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ATF3 repression of BCL-X_L determines apoptotic sensitivity to HDAC inhibitors across tumour types

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Abstract

Purpose—Histone deacetylase inhibitors (HDACi) are epigenome-targeting small molecules approved for the treatment of cutaneous T cell lymphoma and multiple myeloma. They have also demonstrated clinical activity in AML, non-small cell lung cancer and estrogen receptor-positive breast cancer, and trials are underway assessing their activity in combination regimens including immunotherapy. However, there is currently no clear strategy to reliably predict HDACi sensitivity.

In colon cancer cells, apoptotic sensitivity to HDACi is associated with transcriptional induction of multiple immediate-early (IE) genes. Here, we examined whether this transcriptional response predicts HDACi sensitivity across tumour type, and investigated the mechanism by which it triggers apoptosis.

Experimental design—Fifty cancer cell lines from diverse tumour types were screened to establish the correlation between apoptotic sensitivity, induction of IE genes, and components of the intrinsic apoptotic pathway.

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Results—We show that sensitivity to HDACi across tumour types is predicted by induction of the IE genes *FOS*, *JUN* and *ATF3*, but that only *ATF3* is required for HDACi-induced apoptosis. We further demonstrate that the pro-apoptotic function of *ATF3* is mediated through direct transcriptional repression of the pro-survival factor *BCL-X_L* (*BCL2L1*). These findings provided the rationale for dual inhibition of HDAC and *BCL-X_L* which we show strongly cooperate to overcome inherent resistance to HDACi across diverse tumour cell types.

Conclusions—These findings explain the heterogeneous responses of tumour cells to HDACi-induced apoptosis and suggest a framework for predicting response and expanding their therapeutic use in multiple cancer types.

Keywords

HDAC inhibitor; ATF3; immediate-early gene; apoptosis; *BCL-X_L*

Introduction

Histone deacetylase inhibitors (HDACi) are epigenome-targeting anti-cancer therapeutics with established clinical activity in several haematological malignancies (1). A number of distinct chemical classes of HDACi have been identified or developed including short-chain fatty acids (butyrate, valproic acid), hydroxamic acids (trichostatin A, vorinostat, belinostat, panobinostat and pracinostat), tetrapeptides (romidepsin), and benzamides (entinostat) (2). Vorinostat and romidepsin are approved for the treatment of cutaneous T-cell lymphoma (CTCL) (3), and belinostat is approved for the treatment of peripheral T-cell lymphoma (PTCL). In addition, combinatorial use of panobinostat with the proteasome inhibitor bortezomib is approved for refractory multiple myeloma (4) and pracinostat was recently granted breakthrough therapy designation with azacytidine in acute myelogenous leukemia (AML) (5). While responses to single-agent HDACi are limited in solid tumours (6), studies in non-small cell lung cancer and estrogen receptor-positive advanced breast cancer suggest they may have efficacy in combination therapy regimens (7,8).

HDACi's inhibit class I (HDACs 1, 2, 3 and 8) and class II HDACs (HDACs 4, 5, 6, 7, 9 and 10), which deacetylate lysine residues on target proteins (2). HDACi activate gene expression by inducing hyperacetylation of DNA-bound core histones, thereby increasing accessibility of the core transcriptional apparatus to DNA (1), or by hyperacetylating transcription factors, which can either increase or decrease their transcriptional activity (1). In addition, HDACi can elicit cellular effects independent of transcription by acetylating cytoplasmic proteins such as Hsp90 and tubulin (9,10).

While HDACi induce multiple effects on tumour cells including inhibiting proliferation and inducing differentiation (1), their primary mechanism of anti-tumour activity is through the induction of apoptosis (2). In this regard, HDACi induce apoptosis primarily through the intrinsic/mitochondrial pathway (11), although in some tumour cell lines the extrinsic/death receptor pathway is also activated (12,13). HDACi-induced apoptosis has been linked with altered expression of key apoptotic regulators including upregulation of the pro-apoptotic molecules BAX (14), BAK (15), APAF1 (16) BMF (17), BIM (18) and DR5 (19), and down-regulation of the anti-apoptotic proteins SURVIVIN (20), *BCL-X_L* (21), and c-FLIP

(22). However, HDACi regulation of these factors varies between cell type, and has not been systematically linked to apoptotic response (23). Furthermore, the mechanisms by which HDACi regulate the expression of pro- and anti-apoptotic genes are only partially understood.

We previously identified a robust transcriptional response specifically associated with HDACi-induced apoptosis in colorectal cancer cell lines. This response involved the coordinate induction of multiple immediate-early (IE) response genes (*FOS*, *JUN*, *EGRI*, *EGR3*, *ATF3*, *ARC*, *NR4A1*) and stress response genes (*NDRG4*, *MTIE*, *MTIF* and *GADD45B*) (24). The goals of this study were to determine whether this represents a generic transcriptional response which defines HDACi-induced apoptosis across tumour types, including CTCL and multiple myeloma where these agents currently have the greatest clinical activity. Second, we sought to determine whether this transcriptional response underpins HDACi-induced apoptosis by regulating expression of key apoptotic regulators.

Herein we demonstrate that HDACi robustly induce expression of the IE genes *FOS*, *JUN* and *ATF3* in multiple tumour cell types, which correlated significantly with the magnitude of HDACi-induced apoptosis. We also demonstrate induction of these genes in 2 patients with CTCL treated with panobinostat. Functional studies revealed that *ATF3* but not *FOS* or *JUN* was required for HDACi-induced apoptosis across tumour cell lines, and that the effects of *ATF3* were mediated through repression of the pro-survival gene BCL-X_L (*BCL2L1*). These data provided a rationale for combining HDAC and BCL-X_L inhibitors, which successfully overcame inherent resistance to HDACi in a range of tumour types. Our findings establish the induction of *ATF3* and subsequent repression of BCL-X_L as a consistent and key determinant of HDACi-induced apoptosis independent of tumour type. They also define the molecular basis for differential sensitivity to HDACi and identify avenues for predicting response and overcoming inherent resistance to HDACi through rational combination therapy.

Materials and Methods

Cell culture

All cell lines used for this study were obtained from the American Type Tissue Culture Collection (ATCC), or as gifts from collaborators listed in the acknowledgements section. A total of fifty human cancer cell lines derived from multiple tumour types were used: Solid tumour cell lines used were PC-3, DU-145, LNCAP (Prostate); HT-1197, HT-1376, 5637 (Bladder); SK-MEL-3, SK-MEL-5, SK-MEL-28 (Melanoma); MDA-MB-231, MDA-MB-468, MCF-7 (Breast); A549, NCI-H292, NCI-H460, NCI-H358, NCI-H1650, NCI-H1975 (Lung); RKO, LIM1215, Colo320, SW48, HCT116, SW948 (Colon), IGROV1, SK-OV-3, JAM, OVCAR-8, OVCAR-5 (Ovarian), OU-87 (Glioblastoma); PANC-1 (Pancreatic); ACHN (Renal); 293T (Embryonic kidney); A431 (Epidermis); AGS (Gastric) and Hep3B (Hepatoma). Haematological cancer cell lines used were HH, HuT-78, HuT-102, MJ (Cutaneous T-cell lymphoma); Jurkat, Raji, U937 (Lymphoma); LP-1, OPM-2, RPMI-8226, U266 (Multiple myeloma); and K-562, KG-1 and KG-1A (Leukaemia). Cells were maintained at 37°C and 5% CO₂ in base medium DMEM for solid tumour cell lines or RPMI for haematological cancer cell lines. Base medium were supplemented with 10% FCS,

2 mM L-glutamine, 100U/mL Penicillin and 100µg/mL Streptomycin. Wild-type and *Atf3*^{-/-} mouse embryonic fibroblasts were maintained in low glucose DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100U/mL Penicillin and 100 µg/mL Streptomycin at 37°C in 10% CO₂. Methods for cell maintenance have been previously described (25). WT and FLAG-tagged hBCL-X_L transduced mouse embryonic fibroblasts were maintained in DMEM, high glucose media supplemented with 10% (v/v) fetal bovine serum, 250 µM L-asparagine, 50 µM 2-mercaptoethanol, 1 µM HEPES. Cell lines were assessed for mycoplasma status using the MycoAlert assay (Lonza, Switzerland) and mycoplasma negative frozen stocks used for a maximum of 2 months. Authenticity of frozen stocks of the A549, AGS, HCT116, PC3, U87, RPMI-8226, SKMEL28, MCF7, PANC1, HH, RKO, LIM1215, Colo320, SW48 and SW948 cell lines was determined by short-tandem repeat (STR) profiling using the GenePrint 10 system (Promega, USA), and all found to be exact matches with published profiles.

Drug source

Sodium-butyrate and valproate were obtained from Sigma (St. Louis, MO). Vorinostat, belinostat, Depsipeptide, entinostat, ABT-737 and ABT-199 were obtained from Selleck Chemicals (Houston, TX). ABT-263 was obtained from ApexBio (USA). Synthesis of A-1331852 was as described previously (26).

Measurement of apoptosis

Apoptosis assays were performed as previously described by PI staining and FACS analysis (25). Cells were seeded in triplicate in 24-well plates. Seeding densities varied between 30,000 – 90,000 cells per well and were calculated such that control cell density approximated 80% confluence at the completion of the experimental period. Drug treatment was performed for 24–72 hours. Both attached and floating cells were harvested by scraping, washed in cold PBS, and resuspended in 50 µg/ml propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100. Cells were stained overnight at 4°C, and 10,000 cells were analyzed for DNA content using a BD FACS Canto II (BD Biosciences). The percentage of cells with a sub-diploid DNA content was quantified using ModFit LT (Verity Software House, Topsahm, NE).

Clinical trial samples

Whole blood was collected in sodium-heparin tubes from 2 patients diagnosed with cutaneous T-cell lymphoma who participated in a single arm, open-label, institutional phase 2 Panobinostat trial ([Clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01658241) identifier: NCT01658241). Patients received 30 mg Panobinostat orally, three times weekly for up to 4 weeks. Both patients had >70% tumour involvement in PBMCs (Peripheral blood mononuclear cells). PBMC's were isolated by density centrifugation (Lymphoprep™, Norway), according to manufacturer's instructions. RNA from PBMC was purified and subjected to gene expression analysis using qRT-PCR. The clinical protocol, informed consent form, and other relevant study documentation were approved by the institutional review board of the Peter MacCallum Cancer Centre. All patients gave written informed consent prior to study entry.

Quantitative RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse-transcribed using random hexamers and the Transcriptor High Fidelity cDNA Synthesis Kit (Roche), according to manufacturer's instructions. Quantitative RT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems) according to manufacturer's instructions. 10 ng of cDNA was amplified with 75 nM forward and reverse primers in a 15 μ L reaction. Primers used are listed in Supplementary Table 1.

Western Blot

Western blot analysis was performed as previously described (27). The source and dilutions of antibodies used are as follows: Rabbit anti-ATF3 (sc-188, Santa Cruz, 1:1000), rabbit anti-FOS (cst-4384, Cell Signalling, 1:1000), mouse anti-c-JUN (cst-2315, Cell Signalling 1:1000), rabbit anti-Ac Histone H3 (06-599, Merck Millipore, 1:10000), goat anti-Histone H3 (sc8654, Santa Cruz, 1:5000), rabbit anti-beta Tubulin (ab6046, Abcam, 1:20000), mouse anti-actin (A5316, SIGMA, 1:10000) and rabbit-anti-BCL-X_L (54H6, Cell Signalling, 1:1000).

Plasmids and luciferase reporter assays

The ATF3 overexpression vector was provided by Dr. Dakang Xu at Monash University (28). The AP-1 reporter construct was obtained from Clontech (Mountain View, CA), Sp1/Sp3 reporter constructs were provided by Dr. Yoshihiro Sowa (Kyoto Prefectural University of Medicine) and pGL3-BCL-X_L reporter constructs were kindly provided by Dr. Ni Chen, Sichuan University, Chengdu, China (29).

Cell lines were transiently transfected with reporter constructs using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). Transfected cells were treated with HDACi for 24–48 h and luciferase reporter activity determined using the dual-luciferase reporter assay kit from Promega (Madison, WI). Due to the strong effects of HDCAi treatment on TK-Renilla luciferase activity, reporter activity was normalized to total protein.

RNAi-mediated knockdown

siRNAs targeting FOS, JUN, ATF3 and BCL-X_L were obtained from Dharmacon (Denver, CO). siRNA transfection was performed using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instruction. Cells were harvested 24, 48 or 72h post-transfection for subsequent analysis.

Xenograft Studies

Animal studies were performed with the approval of the Austin Health Animal Ethics Committee. Eight-week-old female BALB/c *nu/nu* mice weighing approximately 16g were obtained from the Australian Resources Centre, (ARC, Perth, Australia). U87 cells (3×10^6 cells) were injected subcutaneously into the right and left flank of each animal in a 150 μ L suspension consisting of a 1:1 mixture of DMEM (Invitrogen) and BD Matrigel Basement Matrix (BD Biosciences). Once palpable tumours developed, mice were randomized into 4

groups to receive either vehicle (DMSO by intra-peritoneal injection, and Phosal50 [60% Phosal PG, 30% PEG400 and 10% EtOH] by oral gavage), 50mg/kg Vorinostat via intra-peritoneal injection, 25mg/kg ABT-263 via oral gavage, or the combination. Mice were treated daily for 19 days. Tumour growth was monitored every second day by calliper measurement until the end of the experimental period or when tumours reached 1 cm³ in size. At this point, animals were euthanized and tumours were excised and weighed.

Statistical analysis

In all cases groups were compared using student's t tests, with P<0.05 considered to be statistically significant. Correlation analyses were performed using Pearson's correlation with P<0.05 considered statistically significant.

Results

HDACi sensitivity spectrum of human cancer cells

To identify the molecular mechanisms underlying HDACi-induced apoptosis, we first stratified vorinostat-induced apoptotic responses in 50 human cancer cell lines representing common tumour types, including those displaying significant clinical response to HDACi (CTCL, multiple myeloma, leukemia, breast and lung cancers) (3,7,8). Sensitivity of the cell lines to vorinostat were highly variable (ranging from 2.5% apoptosis in U87 cells to 97.4% in RPMI-8226 cells), enabling separation into strong or weak responders (Figure 1A). As observed clinically (3), vorinostat more potently induced apoptosis in hematological cell lines (Figure 1B). Amongst the solid tumour models, ovarian cancer lines were most sensitive while prostate and bladder lines were most resistant (Figure 1B). This spectrum of anti-tumour responses was replicated using sodium-butyrate (NaBu), a member of the short-chain fatty acid subclass of HDACi (Figure 1C & 1D). Differential sensitivity to HDACi was not due to differences in the extent of HDAC inhibition, as histone H3 acetylation was similarly increased by vorinostat in representative sensitive and resistant lines (Supplementary Figure 1).

HDACi induce sustained immediate-early gene expression in multiple tumour cell types and in CTCL patients *in vivo*

Using our comprehensive profile of HDACi-induced apoptosis, we next investigated the mechanisms likely to underpin HDACi response across multiple cancers. Based on our prior findings in colon cancer cells (24), we investigated whether the induction of the immediate-early (IE) genes *FOS*, *JUN*, *ATF3*, *EGR1*, *EGR3* and *GADD45B* is a general consequence of HDACi treatment, independent of tumour type. Using eight HDACi-sensitive cell lines representing solid and hematological cancers, we found that vorinostat robustly induced these genes by 2–10 fold within 2 hours, and sustained their expression over 48 hours (Figure 2A). Dose-dependent induction of these genes was confirmed in SK-MEL-28 (melanoma) and MCF7 (breast) cells (Figure 2B), and corresponding increase in protein expression of c-FOS, c-JUN and ATF3 was confirmed in 5 sensitive cell lines (Figure 2C). Induction of this transcriptional response was independent of HDACi chemical subclass as *FOS*, *JUN*, *ATF3*, *EGR1*, *EGR3* and *GADD45B* were also induced in the HH CTCL cell

line treated with panobinostat, belinostat, depsipeptide, entinostat and valproic acid (Supplementary Figure 2).

To determine if HDACi induce IE genes in a clinical context, we assessed their expression before and after 4 hours panobinostat treatment in 2 patients with CTCL enrolled in an institutional phase II panobinostat trial ([Clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01658241) identifier: NCT01658241). Both patients had >70% tumour load in their PBMCs, and received 30 mg panobinostat orally. As in cell lines, panobinostat robustly induced IE genes in PBMCs in both patients establishing induction of this transcriptional response as a clinically detectable consequence of HDACi therapy (Figure 2D).

HDACi-induced apoptosis correlates with the magnitude of IE gene induction independent of tumour type

We next examined if HDACi-induced apoptosis was coupled to the magnitude of IE gene induction by assessing HDACi-induction of this transcriptional response in all 50 cell lines. Vorinostat-induced apoptosis across the 50 cell lines correlated significantly with the corresponding magnitude of *FOS*, *JUN* and *ATF3* induction, but not *EGR1*, *EGR3* or *GADD45* (Figure 2E). Similar results were observed following treatment of the 50 cell lines with sodium-butyrate (Supplementary Figure 3A). The preferential induction of *FOS*, *JUN* and *ATF3* in HDACi sensitive cell lines was confirmed at the protein level in 2 representative sensitive and resistant cell lines, derived from different tumour types (Supplementary Figure 3B).

FOS, *JUN* and *ATF3* encode members of the AP-1 family of transcription factors, which control transcription when bound to specific DNA sequences as homo- or hetero-dimers (30). To determine if induction of these genes by HDACi causes AP-1 activation and if the magnitude of AP-1 activation is associated with apoptotic sensitivity, AP-1 reporter gene assays were performed on 5 representative HDACi-sensitive and resistant cell lines derived from multiple tumour types. Consistent with the preferential induction of *FOS*, *JUN* and *ATF3* in sensitive lines, HDACi-induction of AP-1 reporter activity was significantly higher in the sensitive cell lines (Supplementary Figure 3C).

Finally, we have previously demonstrated that the Sp1 and Sp3 transcription factors are required for HDACi induction of IE gene expression, and that HDACi preferentially induce Sp1/Sp3 reporter activity in HDACi-sensitive colon cancer cell lines (24). To determine if the differential induction of IE genes is linked to differential activation of Sp1/Sp3 transcription factors independent of tumour type, the 5 HDACi sensitive and resistant cell lines derived from different tumour types were transfected with an Sp1/Sp3 reporter construct and treated with vorinostat for 24 hours. Consistent with the findings in colon cancer cells, HDACi-induction of Sp1/Sp3 reporter activity was significantly higher in sensitive cell lines, suggesting preferential activation of these transcription factors mediates IE gene induction independent of tumour type (Supplementary Figure 3D).

ATF3 is required for HDACi-induced apoptosis

To define the contributions of c-FOS, c-JUN and ATF3 to HDACi-induced apoptosis, expression of each of these AP-1 proteins was knocked-down in three HDACi-sensitive cell

lines. We found that only ATF3 depletion was sufficient to attenuate HDACi-induced apoptosis (Figure 3A, B). These effects were confirmed using multiple ATF3-targeting siRNAs (Supplementary Figure 4). To test the role of ATF3 in HDACi-induced apoptosis in a different model, mouse embryonic fibroblasts (MEFs) derived from wild-type and *Atf3* knockout mice were treated with HDACi. As expected, vorinostat only induced ATF3 mRNA in wild-type MEFs (Figure 3C). *Atf3*^{-/-} MEFs were significantly less responsive to vorinostat and sodium butyrate-induced apoptosis than wild-type cells (Figure 3D, E), collectively implicating *ATF3* as a key mediator of HDACi-induced apoptosis in multiple cell types.

HDACi-induced ATF3 represses the pro-survival factor BCL-X_L

HDACi-induced apoptosis has been linked with altered expression of regulators of both the intrinsic and extrinsic apoptotic pathways, however the majority of studies indicate a dominant role for the intrinsic (mitochondrial) pathway (2,31,32). To determine if ATF3 induction plays a role in altering expression of the key regulators of this pathway, we first determined the effect of HDACi treatment on expression of all components of the intrinsic apoptotic pathway in 15 cell lines spanning a range of tumour types and HDACi sensitivities. HDACi significantly induced expression of *BIM*, *BIK*, *BMF* and *NOXA* (*PMAIP1*) and downregulated expression of *BCL-w* (*BCL2L2*) in all cell lines, independent of apoptotic response (Supplementary Figure 5). We next investigated if altered expression of any components of the intrinsic apoptotic pathway correlated with the magnitude of HDACi-induced apoptosis and the magnitude of HDACi induction of ATF3. This analysis identified *BCL-X_L* (*BCL2L1*) as a candidate ATF3 repressed gene, whose expression was inversely correlated with both the magnitude of HDACi induced apoptosis and HDACi induction of *ATF3* (Figure 4A, B). Consistent with changes in its transcript levels, BCL-X_L protein was also preferentially repressed by HDACi in sensitive cell lines (Supplementary Figure 6).

To directly determine if ATF3 is required for HDACi-mediated repression of BCL-X_L, we examined the effect of HDACi on BCL-X_L promoter activity using a series of BCL-X_L promoter reporter constructs (Figure 4C) (29). Vorinostat and panobinostat maximally repressed activity of the P1281 reporter (-664 downstream to +617 of the transcription start site) and also repressed the P828 and P1692 reporters (Figure 4D). Conversely, minimal effect was observed on the P621 reporter, implicating key *cis*-acting sequences located between -4 and -664 bp upstream of the transcription start site that are required for HDACi-mediated repression of BCL-X_L promoter activity (Figure 4D). Vorinostat also significantly repressed activity of the BCL-X_L P1281 reporter in two additional HDACi-sensitive cell lines, A549 and AGS (Supplementary Figure 7). To determine if these effects were mediated through ATF3, experiments were repeated following ATF3 knockdown, which resulted in significant attenuation of HDACi-induced BCL-X_L promoter repression (Figure 4E).

To determine if ATF3 can directly repress BCL-X_L promoter activity, we first assessed the effect of ATF3 overexpression alone on BCL-X_L promoter activity. Similar to the effects of HDACi, ATF3 overexpression repressed activity of the P828, P1281 and P1692 reporters but not the P621 reporter (Figure 4F). Analysis of the promoter sequence -4 to -664 bp

downstream of the transcription start site identified the presence of an AP-1 site and three CREB sites, which are putative ATF3 binding motifs (Figure 4C). To directly establish ATF3 binding to this region in response to HDACi treatment, we performed ATF3 chromatin immunoprecipitation experiments which sequentially interrogated ATF3 binding along the BCL-X_L promoter. The most robust enrichment of ATF3 binding following vorinostat treatment was observed at regions R2 and R3 (Figure 4G), overlapping the key regulatory region (−4 to −664) identified in the promoter reporter assays. Notably, HDACi and ATF3 overexpression were able to repress the P828 promoter despite the lack of ATF3 binding to this region suggesting that ATF3 may also indirectly repress BCL-X_L promoter activity.

Finally, to establish the requirement of ATF3 induction for HDACi-mediated repression of BCL-X_L at the endogenous level, ATF3 knock down was performed in three sensitive cell lines prior to HDACi treatment. In each case, ATF3 knockdown markedly attenuated BCL-X_L repression in response to HDACi-treatment (Figure 4H), establishing *ATF3* induction as a critical requirement for HDACi-mediated BCL-X_L repression.

BCL-X_L inhibition overcomes inherent resistance to HDACi-induced apoptosis

We next examined the importance of BCL-X_L repression in HDACi-induced apoptosis. Knockdown of BCL-X_L in HDACi-refractory PANC1, U87 and PC3 cells significantly enhanced HDACi-induced apoptosis, implicating BCL-X_L repression as a key determinant of HDACi response (Figure 5A). Conversely, BCL-X_L overexpression in FLAG-tagged hBCL-X_L MEFs conferred resistance to vorinostat-induced apoptosis compared to WT MEFs (Figure 5B), collectively establishing BCL-X_L repression as a key determinant of HDACi-induced apoptosis.

These findings suggested that therapeutic targeting of BCL-X_L may have similar effects. To test this, the HDACi-resistant cell lines PANC1, PC3 and U87 were treated with vorinostat alone and in combination with the BH3 mimetics ABT-263 (navitoclax), which inhibits BCL-2, BCL-X_L and BCL-w. Combination treatment significantly enhanced apoptosis compared to either agent alone in each cell line (Figure 5C). Similar effects were obtained using its precursor compound, ABT-737 (Supplementary Figure 8A). To directly determine the role of BCL-X_L, we next examined the effects of combining HDACi with the novel BCL-X_L-specific inhibitor, A-1331852 (33). Combination treatment significantly enhanced apoptosis in all 3 cell lines compared to either agent alone (Figure 5D). In contrast, combination treatment with the BCL-2-specific inhibitor ABT-199 (venetoclax) resulted in modest to no enhancement of HDACi-induced apoptosis (Supplementary Figure 8B). We next determined whether this combination could also be utilized in HDACi sensitive cell lines, by enabling each drug to be used at significantly lower concentrations. Treatment of the HDACi-sensitive cell line HCT116 with a 2-fold lower concentration of vorinostat (2.5 μM) and a 100-fold lower concentration of ABT-263 (0.1 μM), or a 10-fold lower concentration of A-1331852 (1 μM) to that used in resistant cells was still sufficient to induce >60% apoptosis (Figure 5E, F).

As A-1331852 is not suitable for use *in vivo*, we next tested the effect of combination treatment with vorinostat and ABT-263 on growth of HDACi refractory U87 xenografts *in vivo*. Daily treatment with the combination significantly inhibited tumour growth compared

to control or either agent alone (Figure 6A–C). Importantly, no differences in body weight were observed in either the single agent or combination treatment arms compared to control (Figure 6D).

DISCUSSION

HDACi are an established treatment for haematological malignancies (CTCL, multiple myeloma) and continue to be tested, mostly in combination, for activity in other tumour types (1). Comparatively, the activity of these agents in solid tumours is more limited. The goal of this study was to define the mechanisms of HDACi action in tumour cells in order to provide a framework for the rational design of drug combinations involving their use, and the identification of molecular determinants of sensitivity.

We previously demonstrated that HDACi-induced apoptosis in colon cancer cells is associated with a specific transcriptional response involving the induction of multiple immediate-early response genes, including 3 members of the AP-1 transcription factor family, *FOS*, *JUN* and *ATF3*. We now demonstrate that this transcriptional response provides a robust and early readout of HDACi-induced apoptosis which transcends tumour cell type.

While HDACi treatment preferentially induces expression of three AP-1 family members in sensitive cell lines, we found that only ATF3 is required for HDACi-driven apoptosis. The pro-apoptotic role for ATF3 identified herein is consistent with ATF3 overexpression alone being sufficient to induce apoptosis in prostate (34) and ovarian cancer cells (35), and the resistance of *Atf3* knockout MEFs to UV-induced apoptosis (36). Furthermore, ATF3 is required for apoptosis induced by ER stress (37), anoxia (38), and the chemotherapeutic agents 5FU, etoposide and cisplatin (39–41). Finally, ATF3 is required for apoptotic sensitization to HDACi combination therapy with cisplatin and agonistic anti-DR5 antibodies (41,42) and for HDACi-induced apoptosis in bladder cancer cells (43). However the subsequent mechanisms of apoptosis induction have not been investigated.

Prior studies have linked HDACi-induced apoptosis with altered expression of a number of pro- and anti-apoptotic genes, particularly components of the intrinsic apoptotic pathway (43). However, these effects have not been investigated in the context of sensitivity across tumour cell type, and the mechanisms which underpin altered expression of these genes have not been systematically addressed. The current study identifies a uniform mechanism which determines HDACi-induced apoptosis, involving ATF3-mediated repression of BCL-X_L, which transcends tumour cell type. The role of ATF3 as a transcriptional repressor is consistent with prior reports (44), and our ChIP and reporter gene analysis indicate that repression of BCL-X_L in HDACi-treated tumour cells involves direct binding of ATF3 to the BCL-X_L promoter. Furthermore, we demonstrate that repression of BCL-X_L is central in HDACi-induced apoptosis, as both molecular and pharmacological inhibition of this pro-survival factor markedly enhanced HDACi-induced apoptosis *in vitro* and *in vivo*, and BCL-X_L overexpression protects cells from HDAC-induced apoptosis, consistent with previous studies (18,45).

However, we note that the molecular or pharmacological inhibition of BCL-X_L alone did not induce apoptosis to the same extent as when BCL-X_L was inhibited in the presence of HDACi, suggesting the requirement for additional HDACi-induced molecular changes to drive apoptosis. In this regard, we did identify consistent induction of the pro-apoptotic BH3-only genes *BIM*, *BIK*, *BMF* and *NOXA* in response to HDACi treatment, several of which has been shown to be required for HDACi-induced apoptosis (32,46). Notably however, induction of these genes occurred uniformly across the cell lines, independent of apoptotic sensitivity, implying their altered expression is not the basis for differential HDACi response. We therefore propose a model whereby HDACi-induced apoptosis involves both the induction of pro-apoptotic factors such as *BIM*, *BIK*, *BMF* and *NOXA* and the ATF3-dependent repression of the pro-survival factor BCL-X_L, of which the magnitude of induction of ATF3 and subsequent repression of BCL-X_L determines apoptotic response.

Delineating BCL-X_L repression as a key determinant for HDACi-induced apoptosis has significant implications for the rational design of strategies to enhance HDACi anti-tumour activity. We exploited this using navitoclax (ABT-263), a BH3-mimetic drug (that inhibits BCL-2, BCL-w and BCL-X_L), and the BCL-X_L-specific inhibitor A-1331852, which significantly enhanced HDACi-induced apoptosis in tumour cells inherently refractory to HDACi. These findings have the potential to enhance the range of tumours amenable to HDACi treatment by overcoming inherent resistance and to potentially reduce toxicities in sensitive cells by enabling HDACi to be used at lower concentrations.

A further application of these findings could be in the selection of patients likely to respond to HDACi by assessment of the magnitude of FOS, JUN and ATF3 induction following short-term HDACi treatment. The feasibility of this approach is supported by our demonstration of induction of these genes following 4 hours panobinostat treatment in 2 patients with CTCL with high circulating tumour load. This approach could potentially be extended to solid tumours where freshly isolated tumour cells in the form of biopsy material, organoids, patient-derived xenografts or circulating tumour cells are assessed for *FOS*, *JUN* and *ATF3* induction following short-term HDACi treatment as a predictor of the likelihood of response.

Our findings also suggest a framework for identifying molecular biomarkers of HDACi response prior to drug treatment through detailed investigation of the molecular determinants of differential *ATF3* induction among tumours. In this regard, our previous studies in colon cancer cells demonstrated that HDACi-induction of immediate-early genes, including *ATF3*, is dependent on the Sp1 and Sp3 transcription factors, and that HDACi preferentially induce Sp1/Sp3 reporter activity in HDACi sensitive colon cancer lines (24). We now extend these findings by demonstrating that HDACi preferentially induce Sp1/Sp3 reporter activity in sensitive cell lines, independent of tumour type. A central role for Sp1 and Sp3 in regulating HDACi-induced apoptosis independent of tumour type is also plausible given their ubiquitous expression (47). However, SP1 and SP3 are not mutated in human cancers and analyses in colon cancer cells suggest basal differences in expression are unlikely to be determinants of HDACi response (24). Notably, both SP1 and SP3 are post-translationally modified by a number of mechanisms including acetylation, ubiquitination and phosphorylation which can alter their activity (48). Exploration of whether such post-

translational modifications occur in response to HDACi treatment and identification of the factors regulating these changes, which may vary between sensitive and resistant cells, may provide novel insight into the basis for differential HDACi response. Notably, several other mechanisms of *ATF3* induction have also been described, including induction by p53 (49), activation of JNK, ERK and p38 signaling (50), and by ATF4 subsequent to activation of the ER stress / unfolded protein response pathway (37). In addition to modulating SP1 and SP3, HDACi can also impact these pathways which may contribute to the differential induction of *ATF3* among tumours.

In summary, we have identified a specific transcriptional response associated with HDACi-induced apoptosis that transcends tumour type, involving the coordinate induction of *FOS*, *JUN* and *ATF3*. We identify the induction of ATF3 and subsequent repression of BCL-X_L as a central mechanism of HDACi-induced apoptosis and applied these findings to develop rational drug combinations which overcome inherent resistance and enhance the activity of HDACi in a range of tumour types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of translational relevance

The study identifies a novel mechanism by which HDAC inhibitors induce apoptosis in tumour cells through induction of the ATF3 transcription factor and subsequent repression of BCL-XL. This mechanism transcends tumour type, is measurable in patient samples *in vivo*, and defines the basis for sensitivity or resistance to HDAC inhibitors.

These findings establish a strategy for overcoming inherent resistance to HDACi by rational combination with BCL-XL inhibitors, and define a framework for the identification of biomarkers predictive of HDACi response, including rapid assessment of ATF3 induction.

These findings have the potential to directly impact the clinical use of HDACi for the approved indications of CTCL and multiple myeloma, and for their ongoing clinical development in multiple malignancies.

Figure 1.

(A) Apoptotic sensitivity of 50 cancer cell lines to Vorinostat. Cells were treated with drug for 72 hrs and apoptosis determined by propidium-iodide (PI) staining and FACS analysis. Cell lines within each tumour type are ordered by increasing sensitivity. Values shown are mean $\pm$ SEM from 2 independent experiments, each performed in triplicate. (B) Separation of the 50 cell lines into solid vs hematological cancer cell lines. (C) Apoptotic sensitivity of 50 cell lines to sodium-butyrate (5 mM, NaBu). Apoptosis was assessed as for vorinostat. (D) Pearson's correlation of Vorinostat and NaBu-induced apoptosis across the 50 cell lines.

Figure 2.

(A) Effect of vorinostat on *FOS*, *JUN*, *ATF3*, *EGR1*, *EGR3* and *GADD45B* mRNA expression in 8 HDACi-sensitive cell lines. Cells were treated with 5 μ M vorinostat for 2–48 hrs and gene expression determined by quantitative real-time PCR. Values shown are average Log2 fold-induction from three biological experiments represented in a heat map. (B) Effect of Vorinostat dose escalation on *FOS*, *JUN*, *ATF3*, *EGR1*, *EGR3* and *GADD45B* mRNA expression in 2 representative HDACi sensitive cell lines (SK-MEL-28 and MCF7). Values shown are average Log2 fold-induction from a representative experiment performed in triplicate. (C) Effect of vorinostat (Vorino 5 μ M) treatment on FOS, JUN and ATF3 protein expression in 5 representative HDACi sensitive cell lines. (D) Effect of Panobinostat on *FOS*, *JUN*, *ATF3*, *EGR1*, *EGR3* and *GADD45B* mRNA expression in PBMCs isolated from 2 CTCL patients. Samples were collected before and 4 hrs after panobinostat treatment. Values shown are mean $\pm$ SEM of the Log2 fold-change in post versus pre-treated samples analysed in triplicate. (E) Correlation of the magnitude of change in expression of *FOS*, *JUN*, *ATF3*, *EGR1*, *EGR3* and *GADD45B* with apoptosis following vorinostat (5 μ M) treatment across the 50 cell lines. The magnitude of gene induction was determined in each cell line 24 hrs post HDACi treatment by q-RT-PCR.

Figure 3.

Effect of *FOS*, *JUN* and *ATF3* knockdown on HDACi-induced apoptosis. HDACi sensitive cell lines (A549, AGS and HCT116) were transiently transfected with a non-targeting siRNA or *FOS*, *JUN* or *ATF3*-targeting siRNAs, and treated with Vorinostat (5 μ M) for 24 hrs. (A) Knockdown efficiency of FOS, JUN and ATF3 protein, and (B) corresponding apoptotic responses determined by PI staining and FACS analysis. Values shown are mean $\pm$ SD from a representative experiment performed in triplicate. (C) Induction of *Atf3* mRNA following 24hrs Vorinostat (5 μ M) treatment of WT and *Atf3*^{-/-} MEFs and (D, E) corresponding apoptotic response to 72 hr (D) vorinostat and (E) sodium-butyrate treatment.

Values shown are mean $\pm$ SEM from three biological experiments performed in triplicate.
* $P < 0.05$, unpaired t-tests.

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Figure 4.

(A) Pearson's correlation of the magnitude of repression of *BCL-X_L* versus induction of apoptosis following HDACi treatment across 15 cell lines. (B) Pearson's correlation showing the inverse relationship between the magnitude of induction of *ATF3* and the repression of *BCL-X_L* mRNA following HDACi treatment across 15 cell lines. (C) *BCL-X_L* promoter reporter constructs used including location of putative AP-1 and CREB binding sites and regions (R) amplified in ChIP analyses. UPS (upstream). (D) HCT116 cells were transiently transfected with a series of *BCL-X_L* promoter reporter constructs and treated with vorinostat (Vor) or panobinostat (Pan) for 24hrs. Luciferase activity was corrected for total cellular protein. (E) Effect of ATF3 knockdown on HDACi-mediated repression of the *BCL-X_L* P1281 promoter activity. Cells were transiently transfected with non-targeting or ATF3-targeting siRNAs overnight and treated with vorinostat for 24 hrs. (F) Effect of ATF3 overexpression on *BCL-X_L* promoter activity. HCT116 cells were transiently transfected with *BCL-X_L* luciferase reporter constructs of varying lengths and an *ATF3* expression vector (*pcDNA-ATF3*) or empty vector control (*pcDNA-EV*) and luciferase activity assessed after 24hrs. All cells were also transfected with *TK-Renilla* as a control for transfection efficiency. Values shown are mean $\pm$ SD from three biological experiments performed in triplicate. ** $P < 0.01$, *** $P < 0.005$, unpaired t-tests. (G) HCT116 cells were treated with

vorinostat (5 μ M) for 24hrs and ATF3 binding to sequential regions of the BCL-X_L promoter determined by chromatin immunoprecipitation. (H) Effect of *ATF3* knockdown on HDACi-induced BCL-X_L repression. The HDACi sensitive cell lines A549, AGS and HCT116 cells were transiently transfected with *ATF3*-targeting siRNAs and treated with Vorinostat for 24 hrs. ATF3 knockdown efficiency is shown in Figure 5.

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Figure 5.

(A) Effect of *BCL-X_L* knockdown on HDACi-induced apoptosis in HDACi resistant cell lines. Cells were transiently transfected with a non-targeting or *BCL-X_L*-targeting siRNA, and treated with Vorinostat (5 μM) for 24 hrs. (Top panels) Knockdown efficiency of *BCL-X_L* protein assessed by western blot. (Bottom panel) Corresponding apoptotic response following treatment with Vorinostat (5 μM) for 72hrs. Values shown are mean ± SD from 3 biological experiments performed in triplicate. **P* < 0.05, ***P* < 0.005, unpaired t test. (B) *BCL-X_L* overexpression (O/E) protects MEFs from HDACi-induced apoptosis. (Top panel) Validation of overexpression of flag-tagged *BCL-X_L* in MEFs by western blot (Endog:

Endogenous BCL-X_L). (Bottom panel) Effect of 72 hours vorinostat treatment (20 μ M) on apoptosis. Values shown are mean $\pm$ SD from a representative experiment performed in triplicate. * P < 0.05, ** P < 0.005, unpaired t test. (C–D) Apoptotic response of HDACi resistant cell lines to combination treatment with vorinostat (5 μ M) and the BH3 mimetic (C) ABT-263 (10 μ M) or the (D) BCL-X_L-specific inhibitor A1331852 (10 μ M). (E–F) Apoptotic response of the HDACi sensitive cell line HCT116 to combination treatment with vorinostat (2.5 μ M) and (E) ABT-263 (0.1 μ M) or (F) A1331852 (1 μ M). All cell lines were treated with either drug alone or in combination for 72 hrs and apoptotic response determined by PI staining and FACS analysis. Values shown are mean $\pm$ SD (n=3). * P < 0.05, ** P < 0.01 and *** P < 0.005, unpaired t-tests.

Figure 6.

Effect of Vorinostat and ABT-263 treatment alone and in combination on tumor growth *in vivo*. HDACi-refractory U87 cells were injected into the right and left flank of BALB/c *nu/nu* mice (day 0). On day 4, mice were randomized to receive vehicle, Vorinostat (50 mg/kg), ABT-263 (25mg/kg) or the combination. Mice were treated daily for 5 days followed by 2 days break for a total of 19 days. (A) Tumour volume was monitored over time by caliper measurement. (B) Representative resected tumours at study completion (Day 19) and (C)

weight of resected tumours. (D) Body weight of mice relative to weight at day 0. Data represented are mean $\pm$ SEM. * P <0.05, ** P <0.005, unpaired t tests.

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