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Entinostat, a novel histone deacetylase inhibitor is active in B-cell lymphoma and enhances the anti-tumour activity of rituximab and chemotherapy agents

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Summary

Histone deacetylases (HDACs) inhibitors are active in T-cell lymphoma and are undergoing pre-clinical and clinical testing in other neoplasms. Entinostat is an orally bioavailable class I HDAC inhibitor with a long half-life, which is under evaluation in haematological and solid tumour malignancies. To define the activity and biological effects of entinostat in B-cell lymphoma we studied its anti-tumour activity in several rituximab-sensitive or -resistant pre-clinical models. We demonstrated that entinostat is active in rituximab-sensitive cell lines (RSCL), rituximab-resistant cell lines (RRCL) and primary tumour cells isolated from lymphoma patients ($n = 36$). Entinostat exposure decreased Bcl-XL (BCL2L1) levels and induced apoptosis in cells. In RSCL and RRCL, entinostat induced p21 (CDKN1A) expression leading to G1 cell cycle arrest and exhibited additive effects when combined with bortezomib or cytarabine. Caspase inhibition diminished entinostat activity in some primary tumour cells suggesting that entinostat has dual mechanisms-of-action. In addition, entinostat increased the expression of CD20 and adhesion molecules. Perhaps related to these effects, we observed a synergistic activity between entinostat and rituximab in a lymphoma-bearing severe combined immunodeficiency (SCID) mouse model. Our

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Supporting Information

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

Author contributions

S.F., J.J.G, G.P.K, Z.S., M.B., Q.H and C.M., performed research; F.J.H.I., J.J.G., L.S., M.S.C. designed the research study; J.S. provided key-pathological material, J.J.G., M.B., and F.J.H.I. analysed the data; S.F., J.J.G., L.S. J.S., M.S.C. and F.J.H.I., wrote the paper.

Conflict of interest

M.S.C. has served on an advisory board for Millennium Pharmaceuticals. F.J.H.I. has served on an advisory board for Seattle Genetics. The remaining authors declare no competing financial interest.

data suggests that entinostat is an active HDAC inhibitor that potentiates rituximab activity *in vivo* and supports its further clinical development in B-cell lymphoma.

Keywords

entinostat; rituximab; lymphoma; histone deacetylase inhibitors

Acquirement of resistance by cancer cells hinders the clinical outcome in a significant number of patients with non-Hodgkin lymphoma (NHL). The early use of rituximab (R) as a single agent or in combination with various systemic chemotherapy regimens (e.g. cyclophosphamide, vincristine and prednisone [CVP]; cyclophosphamide, doxorubicin, vincristine and prednisone [CHOP]; bendamustine, etc.) has resulted in improved response rates, duration of remission, progression-free survival (PFS) and overall survival (OS) in patients with follicular lymphoma (FL) and (in combination with CHOP) in diffuse large B-cell lymphoma (DLBCL) (Czuczman *et al*, 1999; Coiffier *et al*, 2002; Hiddemann *et al*, 2005; Marcus *et al*, 2005; Forstpointner *et al*, 2006; Pfreundschuh *et al*, 2006). The need to develop novel salvage therapeutic regimens after R-CHOP induction failures was demonstrated by the results of the prospective, multicentre, Phase III 'Collaborative Trial in Relapsed Aggressive Lymphoma' (CORAL) study. Investigators demonstrated that prior rituximab exposure in the frontline treatment of DLBCL negatively affected 4-year event-free survival (Thieblemont *et al*, 2011). Scientific efforts need to be focused not only on defining the pathways utilized by lymphoma cells to evade immunochemotherapy, but also to develop novel therapeutic strategies to overcome or circumvent resistance. To this end, we developed several rituximab-resistant cell-lines (RRCLs) and found that the acquirement of rituximab resistance was associated with decreased CD20 (MS4A1) expression and resistance to multiple chemotherapy agents commonly used to treat B-cell lymphoma (Czuczman *et al*, 2008; Olejniczak *et al*, 2008). We found that, related to the acquisition of a multi-chemotherapy resistance phenotype, RRCLs have a deregulation of pro-apoptotic (e.g. loss of Bak/Bax [BAK1/BAX]) and anti-apoptotic (e.g. up-regulation of Mcl-1 [MCL1], Bcl-XL [BCL2L1]) proteins (Czuczman *et al*, 2008; Olejniczak *et al*, 2008; Wenzel *et al*, 2013).

Post-transcriptional histone modification plays an important role in regulating gene expression, and is altered by histone acetyltransferases (HATs) and histone deacetylases (HDACs). In general, acetylation of specific lysine amino acid residues on the histone tails favours uncoiling of chromatin and increases gene transcription. On the other hand, an increase in HDAC activity leads to histone deacetylation, more tightly wound chromatin and a decrease in gene transcription. Unbalanced HAT and HDAC activity has been described in NHL (Glozak & Seto, 2007). To date, 18 HDACs have been identified in humans. HDACs are grouped in two major categories and four classes: zinc-dependent HDACs (Class I, II and IV) and NAD-dependent HDACs (Class III). Class I includes HDAC 1-3, 8 and 11; Class II includes HDAC 4-9 and 10; Class III includes homologues of yeast SIRT 1–7 and Class IV includes only HDAC 11. HDACs regulate several key cellular functions, including cell proliferation, cell cycle, apoptosis (including some members of the Bcl-2 [BCL2] family proteins), angiogenesis, migration, antigen presentation and/or immune regulation (Bolden

et al, 2006; Glozak & Seto, 2007; Buglio & Younes, 2010). The activity spectrum of each HDAC is yet to be defined and there is overlap between the function of different HDACs regardless of their group or class. HATs and HDACs may also interact with non-histone proteins, including transcription factors, tubulin and chaperones, adding to the complexity of their cellular function(s) in normal and malignant cells. Given their influence in multiple regulatory pathways, inhibition of HDACs is an attractive strategy to target cancer cells. Several pan- or selective-HDAC inhibitors (class I and IV) have been developed (Buglio & Younes, 2010). Pre-clinical and clinical studies demonstrate significant anti-tumour activity in various cancer models, including NHL and Hodgkin lymphoma (HL) (Piekarz *et al*, 2009; Kirschbaum *et al*, 2011, 2012; Tiffon *et al*, 2011; Lemoine *et al*, 2012; DeAngelo *et al*, 2013).

Three HDAC inhibitors (vorinostat [Suberoylanilide Hydroxamic Acid, SAHA], belinostat and romidepsin) have been approved by the United States Food and Drug Administration (FDA) for the treatment of relapsed/refractory peripheral T-cell lymphoma (PTCL) or cutaneous T-cell lymphoma (CTCL). The clinical development of HDAC inhibitors in B-cell lymphoma has been limited by: (i) modest single agent anti-tumour activity; (ii) toxicities; and (iii) the rapid development of B-cell receptor pathway inhibitors (e.g. ibrutinib, idelalisib) or anti-CD30 drug conjugates (i.e. brentuximab vedotin). The pre-clinical characterization of new HDAC inhibitors will hopefully lead to the rational design of alternative therapeutic strategies for patients with relapsed/refractory B-cell lymphoid neoplasms.

Entinostat (MS-275, SNDX-275, Syndax, Waltham, MA) is an orally available potent HDAC inhibitor that displays anti-tumour activity in pre-clinical cancer models.(Fandy *et al*, 2009) In contrast to other HDAC inhibitors, entinostat is more selective. It affects only class I and class IV HDACs while SAHA and romidepsin are pan-class inhibitors (I, II and IV) (Buglio & Younes, 2010). Because of its favourable toxicity profile, entinostat appears to be a more suitable HDAC inhibitor to incorporate into currently available immuno-chemotherapy regimens. Here we evaluate entinostat anti-tumour activity as a single agent or in combination with monoclonal antibodies (mAbs) or chemotherapy agents in rituximab-sensitive or -resistant B-cell lymphoma models. Our research demonstrated that entinostat has distinct mechanisms-of-action and by modulating *MS4A1* (*CD20*) expression, cell cycle regulatory proteins and/or adhesion molecules, enhanced rituximab activity *in vivo*.

Materials and methods

Cell lines and culture

A panel of rituximab-sensitive (RSCL) or -resistant (RRCL) cell lines was used for the experiments. The parental, RSCL Raji (Burkitt lymphoma [BL]), and RL (germinal centre B-cell [GCB] DLBCL) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The parental activated B-cell (ABC) DLBCL cell line was acquired from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The RRCL (Raji-4RH, RL-4RH and U2932-4RH) were created and characterized from RSCL as previously described.(Czuczman *et al*, 2008; Olejniczak *et al*, 2008) All cell lines were maintained in RPMI 1640 medium with Glutamax-1

(Invitrogen, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS), HEPES (5 mmol/l), penicillin and streptomycin at 100 iu/ml and sodium pyruvate 1 mmol/l.

Patient samples

Primary neoplastic B-cells were isolated from pre-treatment biopsy tissue obtained from 36 patients with B-cell NHL or lymphocyte-predominant Hodgkin lymphoma (LPHL) receiving therapy at Roswell Park Cancer Institute (RPCI). Samples from patient biopsy specimens were procured under Institutional Review Board (IRB) RPCI protocols I42804 and I42904. Tissue specimens were placed in PBS-containing collagenase type IV (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) and incubated for 15 min at 37°C, followed by manual agitation for 5 min. Next, samples were diluted with RPMI 1640 medium-containing 10% FBS and the cell suspension filtered through a 100-μm cell strainer to remove large clumps. Lymphocytes were enriched by density centrifugation. B-cells were then isolated from enriched lymphocytes by magnetic-activated cell sorting (MACS) separation using a human B-cell Isolation Kit II (Miltenyi Biotec, Gladbach, Germany).

Reagents and antibodies

Entinostat (MS-275, SNDX-275) was provided by Syndax Pharmaceuticals (Waltham, MA, USA). Vorinostat (SAHA) was purchased from Sigma-Aldrich Inc. Cisplatin was purchased from the American Pharmaceutical Partners (Schaumburg, IL, USA); doxorubicin was obtained from Bedford Labs (Bedford, OH); vincristine and bortezomib were provided by the RPCI Pharmacy. Therapeutic antibodies, rituximab (anti-CD20) and trastuzumab (anti-Her-2/neu; used as isotype control) were obtained from Genentech, Inc. (San Francisco, CA, USA) and, unless otherwise specified, were used at a final concentration of 10 μg/ml.

Primary rabbit anti-human antibodies raised against Bak, Bax, Bcl-2, Bcl-xl, checkpoint kinase 1 (CHK1), Cyclin B (CCNB1), p21 and actin (ACTB), as well as primary mouse anti-human antibody raised against Cyclin A (CCNA2), were purchased from Cell Signaling Technologies (Danvers, MA, USA). Primary mouse anti-human antibodies raised against Mcl-1 and PARP, as well as primary rabbit anti-human antibody raised against p53 (TP53), were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). GST77, an anti-rabbit antibody that binds to C-terminal region of the intracellular domain of CD20, was gift from Dr. Julie P. Deans. Alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Ficoll-Hypaque was purchased from Sigma-Aldrich Inc. Sodium chromate^{51} (^{51}Cr) (Perkin-Elmer Life Inc., Boston, MA, USA) was utilized in immunological assays assessing antibody-dependent cellular cytotoxicity (ADCC) and complement mediated cytotoxicity (CMC). Triton X-100, trypan blue and histopaque-1077 were obtained from Sigma-Aldrich Inc. Cell Titer-Glo Luminescent Viability Assay reagent was purchased from Promega (Madison, WI, USA). The caspase inhibitor Q-VD-OPh was obtained from MBL International (Woburn, MA, USA).

In vitro effects of entinostat in the viability of NHL cell lines

RRCL or RSCL were exposed to escalating doses of entinostat (0.1–10 μmol/l) or vehicle control (Dimethyl sulfoxide [DMSO] 0.001%) for 48 or 72 h. Cells were plated at a cell density of 0.5×10^6 cells/ml in 96-well plates and, at each time period, 20 μl of Alamar blue (Invitrogen, Grand Island, NY, USA) was added to each well and then incubated for two additional hours. Cell proliferation was determined as the change in Alamar blue reduction by living cells and measured using a FluoroScan Ascent LF (Thermo Fisher Scientific, Barrington, IL, USA).

In vitro effects of entinostat on the anti-tumour activity of chemotherapy agents or bortezomib

RSCL and RRCL were placed in 384-well plates (1×10^5 cells/well, cell density of 0.5×10^6 cells/ml) and exposed to entinostat (0.25, 0.5 and 1 μmol/l) and/or escalating doses of cisplatin (0–60 μmol/l), doxorubicin (0–10 nmol/l), vincris-tine (0–1 nmol/l), gemcitabine (0–50 μmol/l), cytarabine (0– 100 nmol/l) or bortezomib (0–20 nmol/l). Cells were then incubated at 37°C and 5% CO_2 for 24 and 48 h and changes in adenosine triphosphate (ATP) levels were measured using the CellTiter Glo ® (Promega, Fitchburg, WI, USA) luminescence assay.

^{51}Cr release assay to assess the impact of entinostat in rituximab-mediated complement-mediated cytotoxicity (CMC) and antibody-dependent cellular cytotoxicity (ADCC)

RSCL or RRCL were exposed *in vitro* to entinostat (5 μmol/l) or DMSO and incubated at 37°C and 5% CO_2 for 24 h. Subsequently, 2×10^6 viable cells were labelled with ^{51}Cr at 37°C in 5% CO_2 for 2 h. ^{51}Cr-labelled RSCL or RRCL were then placed in 96-well plates at a cell concentration of 1×10^5 cells/well (CMC assay) or 1×10^4 cells/well (ADCC assay). Cells were then exposed to rituximab (10 μg/ml) or isotype (10 μg/ml) and human serum (CMC, 1:4 dilution) or peripheral blood mononuclear cells (PBMCs) (ADCC, 40:1 effector: target ratio) for 6 h at 37°C in 5% CO_2. ^{51}Cr release was measured as previously described (Brem *et al*, 2011). PBMCs were obtained from healthy donors (IRB-approved protocol CIC-016) and isolated by Histopaque-1077 ultracentrifugation of peripheral whole blood. Pooled human serum was used as the source of complement for CMC assays.

In vitro effects of entinostat in the ATP content of primary tumour cells derived from B-cell lymphoma patients

To further confirm the biological activity of entinostat and to study its effects in a more clinically relevant model, we studied changes in ATP levels following entinostat exposure in 36 tumour specimens obtained from lymphoma patients. Briefly, malignant B-cells isolated from patients were exposed to entinostat (0.5 μmol/l), SAHA (0.5 μmol/l) or RPMI medium at a cell-density of 0.5×10^6 cells/ml. After a 24-or 48-h incubation at 37°C and 5% CO_2, changes in ATP were determined using the Cell Titer-Glo Luminescent Viability Assay reagent (Promega) and read according to assay protocol using the Thermo FluoroScan Ascent FL (Thermo Fisher Scientific, Waltham, MA, USA). Experiments were done in triplicates and the percentage of viable cells was assessed and normalized to controls.

Effect of caspase inhibition in entinostat anti-tumour activity

Primary tumour cells isolated from B-cell lymphoma patients were incubated with entinostat (0.5 μmol/l) or RPMI medium for 24 or 48 h with or without the pan-caspase inhibitor Q-VD-OPh (5 μmol/l). Differences in cell viability following caspase inhibition were determined using the Cell Titer Glo luminescent viability assay reagent.

Changes in the cell cycle of RSCL or RRCL exposed to entinostat

Propidium iodide (PI) nuclear staining was utilized to determine the cell-cycle fractions (Sigma-Aldrich). Briefly, cells were exposed to entinostat (0, 0.25 or 1 μmol/l) or DMSO for 48 h and subsequently harvested, washed three times in PBS, fixed in 70% ethanol for 30 min on ice and then incubated in a PBS/ribonuclease solution for 30 min at 37°C (1 ml PBS and 10 μmol/l ribonuclease). Data was collected on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analysed with Mod Fit LT Version 3.2 software (Verity Software House, Topsham, ME, USA). Results represent the mean value of three independent experiments.

Gene expression profile changes following HDAC inhibition

Cells were exposed to entinostat (0.5 μmol/l) or RPMI medium in 6-well plates for 24 h. The HumanHT-12v4 whole-genome gene expression array was used for expression profiling (Illumina Inc., San Diego, CA, USA). Microarray experiments were performed in triplicates for each cell line tested. The raw intensity of gene expression array was scanned and extracted using BeadScan, with the data corrected by background subtraction in GenomeStudio module. The log2 transformed intensity data were normalized using Quantile normalization function. The Limma program was used to calculate the level of gene differential expression for each comparison. Briefly, a linear model was fit to the data (with cell means corresponding to the different condition and a random effect for array), and selected contrast for each comparison was performed. The differential expression is set with at least 1.2-fold expression change at P value <0.05. The list of differentially expressed genes was used for Gene Ontology (GO) term enrichment analysis with the National Institutes of Health Database for Annotation, Visualization and Integrated Discovery (NIH DAVID) Tools.

Changes in the expression of pro-apoptotic, cell death or cell cycle regulatory proteins in RSCL or RRCL after exposure to entinostat

In order to study the mechanisms responsible for entinostat anti-tumour activity in B-cell lymphomas, a panel of RSCL or RRCL was exposed to entinostat (0.5 μmol/l and 1 μmol/l) or control *in vitro* for 48 h. Changes in the expression of Bak, Bax, Bcl-2, Bcl-xl, Mcl-1, CHK1, CD20, Cyclin A, Cyclin B, p21, p53, and PARP (PARP1) cleavage were determined by Western blotting. RSCL or RRCL (5×10^6 cells) were solubilized with a RIPA buffer containing 2 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin, 1 μg/ml pepstatin and 1 μg/ml aproprotinin. After solubilization at 4°C for 60 min, nuclei and debris were pelleted at 10 000 rpm for 30 min. Protein was quantified using Opsys MR (Thermo LabSystems Inc., Beverly, MA, USA). Lysates were prepared with equal amount of protein, distilled water and 4x laminar buffer. Lysate was loaded onto a 12% sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a nitrocellulose membrane using iBLOT (Invitrogen Technologies, Grand Island, NY, USA). The membrane was blocked for a minimum of 1 h with 5% casein in PBS and then incubated at 4°C overnight with antibodies directed against proteins of interest. After adding the appropriate AP- or HRP-conjugated secondary antibody, detection was performed by Western-lightning Plus ECL chemiluminescent substrate (PerkinElmer, Waltham, MA, USA).

Changes in *MS4A1* (*CD20*) mRNA transcription following entinostat in vitro exposure

To confirm gene expression profiling (GEP), lymphoma cell lines were exposed to entinostat (0.5 or 1 μmol/l) or vehicle for 48 h. Changes in *MS4A1* gene expression were analysed by quantitative real-time polymerase chain reaction (qPCR). Total RNA was extracted and converted in complementary DNA (cDNA) using the Taqman Gene Expression cells-to-CT kit (Life Technologies, Grand Island, NY, USA). Real-time polymerase chain reaction (PCR) reaction was carried out using PCR Master Mix (Life Technologies) on ABI-7900 platform (Applied Bio systems, Grand Island, NY, USA). *MS4A1* was amplified from cDNA using primers designed to amplify its coding region as previously described (Tsai *et al*, 2012).

Changes in total CD20 expression and surface CD20 expression density following entinostat exposure

RSCL and RRCL were exposed to entinostat or vehicle for 48 h. Subsequently, changes in total CD20 were evaluated by Western blotting as previously described (Czuczman *et al*, 2008). In addition, changes in surface CD20 following exposure to entinostat or SAHA were detected using the Image-Stream Technology (Amnis Inc., Seattle, WA, USA) (Tsai *et al*, 2012). In brief, 2×10^6 cells were stained with anti-human CD20-fluorescein isothiocyanate (FITC) or isotype control and then fixed by 2% paraformaldehyde. Cells were illuminated in the ImageStream system by a bright-field lamp and a 488-nm excitation laser for FITC. Data were analysed by ImageStream IDEAS image analysis software. Cells were gated for single, focused populations, which were then analysed for the mean cell size (by mean surface area) and mean surface CD20 expression (by mean CD20-FITC intensity). Mean surface CD20 density was calculated as follows: mean surface CD20-FITC intensity/mean surface area (CD20-FITC per μm^2).

In vivo effects of entinostat in rituximab activity

In vivo studies utilized a disseminated human lymphoma-bearing severe combined immunodeficiency (SCID) mouse xenograft model previously described (Hernandez-Ilizaliturri *et al*, 2003). For *in vivo* experiments, 6- to 8-week-old SCID mice were bred and maintained at the Department of Laboratory Animal Resource (DLAR) facility at RPCI and housed and maintained in laminar flow cabinets or micro isolator units and provided with sterilized food and water. SCID mice were inoculated on day zero with 1×10^6 Raji cells through tail vein injection. After 72 h (to allow tumour engraftment), the animals were then divided into five cohorts. The first cohort (Group A) was used as control and the animals did not receive any treatment. Group B consisted of animals treated with rituximab at 10 mg/kg on days +3, +7, +10 and +14. Groups C and D were treated with entinostat at 5 mg/kg per dose or 20 mg/kg per dose given orally by gastric lavage once daily for 2 weeks. Groups E

and F were treated with combination treatment of both rituximab at 10 mg/kg and entinostat at 5 mg/kg per dose or 20 mg/kg per dose. The end point of the study was survival, defined as the time to development of limb paralysis. Animals that reached the end point or survived after 3 months of observation were sacrificed by cervical dislocation. The experiments were repeated on three separate occasions.

Results

Entinostat induces cell death in RSCL and RRCL

Decreased cell viability in a dose- and time-dependent manner was observed in all cell lines tested. The decrease in cell viability peaked at 72 h and did not change any further after that time (Fig 1). The calculated 50% inhibitory concentration (IC_{50}) for the cell lines tested varied between 0.5 and 1 μmol/l (Fig 1). Based on pharmacokinetics from phase I clinical studies, some of the doses tested (0.25 to 1 μmol/l) are achievable in patients (Ryan *et al*, 2005; Gojo *et al*, 2007; Kummar *et al*, 2007; Pili *et al*, 2012).

Entinostat is more effective in inducing cell death in primary tumour cells isolated from lymphoma patients when compared to SAHA

To further evaluate entinostat anti-tumour activity, we compared it to SAHA in a more-clinically relevant pre-clinical model consisting of 36 primary tumour cells samples isolated from lymphoma patients. The demographic characteristic of the patients is described in Table SI. After incubation for 48 h, a variable degree of cell death was observed in primary tumour cells exposed to either entinostat or SAHA. At equivalent molar concentrations, entinostat was more effective in inducing cell death when compared to SAHA in the majority of primary samples tested (Fig 2). A decrease of cell viability by at least 25% was observed in 28 (78%) samples exposed to entinostat and in 8 (22%) samples exposed to SAHA. Anti-tumour activity was observed in all histological subtypes tested. No difference in entinostat activity was observed between previously untreated or relapsed/refractory primary tumour cells tested. Of interest, consistent anti-tumour activity was observed in all the ABC-DLBCL samples tested ($n = 5$) (Fig 2A–C).

Caspase inhibition diminished the anti-tumour activity of entinostat in some primary tumour cells isolated from B-cell lymphoma patients, suggesting that entinostat may have a dual mechanism of action and can trigger the activation of both caspase-dependent and -independent cell death pathways (Fig 2D).

Changes in GEP following in vitro exposure to entinostat

In order to define the activation of key regulatory pathways following entinostat therapy, we performed GEP studies in GCB- and ABC-DLBCL cell lines. Using the Illumina HumanHT-12v4 whole genome bead chip, we tested changes in gene expression profiles following entinostat drug exposure for 48 h in both RRSCL and RRCL. As expected, *in vitro* exposure to entinostat resulted in significant gene expression changes in all cell lines tested, and we identified a total of 490 genes that were consistently upregulated or down-regulated following entinostat exposure in all 4 cell lines tested (Fig 3A and B). The complete data is available in Table SII. Genes involved in the cell cycle (*CCNA2*, *CDKN1A* or *CHEK1*), cell

adhesion (*ICAM2*, *FCGRT* or *ITGB1*) or the ubiqutin-proteasome system (*PSMB5*, *CUL4A* or *PSMB6*) were consistently increased or decreased in the lymphoma cell lines exposed to entinostat (Table SIII). Changes in protein expression were also confirmed by Western blotting (Fig 3C). In GEP, upregulation of *MS4A1* mRNA was observed in all the cell lines exposed to entinostat; it was statistically significant ($P < 0.05$) in 3 out of the 4 cell lines.

In vitro exposure of RSCL and RRCL to entinostat results in G1 cell-cycle arrest

We evaluated differences in cell cycle distribution between RRCL and RSCL exposed to entinostat. *In vitro* exposure of NHL cell lines to entinostat resulted in a dose-dependent increase of cells in G1 phase and a decrease in S phase. Changes were similar in GCB- or ABC-DLBCL cell lines and in both RSCL and RRCL (Figure S1).

Changes in cell cycle and apoptosis regulatory proteins following entinostat exposure in RSCL and RRCL

To further define the molecular events triggered by entinostat *in vitro*, we compared differences in protein expression levels of cell cycle or apoptotic regulatory proteins in RSCL and RRCL. Following entinostat exposure, RRCL, and to a lesser degree, RSCL, exhibited a down-regulation of Bcl-XL. No significant changes in other anti-apoptotic proteins were observed (Fig 3C). In addition, upregulation of p21 and downregulation of CDC2, cyclin A and cyclin B protein levels were observed in all cell lines exposed to entinostat (Fig 3C).

Upregulation of total and surface CD20 in B-cell lymphoma cell lines exposed to entinostat

GEP suggested that entinostat exposure resulted in upregulation ($P < 0.05$) of *MS4A1* mRNA in 3 out of the 4 cell lines tested (RL-4H, U2932 and U2932-4RH). GEP profile findings were confirmed by qPCR (Fig 4A). Moreover, qPCR demonstrated that entinostat exposure resulted in an increase in *MS4A1*mRNA in all cell lines tested (Fig 4A). Subsequently, there was an upregulation of total and surface CD20 protein expression (as determined by Western blotting and flow cytometry) not only in DLBCL but also in BL cell lines (RSCL or RRCL) (Fig 4B and C).

Entinostat effects on the cytotoxic activity of chemotherapy agents and proteasome inhibitors

Previously published data demonstrated that less potent or pan-HDAC inhibitors have synergistic effects with chemotherapy agents and/or proteasome inhibitors (i.e. BTZ). Entinostat exhibited additive effects when combined with bortezomib or cytarabine against several RSCL and RRCL (Fig 5A and 5B).

Entinostat enhances rituximab activity in vivo

Previous investigators have demonstrated that ADCC is an important mechanism of action of rituximab *in vitro* and *in vivo* (Clynes *et al*, 2000; Hernandez-Ilizaliturri *et al*, 2003). The dynamics of rituximab-mediated ADCC are currently being investigated, but it is known that Fcc receptors play an important role in rituximab activity (Clynes *et al*, 2000; Cartron *et al*, 2002; Hatjiharissi *et al*, 2007). Recently, it has been demonstrated that co-localization of

intercellular adhesion molecule 1 (ICAM1, CD54) and CD20 is necessary for rituximab activity *in vitro* (Rudnicka *et al*, 2013). Based on these recent findings and on the information obtained from our GEP studies, demonstrating an increase in of *MS4A1*, *FCGRT* and *ICAM2* expression following entinostat in all cell lines tested, we evaluated the effects of entinostat in the activity of rituximab *in vitro* and *in vivo*.

Pre-incubation of lymphoma cell lines with entinostat *in vitro* for up to 48 h prior to rituximab exposure did not affect rituximab-mediated apoptosis, complement-mediated cytotoxicity (CMC) or ADCC *in vitro* (Fig 5C and 5D). On the other hand, entinostat enhanced the anti-tumour activity of rituximab *in vivo* (Fig 6). Treatment of human lymphoma-bearing SCID mice with rituximab resulted in prolongation in survival as compared with placebo-treated controls. The median survival time for rituximab-treated animals was 61 days (95% confidence interval [CI], 35.8–76.1) as compared with a median survival of 19 days (95% CI, 20.6–21.3) for those animals receiving placebo (log-rank test, $P = 0.002$). No significant anti-tumour activity was observed in animals treated with entinostat at the two dose-levels tested, and the median survival (18 [95% CI, 18.6–19.3] and 20 [95% CI, 18.64–19.3] days, respectively) was similar to controls (Fig 6A and B). The administration of entinostat at 20 mg/kg per dose (and, to a lesser degree, at 5 mg/kg per dose) in combination with rituximab resulted in the most effective anti-tumour activity and prolongation of survival of human lymphoma-bearing SCID mice. Statistically significant differences were observed between animals treated with rituximab versus entinostat plus rituximab (Fig 6A and B). The median survival time of animals treated with high-dose entinostat (20 mg/kg per dose) and rituximab was longer (not reached after 90 days) than those treated with rituximab monotherapy alone [median survival, 61 days (95% CI, 35.8–76.1); log-rank test, $P = 0.003$ (Fig 6A and B)]. While the median survival of animals treated with low-dose entinostat (5 mg/kg per dose) in combination with rituximab was longer (not reached at 90 days) when compared to rituximab monotherapy, the difference did not reach statistical significance (log-rank test, $P = 0.084$). After a follow-up period of 3 months, survival rates were the highest for animals treated with rituximab plus entinostat at 20 mg/kg per dose or rituximab plus entinostat at 5 mg/kg per dose when compared with animals treated with rituximab alone (30%).

Discussion

GEP studies have demonstrated that lymphoid malignancies are associated with the silencing of key regulatory genes involved in B-cell maturation in particular in HL and primary mediastinal lymphoma (PML) (Eberle *et al*, 2011). In general, gene expression is a tightly regulated process that is influenced by the (i) DNA/mRNA sequence, (ii) expression/activity of transcription factors, (iii) epigenetics (including DNA, chromatin and histone modifications); and (iv) mRNA stability. Post-transcriptional histone modification plays an important role in regulating gene transcription and is mediated by two main groups of enzymes: HATs and HDACs. The balance between HATs and HDACs is crucial in regulating the expression/function of several proteins involved in cell proliferation, cell cycle, apoptosis, angiogenesis and immune regulation. Altered balance between HATs and HDACs has been found to be associated with various malignancies including HL, PML and, to a lesser degree, DLBCL and BL (Shi *et al*, 2007; Eberle *et al*, 2011). As a group, HDACs

are known to regulate gene expression of key regulatory components involved in multiple cellular functions (Zain, 2012). Several pan- or selective-HDAC inhibitors (class I and IV) have been developed. Preclinical studies demonstrated significant anti-tumour activity in various cancer models including NHL and HL (Rosato *et al*, 2003; Shimizu *et al*, 2010; Vilas-Zornoza *et al*, 2012). Laboratory experiments suggest that HDAC inhibitors, including entinostat, can induce cell cycle arrest, apoptosis or autophagy in cancer cell lines and can potentiate the anti-tumour activity of chemotherapy agents, including proteasome inhibitors (Dasmahapatra *et al*, 2011; Harrison *et al*, 2012; Vilas-Zornoza *et al*, 2012).

Entinostat (SNDX-275) is a class I isotype-selective HDAC inhibitor with a long half-life. The exact understanding of how HDAC inhibitors, including entinostat, kill cancer cells or induce cell differentiation is largely unknown (Minucci & Pelicci, 2006). Moreover, the mechanism(s)-of-action may differ across subtypes of HDAC inhibitors, pharmacological properties of a given agent, or the lymphoma pre-clinical model tested. In pre-clinical models (mostly myeloid leukaemia or T-cell lymphoma cell lines), the following biological effects have been observed following entinostat exposure in a dose- and time-dependent manner: (i) down-regulation of Bcl2 and Bcl-XL (Jona *et al*, 2011); (ii) induction of p21-dependent cell cycle arrest (low dose); and (iii) increase in reactive oxygen species (ROS) leading to apoptosis (Rosato *et al*, 2003). Previously, Rosato *et al* (2003) demonstrated that entinostat (MS-275) induced cell-cycle arrest or apoptosis, depending on the dose used, in leukaemia pre-clinical models. Moreover, the combination of entinostat with gemcitabine or bortezomib was found to have synergistic effects. Jona *et al* (2011) found that entinostat down-regulates anti-apoptotic Bcl2 and Bcl-xL expression without altering Mcl1 or Bax levels and its effect was enhanced by two Bcl-2 inhibitors (ABT-737 and obatoclax). Our pre-clinical studies, using rituximab chemotherapy-sensitive and -resistant lymphoma models, demonstrated that this selective HDAC inhibitor was active in B-cell lymphoma. In addition, we found that entinostat enhanced the anti-tumour activity of the anti-lymphoma agents bortezomib and rituximab. Moreover, it appears to execute caspase-dependent (apoptosis) or -independent (cell cycle arrest) cell death pathways. In contrast to other HDAC inhibitors, entinostat is both orally available and minimally toxic, making it a suitable targeted agent for combination with standard chemoimmunotherapy in B-cell lymphoma. Identifying pathways affected by entinostat in lymphoma pre-clinical models has the potential to assist scientists and clinicians in developing strategies to further improve its therapeutic range in the clinical setting.

In our lymphoma pre-clinical models, we found that entinostat induced p21 expression and G1-phase cell cycle arrest rather than apoptosis in both RSCL and RRCL at the doses tested. We previously demonstrated that RRCL have a deregulated expression of *BCL2* family members and an abnormal mitochondrial potential that protect these cell lines from the apoptotic effects of rituximab or chemotherapy drugs (Olejniczak *et al*, 2008). Entinostat, and possibly other HDAC inhibitors, may have clinically relevant anti-tumour activity in highly resistant aggressive B-cell lymphomas bearing a high apoptotic threshold. Changes observed in p21 and other cell cycle regulatory proteins (Cyclin A, B and CDK2) or members of the ubiquitin-proteasome system expression following entinostat exposure may explain the positive effects on the anti-tumour activity of chemotherapy agents and bortezomib. We further studied how entinostat may up-regulate p21 in aggressive B-cell

lymphoma cell lines. Based on the finding of our GEP studies, we found that entinostat down-regulated E2F1, a known transcriptional regulatory factor of p21 (Abbas & Dutta, 2009). On the other hand, entinostat exhibits effects in the tumour micro-environment that may contribute to its anti-tumour activity, such as the up-regulation of OX40L (TNFSF4) in T-cells (Buglio *et al*, 2008).

To our knowledge, we are the first group to demonstrate that entinostat enhances the anti-tumour activity of rituximab *in vivo*. Previously, other investigators demonstrated that valproid acid, a weak HDAC inhibitor, potentiated the anti-tumour activity of rituximab by inducing CD20 expression *in vitro* (Shimizu *et al*, 2010). In addition, Zhao *et al* (2007) demonstrated that suberoylanilide hydroxamic acid (SAHA, vorinostat) potentiated rituximab activity *in vitro* and *in vivo* by down-regulation of nuclear factor (NF)jB activity in B-cell lymphoma pre-clinical models. Similarly, we observed an up-regulation of *MS4A1* gene/CD20 protein expression in BL, GCB- or ABC-DLBCL cells exposed to entinostat that could explain the effects of entinostat on rituximab activity *in vivo*. On the other hand, it is possible that other mechanism(s) could be responsible for the augmented anti-tumour activity observed in lymphoma-bearing SCID mice treated with rituximab and entinostat besides changes in *MS4A1* expression. Perhaps related to this finding, our GEP studies demonstrated an upregulation of ICAM2 CD102) and integrin in all cell lines exposed to entinostat. ICAM2, similar to ICAM1, facilitates the interactions between cells in the immune response (Bryceson *et al*, 2005). It is proposed that ICAM2 mediates the migration of natural killer (NK) cells into the tumour bed by its interaction with the leucocyte functional antigen (LFA1 [CD11a/CD18], ITGB2) (Fuchs & Colonna, 2006). Antibody blocking of LFA1–ICAM interactions impairs ADCC and natural cytotoxicity by human NK-cells (Miedema *et al*, 1984; Perez *et al*, 2004). It is possible that entinostat may also improve rituximab activity by upregulating ICAM2 in cancer cells, facilitating NK-cell dependent rituximab-mediated ADCC (Gross, 2010).

Results from a phase I study seeking to determine the maximum tolerated dose (MTD) of entinostat in patients with haematological malignancies demonstrated that the dose limiting toxicities (DLTs) at the MTD (10 mg/m^2 every 14 days) were nausea and vomiting.(Ryan *et al*, 2005) Our data suggests that entinostat may have therapeutic activity against B-cell NHL. Other HDAC inhibitors have been tested in DLBCL in combination with chemotherapy agents (Kalac *et al*, 2011). While entinostat has not yet been formally tested in patients with NHL, several clinical studies have been conducted in patients with relapsed/refractory HL, breast cancer and lung cancer (Jona *et al*, 2011; Witta *et al*, 2012; Yardley *et al*, 2013). Recently, entinostat received breakthrough therapy designation from the FDA for the treatment of advanced breast cancer. Anti-tumour activity with minimal toxicity has been observed in clinical trials. Pharmacologically, entinostat possesses several advantages over other HDAC inhibitors when designing combination clinical trials, such as: (i) long half-life, facilitating an easier dosing schedule; (ii) safer toxicity profile, permitting its incorporation with cytotoxic agents; and (iii) unique capacity to modulate the biological activity of targeted agents in preclinical models (i.e. modulating hormonal therapy response against breast cancer or rituximab activity against B-cell lymphoma). Our data suggests that entinostat is active against RSCL and RRCL and potentiates the anti-tumour activity of

rituximab. In addition, entinostat appears to have a dual mechanism of action that supports its clinical development in both rituximab-sensitive and -resistant B-cell NHL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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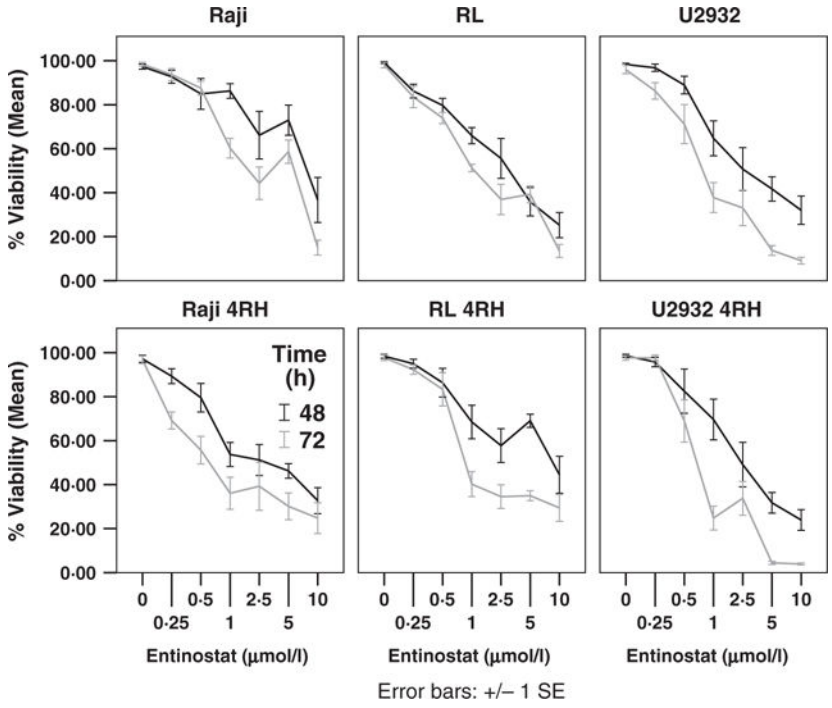


Fig 1.

In vitro exposure of B-cell lymphoma cell lines to entinostat results in dose- and time-dependent cell death in rituximab-sensitive (RSCL) (Raji, RL, U2932) and -resistant (RRCL) (Raji 4RH, RL 4RH and U2932 4RH) cells. RRSL or RRCL were exposed to escalating doses of entinostat (0.25–10 μmol/l) or vehicle (labelled as entinostat 0 μmol/l). Cell death was determined by Cell Titer Glo luminescence assay. Experiments were repeated in three separate occasions and are reported as the median with standard error bars (SE).

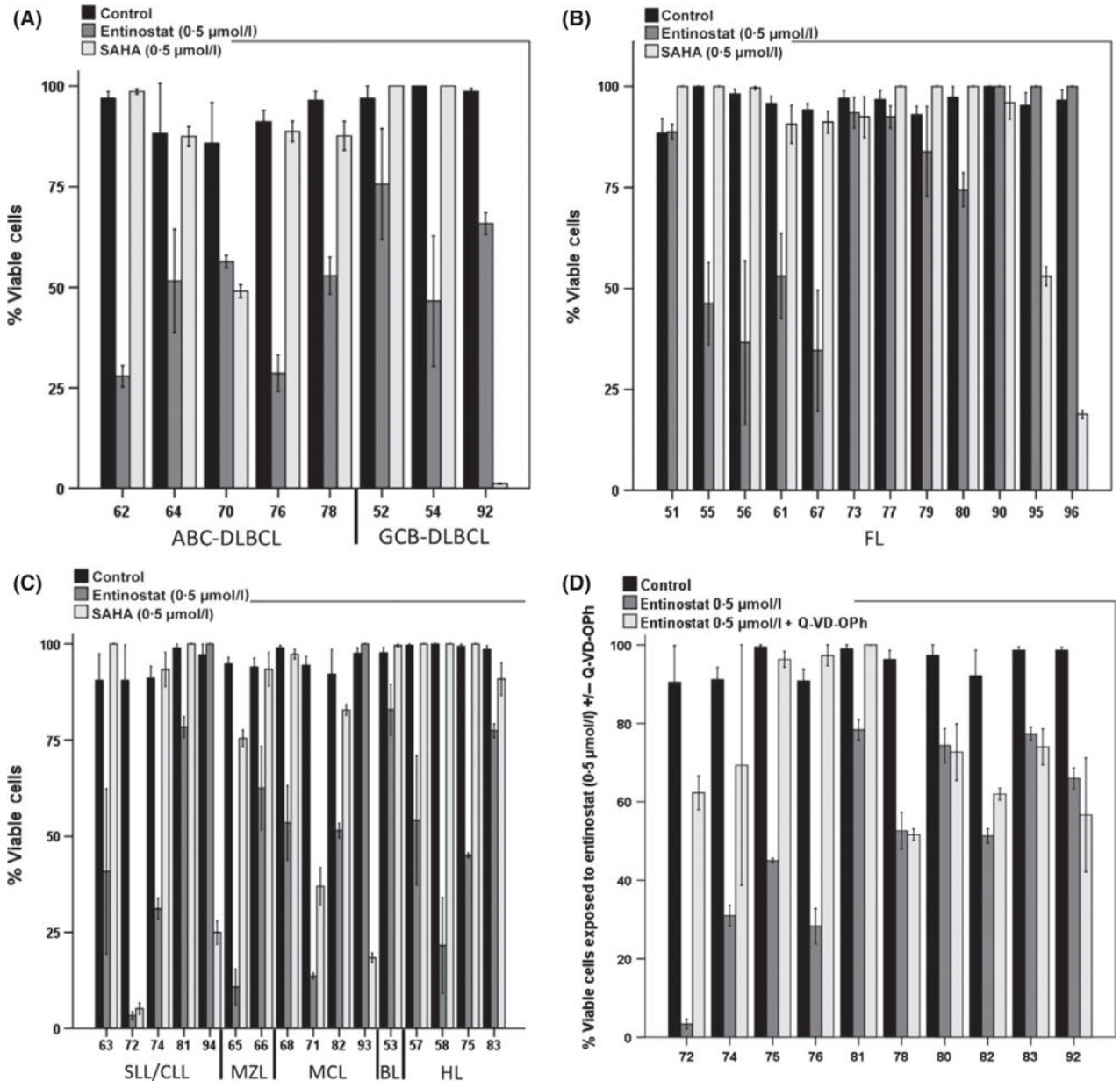


Fig 2.

Ex vivo exposure of lymphoma cells derived from patients with untreated or relapsed/refractory B-cell lymphoma to entinostat or suberoylanilide hydroxamic acid (SAHA) resulted in cell death. Production of ATP was used as a surrogate of viability after incubation for 48 h. Entinostat or SAHA was utilized at 0.5 μmol/l in patients with germinal centre B-cell (GCB) or activated B-cell (ABC) diffuse large B-cell lymphoma (DLBCL) (A), follicular lymphoma (FL) (B), or other subtypes of B-cell lymphoma (C). SLL/CLL = Small cell lymphocytic lymphoma/chronic lymphocytic leukaemia; MZL = Marginal zone lymphoma; MCL = Mantle cell lymphoma; BL = Burkitt lymphoma; and HL = Hodgkin lymphoma. Entinostat induces either caspase-dependent or -independent cell death in primary tumour cells (D). Various primary tumour cells isolated from B-cell lymphoma

patients were exposed *in vitro* to entinostat (0.5 μmol/l) with or without a pan-caspase inhibitor (Q-VD-OPh, 50 μmol/l). Cell viability was detected by ATP generation using the Cell titer Glo luminescent assay after 48 h. Each number in the *X*-axis represents the patient from which the sample was obtained. Percentage of viability was normalized to untreated control for each patient sample.

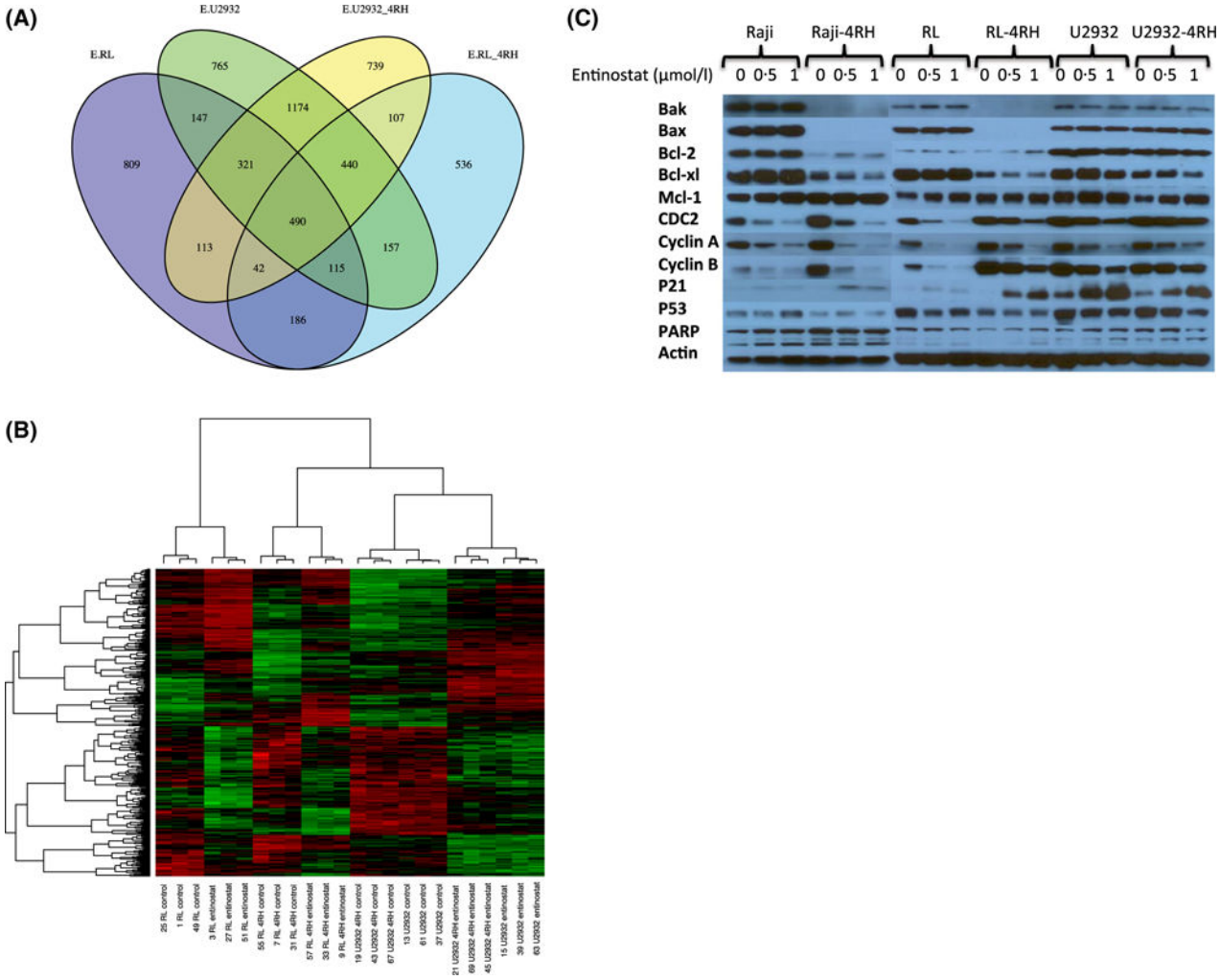


Fig 3.

Gene (A) and protein (B) expression changes of key regulatory pathways in lymphoma cells exposed to entinostat. Rituximab-sensitive (RSCL) (RL and U2932) and rituximab-resistant (RRCL) (RL 4RH and U2932 4RH) cell lines were exposed to Entinostat (0.5 μmol/l) for 24 h and then placed on an Illumina HumanHT-12v4® whole genome expression microarray. A total of 490 genes were found to be differentially expressed in all the cell lines exposed to entinostat (A). Hierarchical clustering of expression profiles of the 490 genes differentially expressed in all the cell lines exposed to entinostat. In the clustering heat map, red indicates upregulation while green indicates down-regulation. (B). Changes in gene expression following entinostat *in vitro* exposure was confirmed by Western blotting in selected target genes using total cell lysates extracted from RSCL and RRCL (C).

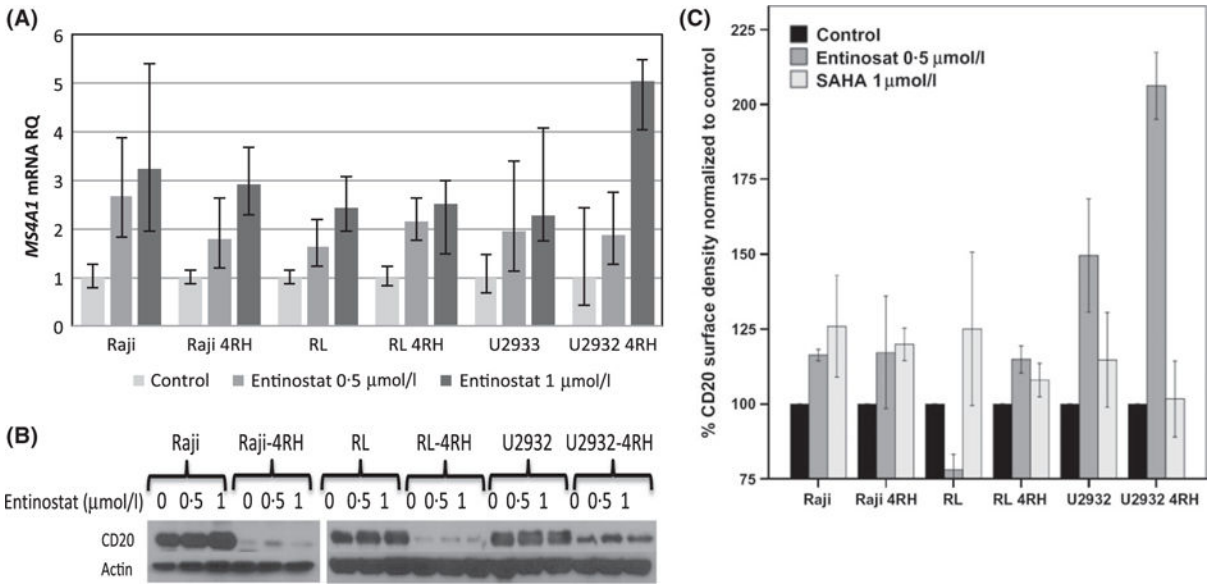


Fig 4.

Upregulation of total and surface CD20 antigen following entinostat exposure in B-cell lymphoma cell lines. A panel of RSCL and RRCL were exposed to entinostat for 48 h and a variable increase in *MS4A1* mRNA/CD20 expression was demonstrated by quantitative polymerase chain reaction (aPCR) (A), Western blotting (B) and flow cytometry (C).

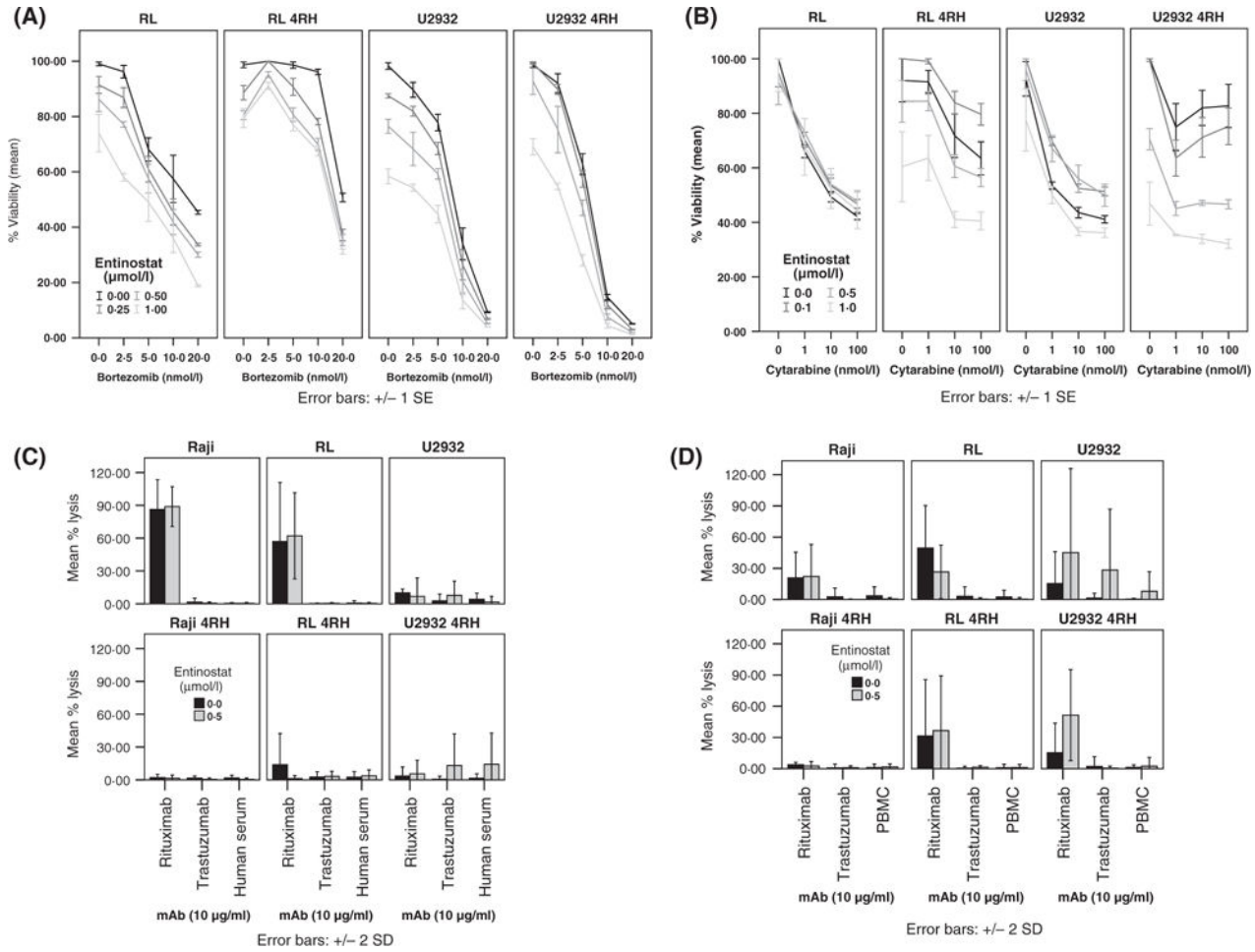
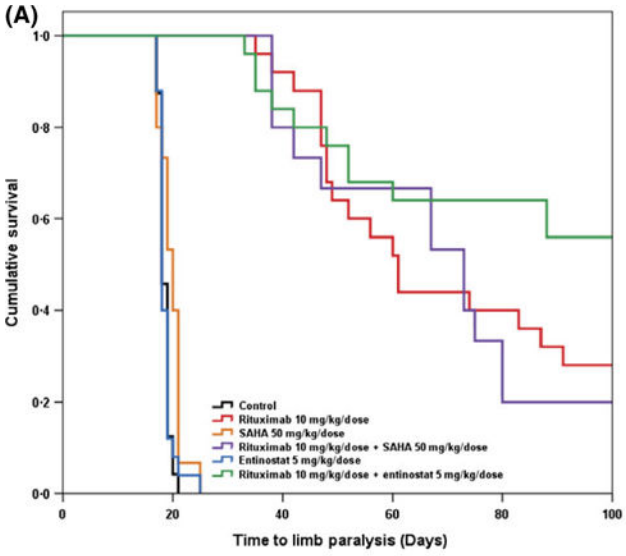
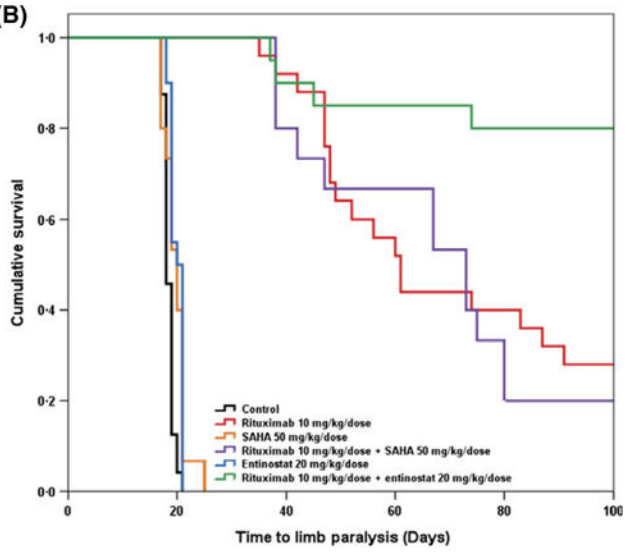


Fig 5.

Effects of entinostat on the anti-tumour activity of bortezomib (A), cytarabine (B) or rituximab (C and D) *in vitro*. Pre-exposure of rituximab-sensitive (RSCL, RL and u2932) or –resistant (RRCL, RL 4RH and U2932 4RH) diffuse large B-cell lymphoma (DLBCL) cell lines to bortezomib (A) or cytarabine (B) for 24 h enhanced entinostat (0–1 μmol/l) anti-tumour activity. Changes in cell viability were determined using the Cell Titer Glo assay. On the other hand, entinostat (0.5 μmol/l) did not alter rituximab-mediated antibody-dependent cellular cytotoxicity (ADCC); or (D) complement-mediated cytotoxicity (CMC); (C) in RSCL or RRCL. Cells were exposed to dimethyl sulfoxide or entinostat (5 μmol/l) for 24 h and subsequently labelled with ^{51}Cr. Labelled cells were then exposed to rituximab or isotype (trastuzumab) and peripheral blood mononuclear cells at an effector:target ratio of 40:1 (a) (ADCC) or 20% human serum pooled from healthy volunteers (CMC) and incubated at 37°C, 5% CO_2 for 6 h. ^{51}Cr-release was measured and the percentage of lysis calculated.





Treatment group (Entinostat dose 5 mg/kg)	Median survival (Days)	95% CI
Control (*N* = 24)	18	17·468 – 18·532
Rituximab (*N* = 25)	61	52·892 – 69·108
SAHA (*N* = 15)	20	18·512 – 21·488
Rituximab + SAHA (*N* = 15)	73	48·828 – 97·172
Entinostat (*N* = 25)	18	17·600 – 18·400
Rituximab + entinostat (*N* = 25)	NR	NR

Statistical differences between treatment groups	*P*-value (Log rank test)
SAHA vs. entinostat (5 mg/kg/dose)	0·038
Rituximab vs. rituximab & entinostat (5 mg/kg/dose)	0·087
SAHA & rituximab vs. rituximab & entinostat (5 mg/kg/dose)	0·029

Treatment group (Entinostat dose 20 mg/kg)	Median survival (Days)	95% CI
Control (*N* = 24)	18	17·468 – 18·532
Rituximab (*N* = 25)	61	52·892 – 69·108
SAHA (*N* = 15)	20	18·512 – 21·488
Rituximab + SAHA (*N* = 15)	73	48·828 – 97·172
Entinostat (*N* = 20)	20	19·203 – 20·797
Rituximab + entinostat (*N* = 20)	NR	NR

Statistical differences between treatment groups	*P*-value (Log rank test)
SAHA vs. entinostat (20 mg/kg/dose)	0·643
Rituximab vs. rituximab & entinostat (20 mg/kg/dose)	0·001
SAHA & rituximab vs. rituximab & entinostat (20 mg/kg/dose)	<0·001

Fig 6.

Entinostat potentiated the anti-tumour activity of rituximab *in vivo*. The combination of entinostat at 5 mg/kg per dose (A) or 20 mg/kg per dose (B) plus rituximab (10 mg/kg per dose) was more effective in controlling lymphoma growth and prolonged survival in severe combined immunodeficiency (SCID) mice inoculated with lymphoma cells (Raji cells) than entinostat single agent, suberoylanilide hydroxamic acid (SAHA) single agent, rituximab alone or rituximab in combination with SAHA. The survival difference between rituximab as a single agent or rituximab + SAHA compared to rituximab combined with entinostat at 20 mg/dose (and to a lesser degree at the 10 mg/kg per dose) was found to be significant. Survival differences between groups were compared using log rank analysis. Experiments were repeated three separate times. NR = Not reached.