Cancer differentiating agent hexamethylene bisacetamide inhibits BET bromodomain proteins

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Running title: HMBA is a BET inhibitor

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ABSTRACT

Agents that trigger cell differentiation are highly efficacious in treating certain cancers, but such approaches are not generally effective in most malignancies. Compounds such as dimethylsulfoxide (DMSO) and hexamethylene bisacetamide (HMBA) have been used to induce differentiation in experimental systems, but their mechanisms of action and potential range of uses on that basis have not been developed. Here we show that HMBA, a compound first tested in the oncology clinic over twenty-five years ago, acts as a selective bromodomain inhibitor. Biochemical and structural studies revealed an affinity of HMBA for the second bromodomain of BET proteins. Accordingly, both HMBA and the prototype BET inhibitor JQ1 induced differentiation of mouse erythroleukemia cells. As expected of a BET inhibitor, HMBA displaced BET proteins from chromatin, caused massive transcriptional changes and triggered cell cycle arrest and apoptosis in Myc-induced B cell lymphoma cells. Further, HMBA exerted anticancer effects in vivo in mouse models of Myc-driven B-cell lymphoma. This study illuminates the function of an early anticancer agent and suggests an intersection with ongoing clinical trials of BET inhibitor, with several implications for predicting patient selection and response rates ot this therapy and starting points for generating BD2-selective BET inhibitors.

INTRODUCTION

The idea of tumor cell differentiation was conceptualized when Charlotte Friend showed that the solvent dimethylsulfoxide (DMSO) was capable of inducing differentiation of virus-induced mouse erythroleukemia (MEL) cells (1). Since the FDA had banned DMSO for human use, Paul Marks and colleagues initiated a search for polar compounds that would be able to induce MEL cell differentiation at lower concentrations (2). The most effective compound was hexamethylene bisacetamide (HMBA) (3). It was developed as far as to the clinic where it showed acceptable toxicity profiles in phase I clinical trials and activity against one quarter of patients with acute myelogenous leukemia (AML) in phase II trials. However, likely due to low response rates, HMBA was not further developed after 1992 (4-7). Parallel to the development of HMBA, Marks and colleagues also discovered the target of a much more potent MEL differentiation agent, suberoylanilide hydroxamic acid (SAHA) (8).

They demonstrated that SAHA is a potent inhibitor of histone deacetylases (HDACs), which spawned active investigations and clinical development. SAHA, or vorinostat, is now FDA-approved for treatment of cutaneous T-cell leukemia (9) and other HDAC inhibitors are approved for use in other tumor types. The target of HMBA remained elusive though (10) since Marks and colleagues could not demonstrate that it inhibited HDACs (8).

The function of BET bromodomain proteins (BRD2, BRD3, BRD4 and BRDT) is to bind acetylated proteins, most notably histones, and thereby regulate transcription (11). BET bromodomain inhibitors (BETi) block this binding, resulting in broad transcriptional changes (12-16). Recently we demonstrated that vorinostat triggers transcriptional changes that are overlapping with those induced by BETi in murine Myc-induced lymphoma cells (17). Given our data that BETi and HDACi can induce similar transcriptional changes (17), we hypothesized that the target of HMBA could be one or several BET bromodomain proteins.

MATERIALS & METHODS

Chemicals

The (+)-enantiomer of JQ1 was purchased from Cayman chemicals. HMBA, C646 and CPTH2 were purchased from Sigma-Aldrich. Vorinostat was purchased from Selleck Biochemicals.

Cell culture and mouse experiments

The B-cell lymphoma cell lines λ663, λ820, Eμ239 and Eμ580 were established 2006 from single cell suspensions of tumors arising in λ-*Myc* or Eμ-*Myc* transgenic mice by serial passage in culture (17) and were grown in RPMI-1640 supplemented with 10% fetal bovine serum, glutamine, 50 μM β-mercaptoethanol, and antibiotics. The only mean of authentication of these lines were by qRT-PCR of *Myc* (human and mouse for λ-*Myc* or Eμ-*Myc* transgenic mice, respectively). The human Burkitt lines Akata and Daudi were obtained from Erik Lundgren (Umeå University, Sweden) and were authenticated by Myc overexpression and p53 mutation status in 2008 as previously described (18). The MEL cells were a kind gift from Dr Olle Heby (Umeå University, Sweden) and were cultured in DMEM supplemented with 20 % fetal bovine serum

and antibiotics. They were only authenticated in this study by showing their differentiation to erythrocytes (Figure 1).

All animal experiments were performed in accordance with regional/local animal ethics committee approval (approval number 287/2011 and 193/2014). C57BL/6 mice were transplanted with 200,000 λ2749 lymphoma cells (17). White blood cell (WBC) counts were measured by blood sampling from vena saphena. When WBC counts were above the normal range (6-15 cells/nl), mice were treated with bi-daily intraperitoneal injections of HMBA or left untreated.

Cell cycle analysis

1 million cells per ml were lysed and stained for 30 min at 37°C in modified Vindelöv's solution (20 mM Tris, 100 mM NaCl, 1 µg/ml 7-AAD, 20 µg/ml RNase, and 0.1 % NP40 adjusted to pH 8.0) followed by analysis of DNA content using the FL3 channel (linear mode, cell cycle) or FL3 channel (logarithmic mode, apoptosis).

RNA preparation and analyses

λ820 (in biological replicates) were cultured in the absence or presence of 5 mM HMBA for 24 h. RNA was prepared using NucleoSpin® RNA II kit (Macherey-Nagel, Germany). The analysis of gene expression was performed using the Illumina BeadChip system at the Genomics Core Facility, University of Gothenburg, Sweden. Mouse Ref-8 v2 Beadchip Arrays (Illumina) were used following the manufacturer's protocol. Primary data were collected from the BeadChips using the Illumina BeadArray Reader (HiScan) and analyzed using the supplied software. Data normalization was performed by cubic spline normalization using Illumina's GenomeStudio software equipped with the necessary modules. Prior to further analysis, FORCEPOS adjustment (+25) was performed to avoid negative data arising during normalization. To compare data with those generated previously from JQ1-treated or vorinostat-treated cells (17) all data from compound-treated cells were normalized to their respective experimental controls (untreated cells for HMBA, 0.1% DMSO-treated cells for JQ1 and vorinostat). Full data sets are available as Supplemental dataset 1. Clustering and visualization of genes was performed using Spotfire. Gene-set enrichment analysis was done using the Qlucore software.

For quantitative reverse transcriptase PCR (qRT-PCR), total RNA was prepared from cultured cells or tumors. cDNA was prepared from 500 ng total RNA

using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). qRT-PCR was performed using KAPA SYBR® FAST ABI Prism® 2X qPCR Master Mix (Kapa Biosystems, Inc, Woburn, MA, USA). Data analyses were performed by comparing ΔΔCt values, using *Ubiquitin* (*Ub*) as the reference gene and with a control sample set as relative expression 1.

Chromatin immunoprecipitation (ChIP)

ChIP was carried out using the manufacturer's protocol (SimpleChIP Plus Enzymatic Chromatin IP Kit; Cell Signaling Technology). Briefly, cells left untreated or treated with HMBA for 18 h were fixed with formaldehyde and lysed; chromatin was partially digested by micrococcal nuclease and then sonicated. Lysates were quantified and 10 μg of chromatin was loaded per IP and the rest was used as input. ChIP was carried out using antibodies directed against Brd2 (rabbit; Bethyl Labs), Brd3 (mouse; Santa Cruz) or Brd4 (rabbit; Bethyl Labs), where anti-rabbit and anti mouse IgG was used as an isotype control. Purified and reverse–cross-linked chromatin were analyzed by quantitative PCR using primers designed against, Cd74 (17), Srm (19) and Vpreb3.

Biochemical assays

HMBA at five different concentrations (5 mM, 0.5 mM, 50 μM, 5 μM, 0.5 μM and 50 nM) was profiled against ten different bromodomain (BRD) protein fragments and rat liver HDAC. For all assays, reference compounds were used to validate the assays. For BRD proteins, binding of recombinant human proteins to biotinylated and acetylated histone peptides was detected using the AlphaScreen detection method as previously described (20). For HDAC, a fluorimetric method was used that had been previously described (21).

Structure determination

Molecular docking of BRD2 bromodomain 2 (PDB# 2E3K) to JQ1 or HMBA was performed using the Swissdock server (www.swissdock.ch/). Input ligands were imported from the ZINC database. The resulting docking results were visualized in Chimera.

For crystallization, His-tagged BRD3 bromodomain 2 (BRD3 BD2) was expressed in *E. coli* and purified on a Ni-NTA column as previously described (20). The His-tag was removed by TEV protease cleavage followed by an additional

purification on Ni-NTA column. BRD3 BD2 was concentrated to 42.1 mg/ml in 20 mM HEPES, 500 mM NaCl, 5 % glycerol, pH 7.5. Crystallizations were then done using a mosquito robot (TTP Labtech). Prior to crystallization, the buffer was changed using a micron centrifugal filter with a cut off of 10kDa. The crystallization buffer contained 10 mM HEPES pH 7.5 and 300 mM NaCl. Crystals were grown by vapour diffusion at 4 °C from a sitting drop consisting of 150 nl protein (10 mg/ml) and 150 nl reservoir solution containing 19% PEG 6000, 0.1M HEPES pH 7.0. The crystals appeared after a few days and had a size of 60 µm x 60 µm x 15 µm. For soaking, the crystal was transferred to a drop containing 19 % PEG 6000, 0.1 M HEPES pH 7.0 and 50 mM hexamethylene bisacetamide (HMBA) for 20h. The crystal was then transferred to a cryoprotectant solution containing 19 % PEG6000, 0.1 M HEPES pH 7.0, 25 % ethylene glycol and 50 mM HMBA and flash-frozen in liquid nitrogen.

A BRD3 BD2 data set was collected to 1.9 Å at 100 K at beam line I911-3, MAX IV laboratory, Lund, Sweden (λ = 1.0000 Å). 165 images in total, each with 1° rotation, were collected. All data were integrated and scaled using XDS and Aimless softwares. The structure of BRD3-BD2 in complex with HMBA was determined by rigid body refinement of an in-house apo structure to 1.5 Åresolution. Ligand conformations were created using software from Schrödinger, LLC. After initial anatomic refinement of the protein coordinates in Refmac5 the coordinates of HMBA were fitted to the electron density using the Coot software. CCP4-style restraints for HMBA were generated using the Jligand program. The structures were refined to convergence using Refmac5. The protein structure quality was checked using the Molprobity server. Hydrogen atoms were added in the riding positions. Water molecules were added to positive difference density peaks more than 5 standard deviations above the mean and present in 2m|Fo|-d|Fc| map at the 1 sigma level.

Accession codes: Coordinates and structure factors have been deposited in the Protein Data Bank under accession code 5A7C.

Hemoglobin measurement

Cells were washed twice in PBS, dissolved in water and freeze-thawed three times. After a 30-min centrifugation at 15,000 x *g* the absorbance spectrum of the

supernatant was measured on a Nanodrop spectrophotometer. Whole blood from a mouse was diluted and processed the same way as the cells and was used a positive control. Presence of hemoglobin in the sample was evident by the presence of peaks at 414, 540 and 576 nm.

Statistical analysis

The bars shown represent the mean ± standard deviation (SD). The Student's *t*-test were performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Statsitically significant differences are indicated with * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) or **** ($p<0.0001$).

RESULTS

HMBA cause MEL cell differentiation by acting as a selective BD2 inhibitor

JQ1 is a potent BETi (16), which binds to bromodomain 1 and 2 (BD1 and BD2) in all BET bromodomain proteins. Consistent with the hypothesis that HMBA could be a BET inhibitor, molecular docking *in silico* shows that HMBA fits the same pocket in BD2 of BRD2 as JQ1 (Figure S1A). Since HMBA is a small polar molecule that potentially could fit many pockets we also measured binding of bromodomain-containing proteins to acetylated histone peptides in the presence of increasing concentrations of HMBA or known ligands. This demonstrated that HMBA could displace BD2 in BRD2, BRD3 and BRD4, with the highest affinity to BRD3 (Table 1 and Figure 1A). On the other hand, as previously shown (8), HMBA does not inhibit HDACs (Table 1).

In order to assess whether HMBA's interactions with BET proteins were specific we soaked crystals of the second bromodomain of BRD3 with HMBA and determined the X-ray crystal structure of the complex. Electron density maps revealed unambiguous density for bound HMBA (Figure 1B and S1B). There are four molecules of BRD3 BD2 in the asymmetric unit. HMBA is fully visible in the electron density for subunit A and is bound in the acetyl-lysine pocket of BRD3 BD2 (Figure 1B). For the other three subunits the acetamide binding to Asn391 and the conserved water molecules is very similar, but the tail and the second acetamide are poorly visible in the electron density, indicating flexibility. Furthermore, in subunit A there are two water molecules from a symmetry related molecule interacting with the

second acetamide which could explain why the entire ligand is only visible in the electron density for this subunit. The surrounding crystallographic environment is not the same for the other subunits. When superimposing all subunits it becomes clear that the linker and the second acetamide are flexible (Figure S1C and S1D). Of interest is also that HMBA makes a hydrogen bond with His395 in one of the subunits. Since this position is conserved in the BC-loop of the BD2 domain of all BET proteins, and since BD1 has an Asp at the corresponding position (20), it is tempting to speculate that this is the reason behind the apparent BD2 selectivity of HMBA.

BET proteins belong to a larger family of bromodomain-containing proteins, the BRD family (20). Given HMBA's small size it requires 3-5 mM in concentration to exhibit most effects in cell culture. Hence, it was of importance to investigate whether HMBA could bind more proteins than the BD2 domain of BET proteins. Analyzing representative proteins of different branches of the BRD family tree, we found that the bromodomain of the histone acetyltransferase EP300 was also inhibited from binding acetylated histone (Table 1).

Next we evaluated the effects of HMBA and JQ1 on mouse erythroleukemia (MEL) cells. We reasoned that if HMBA induced differentiation by blocking BET proteins then the prototype BET inhibitor JQ1 should also induce differentiation of MEL. Indeed, treatment of MEL cells with either 5 mM HMBA or 0.1 μM JQ1 resulted in formation of red cells, indicative that hemoglobin has been produced (Figure 1C). Confirming this, lysates of HMBA or JQ1-treated MEL cells exhibited the same absorbance spectra as did mouse blood (Figure 1D) and qRT-PCR analysis showed that both JQ1 and HMBA induce the expression of the hemoglobin-encoding gene *Hba-a1* (Figure 1E). These data suggest that BET inhibition is sufficient to trigger differentiation of MEL cells. However, inhibiting EP300 with the selective inhibitor C646 (22), or histone acetyltransferase GCN5 with the selective inhibitor CPTH2 (23), does not result in differentiation (Figure 1C). This suggests that HMBA's effect on differentiation is mediated by inhibiting BET proteins and not by inhibiting acetyltransferases in general.

HMBA kills Myc-induced lymphoma cells in vitro and in vivo

In the clinic, the therapeutic effects of HMBA were only modest. Since we previously demonstrated that BETi such as JQ1 and RVX2135 arrest and kill Myc transgenic

murine lymphoma cells (17), we were interested to investigate if Myc-overexpressing cells would be sensitive to HMBA. To that end, cultured λ820 and λ663 cells, developed from λ-*Myc* transgenic mice, were treated with HMBA. Similar to the effects seen with other BETi (17), HMBA induced a cell cycle arrest at 24 h followed by apoptosis of all cells by 48 h (Figure 2A and data not shown). We also treated two lymphoma lines developed from Eμ-*Myc* transgenic mice (24), and two human Burkitt lymphoma cell line. We observed robust anti-proliferative effects after 24 h in the murine lines (Figures 2A) whereas the effects in Burkitt lymphoma cell line were slower (Figure 2B).

Next, we performed microarray analyses of λ820 cells treated with HMBA and compared the data with those of previously performed expression profiles of JQ1 or the HDACi vorinostat (SAHA) treated cells (17). As shown in Figure 3A, unsupervised hierarchical clustering analysis of the 2-fold up- and downregulated genes (by all compounds, Supplemental dataset 1) shows that HMBA clusters more closely with JQ1 than with vorinostat. Moreover, 56 % of genes suppressed and 68 % of genes induced (>2-fold) by JQ1 are also regulated in the same way by HMBA, whereas that similarity was lower for vorinostat-regulated genes (36% similarity of both induced and suppressed genes, Figure 3B). In our previous study it was demonstrated that HDACi and BETi regulate a few genes in each other's opposite directions (17). Indeed, the expressions of genes like *Cd74*, which are *induced* by vorinostat but *repressed* by JQ, were also repressed by HMBA (Figure 3A *inset* and Figure 3C). Furthermore, ingenuity pathway analyses of 2-fold up- and down-regulated genes showed high similarities between JQ1 and HMBA. Of the ten pathways most affected by both compounds, six were of lymphocyte development or signaling (e.g. B-cell development shown in Figure S2), and four were of cell cycle regulation (e.g. Figure S3). Accordingly, investigation of the MutSigDB database and follow-up gene-set enrichment analysis showed that genes repressed by HMBA were enriched in genes induced in S-phase by E2f (Figure S4), as previously shown for JQ1 (25). Finally, a ChIP assay experiment demonstrated that Brd2 and Brd4, but not Brd3, was clearly displaced by HMBA from the regulatory regions of the *Spermidine synthase* (*Srm*) gene (Figure 3D) – a gene whose expression was suppressed by HMBA (Supplemental dataset 1). Taken together, the data strongly suggest that HMBA is a BETi also in Myc-induced lymphoma cells.

HMBA was never shown to be efficacious in mouse models of cancer, but was moved into clinical trials based on overwhelming *in vitro* data. Having established that HMBA kills Myc-induced lymphoma cells *in vitro*, we were interested in investigating if anti-tumoral activity could be observed in this tumor type. We transplanted mice with λ2749 cells, which originally developed in a λ-*Myc* transgenic mouse and has been maintained since by serial transplantation in syngenic recipient B6 mice. Mice transplanted with these cells develops a lethal lymphoma that can be monitored by measuring if the white blood cell count (WBC) is above the normal range (6-15 cells/nl) (17). Treatment with the BETi RVX2135 results in a rapid reduction of WBC (17) and prolonged survival, making this an optimal model to assess effects of HMBA *in vivo*. As seen in Figure 4A, treatment of mice with HMBA carrying λ2749 cells resulted in a normalization of WBC, and this translated to a prolonged survival of these mice (Figure 4B). To investigate if HMBA exhibited activity against other Myc-driven B-cell malignancies, λ663 cells were injected into mice. The λ663 cells do not display traditional growth pattern with growth primarily in lymph nodes and spleen. Instead the mice transplanted develop hind leg paralysis. Labeling the cells with luciferase demonstrated that the lymphoma cells in these mice indeed propagate in the bone marrow (Figure 4C). Although the disease was not ameliorated by HMBA, the progression was significantly blunted (Figure 4C). Taken together, we here demonstrate for the first time that HMBA has activity against Myc-driven lymphoma *in vivo.*

DISCUSSION

In this report we have established that HMBA has the ability to inhibit at least four members of the BRD family. Our data also suggest starting points for synthesis of BD2-selective BET inhibitors. Identification of the targets is important since HMBA continues to be used as a differentiation agent and continues to be instrumental for identifying mechanisms of transcriptional elongation (26). Our data suggest that HMBA can be exchanged for more selective and potent BET bromodomain inhibitors in the study of differentiation.

More than twenty-five years ago, HMBA was used in patients suffering from many different malignancies, most notably AML. Current clinical investigators could learn

from the HMBA trials that certain toxicities, e.g. thrombocytopenia, need to be managed, and, unfortunately, few patients are likely to respond. The presented data suggest that *MYC* overexpression could be a biomarker of response to BET inhibitors. This was not known when the HMBA trials were conducted, so it is plausible that the trials would have generated better results if patients suffering from Myc-driven diseases had been recruited. Re-evaluating HMBA as a drug to treat Myc-induced cancers such as multiple myeloma, glioblastoma, neuroblastoma and small cell lung cancer is ongoing in animal models in our laboratory.

Although not formally proven here, our data also suggest that BET inhibition could be the reason for the induction of differentiation by DMSO. Indeed, DMSO is generally advised against as solvent of compounds for use in bromodomain protein binding assays or in crystallization studies, since it can bind and inhibit bromodomains by displacing essential water molecules (27). It is tempting to speculate that BET protein inhibition could explain why high concentrations (>1%) of DMSO have many biological effects such as inducing differentiation or killing tumor cells or blocking inflammation. Speculatively, it could also explain why cultured cells need time to recover after cryopreservation – they have been soaked in 10 % of a BET inhibitor.

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Table 1. Profiling of HMBA (and indicated known inhibitors) was performed by AlphaScreen assay (bromodomain) or enzyme assay (rat liver HDAC). Assays were performed in duplicates over a 10-fold dilution series with 5 mM being the highest concentration. Inhibition was observed in five assays, in the rest an IC50 was not reached (NR).

BRD Assay	Reference Cpd	IC50 (M) Reference	IC50 (M) HMBA
SMARCA4	I-BET	4.5E-04	NR
ASHL1	JQ1	2.3E-05	NR
ATAD2	JQ1	8.8E-05	NR
EP300	JQ1	2.9E-05	5.5E-04
BRD2(BD1)	JQ1	1.5E-06	NR
BRD2(BD2)	JQ1	1.2E-07	1.6E-03
BRD3(BD1)	JQ1	4.8E-07	NR
BRD3(BD2)	JQ1	3.6E-08	1.8E-04
BRD4(BD1)	JQ1	2.0E-07	NR
BRD4(BD2)	JQ1	2.9E-07	1.4E-03
HDAC	TSA	4.1E-9	NR

Figure legends

Figure 1. HMBA cause MEL cell differentiation by acting as bromodomain 2 (BD2) selective BET bromodomain inhibitor. a) Dose-response curves showing inhibition of BET BD2 domains. b) Co-crystal structure of BRD3 BD2 (green) and HMBA (yellow). Bound HMBA is shown as a stick and water molecules are shown as spheres coloured in red respectively. The electron density of the $2m|F_o|-d|F_c|$map is shown around the HMBA contoured at 1.0σ. c) MEL cells were cultured in the presence or absence of 5 mM HMBA, 0.1 μM JQ1, 1 or 10 μM C646, 1 μM CPTH2 or vehicle (DMSO 0.1 %) for 4 days. Cells were pelleted by centrifugation and photographed. d) Whole mouse blood or untreated (control) or JQ1/HMBA-treated MEL cells were lysed and the cleared supernatant was measured in a Nanodrop spectrophotometer. e) MEL cells were grown in the absence (C) or presence of 5 mM HMBA (H) or 0.1 μM JQ1 (J) for the indicated times. Cells were harvested and analyzed for *Hba-a1* mRNA expression by qRT-PCR. Shown is a representative experiment performed with three biological replicas. The PCR was run with three technical replicas and all data points are shown.

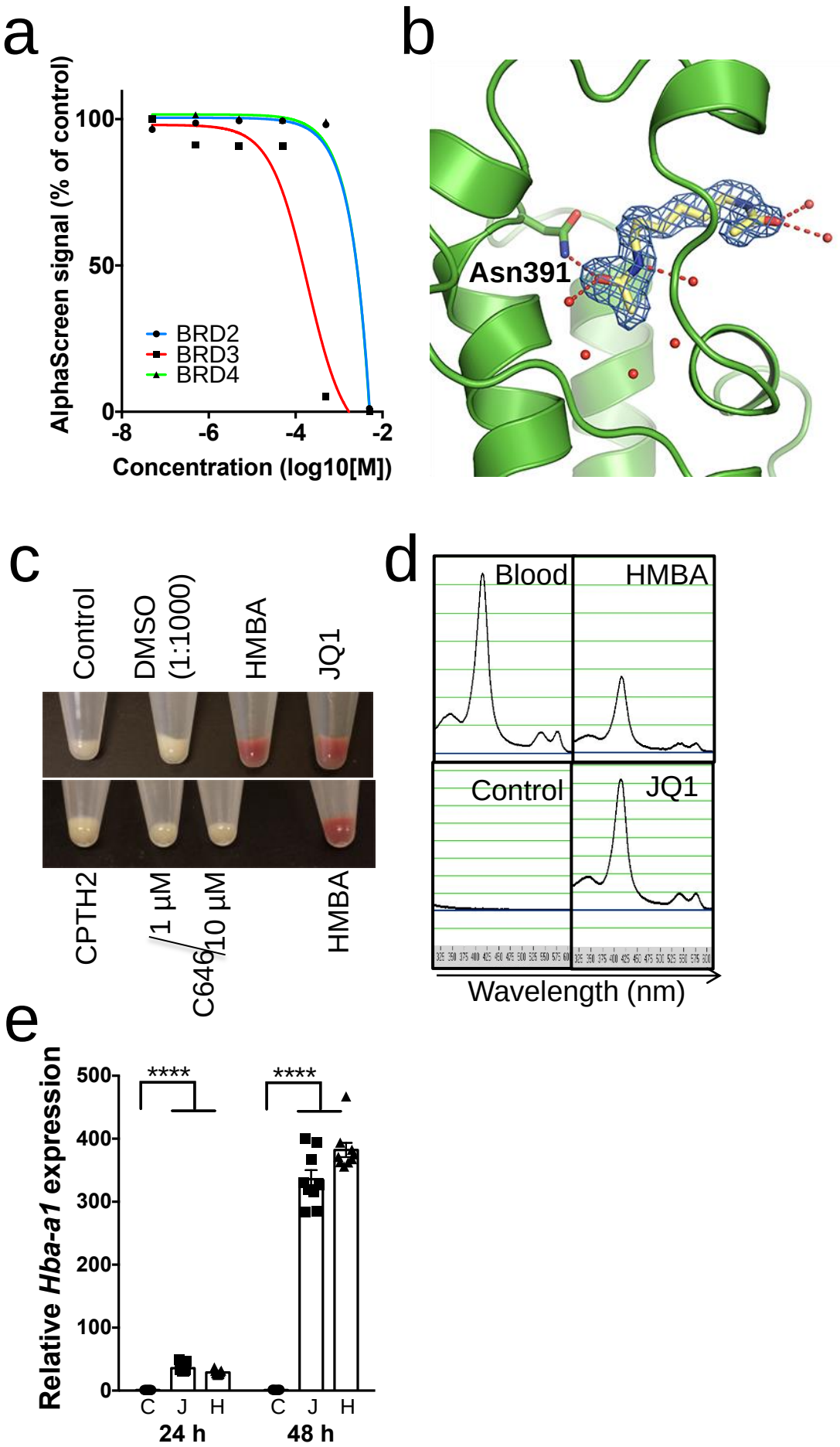
Figure 2. HMBA suppresses growth and induces apoptosis in Myc-induced lymphoma cells. a) Indicated cells were grown in the absence (C) or presence of 5 mM HMBA (H) for 24 h. Cells were harvested and analyzed for DNA content by flow cytometry. Apoptotic cells were defined as those having less than diploid DNA content (sub-G1). Shown are mean values of six different experiments. b) Human Daudi and Akata Burkitt lymphoma cell lines were grown in the absence or presence of 5 mM HMBA for 24, 48 and 72 h. Cells were harvested and analyzed for DNA content by flow cytometry.

Figure 3. HMBA triggers a BETi-like transcriptional program in Myc-induced lymphoma cells. a) λ820 cells were grown in the presence or absence of 5 mM HMBA, 1 μM JQ1 or 1 μM vorinostat for 24 h. RNA was prepared and analyzed by Illumina bead arrays encompassing all mouse RefSeq genes. Shown are unsupervised hierarchical clustering analyses comparing untreated/0.1 % DMSO-treated cells (C) with those repressed >2-fold by 5 mM HMBA (H), 1 μM JQ1 (J) or 1 μM vorinostat (V). Zoomed in is a cluster of genes that are regulated in opposite directions by the BETi JQ1 and the HDACi vorinostat, including *Cd74*. b) Venn diagrams of down-regulated genes (>2-fold) or up-regulated genes (>2-fold) by indicated compounds as compared to control samples (untreated for HMBA; 0.1 % DMSO for JQ1 or vorinostat). c) λ820 cells were grown in the presence or absence (C) of 5 mM HMBA (H), 1 μM JQ1 (J), 1 μM vorinostat (V) for 24 h. Cells were harvested and analyzed for *Cd74* mRNA expression by qRT-PCR. The PCR was run with three technical replicas and all data points are shown. d) ChIP assay experiment of cells treated with HMBA. Following ChIP with indicated antibodies, qPCR analyses were performed on the immune precipitate and the input DNA using primers directed against the promoter regions of *Cd74*, *Srm* and *Vpreb3* and the E-box containing region of intron 1 of the *Srm* gene. Relative expression was calculated using the equation Relative expression = $2^{(Ct\ input\ -\ Ct\ ChIP)}$ x % input x 100. Shown are the mean +/- standard deviation of four values generated from two biological replicas run in two technical repetitions.

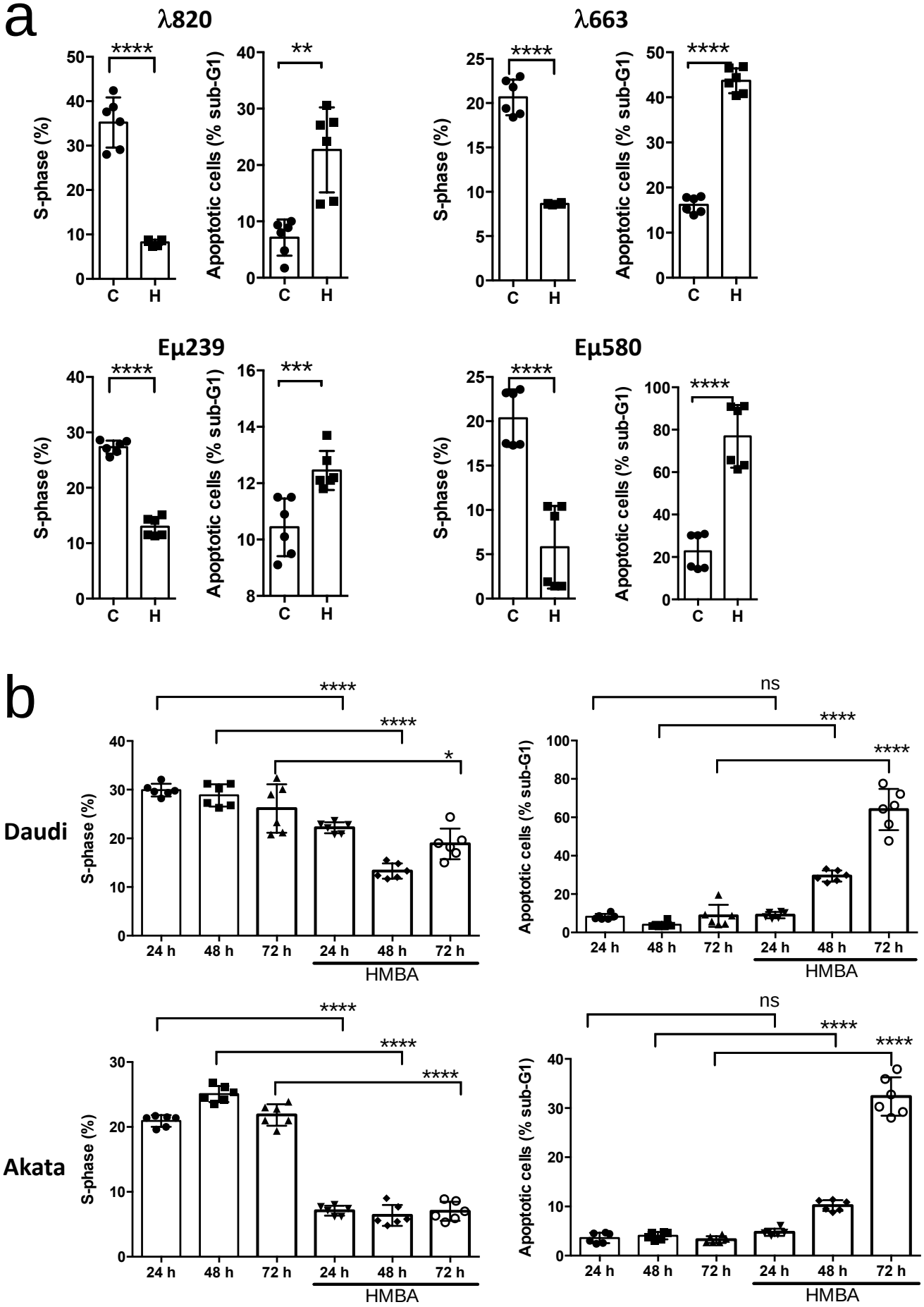
Figure 4. HMBA suppresses growth of established Myc-induced lymphoma in mice. a) Mice carrying λ2749 lymphoma cells were treated by intraperitoneal doses of 1, 1.5 or 2 g/kg/day HMBA (four mice per treatment group) when showing WBC counts suggestive of leukocytosis. WBC counts were measured at indicated timepoints. b)

Kaplan-Meier-like survival curve showing long-term consequences of HMBA-treatment mice (n=7 per treatment group; $p<0.0001$). c) 20 mice were injected with λ663 cells and randomized to two groups, one receiving 1500 mg/kg/day of HMBA, and the other left untreated. Mice were monitored for the development of hind leg paralysis, at which time they were sacrificed. *Inset:* λ663 cells were labeled with luciferase using retroviral transduction (pBABE-luc-hygro) and were transplanted into C57BL/6-*Tyrosinase* knockout mice (B6 albino) via tail-vein injection. 12 days after transplantation mice were injected with 100 µl of 30 mg/ml D-luciferin. Mice were sedated in an isofluran administrating chamber and then placed in an IVIS Lumina III XR machine (Perkin-Elmer).

Nilsson Fig 1



Nilsson Fig 2



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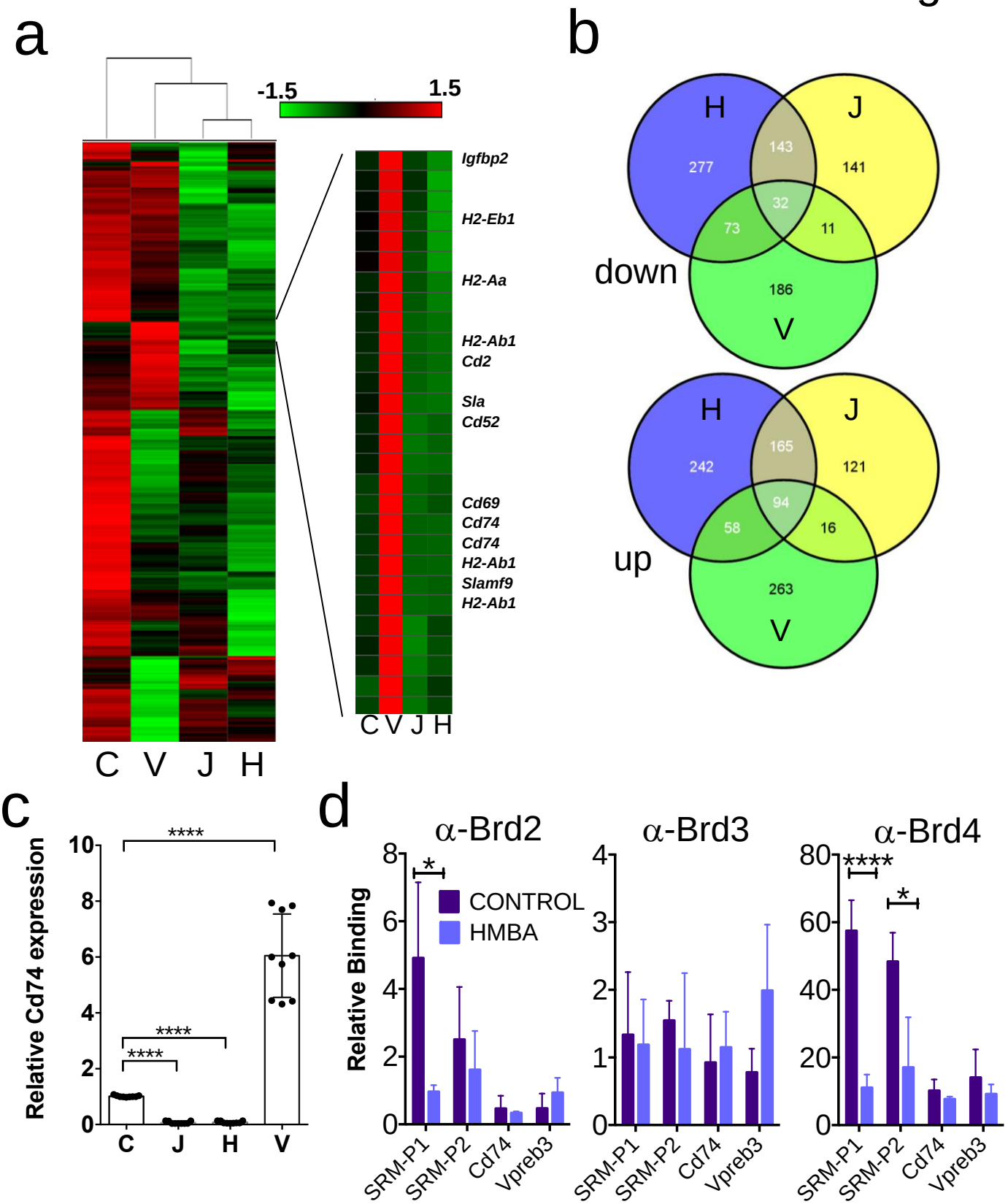
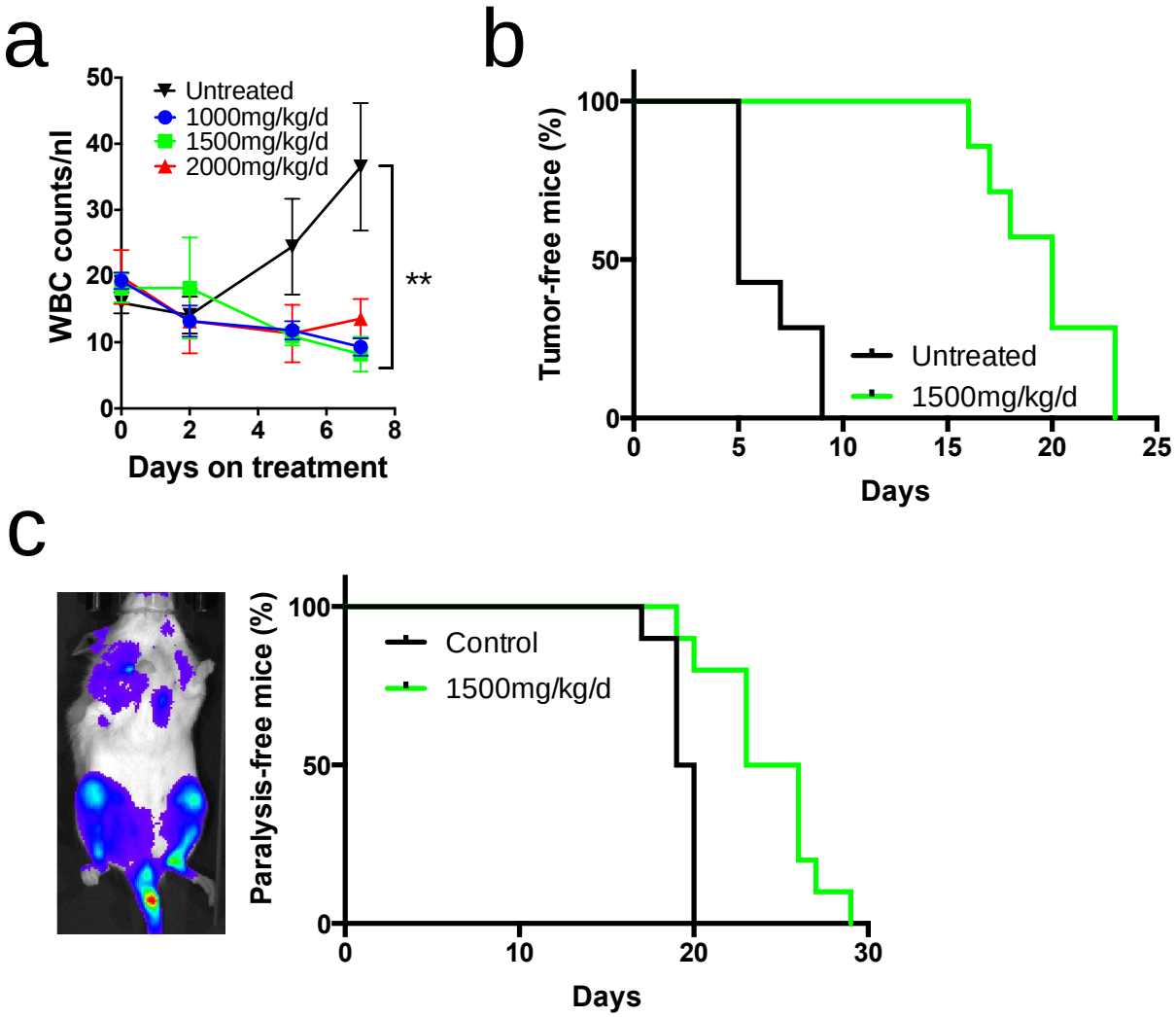


Nilsson Fig 4



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Cancer differentiation agent hexamethylene bisacetamide was likely the first BET bromodomain inhibitor in clinical trials

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