correlated with clinical response to treatment determined at 1, 3 and 6 months. n=23, $p<0.05$ deemed significant. Responses were defined according to International Working Group criteria for AML and MDS (15).

ated complications and delays in commencement of other potentially effective therapies a significant demand exists for molecular markers to improve early prediction of response to epigenetic therapy, particularly as baseline and early treatment epigenetic modifications including methylation and acetylation status may not inform on this issue (9,10,17,18).

LBH589 is a novel cinnamic hydroxamic acid analog with established antitumor activity in pre-clinical models (19). LBH589 has demonstrated limited single-agent activity in advanced acute leukemia (20). Interestingly our *in vitro* observations identified LBH589 as a more potent single agent than AZA with the combination of LBH589 and AZA demonstrating a non-significant increase in potency in inhibition of cell growth over single agent treatment. Single agent treatment demonstrated induction of p15 ^{INK4B}, p21$^{WAF1/CIP1}$, *NUR77*, Nor1 and caspase-3 together with suppression of Bcl-xl expression with LBH589 demonstrating greater potency and a non-significant trend to enhanced modulation of gene expression with combination treatment.

Having identified significant *in vitro* modulation of several genes implicated in the pathogenesis of MDS/AML we were interested to identify temporal expression profiles for these genes in PBMC samples from high-risk MDS/AML patients receiving combination AZA and LBH589 therapy in our recently reported phase 1b/II clinical trial (14). Analysis of *NUR77* and p21$^{WAF1/CIP1}$ expression in patients compared with healthy controls identified significantly reduced expression of both *NUR77* and p21$^{WAF1/CIP1}$ compared with healthy controls as has been previously demonstrated (16,21). Screening levels of both *NUR77* and p21$^{WAF1/CIP1}$ demonstrated no significant correlation with clinical response to therapy (Fig. 3B) similar to several previous studies undertaking analysis of other epigenetic markers in response to single or combination EGT (18). However determination of *NUR77* and p21$^{WAF1/CIP1}$expression levels, compared with other genes implicated in the pathogenesis of MDS/AML, from early treatment time-points (day 25), demonstrated a significant correlation with clinical response (Fig. 3C and Table I). Whilst our observation is not unprecedented, for example Link and colleagues demonstrated a statistically significant concordance between response to AZA therapy and induction of p53-inducible-ribonucleotide-reductase (p53R2) (22) these findings were only noted post several cycles of therapy. Identification of *NUR77* as a primary inhibitor of leukemogenesis (16), upregulation of *NUR77* in primary AML cells and leukemic stems cells in response to epigenetic treatment (23) and early response of *NUR77* expression to EGT together with strong correlation with clinical response suggests the identification of a potentially novel molecular marker of early response to combination EGT in the setting of MDS/AML. Unlike other potential indirect molecular markers of EGT treatment response e.g., global or gene specific, methylation/acetylation status the delineation of the critical molecular involvement of *NUR77* in the leukemogenesis process, its ubiquitously reduced expression in AML/MDS compared to healthy controls and its early upregulation and correlation with clinical response posits a compelling argument for an early molecular marker of response to combination EGT in MDS/AML. Interestingly, whilst *in vitro* expression levels of the related orphan nuclear receptor Nor-1 were significantly upregulated by combination EGT and previous *in vivo* studies have demonstrated downpregulation of Nor-1 in patient leukemic blasts (16) no significant correlation between clinical response and early Nor-1 expression was observed in our study suggesting that *NUR77* has a more central role in the early and subsequent clinical response to combination EGT.

Our *in vitro* and *in vivo* observations identified significant modulation of expression of apoptosis machinery molecules including Bcl-xL and caspase-3, previously identified as important in mediating the therapeutic effects of EGT (24), although neither demonstrated a significant correlation with clinical response (Figs. 1-3). *NUR77* has also been identified to play a critical upstream role in mitochondrial-mediated apoptotic events translocating from nucleus to mitochondria, binding Bcl-2 and releasing cytochrome *c* with resultant induction of apoptosis (25,26). The critical molecular and upstream role of *NUR77* in the leukemogenic process as opposed to the effector functions of Bcl-xL and caspase-3 may potentially explain why EGT-mediated restitution of *NUR77* and not Bcl-xL and caspase-3 expression levels is associated with subsequent clinical response.

Our results also identified a trend toward early upregulation of p15^{INK4B} and clinical response. The effects of AZA alone and in combination with HDACi on p15^{INK4B} expression are well documented and have been thought pivotal in the therapeutic activity of this agent in MDS (8,13,27-29). Indeed early studies showed that promoter methylation of the p15^{INK4B} gene was associated with disease progression in MDS and that the p15^{INK4B} promoter was demethylated during treatment with AZA at an early time-point (28). However, baseline and early treatment methylation status has subsequently been demonstrated not to predict response to epigenetic therapy (9,10) and early re-expression of p15^{INK4B} is not associated with clinical response (30,31). Together these results suggest whilst p15^{INK4B} expression may be modulated in response to EGT it is unlikely to serve as a robust molecular marker of early epigenetic treatment response and subsequent clinical response.

In conclusion, our observations identify potential molecular mechanisms associated with combination AZA and LBH589 treatment in *in vitro* and *in vivo* settings and posit an argument for prospective clinical investigation of orphan nuclear receptor *NUR77* and cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ as potential molecular markers of early response to combination AZA and HDACi EGT in the setting of MDS and AML.

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