Epigenetic reprogramming reverses the relapse specific gene expression signature and restores chemosensitivity in childhood B-lymphoblastic leukemia

Running Title: Epigenetic reprogramming in relapsed childhood ALL

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Abstract

While the improvement in outcomes for children with acute lymphoblastic leukemia (ALL) has been gratifying, the poor outcome of patients who relapse warrants novel treatment approaches. Previously, we identified a characteristic relapse-specific gene expression and methylation signature associated with chemoresistance using a large cohort of matched diagnosis-relapse samples. We hypothesized that “reversing” such a signature might restore chemosensitivity. In this study, we demonstrate that the histone deacetylase inhibitor (HDACi) vorinostat not only reprograms the aberrant gene expression profile of relapsed blasts by epigenetic mechanisms but is synergistic when applied prior to chemotherapy in primary patient samples and leukemia cell lines. Furthermore, incorporation of the DNA methyltransferase inhibitor (DNMTi) decitabine led to re-expression of genes shown to be preferentially methylated and silenced at relapse. Combination pretreatment with vorinostat and decitabine resulted in even greater cytotoxicity compared to each agent individually with chemotherapy. Taken together, our results indicate that acquisition of chemoresistance at relapse may be driven in part by epigenetic mechanisms. Incorporation of these targeted epigenetic agents to the standard chemotherapy backbone is a promising approach to the treatment of relapsed pediatric ALL.
Introduction

Relapsed ALL is one of the leading causes of death among children with cancer. A hallmark of relapsed blasts is their intrinsic chemoresistance compared to what is observed at initial diagnosis. Given the frequent failure of conventional salvage chemotherapy including intensified drug schedules and stem cell transplantation in the treatment of relapsed ALL, innovative strategies are urgently needed.

In recent years it has become clear that cancer can be driven by patterns of altered gene expression mediated by mechanisms that do not affect the primary DNA sequence, but through the “epigenetic” processes of DNA promoter methylation and histone modification. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) and has been shown to be an important contributor to carcinogenesis by silencing tumor suppressor genes in many tumor types, including hematologic malignancies. Chromatin structure is also regulated by the “histone code”, which refers to post-translational modifications (methylation, acetylation, phosphorylation, ubiquitination) of key lysine residues on core histone proteins. These two epigenetic processes of DNA promoter methylation and histone modifications are clearly interdependent and coordinated.

DNMTi such as 5-azacitidine and decitabine have the potential to reverse promoter hypermethylation in tumor cells, leading to re-expression of aberrantly silenced genes, and induce tumor cell death. DNMTi’s have been demonstrated to be effective therapy for myelodysplastic syndrome (MDS), which is characterized by global promoter hypermethylation. Likewise, inhibition of histone deacetylases with HDACi’s such as vorinostat can alter the balance in favor of histone acetyltransferases (HATs), resulting in an increased acetylation of histone H3K9 and H3K14 and gene transcription.

Recently, we performed an integrated analysis to identify biological pathways underlying relapsed ALL using genome wide gene expression arrays, SNP arrays and DNA methylation arrays. We have identified a relapse specific gene expression signature characterized by upregulation of genes involved in regulation of cell cycle and apoptosis (BIRC5, FOXM1,
GTSE1), DNA replication and repair (FANCD2) and nucleotide biosynthesis (TYMS, CAD, PAICS, ATIC, DHFR); and by downregulation of genes involved in sensitivity to thiopurines and alkylators (MSH6) and glucocorticoids (BTG1 and NR3C1). In addition, our genome wide methylation analysis revealed a distinctly higher CpG methylation level in the relapsed cohort compared to diagnosis, and for a subset of differentially methylated genes, concordant downregulation of mRNA expression was observed, implicating epigenetic dysregulation in the acquisition of chemoresistance at relapse.

We hypothesized that there may be an existing drug whose effect on gene expression might mimic reversal of the relapse signature and may therefore functionally restore chemosensitivity in leukemic blasts. We searched the Connectivity map database to “query” our relapse signature, and identified the HDACi vorinostat as the top candidate agent which could potentially endow a chemosensitive gene expression profile. We validated this finding by demonstrating reversal of the relapse signature in primary B-lymphoblastic leukemia patient samples and cell lines treated with vorinostat that correlated with modulation of key epigenetic histone modifications. We further questioned whether a DNMTi could reverse relapse-specific promoter hypermethylation and re-express aberrantly silenced genes, and confirmed this in ALL cell lines treated with the DNMTi decitabine. Finally, we tested whether “epigenetic reprogramming” with each agent alone or the combination of HDACi and DNMTi may functionally restore chemosensitivity. By treating primary patient samples and cell lines with vorinostat and decitabine, we demonstrated enhanced chemosensitivity that correlated with modulation of aberrant relapse-specific signature. Our data suggests that incorporation of epigenetic agents into conventional treatment regimens may improve the outcome of relapsed childhood ALL.
Materials and Methods

Cells
Primary patient samples were collected from patients treated at the New York University Medical Center and from the Children’s Oncology Group (COG) cell bank. Samples were collected under the respective center’s institutional review board–approved cell procurement protocols for children with ALL. Informed consent was obtained in accordance with the Helsinki protocol. Of the total 7 samples, 4 were from initial diagnosis and 3 were collected at the time of relapse. Samples were enriched from diagnostic bone marrow collections by Ficoll-Hypaque centrifugation and resuspended in fresh culture medium (RPMI1640) containing 20% fetal bovine serum, 0.01% ITS (insulin, transferrin and sodium selenide- Sigma-Aldrich, St. Louis, MO) solution and 1% penicillin-streptomycin. The diagnosis of ALL was based on morphology and flow cytometric analysis of the immunophenotype.

B-lineage leukemia cell lines, Reh, RS4:11 and UOCB1 cells, B-lymphoma cell lines, Raji cells and B-myelomonocytic leukemia cell lines, MV4:11 were grown in RPMI1640 medium supplemented with 10% fetal bovine serum, 10 mM HEPES buffer, 1% Penicillin/Streptomycin under 5% CO₂ at 37°C. All cell lines were purchased from American Type Culture Collection and are authenticated according to their protocols (http://www.atcc.org). Stock solutions of vorinostat (Cayman Chemical, Ann Arbor, MI) and etoposide were prepared in dimethyl sulfoxide while doxorubicin, cytarabine and decitabine (Sigma-Aldrich, St. Louis, MO) were prepared in ddH₂O. Prednisolone (Pharmacia, St Paul, MN) was suspended in 0.9% NaCl. Drugs were serially diluted in RPMI and added to the culture media at indicated concentrations. Cells were incubated with chemotherapy for 24-48 hours.

Connectivity Map
The Connectivity map (cmap) is a collection of genome-wide transcriptional expression data from cultured human cells treated with various bioactive small molecules. Using a nonparametric, rank-based pattern-matching strategy based on the Kolmogorov-Smirnov statistic, this analysis provides a ranked order of individual treatment instances based on their similarity to a given gene expression profile. The current version of the cmap data set (build02)
(www.broadinstitute.org/cmap) was used to “query” our relapse specific gene expression signature. The relapse gene expression profile was derived from 49 diagnosis/relapse patient pairs analyzed on Affymetrix U133plus2.0 microarrays.\textsuperscript{16} 300 top ranking genes (150 down-regulated and 150 upregulated genes at relapse compared to diagnosis) were chosen based on a false discovery rate (FDR) <10\% and a p-value <0.002. As cmap contains gene-expression signatures derived from the Affymetrix U133A platform, close to half of our probe-sets could not be analyzed. Thus the final list included 154 probe sets consisting of 56 upregulated and 99 downregulated genes (Table S1).

**Gene Expression Analysis**

RNA was isolated from three primary patient samples and three leukemia cell lines with or without treatment with 1 \(\mu\text{M}\) of vorinostat for 24 hours using Qiagen RNeasy Mini Kits (Valencia, CA) and quality was verified by an Agilent 2100 Bioanalyzer (Palo Alto, CA). In vitro transcription was completed with biotinylated UTP and CTP for labeling using the ENZO BioArray HighYield RNA Transcript Labeling kit (Enzo Diagnostics, Farmingdale, NJ). Thirteen micrograms of labeled cRNA was fragmented and hybridized to Affymetrix U133Plus2 microarrays according to Affymetrix protocol (Santa Clara, CA). Raw Affymetrix CEL files were processed with the standard Affymetrix probe modeling algorithm RMA. Background correction and quantile normalization were applied during the process. The processed data was stored in a matrix containing one intensity value per probe set in the GCT (gene cluster text) format file. Analyses were based on the GCT format files with a class label file created in the CLS (categorical class file) format. Different versions of the CLS files were created when comparing different subsets of samples using applications of gene pattern. Two independent statistical methods, Chi-square test and random sampling, were used to test the significance of changes observed in the relapse specific signature (154 probes) compared to the whole gene set (54,676 probes). Expression data discussed in this publication have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; http://www.ncbi.nlm.gov/geo) and are accessible through GEO Series accession number GSE34880.
**Quantitative Reverse Transcription PCR Analysis**

To determine the relative expression of each gene of interest, total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA), and reverse transcription (RT)-PCR was performed using the I-Script II complementary DNA Synthesis kit (Biorad, Hercules, CA) and the PerfeCTA SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD). Synthesis of PCR products was monitored by the DNA Engine Opticon System (MJ Research, Waltham, MA) and normalized to β2 microglobulin levels. Data were plotted relative to mRNA levels in control samples using the ΔCt or ΔΔCt method. PCR primers are listed in the supplemental data (Table S2). Wilcoxon paired signed-rank test was used to test the significance of change.

**Western Blotting**

Reh cells were treated with or without vorinostat (1 µM) and cell lysates were prepared using the histone extraction protocol (acid extraction) following the manufacturer’s instructions (Abcam, Cambridge, MA). Lysates were probed with purified rabbit polyclonal anti-acetyl histone H3 at 1:5000 dilution (# 06-599, Millipore, Bellerica, MA) and the levels of acetyl-H3 was determined relative to a housekeeping gene, actin, at 1:2000 dilution (Abcam, Cambridge, MA). Signals were visualized using the Odyssey infrared imaging system (LI-COR, Lincoln, NE).

**Chromatin Immunoprecipitation**

Chromatin immunoprecipitation was carried out following the protocol provided by EZ-ChIP (Millipore) with few modifications. Briefly, Reh cells (1 x 10^6) were grown in RPMI with 10% FBS with or without vorinostat (1 µM) for 24 hours. Proteins were cross-linked to DNA with 1% formaldehyde added directly to culture medium for 10 min at 37°C, followed by cell lysis with SDS. The cell lysates were sonicated to shear DNA to the length of 200-1000 base pairs (bp). Chromatin solutions were precipitated overnight at 4°C using 2 µg of anti-H3K9ac (# 334481, Qiagen, Valencia, CA), anti-H3K27me2 (#ab24684, Abcam), anti H3K4me2/me3 (#ab6000, Abcam) and antiH3K9me3 (# ab8898, Abcam). For the negative control, species specific IgG was used. For a positive control, anti-RNA polymerase II (# 05-623, Millipore) was used. DNA from protein-associated complexes and corresponding input samples were recovered using the QIAquick PCR purification kit (Qiagen) and assayed by real time PCR under standard...
conditions. Primers were targeted to the transcription start site (TSS) and the upstream promoter regions of the genes of interest. Full details, including the primer sequences are provided in supplemental data (Table S2).

**Methylation Specific PCR**
Genomic DNA was extracted from B- lineage leukemia (Reh and UOCB1) and B-lymphoma cell lines (Raji); and sodium bisulfite modification was performed using Epitect bisulfite kit (Qiagen) according to manufacturer’s protocol. After this, methylation specific PCR (MSP) was performed as described previously.\(^{19}\) Completely methylated and unmethylated control DNA (Epitect PCR control DNA set-Qiagen) was tested for each primer pair. PCR products were analyzed following electrophoresis on 2% agarose gels containing ethidium bromide. MSP primers are described in supplemental data (Table S2).

**Cell Viability Assays**
CellTitre-Glo Luminescent Cell Viability assays (Promega, Madison, WI) were performed on primary patient samples and cell lines grown in appropriate survival conditions as described above. Briefly, cells were seeded in 96-well plates and treated with vorinostat and/or decitabine (0, 0.1, 0.5, 1, 2.5 and 5 µM). After 24 or 48 hours incubation, prednisolone, etoposide, doxorubicin and cytarabine was added to the media for additional 24 hours incubation. Finally, CellTitre-Glo reagent was added and luminescence was recorded using a Synergy HT multidetection microplate reader (Biotek, Winooski, VT). Drug combination effects were analyzed by calcsyn software (Biosoft, Cambridge, GB, UK) using the combination index (CI) equation of Chou-Talalay.\(^ {20}\) This application describes the drug interaction as: CI >1.1 antagonism, 0.9-1.1 additive, 0.85-0.9 slight synergism, 0.7-0.85 moderate synergism, 0.3-0.7 synergism, 0.1-0.3 strong synergism, <0.1 very strong synergism.
Results

Connectivity map analysis identifies vorinostat as top candidate agent which could reverse the relapse specific gene expression signature

Following the identification of the relapse specific drug resistant signature, we searched the current version of the cmap database to identify agents that could potentially reverse this signature and restore chemosensitivity. All treatment instances from the database were ranked according to their negative connectivity scores with a p-value <0.05. Remarkably, compounds which ranked amongst the top were the HDAC inhibitors vorinostat and trichostatin A. The average connectivity score of the 12 treatment instances of vorinostat in various cancer cell lines in the cmap database was -0.659 (p value=0) while the 182 instances of trichostatin A had a connectivity score of -0.452 (p value=0) (Figure S1). This analysis indicated that the genetic make-up of relapsed ALL might be epigenetically driven and hence its reversal by histone deacetylase inhibitors could potentially restore chemosensitivity.

Gene expression microarrays and RT-PCR validates the Connectivity map results

To validate and confirm the Connectivity map findings, primary patient samples (n=3) and leukemia cell lines (Reh, RS4:11, MV4:11) were treated with 1 µM of vorinostat and incubated for 24 hours. RNA was extracted and gene expression profiling was performed on Affymetrix U133Plus2 array. The expression of 38.7% (mean) (95% confidence interval across the 6 samples being 30.51-46.18%) of genes differentially expressed at relapse was reversed following vorinostat treatment using the fold change of 0.8 and 1.2 for down and up regulated genes respectively and a p value <0.05 (Figure 1). Details of individual genes and corresponding reversal upon vorinostat treatment have been outlined in supplemental data (Table S1). To determine whether the relapse signature is enriched for genes whose expression is epigenetically regulated as compared to the whole genome, we examined expression changes in genes using a more stringent fold change of 2. The expression of 17.8% (mean) (95% confidence interval across the 6 samples being 12.7-22.8%) of the relapse specific genes were reversed after vorinostat treatment, compared with only 12.8 % (mean) (95% confidence interval across 6 samples being 8.8-16.7%) genes were affected in the whole genome analysis (p = 0.025 by chi square test). This result was also validated using a random sampling method where 10,000
random combinations showed that in only 185 instances could such a finding be attributable to chance alone (p = 0.019). These two independent methods show a specific effect of vorinostat on the genes included in the relapse specific signature. Microarray data were then validated by RT-PCR in a selected set of candidate chemoresistance genes from our previous publication, which showed significant downregulation of the expression of BIRC5, FOXM1, TYMS and FANCD2 (genes upregulated at relapse) (Figure 2A), while an increase in the expression of NR3C1, HRK and SMEK2 (genes downregulated at relapse) (Figure 2B).

Association of histone marks correlates with vorinostat exposure as histone deacetylase inhibitor

The biological impact of vorinostat was validated by showing an increase in global histone acetylation at the protein level as measured by western blot in Reh cells (Figure 3A). Chromatin Immunoprecipitation (ChIP) experiments were performed to examine the effect of vorinostat on specific activating (H3K4me2/3 and H3K9ac) and repressive (H3K9me3 and H3K27me3) histone marks that modulate gene expression. Chromatin from the vorinostat treated and untreated Reh cells were immunoprecipitated with these antibodies, and DNA was subjected to PCR amplification using primer sets designed to amplify the transcriptional start site and/or 5´ region of the genes of interest. We observed significant enrichment of the acetylation histone mark H3K9ac on the promoters of all the genes tested (NR3C1, HRK, BIRC5 and FOXM1) indicating a direct effect of vorinostat in accumulation of acetylated lysine 9 of histone H3 (Figure 3B). This observation correlated with the transcriptional activation of NR3C1 and HRK in patient samples and cell lines after vorinostat treatment. In addition, we observed modest enrichment of H3K4me2/3 (activation mark) at the NR3C1 and HRK promoters supporting the activation of these genes following vorinostat exposure. Of the repressive marks, we observed a decrease in the H3K9me3 mark on the promoters of all genes except in FOXM1. The interplay of post translational methylation and acetylation at the same lysine residue (K9) further supports the effect of vorinostat in regulating expression of NR3C1 and HRK, though this effect is not consistent with the observed down-regulation of BIRC5 and FOXM1. The H3K27me3 mark did not show significant modulation with vorinostat treatment in any of the genes (Figure 3B).
Treatment with demethylating agent decitabine leads to re-expression of relapse specific hypermethylated genes

Having identified the “methylome signature” of relapse ALL previously, we sought to determine the impact of the demethylating agent decitabine in re-expression of relapse specific silenced hypermethylated genes. Reh and UOCB1 cell lines were treated with 1µM of decitabine and incubated for 48 (Reh) or 72 (UOCB1) hours. RNA was extracted and RT-PCR was performed on relapse specific target genes. We focused on the genes which were identified from the integrated results of cross platform analysis, CDKN2A, COL6A2, PTPRO and CSMD1, that were hypermethylated and down regulated at relapse as well as focally deleted in a subset of samples. In addition, we also examined other gene promoters which showed concordant hypermethylation and downregulation in our relapse cohort such as WT1, APC, GATA4 and HOXA9. We observed a 2-200 fold induction of expression of PTPRO, COL6A2, WT1, GATA4 and HOXA9 (Figure 4A). Re-expression of CDKN2A was examined in Raji (B-lymphoma cell lines) cells as both alleles are deleted in Reh cell lines, while its expression is lost in UOCB1 cell lines. Again, we observed 2-4 fold induction in its expression. A six fold increase in expression of CSMD1 was observed in UOCB1 cells, while no change was observed in its expression in the Reh cell line. No change in the expression of APC was observed in either cell line tested. As a negative control, we tested the NR3C1 gene which is down regulated at relapse and not found to be hypermethylated in our relapse cohort, although it has been shown to be hypermethylated in other cancer types. Again, no change in expression of NR3C1 was observed following decitabine treatment in either cell line.

MSP demonstrates the effect of decitabine on promoter hypermethylation and validates RT-PCR results

To demonstrate the direct effect of decitabine on promoter hypermethylation, MSP was performed with cell lines before and after treatment with decitabine. The PTPRO and HOXA9 genes were completely methylated in Reh and UOCB1 cell lines prior to treatment and an enrichment of the unmethylated amplicon was observed following treatment with decitabine (1 µM). The GATA4 promoter was preferentially methylated in both cell lines, though some unmethylation was also observed at baseline which was significantly enriched after treatment
with decitabine. The APC promoter was preferentially unmethylated in both Reh and UOCB1 cell lines but had a faint methylation band in Reh cells at baseline. Following decitabine treatment, the intensity of the methylation band was decreased and unmethylated product was modestly increased in Reh cells, while it was unchanged in the UOCB1 cells. Preferential unmethylation of this promoter at baseline in these cell lines could be the reason for unaltered expression of this gene as determined by RT-PCR. The NR3C1 promoter was completely unmethylated before and after exposure to decitabine in both the cell lines, which is the likely explanation for its insensitivity to decitabine treatment (Figure 4B). For the reasons cited above, MSP for CDKN2A promoter performed in Raji cell lines showed enrichment of unmethylated product after decitabine exposure, which correlates with the increase in expression as shown by RT-PCR.

**Pretreatment with vorinostat and/or decitabine induces chemosensitivity in primary patient samples and leukemia cell lines**

Finally, to examine whether epigenetic priming translates to increased chemotherapy-induced cytotoxicity of leukemic blasts, we assessed cytotoxicity with a panel of chemotherapeutic agents commonly used in ALL therapy. Primary patient samples (n=7) and Reh and RS4:11 cell lines were treated sequentially with increasing concentrations of vorinostat and/or decitabine at hour 0, followed by application of conventional chemotherapeutic agents (prednisolone, doxorubicin, cytarabine and etoposide) at varying concentrations at hour 24 or 48. Cytotoxicity assays were performed at hours 48 or 72. Vorinostat alone induced 40-60% of cytotoxic effect at the maximal concentration of 2.5 µM. An additive or synergistic effect was observed when cells were pretreated with vorinostat followed by chemotherapy in Reh and RS4:11 cell lines (CI=0.5-1.05) (Figure S2A and S2B). Similarly, decitabine alone induced 25-40% of cytotoxic effect at its maximal concentration used and addition of chemotherapy following decitabine pretreatment significantly increased the amount of cytotoxicity (CI 0.34-0.9) (Figure S2C and S2D). The two epigenetic drugs by themselves showed mostly additive effect to moderate synergism (CI=0.57-1.1), while pretreatment with both vorinostat and decitabine followed by prednisolone had the most robust cytotoxicity compared to any other combination, showing strong synergism (CI=0.17-0.5) (Figure 5A-D). As predicted from the Connectivity Map results, we did observe
increased cytotoxicity in the relapse samples (n=3) when vorinostat alone was followed by prednisolone (CI=0.36-0.6) as compared to the diagnosis samples (n=4) (CI=0.43-1.0) (Figure 5C and 5D), though the comparison was limited due to small sample size. This data suggests that although both the diagnosis and relapse blasts can be sensitized to chemotherapy, the effect of vorinostat was more pronounced in the relapsed blast population. We did not see this effect with decitabine where the impact was equivalent in both initial diagnosis and relapse.

**Discussion**

ALL is the most common childhood cancer and the majority of children can be cured with current therapies but up to 20% children relapse and their outcome remains dismal. Historically there have been several obstacles to the successful treatment of relapsed ALL and numerous efforts to improve outcome after relapse have been unsuccessful. Contemporary re-induction regimens have relied on significantly more aggressive approaches with higher doses and/or compacted drug schedules. These have not only failed to improve remission rates, but have also reached tolerability limits with toxic death rates generally ranging from 3-8%, but with reports of rates up to 19%. Thus further dose intensification is not a viable option for improving outcome. One of the major challenges faced at relapse is intrinsic chemoresistance. Our data indicate that the chemoresistance that drives relapse in ALL may be reversible by epigenetic reprogramming with DNMTi and HDACi. Our results indicate that vorinostat significantly reverses the expression of genes which are differentially regulated at relapse, some of which are linked to tumor formation and progression and, most importantly, may have a role in chemoresistance. Of particular interest are BIRC5 and FOXM1, which are associated with a wide range of cancers and were upregulated in our relapse signature. We demonstrated that expression of both these genes could be decreased after vorinostat exposure. Improved chemosensitivity by performing knockdown experiments of BIRC5 by our group and others, have further supported its role in chemoresistance. Similarly, downregulation of genes such as NR3C1 and BTG1 have been linked to glucocorticoid resistance and we observed increased expression of these genes upon vorinostat treatment. In addition, upregulation of genes involved in nucleotide biosynthesis and folate metabolism identified in the late relapse (relapse>36 months from diagnosis) cohort such as TYMS, CAD and DHFR was reversed upon treatment.
with vorinostat. The effect of HDAC inhibition on BIRC5, TYMS and DHFR expression in B-ALL patient samples and cell lines is in agreement with previous studies in variety of other tumor types.\textsuperscript{31-33} Thus, we have demonstrated that many genes differentially expressed at relapse as compared to diagnosis are epigenetically regulated and can be reprogrammed with HDACi. This reversal of gene expression results functionally in enhanced chemosensitivity of leukemic blasts.

Post-translational modifications of histone tails, especially acetylation and methylation on lysine residues, play a pivotal role in regulating gene expression by controlling the access of key regulatory factors and complexes to chromatin.\textsuperscript{34,35} Although HDACi’s are known to activate gene expression by antagonizing histone deacetylases and opening up the chromatin structure, their effect on global gene expression patterns remain elusive and needs further investigation. While our ChIP experiments offer an explanation of vorinostat-mediated activation of repressed genes such as NR3C1 and HRK, we did not observe enrichment of repressive histone marks as a possible mechanism of vorinostat-induced down-regulation of BIRC5 and FOXM1. On the other hand, we observed reciprocal interaction of acetylation and methylation marks at lysine 9 on histone H3, which is in agreement with some of the previous studies showing their mutual exclusivity.\textsuperscript{35} In light of several reports suggesting the effect of these agents on various non-histone proteins and transcription factors, we speculate that down regulation of these genes may be mediated by an intermediate transcriptional regulator restored by HDACi exposure. For instance, p21 expression increases with vorinostat treatment in a dose dependent manner\textsuperscript{36} which in turn might repress FOXM1.\textsuperscript{37} Resultant decrease in the levels of FOXM1 may affect the levels of BIRC5, CCNB1, FANCD2 as mentioned previously.\textsuperscript{38} The fact that similar alterations of gene expression patterns were seen repeatedly and consistently in different primary patient samples and cell lines strongly favors its unique and specific action. Recently, Stumpel et al has also demonstrated HDACi-mediated downregulation of a subset of hypomethylated proto-oncogenes, and resultant chemosensitivity in MLL-rearranged ALL which supports our findings though clear underlying mechanisms remains undetermined.\textsuperscript{39} Future studies involving ChIP-sequencing might provide further insight to understand this complex network.
Genome-wide methylation assays have identified many cancer-related genes as substrates of DNMTs leading to epigenetic silencing. In this study, we were able to show re-expression of many genes which were hypermethylated at relapse by both RT-PCR and concordant promoter hypomethylation by MSP. Of these, CDKN2A, PTPRO and CSMD1 have previously been identified as tumor suppressors in many cancers and reactivation with demethylating agents is a logical approach. Furthermore, WTI and APC are inhibitors of the β-catenin/TCF/LEF complex and were differentially down-regulated and hypermethylated in the relapse cohort. In the current study, a significant up-regulation of WTI after decitabine exposure is demonstrated. However, there was no significant baseline hypermethylation of APC in the Reh and UOCB1 cell lines, and hence failed to demonstrate decitabine effect. Finally and most importantly, not only was aberrant gene expression reprogrammed by these two agents, but combination treatment with vorinostat and decitabine synergized with standard chemotherapy agents to result in enhanced chemosensitivity. Larger sample size would be needed to further compare the effect of these agents on the diagnosis versus relapsed blasts.

The combination of vorinostat and decitabine has previously been tested in human leukemia showing evidence of cell growth inhibition and gene silencing. Recently, Kalac et al has also shown highly synergistic combination of HDACi (panobinostat) and DNMTi (decitabine) and association with unique gene expression and epigenetic profiles in large B cell lymphoma. Although newer HDAC and DNMT inhibitors are under active investigation, we focused on vorinostat and decitabine not only because they are FDA approved, but also because Phase 1 trials using these agents have shown their safety in the pediatric population. Reversal of gene expression and methylation signature in our study was seen with 1 µM concentration of vorinostat and decitabine respectively, a plasma concentration which was shown to be clinically achievable and tolerable in these phase 1 clinical trials. Ravandi et al reported this combination to be well tolerated in a phase 1 study in hematologic malignancies in adults. Additional support for the feasibility of this combination has recently been published in adults with solid tumors and Non-Hodgkin’s lymphoma. Moreover, it has been shown in previous reports that apoptosis induced by these epigenetic agents are specific to the malignant cell population, and have comparatively little or no activity against their normal counterparts (non malignant cells).
suggesting that incorporating these agents episodically in the standard reinduction chemotherapy backbone for the treatment of relapsed ALL is feasible.

Overall, our data reveals an attractive approach to reverse the drug resistance gene signature to restore chemosensitivity in relapse ALL. Our study validates this approach in pre-clinical assays. Data described in this manuscript has led to a multi-institutional Phase 1 clinical trial evaluating the feasibility and safety of using the combination of these epigenetic agents that is set to accrue patients through the Therapeutic Advances in Childhood Leukemia and Lymphoma Consortium (http://Clinicaltrials.gov:NCT01483690). Gene expression and methylation arrays and subsequent validation studies will be undertaken as part of correlative biology assays for this clinical protocol. Our data provides a strong rationale for undertaking such a trial to improve the cure-rates in relapsed childhood ALL, which otherwise has a dismal prognosis.

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Author Contribution
TB and DM designed research, performed research, collected data, analyzed and interpreted data, performed statistical analysis and wrote the manuscript. JW analyzed and interpreted data and performed statistical analysis. ER, MB and PB designed the research and wrote the manuscript. WLC designed and directed the research, analyzed and interpreted data, and wrote the manuscript.

Disclosure of Conflicts of Interest
There are no conflicts to declare.
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Figure legends:

Figure 1. Gene expression microarrays in primary B-lymphoblastic leukemia patient samples and cell lines reveal reversal of relapse specific signature

Heat map showing the effect of vorinostat in reversing the relapse specific gene expression profile. Rows represent individual genes (154 probes) where relative overexpression is shown in red and underexpression in green. First 3 columns are primary patient samples (1, 2 and 3) followed by Reh, RS4:11 and MV4:11 cell lines respectively before treatment with vorinostat; the next 6 columns correspond to each sample after treatment.

Figure 2. Relative mRNA expression of genes differentially expressed at relapse after exposure to vorinostat

Quantitative Real-time PCR (qRT-PCR) validation of selected targets on 3 primary patient samples and Reh, RS4:11 and MV4:11 cell lines. The x-axis represents vorinostat concentration. The y-axis represents normalized ∆CT values (CT of gene of interest - CT of housekeeping gene). CT indicates threshold cycles for amplification. Each experiment was performed in triplicate. Mean expression is indicated by the horizontal bars. The P-values correspond to Wilcoxon signed rank test comparing the expression levels before and after treatment.

Figure 3. Histone modifications at the promoters of genes of interest

(A) Western blot analysis of acetyl-H3 (and actin loading control) in Reh cells with and without vorinostat exposure. (B) ChIP was carried out with antibodies specific for key H3 modifications associated with transcriptional activation (K9ac, K4me2/3) and transcriptional repression (K9me3, K27me3) and purified DNA was subjected to RT-PCR using primer sets designed to amplify transcriptional start site (TSS) and/or 5′ region of NR3C1, HRK, BIRC5 and FOXM1. Each graph shows the modification of these marks post vorinostat exposure normalized to log input (1%) and the corresponding mRNA expression of the gene by qPCR.

Figure 4. Decitabine treatment of ALL cell lines shows re-expression of hypermethylated genes at relapse.
Real-time PCR was performed on Reh, UOCB1 (B-lineage) and Raji (lymphoma) cells treated with decitabine at 1 µM concentration for 48 h (Reh and Raji) or 72 h (UOCB1). Results are expressed relative to the levels observed at baseline without decitabine exposure. Each experiment was performed in triplicate. Error bars represent standard deviation of the mean. Determination of concordant changes in promoter methylation levels by Methylation specific PCR (MSP) in Reh and UOCB1 cell lines. *CDKN2A mRNA expression levels as well as MSP was performed in Raji cells only.

**Figure 5. In vitro cell viability assays in primary patient samples and cell lines (Reh and RS4:11).**

Summary graph representing the effect of all agents at 48 h time point either individually or in combination in (A) Reh cell lines, (B) RS4:11 cell lines, (C) relapse patient samples and (D) diagnosis patient samples. X-axis represents the vorinostat and decitabine concentrations at 0, 0.1, 0.5, 1, 2.5 and 5 µM and prednisolone at 0, 100, 200, 300, 400 and 500 µg/ml respectively, whereas Y axis shows % survival.
Figure 1

A. Untreated

Treated
Figure 2

A.

**BIRC5**

![Graph showing BIRC5 expression levels with mean values at 0 µM (Mean-6.9) and 1 µM (Mean-9.1).]

**FOXM1**

![Graph showing FOXM1 expression levels with mean values at 0 µM (Mean-6.4) and 1 µM (Mean-8.3).]

**TYMS**

![Graph showing TYMS expression levels with mean values at 0 µM (Mean-4.9) and 1 µM (Mean-7.0).]

**FANCD2**

![Graph showing FANCD2 expression levels with mean values at 0 µM (Mean-7.3) and 1 µM (Mean-8.6).]
Figure 2

B.

NR3C1

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<th></th>
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<th>Pt 2</th>
<th>Pt 3</th>
<th>Reh</th>
<th>RS4:11</th>
<th>MV4:11</th>
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HRK

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SMEK2

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Figure 3

A. Vorinostat 1 µM

Acetyl H3
Actin

B. NR3C1

Fold enrichment normalized to log input

H3K9Ac
H3K4Me2/3
H3K9Me3
H3K27Me3

qPCR normalized to β2M

1500 bp upstream
TSS

BIRC5

Fold enrichment normalized to log input

H3K9Ac
H3K4Me2/3
H3K9Me3
H3K27Me3

qPCR normalized to β2M

5000 bp upstream
2000 bp upstream

HRK

Fold enrichment normalized to log input

H3K9Ac
H3K4Me2/3
H3K9Me3
H3K27Me3

qPCR normalized to β2M

1500 bp upstream

FOXM1

Fold enrichment normalized to log input

H3K9Ac
H3K4Me2/3
H3K9Me3
H3K27Me3

qPCR normalized to β2M

1500 bp upstream
TSS
Figure 4

A.

For personal use only.
Figure 4

B.

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*CDKN2A
Figure 5

A. Reh

B. RS4:11

C. Relapse patient samples (n=3)

D. Diagnosis patient samples (n=4)

- Decitabine
- Prednisolone
- Decitabine → Prednisolone
- Vorinostat
- Vorinostat + Decitabine
- Vorinostat → Prednisolone
Epigenetic reprogramming reverses the relapse-specific gene expression signature and restores chemosensitivity in childhood B-lymphoblastic leukemia

Teena Bhatla, Jinhua Wang, Debra J. Morrison, Elizabeth A. Raetz, Michael J. Burke, Patrick Brown and William L. Carroll