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## SAHA and cladribine synergistically induce apoptosis in NK-LGL leukaemia

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### Summary

Natural killer (NK) large granular lymphocyte (LGL) leukaemia features a clonal proliferation of CD3<sup>-</sup> NK cells that can be classified into either aggressive or chronic categories. The NK cell line, derived from an aggressive Asian NK cell leukaemia, and patient samples from chronic NK-LGL leukaemia were used in our study to probe for synergistic efficacy of the epigenetic drugs vorinostat (SAHA) and cladribine in this disease. We demonstrate that histone deacetylases (HDACs) are over-expressed in both aggressive and chronic NK leukaemia. Administration of the HDAC inhibitor SAHA reduces class I and II HDAC expression and enhances histone acetylation in leukaemic NK cells. *In vitro* combination treatment with SAHA and cladribine dose-dependently exerts synergistic cytotoxic and apoptotic effects on leukaemic NK cells. Expression profiling of apoptotic regulatory genes suggests that both compounds led to caspase-dependent apoptosis through activation of intrinsic mitochondrial and extrinsic death receptor pathways. Collectively, these data show that combined epigenetic therapy, using HDAC and DNA methyltransferase inhibitors, may be a promising therapeutic approach for NK-LGL leukaemia.

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### Authorship Contributions

X.L., Z.S.H, D.J.F., H.W., X.S., D.Z., T.P.L. and K.X. designed the experiments; X.S. performed and analysed the experiments; A.C. performed the statistic studies; and T.P.L., X.S., Z.S.H. and D.J.F. wrote the manuscript.

### Conflict of Interest

The authors declare no competing financial interest.

### Supporting information

Additional Supporting information may be found in the online version of this article:

## Keywords

natural killer large granular lymphocyte leukaemia; vorinostat (SAHA); cladribine; histone deacetylation; combined epigenetic therapy

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## Introduction

Large granular lymphocyte (LGL) leukaemia is a rare haematological malignancy characterized by excessive proliferation of cytotoxic CD3<sup>+</sup> T cells or CD3<sup>-</sup> natural killer (NK) cells (Lamy & Loughran, 1998). In 2008, the World Health Organization (WHO) classification included a new provisional entity to make a clear distinction between aggressive and chronic NK-cell leukaemias (Swerdlow *et al* 2008). Though both types display a CD3<sup>-</sup>CD56<sup>+</sup> immunophenotype, the chronic lymphoproliferative disorder of NK cells (also known as chronic NK-LGL leukaemia) is characterized by a more indolent course and is more common in the West (Lim *et al*, 2009). There is no known curative therapy for patients with LGL leukaemia, making development of novel therapeutics essential to improve the outcome of this disease.

Epigenetic modifications control gene expression without altering the underlying DNA sequence and have been implicated in aberrant oncogene expression in multiple cancers (Marks *et al*, 2000; Sharma *et al*, 2009). A previous study showed that the demethylating agent 5-aza-2'-deoxycytidine (DAC) interferes with activation of the JAK/STAT signalling pathway, which plays a central pathogenic role in LGL leukaemia (Teramo *et al*, 2013). Such data suggest a potential role for epigenetic modulators of chromatin structure, including histone modifications and DNA methylation, in the treatment of LGL leukaemia.

The histone deacetylases (HDACs) are a class of enzymes that deacetylate the  $\epsilon$ -amino group of histones to promote a transcriptionally inactive chromatin configuration (Kalyaanamoorthy & Chen, 2013) and have been shown to play an integral role in cancer progression through a wide variety of signalling pathways (Chowdhury *et al*, 2011). HDAC inhibitors (HDACi) promote cell differentiation, apoptosis and cell cycle arrest in multiple transformed cell types (Marks *et al*, 2000). SAHA (suberoylanilide hydroxamic acid, Vorinostat) was the first U.S. Food and Drug Administration (FDA)-approved pan-HDACi, for use in cutaneous T-cell lymphoma. HDACi decrease histone-DNA interactions by promoting acetylation of histones. Acetylation neutralizes the positive charges on histones, thus leading to less compact and more transcriptionally active chromatin (Cohen *et al*, 2011). Numerous *in vitro* and *in vivo* studies have been reported in which various HDACi exhibit anti-cancer activities against several tumour types through changes in transcriptional gene regulation (Bolden *et al*, 2006; Butler *et al*, 2000; Thurn *et al*, 2011; Wang *et al*, 2013). In addition, emerging data suggest that SAHA has little toxicity against normal tissues (Blattmann *et al*, 2012; Chen *et al*, 2013; Dong *et al*, 2008), making it a promising therapeutic agent for human cancer.

Another drug that utilizes a different mechanism to exert epigenetic regulation is cladribine (2-chlorodeoxyadenosine, 2-CdA). Cladribine received FDA approval in the late 1980s and has been used to treat a range of different leukaemias (Robak & Robak, 2012), including

LGL leukaemia (Edelman *et al*, 1997; O'Brien *et al*, 1994). Its main mechanism as an anti-cancer agent is through chain termination during DNA synthesis. Therefore, it exerts the strongest effect on rapidly dividing tissues, including tumours and immune cells. At lower doses though, cladribine possesses hypomethylating and epigenetic activities, especially in haematological malignancies. It inhibits histone and DNA methylation, leading to de-repression of silenced genes in tumour cells (Byrd *et al*, 2003; Fenaux *et al*, 2009; Spurgeon *et al*, 2011). These epigenetic effects occur at low doses and can avert much of the toxicity that is observed at high doses, such as death of normal lymphocytes, suppression of bone marrow and irreversible neurological damage as well as permanent kidney damage and debilitating cytopenias (Betticher *et al*, 1998; Cheson *et al*, 1994; Kluin-Nelemans *et al*, 2003; Romine *et al*, 1997; Sigal *et al*, 2010). These severe side effects strictly limit dosing in patients, making treatment with low dose cladribine as an epigenetic adjuvant to other therapies that much more appealing.

Deacetylated histone lysine tails and hypermethylated CpG islands are correlated with transcriptionally inactive or condensed chromatin, while acetylated histone lysine tails and unmethylated CpG islands are indicators of transcriptionally active or open chromatin. Thus, DNA methyltransferase inhibitors (DNMTi) and HDACi facilitate changes between these chromatin conformations (Griffiths & Gore, 2008; Leone *et al*, 2008). In order to take advantage of the epigenetic effects of cladribine without invoking its significant side effect profile, SAHA was used in combination with low dose cladribine to affect chromatin remodelling and un-silence repressed genes in NK-LGL cells. The aim of our study was to determine the efficacy of these epigenetic drugs in NK-LGL. We investigated the individual and combined effects of SAHA and cladribine on cell viability and apoptosis, and evaluated differences in apoptotic gene expression, HDAC expression and histone acetylation status in leukaemic NK cells. Here we show for the first time that the combination of SAHA and cladribine had synergistic efficacy in inducing cell death through apoptosis in leukaemic NK cells.

## Methods and Materials

### Cell culture and therapeutics

The human NK-LGL cell line, NKL, was kindly provided by Dr. Howard Young [National Cancer Institute (NCI), Frederick, MD]. Cells were cultured in RPMI 1640 medium with 15% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA) and 10 u/ml human recombinant interleukin 2 (IL2; Allergy and Infectious Diseases [AIDS] Research and Reference Reagent Program, National Institutes of Health [NIH], Bethesda, MD) at 37°C and 5% CO<sub>2</sub>. Cells were harvested during exponential growth phase. SAHA was purchased from Selleckchem (Houston, TX) and cladribine from Sigma-Aldrich (St Louis, MO).

### Patient characteristics and preparation of peripheral blood mononuclear cells (PBMCs)

All patients met the clinical criteria for NK-LGL leukaemia, mainly increased counts (>80%) of CD3<sup>-</sup>, CD16<sup>+</sup>/CD56<sup>+</sup> NK cells in the peripheral blood. All patients were diagnosed with clinically stable chronic NK-LGL leukaemia (n=8) and were treatment-naïve during sample acquisition. Peripheral blood specimens were obtained with informed

consent, in accordance with protocols approved by the Institutional Review Board of the Penn State Hershey Cancer Institute (Hershey, PA). White blood cell buffy coats from anonymous normal donors were obtained from the blood bank of Milton S. Hershey Medical Center (Hershey, PA).

PBMCs were isolated by Ficoll-hypaque gradient separation as described previously (Lamy *et al*, 1998). Cell viability was determined by Trypan blue exclusion assay. A cut-off of >95% viability was used as a quality control. Normal NK cells from anonymous age- and gender-matched healthy donors were isolated by a negative selection process (StemCell Technologies, Vancouver, Canada) as described previously (Epling-Burnette *et al*, 2004). The purity of freshly isolated CD3<sup>-</sup>CD56<sup>+</sup> cells ( $2 \times 10^5$ /sample, in triplicate) was determined by flow cytometry for positive staining of surface CD56<sup>+</sup> on NK cells. The purity for normal purified NK cells was 85–90%.

### HDAC gene expression: real-time quantitative RT-PCR

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using primer sets specific for 11 human HDACs and an internal standard, *RPS18* rRNA, in an ABI PRISM 7900 sequence detector (Applied Biosystems, Foster City, CA) as described previously (Liu *et al*, 2010). Total RNA was isolated from PBMCs ( $5 \times 10^6$ ) from NK-LGL leukaemia patients and healthy donors, and SAHA- or dimethylsulphoxide (DMSO)-treated NKL cells, as described previously (Liu *et al*, 2010). Primer sequences are shown in Supplemental Table S1 and were purchased from RealTimePrimers.com (Elkins Park, PA).

### In vitro cell viability and apoptosis assay

Cell viability was determined using the CellTiter 96 Aqueous One Solution assay kit (Promega, Madison, WI). Colour change (490 nm) was detected with the Synergy HT Multi-Detection Microplate Reader (Bio-TEK, Winooski, VT). Apoptosis was determined by 2-colour flow cytometry with annexin V (5  $\mu$ l/sample) and 7-amino-actinomycin D (7-AAD; 10  $\mu$ l/sample, BD Pharmingen, San Jose, CA) staining using  $5 \times 10^5$  cells/sample on a BD Biosciences FACS Calibur instrument in the flow cytometry core at Penn State Hershey College of Medicine. The percentage of specific apoptosis was calculated using the following formula: Apoptosis (%) = ((% annexinV–fluorescein isothiocyanate [FITC] conjugate positive in assay well - % annexinV–FITC positive in control well)/(100 - % annexinV–FITC positive in control well)) $\times$ 100.

### Apoptosis RT<sup>2</sup> profiler PCR microarray

Human apoptosis PCR array (Cat. PAHS-012ZA; SABiosciences, Valencia, CA) was performed to analyse the mRNA expression of 84 key genes. NKL cells were treated with SAHA (5  $\mu$ M), cladribine (0.125  $\mu$ M), both or DMSO vehicle for 24 h. Total RNA was extracted and reverse transcribed into cDNA by the RT<sup>2</sup> First Strand Kit (SABiosciences). Equal aliquots of the mixture (10  $\mu$ l) were added into wells of the PCR Array plate, which contains pre-dispensed primer sets. RT<sup>2</sup> SYBR Green/ROX PCR Master Mix (SABiosciences) was used to monitor the fluorescent signal during each cycle of PCR reactions as described above.

## Western blot analysis

NKL cells ( $10^7$ ) were treated with various concentrations of SAHA and cladribine for 24 h and then lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich) with protease inhibitor cocktail P8340 (Sigma-Aldrich) and phosphatase inhibitor cocktail 2 (Sigma-Aldrich). Proteins (30  $\mu$ g) were electrophoresed and transferred to polyvinylidene difluoride membrane, blocked, probed with primary antibodies diluted in 5% non-fat dry milk overnight, then washed, incubated with secondary antibody and visualized using enhanced chemiluminescence (ECL; Thermo Scientific, West Palm Beach, FL). Protein bands were subjected to densitometric scanning and were quantified using the ImageJ software (NIH). All primary and secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA).

## Statistical Analysis

All data are presented as mean  $\pm$  standard error of the mean (SEM). Two-tailed paired Student *t* test and two-way analysis of variance tests were used to determine statistical significance.  $P < 0.05$  was considered statistically significant. Synergy was analysed with CalcuSyn software (Biosoft, Cambridge, UK) using median-effect methods of Chou and Talalay (Chou & Talalay, 1984). Combination index (CI) values determined the combination effect as synergistic ( $<1$ ), additive ( $=1$ ), or antagonistic ( $>1$ ).

## Results

### HDACs are highly expressed in leukaemic NK cells

We characterized the mRNA expression levels of the 11 HDACs in purified chronic NK-LGL patient samples and normal NK cells by quantitative RT-PCR. The mRNA levels of *HDAC2*, *HDAC3*, *HDAC5*, *HDAC9* and *HDAC10* are significantly over-expressed in leukaemic cells compared to normal NK cells (Figure 1A). Aggressive NK-LGL leukaemia is rare in the West and access to primary patient materials is limited. Therefore, we used NKL cells (an aggressive human NK-LGL leukaemia cell line) (Robertson *et al*, 1996) for further mechanistic studies. Quantitative RT-PCR results demonstrate that *HDAC1*, *HDAC3* and *HDAC8*, belonging to HDAC class I, are significantly up-regulated in NKL cells compared to normal NK cells (Figure 1B). Collectively, we found that *HDAC* transcripts are significantly up-regulated in chronic leukaemic NK samples as well as the NKL cell line. To evaluate the effects of HDACi in leukaemic NK cells, cell survival was determined in SAHA-treated NKL cells (Figure 1C). SAHA inhibits cell viability in a time and dose-dependent manner, with a 50% inhibition concentration ( $IC_{50}$ ) of 2.00  $\mu$ M, 0.93  $\mu$ M and 0.44  $\mu$ M at 24, 48 and 72 h.

### SAHA and combination treatment with low-dose cladribine induces dose-dependent cytotoxic effects in leukaemic NK cells

Due to the potential benefit of DNA hypomethylation concurrent with histone acetylation, cells were co-treated with cladribine and SAHA in order to evaluate synergistic effectiveness. NKL cells were treated with SAHA and cladribine, and cell survival was determined from 24 to 72 h (single viability inhibition curves for cladribine are shown in

Supplemental Figure 1). Based upon the significant relationship that cell viability has with time and dose, we chose to stratify using these two covariates in our analysis. Due to the strong cytotoxicity of SAHA and cladribine at 72 h, data from 24 and 48 h was selected for analysis. NKL cells were exposed to combinations of the indicated concentrations of SAHA (1, 2, 5 and 10  $\mu\text{M}$ ) and cladribine (0.125  $\mu\text{M}$ ). This dose of cladribine reduces cell viability by 18% and 74% in NKL cells when applied alone for 24 and 48 h, respectively. Combination therapy induces significantly greater cytotoxicity than SAHA and cladribine administered alone (Figure 2A, Supplemental Figure 2A). To extend these findings to clinically relevant samples, experiments were repeated using PBMCs from six chronic NK-LGL leukaemia patients. Both SAHA and combination treatment result in dose-dependent growth inhibitory effects in NK-LGL leukaemic cells. The combination treatment causes greater decreased viability compared to SAHA alone at 24 and 48 h (Figure 2B, Supplemental Figure 2B). SAHA and combination treatment also exerts no detectable cytotoxic effect on PBMCs from healthy donors (Figure 2A, Supplemental Figure 2A). Overall, our data show that cellular growth inhibition is significantly increased in a dose-dependent manner in leukaemic NK cells treated with combined SAHA and cladribine when compared to normal controls.

### **SAHA and combination treatment with low-dose cladribine induce dose-dependent apoptotic cell death in leukaemic NK cells**

To explore whether the effects of SAHA and cladribine were associated with cellular apoptosis, NKL cells were treated with SAHA alone or in combination with cladribine for 24 and 48 h. A dose-dependent increase in apoptosis was detected with SAHA treatment (1, 2, 5 and 10  $\mu\text{M}$ ) alone and in combination with 0.125  $\mu\text{M}$  cladribine at both 24 and 48 h. Combination therapy significantly increases apoptosis compared to SAHA alone (Figure 3A, Supplemental Figure 3A), and synergistic effects were seen all doses of SAHA combined with 0.125  $\mu\text{M}$  cladribine at both 24 and 48 h (Figure 4). Both SAHA and combination treatment induce apoptotic cell death in patient-derived NK-LGL leukaemic cells in a dose-dependent manner, with combination therapy showing greater effects (Figure 3B, Supplemental Figure 3B). Minimal apoptosis is seen in PBMCs from healthy controls with the same treatment (Figure 3A, Supplemental Figure 3A). These results demonstrate a strong correlation between the degree of apoptosis and viability inhibition and indicate that cell death induced by these compounds is mostly apoptotic in nature.

### **SAHA inhibits HDAC expression and enhances histone acetylation in leukaemic NK cells**

Next we determined the effect of HDACi treatment on *HDAC* mRNA levels in NKL cells. After incubation with SAHA (1, 5 and 10  $\mu\text{M}$ ) for 24 h, relative mRNA levels of nearly all HDACs, including isoforms that are highly expressed in NKL cells (*HDAC1*, *HDAC3* and *HDAC8*), are significantly decreased by SAHA in a dose-dependent manner (Figure 5A). Importantly, *HDAC* mRNA levels were also reduced after 12 or 18 h of SAHA treatment (10  $\mu\text{M}$ ), which demonstrates that these changes precede the induction of apoptosis (Supplemental Figure 4). Class I (HDACs 1, 2, 3 and 8) and the extensively studied class II HDAC enzymes (HDACs 4, 5 and 6) were selected for Western blot analysis. As predicted by mRNA expression results, SAHA also reduces the protein levels of class I and II HDAC enzymes dose-dependently (Figure 5B). Moreover, all four of the core histones become



hyper-acetylated and show a dose-dependent increase apparent at 24 h of SAHA treatment for H2A, H2B and H3 (Figure 5C) and 48 h for H4 (Figure 5D).

### Effects of SAHA and/or cladribine treatment on apoptotic gene expression in leukaemic NK cells

To investigate effects of SAHA and cladribine treatment on apoptotic signalling events and regulatory proteins, PCR microarray and Western blot analyses were carried out in NK cells after incubation with 5  $\mu$ M SAHA or/and 0.125  $\mu$ M cladribine for 24 h. Our data suggest that either single or combination therapy with SAHA and cladribine triggers apoptosis through activation of both intrinsic mitochondrial and extrinsic death receptor pathways in NK-LGL. As illustrated in Table I, SAHA and cladribine treatment leads to increases in expression of *AIFM1* (apoptosis inducing factor), *CYCS* (cytochrome complex) and *APAF1* (apoptotic protease activating factor-1). Increases in pro-apoptotic regulators including *BBC2*, *BAX*, *BAK1* and *BCL2L11* accompanied by decreases in anti-apoptotic genes like *BCL2* and *BCL2L1* also suggest activation of the intrinsic apoptotic pathway (Table I). Moreover, gene expression levels of *FAS*, *FASLG*, *FADD*, *TNFSF10*, *TNFRSF1A*, *TNFRSF10B*, initiator and effector *CASP* (caspases) are greatly enhanced in response to SAHA and/or cladribine (Table I), suggesting that epigenetic combination therapy may also activate the extrinsic pathway. Additionally, combination therapy shows strong synergistic effects in gene expression of *CYCS*, *ABL1*, *BCL2L11*, *CASP4*, *CASP8*, *CD40L*, *CD70*, *FAS*, *FASLG*, *TNFSF10*, *NOL3* and *RIPK2* compared to SAHA or cladribine administered alone (Table I).

Consistent with this mRNA expression data, either single or combination therapy of SAHA and cladribine for 24 h alters protein levels of key apoptosis regulators in NK cells (Figure 6A). Combination therapy increased expression of the pro-apoptotic proteins BCL2L11, BAX and BAK1 and suppressed anti-apoptotic proteins like BCL2, BCL2L1 and MCL1. Protein quantification data shows that combination therapy exerts significantly greater effects on some apoptotic regulators, such as BCL2, BCL2L1 and BAX, compared to SAHA alone. There are significant differences between combination therapy and cladribine treatment on BCL2, BCL2L1, MCL1 and BCL2L11 expression (Figure 6A). Both compounds and their combination subsequently induced activation of CASP3 and poly(ADP-ribose) polymerase (PARP) cleavage in leukaemic NK cells (Figure 6B). Combination therapy also reduced activation of the ERK and STAT3 cellular signalling pathways (Figure 6C), both of which are strongly implicated in the pathogenesis and cell survival of LGL leukaemia (Epling-Burnette *et al*, 2001; Yue & Turkson, 2009). Collectively, these results show that SAHA and SAHA/cladribine combination treatment alters the aberrant survival signalling pathways in LGL leukaemia.

## Discussion

Here we find dramatic synergistic effects in induction of apoptosis in leukaemic NK cells using low doses of cladribine (0.125  $\mu$ M) in combination with SAHA *in vitro*. Cell viability and cell death assays reveal that both agents exert cytotoxic and apoptotic effects dose-dependently in NK-LGL leukaemia patient samples and NK cells, while sparing normal

NK cells. The mechanisms underlying the responses to combination therapy with DNMTi and HDACi in cancer are not yet fully understood but appear to involve global epigenetic changes that affect many cellular pathways. Recent reports have highlighted that both DNMTi and HDACi can synergistically create a more transcriptionally active chromatin configuration through demethylation of CpG islands and acetylation of histone lysine tails, resulting in global alterations in the expression of genes responsible for cell proliferation, differentiation and death (Griffiths & Gore, 2008; Leone *et al*, 2008; Maes *et al*, 2013). Additionally, Arzenani *et al.* (2001) revealed that synergistic effects of DNMTi and HDACi are achieved through a common target, DNMT1. Moreover, DNMT recruits both methyl-binding proteins and HDACs to promoter regions, leading to chromatin condensation and suppressed gene expression (Jones *et al*, 1998; Nan *et al*, 1998; Sarkar *et al*, 2011; Sharma *et al*, 2009). The role of DNMT in NK-LGL responses to DNMTi and HDACi will be further explored in future studies. Collectively, these data suggest that combined epigenetic therapy with HDACi and DNMTi, especially SAHA and cladribine, should be considered as investigational therapy for NK-LGL leukaemia.

We also find a general up-regulation of *HDAC* gene expression in leukaemic NK cells. The HDAC family plays an essential role in the control of gene expression and is aberrantly expressed in multiple human cancers (Arzenani *et al*, 2011; Jones *et al*, 1998; Sarkar *et al*, 2011). We show significant over-expression of *HDAC2*, *HDAC3*, *HDAC5*, *HDAC9* and *HDAC10* in patient samples and *HDAC1*, *HDAC3* and *HDAC8* in NKL cells. Differences between the patient samples and cell line are probably caused by varying isoform over-expression in the NK-LGL patient population (n = 5) as opposed to very specific changes in the clonal NKL cell line. Collectively, these results suggest that HDAC over-expression is a common feature in NK-LGL leukaemia, and provide a strong rationale for targeting HDAC as a therapeutic strategy. We also demonstrate that the HDACi SAHA induces hyperacetylation of core histones and dose-dependently inhibits gene expression and reduces protein levels of class I and II HDAC in leukaemic NK cells. Modest differences between *HDAC* mRNA and HDAC protein levels, such as for *HDAC1*, are probably related to the additional time required for transcriptional changes to be reflected in altered protein content. Of note, selective inhibition of *HDAC3* activity, the isoform most highly expressed relative to normal NK cells, was unable to recapitulate the downregulation of HDAC proteins observed with SAHA treatment (data not shown). More specific characterization of HDAC activity in NK-LGL leukaemia will require fluorometric activity assays that are selective for the individual HDAC isoforms.

Our data suggest that both intrinsic and extrinsic apoptotic pathways may be involved in the robust and synergistic death response induced by cladribine and SAHA treatment of leukaemic NK cells. The mechanisms responsible for HDACi and DNMTi-induced apoptosis seem to be complex and vary among different cell types. Activation of caspases and over-expression of pro-apoptotic genes accompanied by suppression of anti-apoptotic genes has been commonly observed following treatment with SAHA or cladribine in various tissues (Bosanquet *et al*, 2002; Ceruti *et al*, 2003; Fandy *et al*, 2005; Henderson *et al*, 2003; Perez-Galan *et al*, 2002; Zhang *et al*, 2005). Indeed we observe similar findings in leukaemic NK cells treated with combination therapy. Using PCR microarray, we find up-



regulation of pro-apoptotic *BCL2L11*, *BAX* and *BAK1* while *BCL2* and *BCL2L1* are down-regulated (Table I). Additionally, Western blot data show greater alternations caused by combination therapy than either drug alone, indicating synergistic effects of HDACi and DNMTi in regulating expression of key apoptotic proteins (Figure 6A). Of interest, SAHA alone minimally alters the mRNA expression of *BCL2* and *BCL2L1*, while Western blot analysis shows SAHA suppresses protein levels of these factors. Possible explanations for the differential expression may be the post-transcriptional regulation of the BCL2 family after HDACi treatment in NK-LGL. HDACi have been reported to regulate gene expression at transcriptional and post-transcriptional levels (Hirsch *et al*, 2010; Kretzner *et al*, 2011). Similar results were seen in multiple myeloma and acute lymphoblastic leukaemia, suggesting that expression of apoptosis-related proteins may be down-regulated by SAHA at a post-transcriptional level (Liu *et al*, 2013; Willimott & Wagner, 2010).

It is noteworthy that the BH3-only pro-apoptotic gene, *BCL2L11*, was enhanced 35-fold following SAHA treatment at the mRNA level, and similar results were observed at the protein level. Recent studies show that BCL2L11 is involved in the regulation of programmed cell death of various cancer types (Akiyama *et al*, 2009; Gillings *et al*, 2009; Rahmani *et al*, 2009), and over-expression of *BCL2L11* and enhanced BCL2L11-mediated apoptosis are commonly found in response to oncogene-targeted therapeutics (Gillings *et al*, 2009). Our data also suggest an important role of BCL2L11 in HDACi-induced intrinsic apoptosis in leukaemic NK cells.

We observed synergistic effects of SAHA and cladribine on gene expression of *CD40LG*, *CD70*, *FASLG*, *FAS* and *CASP8*. *CD40LG* and *CD70*, both inflammatory cytokines of the tumour necrosis factor (TNF) superfamily, play important roles in the cytotoxic function of NK cells (Carbone *et al*, 1997; Yang *et al*, 1996). *FASLG* and *FAS* are important in regulating lymphocyte homeostasis and in mediating extrinsic pathway apoptosis through the recruitment of FADD and the activation of *CASP8* and effector caspases (Holler *et al*, 2000). Over-expression of *FASLG*, *FAS* and *FADD* is reported to enhance FAS-mediated apoptosis (De Paepe *et al*, 2008; Huang *et al*, 1999; Kim *et al*, 2003; Roger *et al*, 1999). Here, the 32-fold up-regulation of *FASL* expression suggests that FAS-mediated apoptosis may contribute to the synergistic efficacy of SAHA and cladribine in inducing cell death in NK-LGL. Interestingly, one of our previous studies demonstrated that leukaemic NK cells are relatively resistant to FAS-mediated apoptosis, but MEK inhibition increased the sensitivity to FAS-induced apoptosis in NK-LGL cells (Epling-Burnette *et al*, 2004). Similar to MEK inhibition increasing sensitivity, we find reduced ERK pathway activation in SAHA and combination therapy in our study (Figure 6C). Whether the observed synergistic apoptosis is due to the enhanced FAS-sensitization by epigenetic therapy with HDACi and DNMTi in NK-LGL cells needs to be further explored in future studies.

In summary, we show that the HDAC family is a key regulator of cell survival in NK-LGL leukaemia. The pan-HDACi SAHA targets all HDAC enzymes and efficiently reduces expression of HDAC class I and II and increases histone acetylation, thereby leading to apoptotic cell death. We also demonstrate that combination therapy with SAHA and cladribine exerts synergistic cytotoxic and apoptotic effects, potentially through both the intrinsic and extrinsic-mediated apoptotic pathways, and that these effects were selective for

leukaemic rather than normal NK cells. Collectively, SAHA in combination with low-dose cladribine may be a promising therapeutic strategy for NK-LGL leukaemia.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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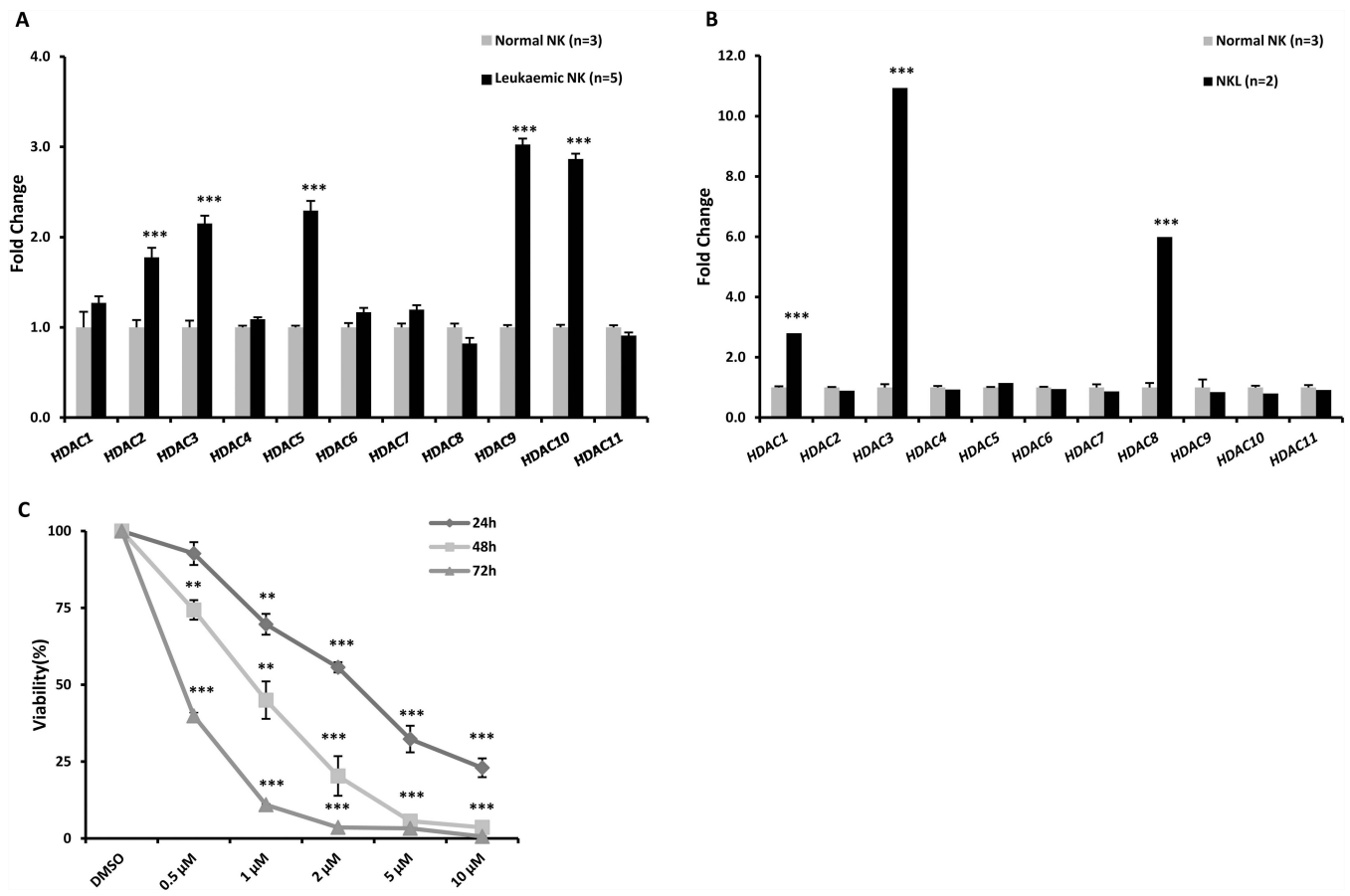
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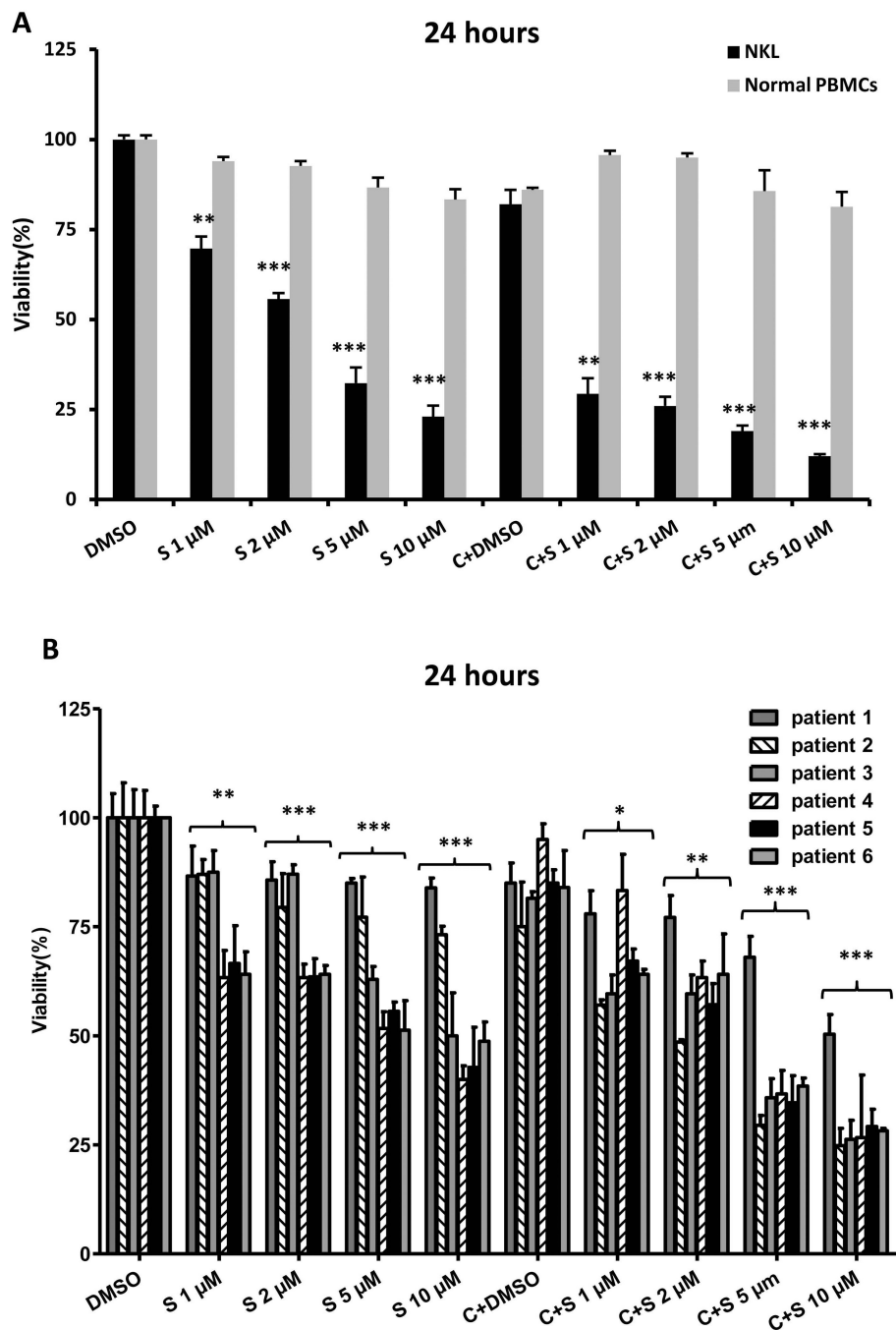
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**Figure 1.**

HDACs are over-expressed in leukaemic NK cells. (A) Real-time quantitative PCR was performed to measure mRNA levels in NK cells from five patients with chronic NK large granular lymphocyte (LGL) leukaemia (NK-LGL) ( $CD3^+CD56^+$  NK cells > 80%) and three normal donors. Data are representative of two independent experiments performed in triplicate and normalized against 18S as a control gene.  $***P < 0.0005$  vs. normal NK cells. (B) Real-time quantitative PCR was performed to measure mRNA levels in the NKL cell line and three normal donors. Data are representative of three independent experiments performed in triplicate and normalized against RPS18 as a control gene.  $***P < 0.0005$  vs. normal NK cells. (C) NKL cells were treated with SAHA (1, 2, 5 and 10  $\mu$ M) for 24, 48 and 72 h and cell survival was determined by MTS assay. Results are expressed as the average of three independent experiments performed in triplicate.  $*P < 0.05$ ,  $**P < 0.005$ ,  $***P < 0.0005$  vs. dimethylsulphoxide (DMSO) control.

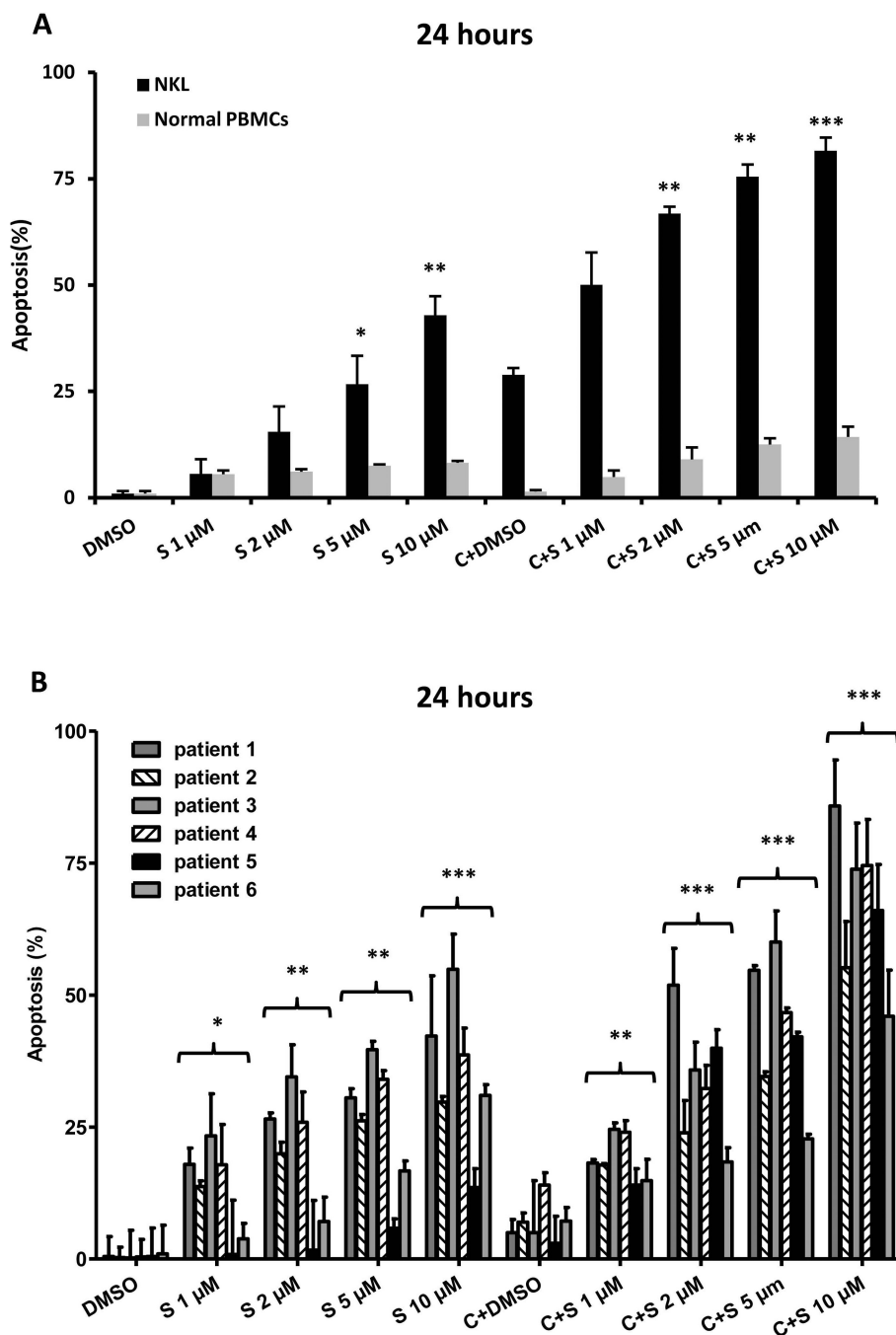




**Figure 2.**

SAHA treatment alone and in combination with cladribine induces dose-dependent cytotoxic effects in leukaemic NK cells. (A) NKL cells and normal peripheral blood mononuclear cells (PBMCs) from three healthy donors were treated with SAHA (1, 2, 5 and 10  $\mu$ M) and 0.125  $\mu$ M cladribine for 24 h and cell survival was determined by MTS assay. Results are expressed as the average of three independent experiments performed in triplicate. (B) Leukaemic NK cells from six chronic NK-LGL patients ( $CD3^+CD56^+$  NK cells > 80%) were treated with SAHA (1, 2, 5 and 10  $\mu$ M) and 0.125  $\mu$ M cladribine for 24 h

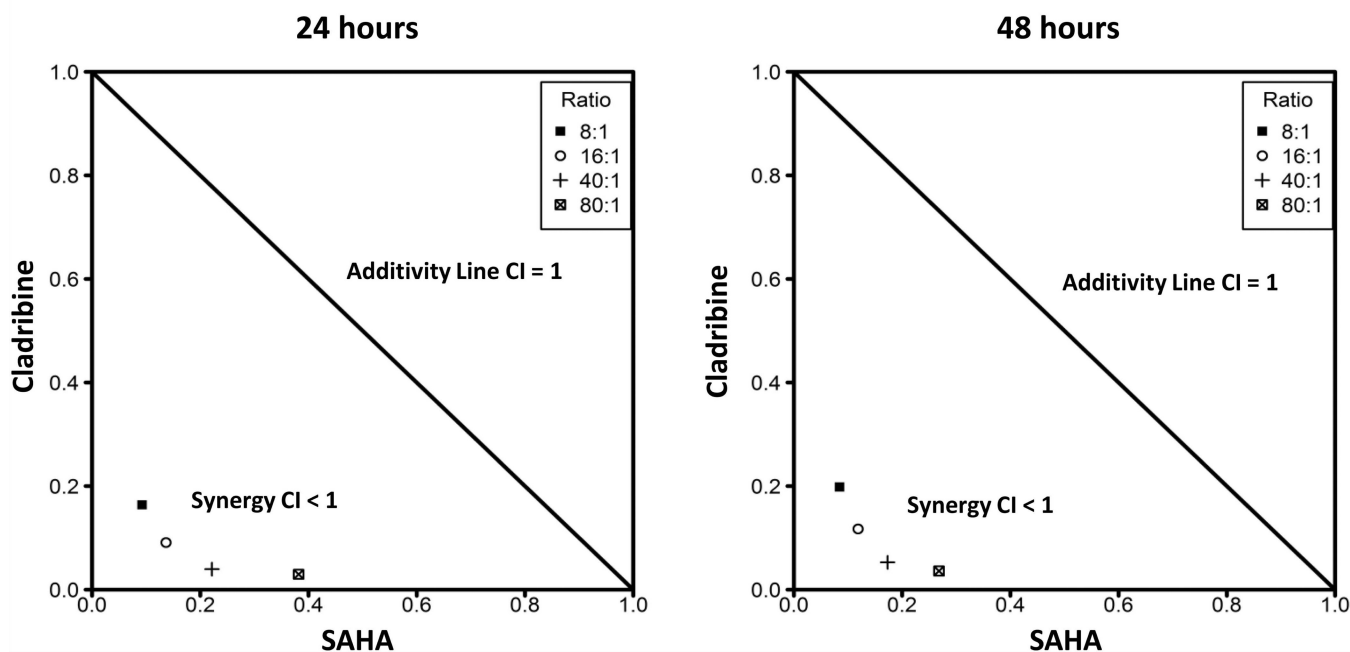
and cell survival was determined by MTS assay. Each sample was assayed in triplicate. S: SAHA, C: cladribine and C+S: cladribine + SAHA. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$  vs. dimethylsulphoxide (DMSO) control.



**Figure 3.** SAHA treatment alone and in combination with cladribine induces apoptotic cell death in leukaemic NK cells. (A) NKL cells and normal peripheral blood mononuclear cells (PBMCs) from three healthy donors were treated with SAHA (1, 2, 5 and 10  $\mu\text{M}$ ) and 0.125  $\mu\text{M}$  cladribine for 24 h and apoptosis was detected as described in Methods and Materials. Results are expressed as the average of three independent experiments performed in triplicate. (B) Leukaemic NK cells from six chronic NK-LGL patients ( $\text{CD3}^-\text{CD56}^+$  NK cells > 80%) were treated with SAHA (1, 2, 5 and 10  $\mu\text{M}$ ) and 0.125  $\mu\text{M}$  cladribine for 24 h

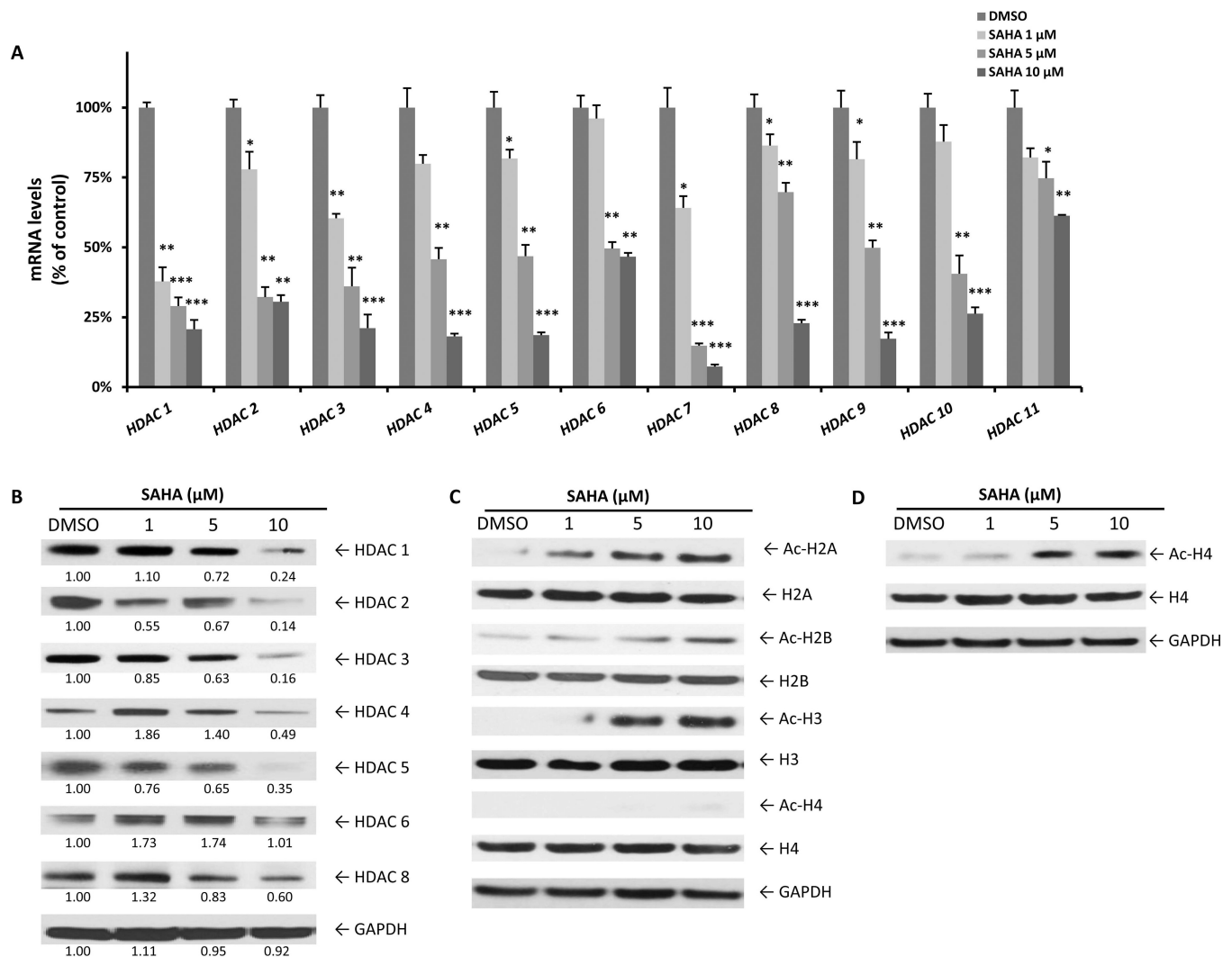
and apoptotic cells were determined as in (A). S: SAHA, C: cladribine and C+S: cladribine + SAHA. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$  vs. dimethylsulphoxide (DMSO) control.

SAHA ( $\mu\text{M}$ )	Cladribine ( $\mu\text{M}$ )	Ratio	24 hours			48 hours		
			CI	Confidence Interval	Combo Effect	CI	Confidence Interval	Combo Effect
1	0.125	8:1	0.26	0.17-0.38	Synergy	0.28	0.20-0.40	Synergy
2	0.125	16:1	0.23	0.16-0.33	Synergy	0.24	0.17-0.33	Synergy
5	0.125	40:1	0.26	0.17-0.40	Synergy	0.23	0.15-0.33	Synergy
10	0.125	80:1	0.41	0.26-0.65	Synergy	0.30	0.20-0.47	Synergy



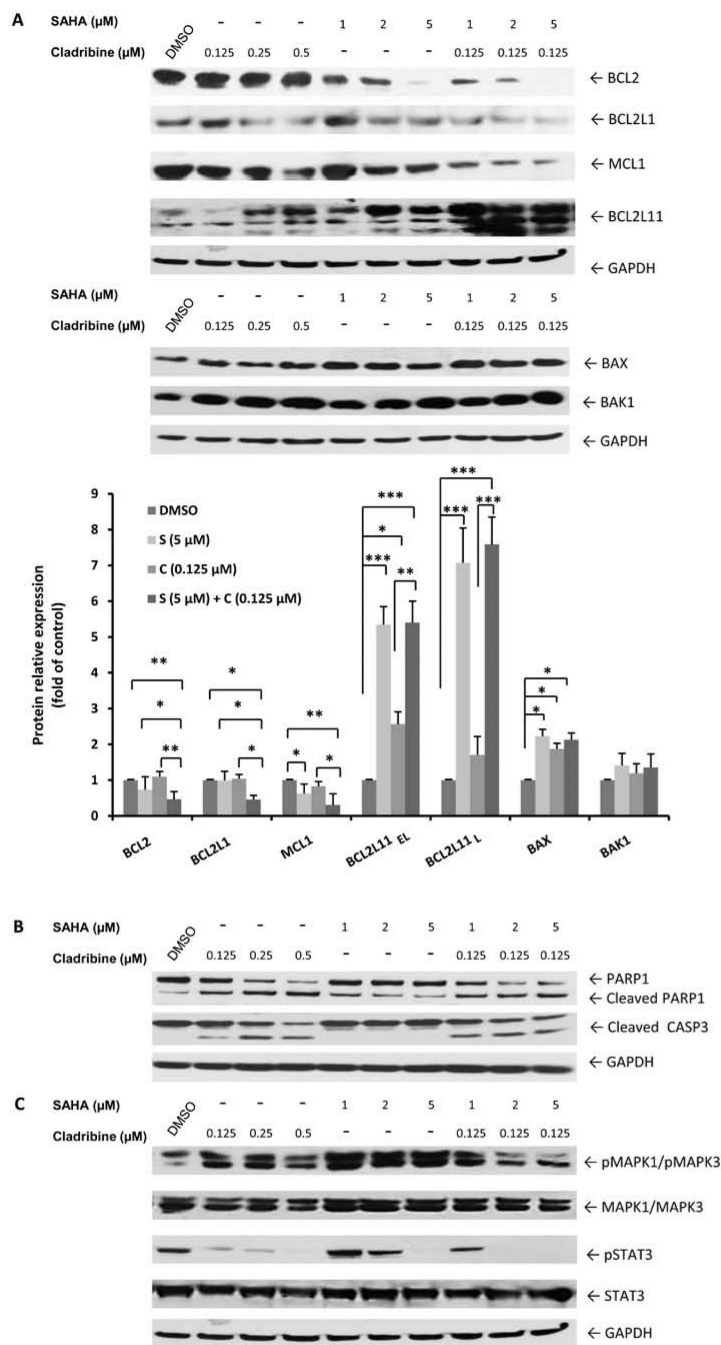
**Figure 4.**

Combination of SAHA and cladribine synergistically induces apoptosis in leukaemic NK cells. Combination index (CI) values and corresponding 95% confidence intervals represent interaction effects of combination therapy (Combo Effect) in leukaemic NK cells. For a CI < 1 (95% confidence interval < 1), the combination is defined as synergism; a CI = 1 (95% confidence interval includes 1), the combination is defined as additive; a CI > 1 (95% confidence interval > 1), the combination is defined as antagonism. The normalized isobologram analysis presents a graphical depiction of the combination index analyses. The analysis was performed with CalcuSyn software, which conducts the drug dose-effect calculation with the median effect method of Chou and Talalay. Data below the additivity line in the isobologram indicate synergy, whereas data above the additivity line indicate antagonism. All combination groups were below the additivity line, indicating synergism between SAHA and cladribine in NK-LGL cells.



**Figure 5.** SAHA reduces HDAC expression and enhances histone acetylation in leukaemic NK cells. (A) NKL cells were treated with SAHA (1, 5 and 10  $\mu$ M) for 24 h and RNA was extracted for qRT-PCR analysis. Data are representative of two independent experiments performed in triplicate and normalized against 18S as a control gene. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$  vs. dimethylsulphoxide (DMSO) control. (B) Western blot analysis was performed for HDACs after treatment with SAHA (1, 5 and 10  $\mu$ M) for 24 h. Data were quantified by densitometry and ratios of HDAC/GAPDH are shown under each band. Results are representative of three independent experiments. (C) Western blot analysis was performed for acetyl-Histone after treatment with SAHA (1, 5 and 10  $\mu$ M) for 24 h or (D) 48 h. Results are representative of three independent experiments. Equal loading of protein lysates was confirmed by re-probing membranes for GAPDH.





**Figure 6.** Effects of SAHA and cladribine on apoptotic regulatory proteins and signalling pathways. (A) Western blot analysis was performed for the indicated apoptotic regulatory proteins after treatment with SAHA (1, 2 and 5 μM), cladribine (0.125, 0.25 and 0.5 μM) or both for 24 h. Upper panels show representative Western blots, and the bar graph indicates average relative protein expression determined by densitometric analysis of three independent experiments. \**P* < 0.05, \*\**P* < 0.005, \*\*\**P* < 0.0005 vs. C+S. S: SAHA, C: cladribine and C+S: cladribine + SAHA. (B) Apoptosis induction was detected by Western blot analysis of

caspace-3 and PARP cleavage after treatment as described in (A). Results are representative of three independent experiments. (C) Western blot analysis was performed for phosphorylated (p)-ERK, total ERK, p-STAT3 and total STAT3 after treatment as described in (A). Results are representative of three independent experiments. Equal loading of protein lysates was confirmed by re-probing membranes for GAPDH.

**Table I**

Analysis of gene expression for apoptotic regulators in SAHA-and cladribine-treated leukaemic NK cells

Symbol*	Previous/alternative symbols	Fold change		
		SAHA	Cladribine	Combination
<i>ABL1</i>	ABL, JTK7, bcr, abl, c-ABL, p150, v-abl	1.68	1.82	7.16
<i>APAF1</i>	APAF-1, CED4, DKFZp781B1145	3.41	2.26	2.71
<i>AIFM1</i>	AIF, COXPD6, MGC111425	6.10	2.12	5.26
<i>BAD</i>	BBC2, BCL2L8	1.3	2.32	1.16
<i>BAK1</i>	BAK, BAK-LIKE, BCL2L7, CDN1	2.59	1.59	3.5
<i>BAX</i>	BCL2L4	2.05	1.78	1.47
<i>BCL2</i>	Bcl-2	1.02	-2.28	-1.86
<i>BCL2L1</i>	BCL-XL, BCL2L, BCLX, BCLXS, Bcl-X	1.45	-5	-5.41
<i>BCL2L11</i>	BAM, BIM, BimEL, BimL	34.86	12.79	38.1
<i>BCL2L2</i>	BCL-W, BCL2-L-2, BCLW	2.09	-3.49	-1.25
<i>CASP1</i>	ICE, IL1BC, P45	4.37	1.42	6.75
<i>CASP3</i>	CPP32, CPP32B, SCA-1	1.95	2.93	1.65
<i>CASP4</i>	ICE(rel)II, ICEREL-II, ICH-2, Mih1, TX	1.65	1.85	4.87
<i>CASP8</i>	ALPS2B, CAP4, Casp-8, FLICE	2.62	1.49	5.02
<i>CASP9</i>	APAF-3, APAF3, CASPASE-9c	2.71	1.91	3.78
<i>CD40LG</i>	CD154, CD40L, HIGM1, IGM, IMD3	1.11	1.15	4.19
<i>CD70</i>	CD27L, CD27LG, TNFSF7	6.58	2.08	19.45
<i>CYCS</i>	CYC, HCS, THC4	2.72	10.32	15.06
<i>FADD</i>	MGC8528, MORT1	1.61	1.85	2.76
<i>FAS</i>	APO-1, APT1, CD95, FAS1, TNFRSF6	2.77	1.35	5.04
<i>FASLG</i>	CD178, CD95-L, CD95L, FASL, TNFSF6	6.84	5.13	31.72
<i>NOL3</i>	ARC, FLJ35304, MYP, NOP, NOP30	1.47	1.11	7.01
<i>RIPK2</i>	CARD3, CCK, GIG30, RICK	4.26	1.18	9.93
<i>TNFRSF10B</i>	CD262, DR5, KILLER, TRAILR2, TRICK2	4.3	2.04	2.69
<i>TNFRSF1A</i>	TNF-R, TNFR1, TNFR55, TNFR60, p55, p60	1.13	2.05	2.09
<i>TNFSF10</i>	APO2L, Apo-2L, CD253, TL2, TRAIL	3.73	2.74	7.63

Gene expression levels in NKL cells cultured with 5  $\mu$ M SAHA or/and 0.125  $\mu$ M cladribine for 24 h are illustrated as fold changes in reference with the control samples (without drugs). Samples were quantified in duplicate and all duplicates agreed within  $\pm$  10%.

\* HGNC (Human Genome Organization [HUGO] Gene Nomenclature Committee symbol (<http://www.genenames.org>))