Vorinostat interferes with the signaling transduction pathway of T-cell receptor and synergizes with phosphoinositide-3 kinase inhibitors in cutaneous T-cell lymphoma

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Supplementary Design and Methods

Reagents

Vorinostat (SAHA) was provided by Merck (Boston, MA, USA). LY294002 was purchased from Calbiochem (La Jolla, CA, USA) and 17-AAG was obtained from Sigma (St Louis, MO, USA). ETP-45628 (3-(1-methyl-4-morpholin-4-yl-pyrazolo[5,4-d]pyrimidin-6-yl)phenol) and ETP-39010 (4-[6-[(4-fluorophenyl)methylamino]imidazo[2,3-f]pyridazin-3-yl]phenol) were developed by Experimental Therapeutics Program at CNIO (Madrid, Spain). All the drugs were dissolved in dimethylsulfoxide (DMSO) to stock concentrations of 50 mM or 10 mM and stored at –20°C. Serial dilutions were made freshly in RPMI 1640.

Cell lines and cell culture

Human MJ, HuT78 and HH CTCL cell lines, derived from the peripheral blood of patients with mycosis fungoides, Sézary syndrome and non-mycosis fungoides/Sézary syndrome aggressive CTCL, respectively,[1-3] were obtained from the American Type Culture Collection (Rockville, MD, USA). The cell line Myla[4] (mycosis fungoides) was obtained from the European Collection of Cell Cultures (Wiltshire, UK); and SeAx[5] (Sézary syndrome) was a kind gift of Dr Reinhard Dummer, University Hospital of Zurich, Switzerland. The MJ cells produce human T-cell leukemia virus (HTLV-1) and HTLV related antigens. Cells were grown in RPMI 1640 (Sigma Chemical, St. Louis, MO, USA) supplemented with 10-20% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine (Life Technologies, Inc., Grand Island, NY, USA), 100 mg/mL penicillin/streptomycin (Life Technologies, Inc.), and 2.5 μg/mL fungizone (Life Technologies, Inc.) and grown in a humidified atmosphere at 37°C with 5% CO_2.

Cell viability

Cell viability was measured by a CellTiter-Glo Luminescent Cell Viability Assay (a luminescent assay measuring intracellular ATP content, which is directly proportional to cell number) according to the manufacturer's instructions (Promega, Madison, WI, USA). Aliquots of 1×10^4 cells per well were distributed in 96-well flat-bottomed tissue culture plates (BD Falcon, MJ, USA) in 100 μL of medium and incubated at 37°C in the presence or absence of the drug for 72 h (unless stated otherwise). Following incubation for the indicated time period, CellTiter-Glo reagent was added to each well and the lumi-

Online Supplementary Table S1. Connectivity Map (cMap, version 2) hits - molecules potentially able to reverse the resistance signature treatment with PUVA with or without interferon-α.

cMap Molecules	Dose [μM]	Cell lines	# instances	Enrichment score	*P* value
H-7	100	PC3, MCF7	4	-0.96	0
Geldanamycin	1	HL60, PC3, MCF7	15	-0.586	0
Tanespimycin (17-AAG)	0.1-1	HL60, PC3, MCF7	62	-0.373	0
Vorinostat (SAHA, Zolinza™)	10	HL60, PC3, MCF7	12	-0.591	0.0002
GW-8510	10	MCF7, PC3	4	-0.854	0.00084
Ciclosporin	1-3	PC3, MCF7	6	-0.708	0.00153
Alsterpaullone	10	MCF7,PC3	3	-0.863	0.00527
Dantrolene	12	HL60, PC3, MCF7	6	-0.647	0.00534
Scriptaid	10	MCF7, PC3	3	-0.849	0.00673
Cefotetan	7	HL60, PC3, MCF7	3	-0.84	0.00815

nescent signal was recorded.

Synergy studies of vorinostat and combination drugs (PI3K, PIM, HSP90 inhibitors)

Cells were seeded in 96-well plates and treated with serial dilutions of each drug individually or with both drugs simultaneously at fixed ratios of doses 0.25, 0.5, 1, 2, 4 times the individual IC_{50} values. After 72 h of exposure, luminescence was measured using the CellTiter-Glo Assay.

Flow cytometry analysis of cell cycle

HH, HuT78, MJ, Myla and SeAx cells (1×10^6 cells) were incubated with DMSO or vorinostat at indicated concentrations for 24 and 48 h. Cells were collected, washed with cold phosphate-buffered saline (PBS), fixed in ice 70% ethanol, treated with DNase-free Rnase (Quiagen, Inc., Valencia, CA, USA), and stained with 50 μg/mL propidium iodide (PI). Distribution of the cell-cycle phase by different DNA content was determined with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). For each sample, 10,000-gated events were acquired. Analyses of cell-cycle distribution (including apoptosis: sub-G0) were performed using CellQuest Pro software (Becton Dickinson).

Annexin V binding staining

Evaluation of the early apoptotic populations (annexin V^+/PI^-) and late apoptotic or secondary necrotic population (annexin V^+/PI^+) was performed by annexin V-allophycocyanin/PI surface staining according to the manufacturer's instructions. Briefly, cells were collected, washed twice with cold PBS, and centrifuged at 1200 rpm for 5 min. Cells were resuspended in 1x binding buffer at a concentration of 1×10^6 cells/mL, 100 μL of the solution were transferred to a 5 μL culture tube, and 5 μL of annexin V-allophycocyanain and 5 μL of PI were added. Cells were incubated for 15 min at room temperature in the dark. Finally, 400 μL of 1x binding buffer were added to each tube and samples were analyzed with a FACSCalibur flow cytometer (BD). For each sample, 20,000 gated events were acquired and the data were analyzed using CellQuest Pro software (BD).

Histone extraction

Histones were prepared according to established protocols.[6] We obtained histones from treated cell lines and their respective DMSO controls. The nuclei were extracted with 0.25 M HCl and precipitated with eight volumes of acetone.

Western blotting

Histone acetylation was determined by western blotting against acetyl forms of histones H2B (Abcam, Cambridge, UK), H2A, H3 and H4 (Upstate, Lake Placid, NY, USA). Histones H3 (Abcam), H2A, H2B and H4 (Upstate) were used as loading controls.

Total protein extracts were prepared by lysing cells in RIPA lysis buffer (Sigma-Aldrich) containing protease inhibitor cocktail set III (Calbiochem, San Diego, CA, USA) plus protease inhibitor cocktail set VIII (Calbiochem) for 20 min on ice. Cell debris was removed by centrifugation (10 000 *g*, 10 min). Protein concentration was measured using protein assay reagents A, B, and S (BioRad, Hercules, CA, USA) following the manufacturer's instructions. For phosphorylation studies the following primary antibodies were used: p-ZAP70(Tyr493), p-ZAP70 (Tyr319)/SYK (Tyr352), p-AKT (Ser473), AKT (Cell Signaling Technology, Beverly, MA, USA) and ZAP70 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibody to α-tubulin was from Sigma Chemicals Co. (St. Louis, MO, USA). Proteins were electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and wet-transferred onto nitrocellulose membranes (Whatman, Dassel, Germany). Membranes were blocked in 5% milk PBS-T (phosphate-buffered saline with 0.1% Tween-20) and sequentially immunoprobed with primary antibodies to acetylated histones. Antibodies were detected using fluorescent-labeled secondary antibodies (Alexa 680λm and Alexa 800λm, Rockland, Gilbertsville, PA, USA) and scanning with an Odyssey Infrared System Scanner (LI-COR Biosciences, Lincoln, NE, USA).

RNA extraction

Total cellular RNA was extracted from cells using a QIAshredder spin column (homogenization) and purified with an RNeasy Mini-Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's RNA Clean-up protocol with the optional on-column digestion with RNase free DNase I. RNA quality and integrity were verified using RNA 6000 Nano Chips, Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Complementary DNA synthesis

First-strand cDNA was synthesized from 500 ng of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol. Reverse transcription reactions were performed in a total volume of 20 μL containing 500 ng DNase treated total RNA, 1x reaction buffer, dNTP mixture, random primers, Rnase inhibitor and 50 U MultiScribe reverse transcriptase. Reaction mixtures were incubated at 25°C for 10 min then incubated at 37°C for 2 h and for 5 sec at 85°C.

Gene expression profiling

Hybridization

The gene expression profile of CTCL cells (Hut78, Myla, MJ, HH, SeAx) treated with 5 μM vorinostat for 0, 1, 2, 4, 8, 12 and 24 h was determined by hybridization to 60-mer 44k oligonucleotide microarrays (Agilent Technologies, Inc., Santa Clara, CA, USA), using Stratagene Universal Human Reference RNA. Briefly, for each sample, 2 μg of total RNA were mixed with 2 μL of a 5,000-fold dilution of Agilent's Two-Color Spike-in RNA control. The mixture was amplified using the low-input RNA amplification kit (Agilent). Following amplification and labeling with Cy3, each sample was assessed on the Nanodrop ND-1000 to measure yield and specific activity. The amplified and labeled samples were hybridized in a rotating oven to Agilent 44K Human Whole Genome microarrays according to the manufacturer's instructions. The images were scanned with a G2565BA Microarray Scanner System (Agilent Technologies, Palo Alto, CA, USA).

Normalization

The data were processed and normalized with the use of Feature Extraction (v.9.0) software. Each time point was additionally normalized to time 0 and genes with more than 30% of missing values were discarded.

Data analysis

Assignment of genes to temporal expression profiles and detection of statistically enriched gene families within each profile was conduct-

ed using the Short Time Series Expression Miner (STEM).[7] Briefly, STEM implements a novel clustering method that depends on a set of distinct and representative short temporal expression profiles and each probe in the dataset is assigned to a profile with closest match. The expected number of probes assigned to each profile is estimated by permutation and the statistically significantly over-expressed profiles are then identified. Genes were deemed to be up-regulated or down-regulated if the expression change was at least 2-fold and the correlation between the duplicate arrays for each gene was at least 0.8. The *P* values derived from STEM analysis were corrected for multiple hypothesis testing using a false discovery rate (FDR) of less than 5%. The significant genes were annotated and classified using FatiGO+[8] and Ingenuity Pathway Analysis software v.6.3 (Ingenuity Systems, Inc., Redwood City, CA, USA)

The significant genes were clustered by average linkage clustering using Cluster[9] and visualized by TreeView v.1.6 (EisenSoftware, Berkley, CA, USA).

Connectivity map

Identification of molecules (drugs) that could reverse the gene expression signatures associated with poor prognosis and drug resistance was performed using Connectivity Map[10] (also known as cMap). cMap is a collection of genome-wide transcriptional expression data from cultured human cells treated with bioactive small molecules and simple pattern-matching algorithms that together enable the discovery of decisive functional connections between drugs, genes and diseases through the transitory feature of common gene expression changes. Enrichment of both the up- and down-regulated genes from the submitted gene expression profiles for each treatment was estimated using a metric based on the Kolmogorov-Smirnov statistics and combined to produce the connectivity score.[10]

Real-time quantitative polymerase chain reaction

Expression of mRNA for eight differentially expressed genes associated with the T-cell receptor pathway was measured with the use of TaqMan Gene Expression Assays on demand (Applied Biosystems, Foster City, CA, USA). The following assays were used: FYN (Hs00941608_g1), IFNGR1 (Hs00988302_m1), STAT3 (Hs00374286_g1), ZAP70 (Hs00896347_m1), CD3G (Hs00962185_g1), LAT (Hs01065378_g1), IL4R (Hs00965052_m1) and CD3E (Hs99999153_m1). Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis was performed in triplicate with the ABI Prism 7900 Sequence Detector System (Applied Biosystems). The PCR cycling conditions were the standard 95°C for 10 min for one cycle, 95°C for 15 sec, and 60°C for 1 min for 40 cycles. The relative changes in gene expression were calculated by the ΔΔCt method using Sequence Detection System 2.1 software (Applied Biosystems). The ΔΔCt method gives the amount of target normalized to an endogenous reference and relative to a calibrator. The expression of each of the genes in all specimens was related to its expression in a reference RNA pool (Stratagene) used as a calibrator. The level of each transcript was quantified by the cycle at which the PCR amplification was in log phase where there was significant fluorescent signal (Ct) with *HGUSB* as the endogenous control. All standards and samples were run in a total volume of 15 μL in triplicate.

Statistical analysis

Group comparisons of parametric data were made by Student's t-test. Statistical analysis was performed with the SPSS software package, version 13.0. The mean inhibitory concentration 50% (IC_{50}) was calculated using Graph Pad Prism software (sigmoidal dose response, variable slope). Isobologram analyses using the method of Chou and Talalay with CalcuSyn (Biosoft, Ferguson, MO, USA) were used to determine synergism.[11] A combination index (CI) value less than 1.0 indicates synergistic effects. A CI value equal to 1.0 indicates additive interactions. A CI value greater than 1 indicates antagonistic interactions.

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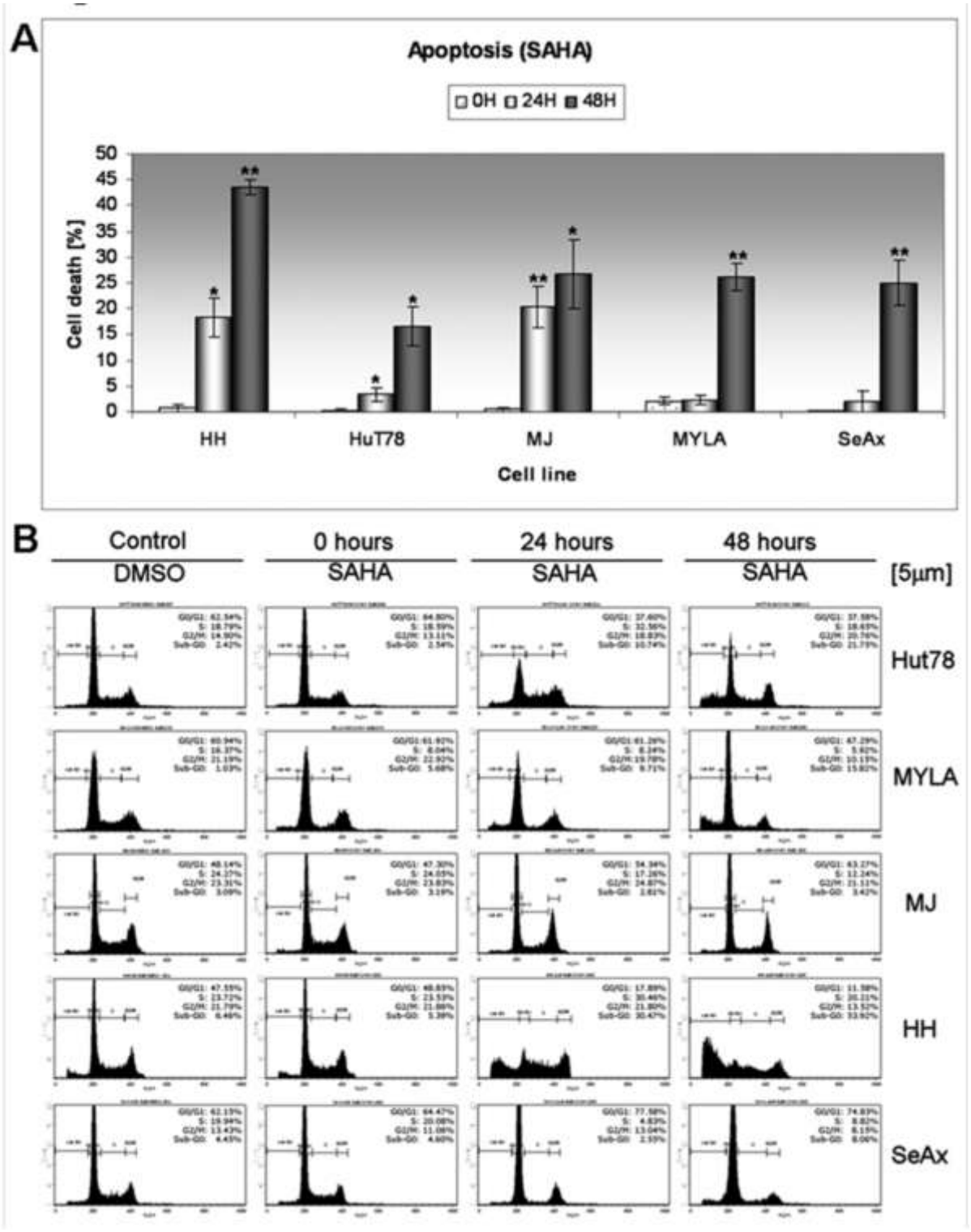
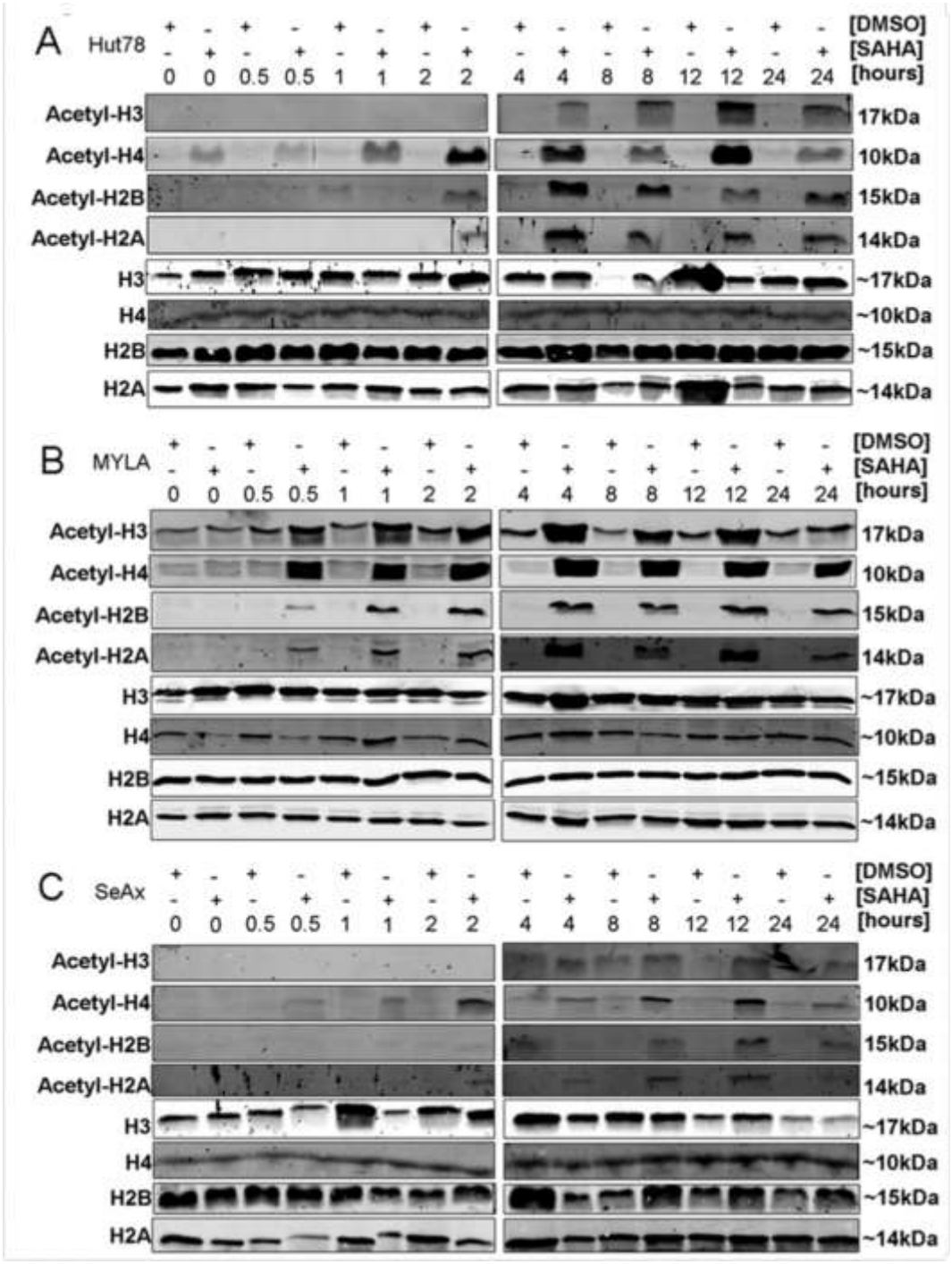
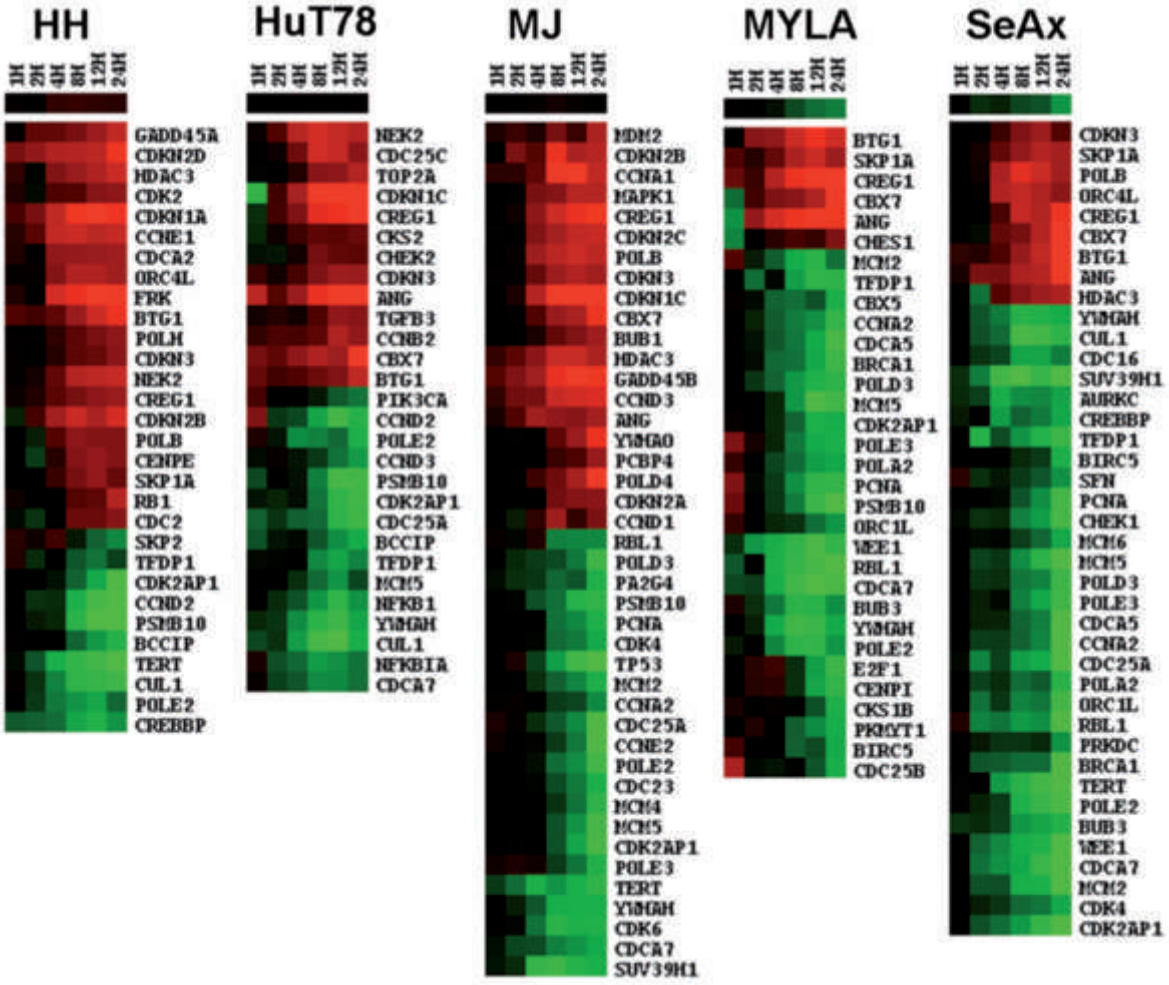


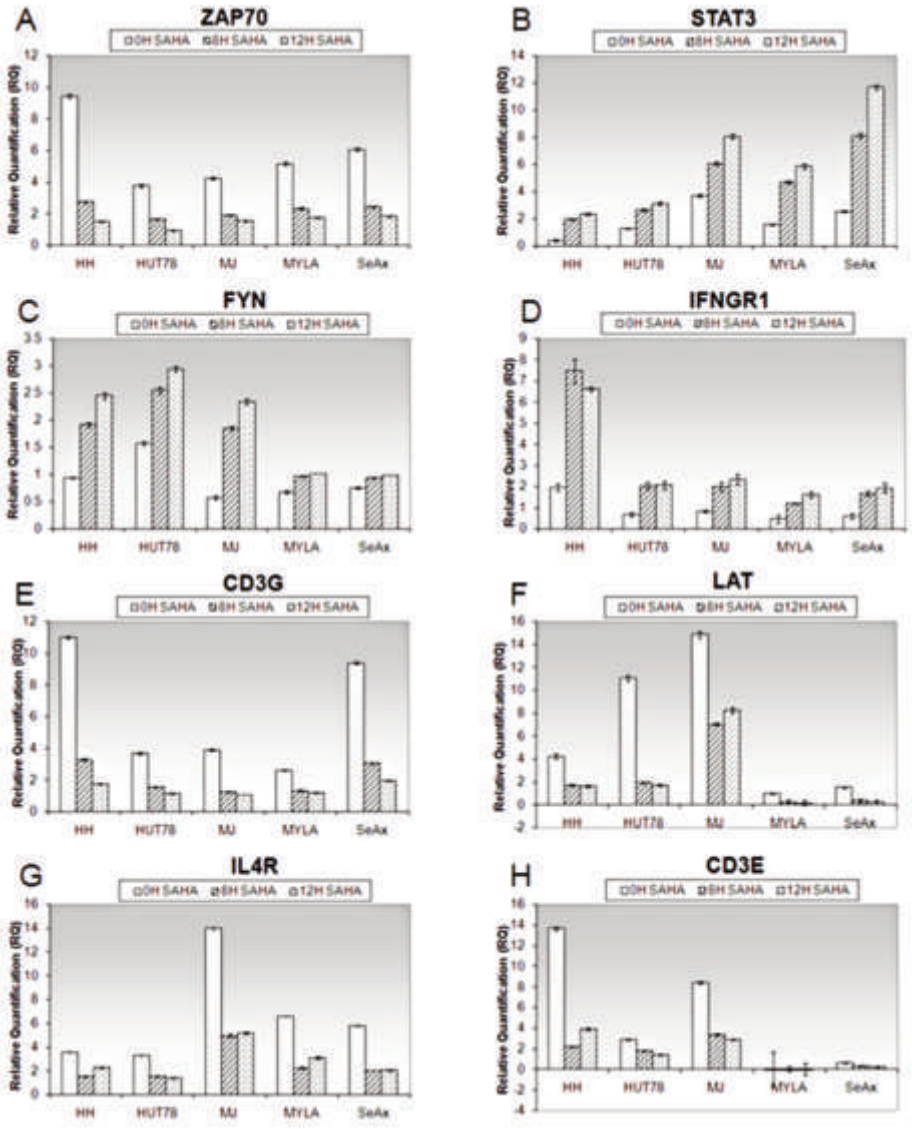
Online Supplementary Figure S1. Effect of vorinostat (SAHA) on apoptosis and cell cycle in cutaneous T-cell lymphoma (CTCL) cell lines. HH, HuT78, MJ, Myla, and SeAx cell lines were treated with or without vorinostat (5 μM) for 24 and 48 h. (A) Apoptosis: each point represents the percentage of annexin V^+PI^-, annexin V^+PI^+ and annexin V^-PI^+ cells. The cell death associated with addition of control DMSO was subtracted from each value (error bar represents mean SD of three to eight determinations). *P<0.05, **P<0.001. (B) Cell-cycle distributions were determined by DNA content analysis with propidium iodide staining and flow cytometry.



Online Supplementary Figure S2. Effect of vorinostat on acetylation of histones. CTCL cell lines: HuT78, Myla and SeAx were exposed to vorinostat (SAHA) (5 μM) for 0.5, 1, 2, 4, 8, 12, and 24 h. Histones were extracted and analyzed by western blot. The acetylation of histones H3, H4, H2B and H2A was determined. Non-acetylated histones H3, H4, H2A and H2B were used as loading controls.



Online Supplementary Figure S3. Functional clustering of vorinostat-regulated cell cycle-associated genes.



Online Supplementary Figure S4. Validation of microarray data by TaqMan quantitative real time PCR. The influence of vorinostat (SAHA) on mRNA expression of *ZAP70* (A), *STAT3* (B), *FYN* (C), *IFNGR1* (D), *CD3G* (E), *LAT* (F), *IL4R* (G) and *CD3E* (H) for all cell lines before and after 8 and 12 h of treatment. Values for each gene were normalized to expression levels of *HGUSB*, an endogenous control. Experiments were performed in triplicate.